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BINDING, INTERNALIZATION, AND RETROGRADE TRANSPORT OF ^{125}I-NERVE GROWTH FACTOR IN CULTURED RAT SYMPATHETIC NEURONS[1]

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Abstract

Sympathetic neurons internalize nerve growth factor (NGF) and transport it retrogradely to their cell bodies where it appears to serve a trophic function in maintaining neuronal survival. We have characterized the binding, internalization, and retrograde transport of ^{125}I-NGF by cultured rat sympathetic neurons. After 3 to 4 weeks in culture, sympathetic neurons possessed approximately 2×10^7 specific, cell surface NGF binding sites per neuron with an apparent affinity constant of 2 to 5×10^{-9} M. The density of binding sites on the plasma membrane of the neurites was approximately twice that on the plasma membrane of the cell bodies. Because of the extensive network of neuronal processes, the neurites probably account for more than 99.5% of the total binding in mature cultures. Using electron microscope autoradiography, we localized the distribution of ^{125}I-NGF in the cell body following a 1-hr exposure to ^{125}I-NGF. The majority of silver grains were associated with lysosomal organelles, including secondary lysosomes, residual bodies, and multivesicular bodies (MVB). The MVB were the most heavily labeled, with a labeling density (L.D.) of 21, while the lysosomes had a L.D. of 3.1. To study the retrograde transport of ^{125}I-NGF, neurons were grown in compartmentalized culture dishes and their distal processes were exposed to ^{125}I-NGF. Radioactive material was transported to the cell bodies at the rate of approximately 3 mm/hr. The transport mechanism was sensitive to colchicine and was saturable with respect to NGF. After 8 hr of transport, when the radioactivity in the cell bodies had reached a steady state, the label again was localized primarily to the MVB (L.D. = 16.8) and the lysosomes (L.D. = 3.8). The nuclei were not labeled significantly and had an overall L.D. of 0.47. We saw no evidence for the accumulation of NGF by the nuclear membrane, the nucleolus, or chromatin.

A number of polypeptide hormones, peptides, asialoglycoproteins, and lysosomal enzymes are taken up into cultured cells rapidly by a process referred to as receptor-mediated endocytosis or adsorptive endocytosis (Goldstein et al., 1979; Pastan and Willingham, 1981). The retrograde transport of nerve growth factor (NGF) in sympathetic axons *in vivo* after injection into the anterior chamber of the eye (Hendry et al., 1974; Johnson et al., 1978) suggested that NGF can be taken up into neurons selectively via their terminals, presumably also by a receptor-mediated process. It has been proposed, furthermore, that the uptake and retrograde transport of NGF, produced by innervated target organs, may play an important role in the development and maintenance of at least the sympathetic nervous system and possibly also the sensory nervous system (Thoenen et al., 1978).

The biochemical basis underlying the mechanisms by which NGF produces its actions is unknown. A similar situation applies for the other polypeptide hormones, such as insulin and epidermal growth factor (EGF), which are internalized via receptor-mediated endocytosis (Greene and Shooter, 1980; Czech, 1977; Carpenter and Cohen, 1979). A prerequisite for this type of endocytosis is the presence of high affinity surface binding sites. A variety of target cells, responsive to NGF action, contain

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high affinity receptors for NGF (Greene and Shooter, 1980).

Internalization of NGF may be required for some of the actions of NGF in target cells. It may be that plasma membrane-localized binding of NGF mediates a set of rapid responses, such as the efflux of Na^+ ions (Skaper and Varon, 1980), which then give rise to other responses, such as the observed increased adhesivity in PC12 cells (Schubert et al., 1978) and the transient activation of the cell surface into ruffles and pits (Connolly et al., 1979). In contrast, the more long term, growth-promoting effects of NGF could be a consequence of the internalization of NGF and potentially an interaction, either directly or indirectly, with the genome (Yankner and Shooter, 1979; Marchisio et al., 1980, 1981). Alternatively, the sole purpose for internalization may be the removal and degradation of bound NGF.

Unlike many of the polypeptide hormones which primarily elicit acute responses in target cells, NGF is required continuously for the survival of rat sympathetic neurons in culture (Mains and Patterson, 1973; Chun and Patterson, 1977a, b, c). There is also evidence that NGF is required in adult rats for the continued integrity and maintenance of the sympathetic nervous system (Gorin and Johnson, 1980). Since it is possible that chronically required factors, such as NGF, may be processed differently than previously described factors and because of the diverse biological actions of NGF, we set out to characterize, using combined biochemical and autoradiographic techniques, the interaction, under defined conditions, of ^{125}I-NGF with cultured rat sympathetic neurons.

Recent studies with neuronal primary cultures and with the pheochromocytoma cell line, PC12, indicate that NGF becomes internalized after the initial binding to the cell surface (Claude et al., 1979; Levi et al., 1980; Marchisio et al., 1980, 1981). It has been further proposed that the internalized NGF becomes associated with the cell nucleus or with nuclear elements (Andres et al., 1977; Yankner and Shooter, 1979; Marchisio et al., 1980). To date, a direct, high resolution morphological demonstration of NGF-nuclear interaction has not been made. It is the purpose of this paper to describe, using both biochemical methods and high resolution electron microscopic autoradiography, the association of ^{125}I-NGF with rat sympathetic neurons grown in primary culture. The distribution of cell surface NGF receptors over the cell body and neurites is described and the internalization and retrograde transport of ^{125}I-NGF is demonstrated under defined conditions. Furthermore, the various organelles involved in the subcellular processing of ^{125}I-NGF within the cell body are characterized. Significantly, no evidence was obtained for an appreciable accumulation of ^{125}I-NGF in the nucleus or on the nuclear membrane of cultured rat sympathetic neurons. Preliminary reports of some of this work have appeared elsewhere (Campenot et al., 1979; Claude et al., 1979; Hawrot et al., 1980).

Materials and Methods

Cell culture. Sympathetic neurons were dissociated mechanically from superior cervical ganglia of neonatal rat pups. The dissociated cells were plated into L15-CO_2 medium and grown as a monolayer on a substratum of either air-dried rat tail collagen or fixed rat heart cells using modified 35-mm culture dishes (Hawrot and Patterson, 1979; Hawrot, 1980). The growth of non-neuronal cells was prevented by treatment with an antimitotic agent, cytosine arabinoside. Under these conditions, rat sympathetic neurons elaborate an extensive axonal network in culture and their neurotransmitter synthetic ability increases dramatically (Mains and Patterson, 1973; Patterson, 1978). These cultured rat sympathetic neurons require NGF for survival and thus all cultures contained a partially purified preparation of 7 S NGF (Varon et al., 1967) added at a concentration of 1 μg/ml. Typically, there were 1,000 to 3,000 neurons per culture dish and these cultures are referred to as mass cultures as opposed to the compartmentalized cultures described below.

Preparation of NGF. 2.5 S NGF was purified from the salivary glands of postpubertal male mice (retired breeders, Charles River) following the procedure of Bocchini and Angeletti (1969) except that a Sephacryl S-200 column was used in place of the Sephadex G-100 column. By SDS-gel electrophoresis, this preparation of NGF was greater than 95% pure. NGF was labeled with ^{125}I using a lactoperoxidase-mediated procedure similar to that of Sutter et al. (1979). However, instead of adding H_2O_2 directly, peroxide was generated within the reaction vessel by the action of glucose oxidase (Hubbard and Cohn, 1972). NGF (40 μg) was incubated with 2 mCi of $Na^{125}I$ (New England Nuclear, NEZ-033L), 6 mM glucose, 0.33 μg of lactoperoxidase (Boehringer Mannheim), 1.2 μg of glucose oxidase (Worthington, 110 units/mg), in 0.15 M sodium phosphate buffer, pH 7.0, in a final volume of 0.07 ml. After 1 to 2 hr at room temperature, an equal volume of 1% acetic acid was added and an amount of buffer A (50 mM sodium acetate buffer, pH 4.0, containing 0.5 M NaCl and 1 mg/ml of bovine serum albumin) was added to make the final volume 0.9 ml. An aliquot was removed for trichloroacetic acid (TCA) precipitation and the determination of specific activity; the remainder was dialyzed overnight against buffer A. Typically, the incorporation of ^{125}I into protein was 50 to 60% efficient and the final specific activity was comparable to other preparations (Sutter et al., 1979) (50 to 75 cpm/pg of NGF). The dialyzed ^{125}I-NGF usually was filtered through a 0.2-μm sterile nitrocellulose filter (Swinnex) or, in early experiments, was filtered through a Centriflo CF 50A (Amicon) filter (Sutter et al., 1979). Greater than 95% of the final ^{125}I-NGF could be precipitated by TCA or by rabbit antiserum to NGF. The iodinated NGF, stored at 4°C, retained full biological activity, as measured by survival of rat sympathetic neurons in culture, for at least 2 weeks. For the autoradiographic experiments described here, ^{125}I-NGF was used within 1 week of iodination.

Incubations. Mass cultures of sympathetic neurons were grown for 10 to 28 d in complete medium containing NGF. Prior to incubation with ^{125}I-NGF, cultures were incubated for 8 hr in medium lacking NGF. During this time, no cell damage was detectable. Cultures then were washed with NGF-free medium and incubated with medium containing ^{125}I-NGF. A concentration of 20 to 30

ng/ml (1×10^{-9} M) usually was used for those cultures chosen for autoradiographic analysis. Nonspecific binding was determined by incubating similar cultures in medium containing the same concentration of ^{125}I-NGF but also a 50- to 100-fold excess of unlabeled NGF. In early experiments, the incubation was terminated by removing the ^{125}I-NGF-containing medium and rapidly washing the cultures three times with 2 ml of NGF-free medium at 24°C. In later experiments, the nonspecific binding could be reduced further without substantially affecting the specific binding by incubating the washed cultures in NGF-free medium for 1 hr at 24°C. Cell-associated radioactivity was determined by dissolving the cultures in 0.1 N NaOH and measuring the radioactivity in a Gamma counter.

Compartmentalized cultures. Dissociated sympathetic neurons were plated onto a collagen substrate in the central well of three-compartment dishes (Campenot, 1977). The neuronal processes grow under the partitions into the outer two compartments, where the medium could be changed independently of the medium bathing the central compartment. These cultures were grown in complete L15-CO_2 medium for about 4 weeks prior to the experimental incubations with ^{125}I-NGF. Usually, the central compartment was maintained in NGF-free medium for the last 2 weeks of culture in order to prevent neuronal processes from looping back into the central compartment from the side chambers. Just prior to incubation with ^{125}I-NGF, the medium in the outer wells was removed and the neuronal processes were incubated in medium lacking NGF for 8 to 12 hr. This medium then was replaced with medium containing ^{125}I-NGF. After the appropriate time at 36°C, the incubation was terminated by flooding each compartment with NGF-free medium, washing each compartment several times, and then either fixing the cultures for autoradiography or disassembling the cultures to determine directly the radioactivity associated with the neuronal cell bodies in the central compartment. The nonspecific association of radioactivity with the cell bodies was determined by incubating the cultures with ^{125}I-NGF and a large (50- to 100-fold) excess of unlabeled NGF. Furthermore, retrograde labeling experiments with horseradish peroxidase indicate that the majority of individual neurons give rise to axons which cross into both outer compartments (R. B. Campenot, unpublished observations).

Processing for autoradiography. Cultures were fixed at 22°C with 2.5% glutaraldehyde in 0.12 M sodium phosphate buffer, pH 7.3, rinsed, and postfixed in 1% OsO_4 for 1 hr at 4°C. The cultures were dehydrated in graded alcohols to 100% ethanol before being embedded in Epon. Autoradiograms were prepared using a flat substrate method (Kopriwa, 1973) and a high resolution nuclear track emulsion, Kodak 129-01 (Salpeter and Szabo, 1976). After 2 months exposure, the autoradiograms were developed in Dektol and stained with 2% uranyl acetate in 100% ethanol and 0.4% aqueous lead citrate.

Autoradiographic analysis. To ensure adequate sampling of each culture, we sectioned blocks from three of four different areas containing cells. Several grids were made from each block. We determined the background level of silver grains on each grid by photographing, at × 5,000, nine or more areas of sections where there was no tissue. Background levels, grains per μm^2, were constant over each individual grid; these values were used where necessary to correct for background in determining real grain counts. From each grid, one or two grid squares were analyzed. In each square, all of the cellular material was photographed, and a montage of micrographs (× 40,000) was constructed.

To analyze the grains associated with the plasma membrane, we counted grains that lay on the membrane or within an area approximately 100 nm outside of the membrane. This distance corresponds to two half-distances. The half-distance is the distance from a line source of radioactivity within which 50% of the grains are predicted to lie (Salpeter et al., 1978).

To analyze the grains lying over the cell body, we centered a circle with a diameter corresponding to 170 nm (50% circle) around each grain and scored all of the organelles that fell within the circle. The 50% circle has a 50% probability of containing the structure responsible for the grain (Salpeter et al., 1978). The fractional areas occupied by different organelles were obtained from the same micrographs on which the grains were scored. A transparent overlay was placed onto the micrograph and the organelles that fell under the intersections of a grid were scored. The fractional area occupied by a class of organelles is the percentage of the total intersections scored that fell on that organelle. The tabulated *labeling density* (L.D.) of an organelle represents the percentage of total grains attributed to that organelle divided by the fractional area of that organelle and is an indication of the relative intensity of labeling. A completely random distribution of label should give a labeling density of 1 for each organelle. Because of the nature of the calculation, a nonrandom distribution sometimes will produce labeling densities less than 1.

Results

Binding of ^{125}I-NGF to cultured sympathetic neurons

Upon examining the binding of ^{125}I-NGF to intact monolayer cultures of rat sympathetic neurons, we found that these neuronal cultures specifically bind NGF with high affinity. Binding studies performed at 25°C (Fig. 1) indicated that there were approximately 2×10^7 NGF receptors per neuron with an apparent affinity constant of 2 to 5×10^{-9} M. Similar results were obtained when binding was carried out at 1°C, a condition in which endocytosis is curtailed and only true surface binding is measured (E. Hawrot, unpublished observations). It should be noted that, in mature sympathetic neuron cultures, the cell body plasma membrane represents approximately 0.2 to 1% of the total cellular plasma membrane; the remainder is due to the extensive neuritic network. The number of NGF receptors per neuron therefore represents a density of approximately 15 to 70 receptors per μm^2 of surface membrane.

The binding of ^{125}I-NGF to neuronal cultures was reduced by 80% when excess unlabeled 2.5 S NGF was added to the incubation. The binding of ^{125}I-NGF was not displaced by either a 3,000-fold excess of insulin or a 120-fold excess of EGF. The specific binding of ^{125}I-NGF

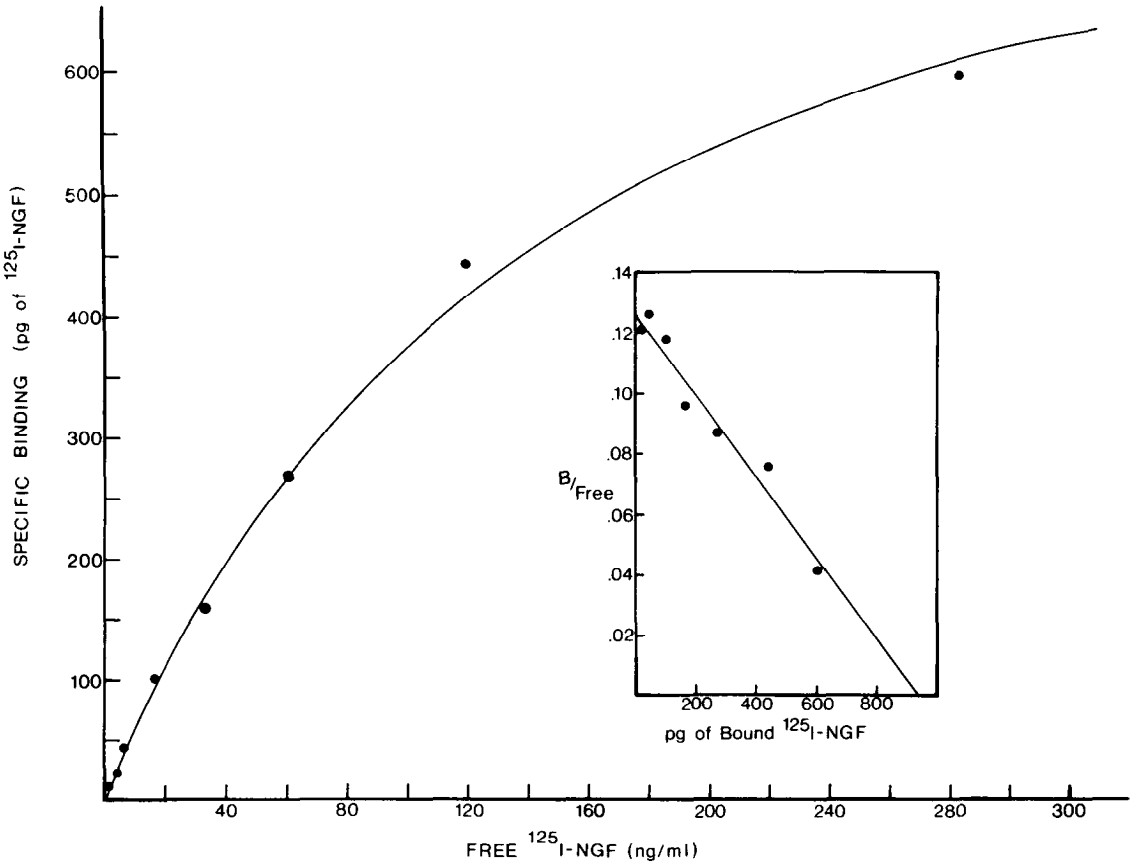

Figure 1. Steady state binding at 25°C of ^{125}I-NGF to neuronal cultures. Sympathetic neuron cultures (36 d in culture; approximately 1,000 neurons per culture dish) were incubated for 140 min with ^{125}I-NGF in L15-air containing 1 mg/ml of bovine serum albumin, 1 mM NaI, and 0.5 mg/ml of gelatin. Cultures were washed and counted as described under "Materials and Methods." The amount of cell-associated radioactivity is given in terms of picograms of ^{125}I-NGF bound per culture dish. Nonspecific binding was determined in the presence of 20 μg/ml of unlabeled NGF and varied linearly with the concentration of ^{125}I-NGF, reaching a level of 32% of total binding at the highest concentration examined. Nonspecific binding was subtracted from the total binding to give the values for specific binding. *B*, bound.

to monolayer cultures of rat heart cells was less than 10% of the binding seen with comparable monolayer neuronal cultures, indicating that ^{125}I-NGF binding shows cell specificity. Cell specificity also could be demonstrated in whole mount autoradiographic studies at the light microscope level. The few non-neuronal, flat, fibroblast-like cells present in neuronal cultures incubated with ^{125}I-NGF at 1×10^{-9} M had no grains associated with them, whereas neurons had grains covering both cell bodies and processes (E. Hawrot, unpublished observations). The grains associated with neuronal elements were specific in that the bulk of these grains were not present when the cultures had been incubated with 1×10^{-9} M ^{125}I-NGF but in the presence of a 100-fold excess of unlabeled NGF. To analyze further the association of ^{125}I-NGF with rat sympathetic neurons, similar cultures were processed for electron microscopic autoradiography.

Electron microscopic autoradiography of mass cultures

Dissociated sympathetic neurons were grown for 10 d in medium containing NGF. After washing out the NGF, the cultures were incubated in 1×10^{-9} M ^{125}I-NGF in normal growth medium with or without a 100-fold excess of unlabeled NGF. After 1 hr at 36°C, the cultures were washed and fixed. Many grains were associated with the loose collagen network forming the culture substratum. The binding to collagen was not reduced substantially by incubation in excess unlabeled NGF. This finding was consistent with direct biochemical determination of ^{125}I-NGF binding to plain, collagen-coated culture dishes. In fact, the bulk of the nonspecific binding seen in these neuronal cultures is attributable to the collagen substratum. Unfortunately, the collagen is required for well attached, intact neuronal cultures that can withstand numerous washings. In scoring grains associated with plasma membranes, we analyzed areas where there was no visible collagen network in sections taken at a level well above the substratum.

The neurites of these cultured neurons are usually extremely slender (less than 1 μm in diameter) and they are often associated in bundles (Fig. 2). Therefore, in order to score the plasma membranes of the neurites for ^{125}I-NGF binding, we initially scored only the plasma membranes of the processes on the outer edge of the bundles ("edge" membranes) in order to reduce any ambiguity about which membrane in the bundle would have been associated with which grain. Using this

method of scoring grains, the density of specific ^{125}I-NGF binding (grains per 100 μm of membrane) to the plasma membrane of the neuritic processes was nearly twice that of the binding to the cell body plasma membrane (Fig. 3). Of the grains associated with neuronal plasma membranes, one-half to two-thirds were specific under the conditions of this experiment. When all of the grains appearing over a bundle of processes were scored and the total length of the plasma membrane was measured ("total" membrane), the number of specific grains per 100 μm of "total" plasma membrane was twice as great as that for the "edge" membrane in the same section (Fig. 3). In this situation, more of the grains appeared to be specific (i.e., displaced by incubation in excess unlabeled NGF). The additional grains associated with "total" membrane probably reflect ^{125}I-NGF internalized during the 1-hr incubation at 36°C and not extracellular trapping of ^{125}I-NGF, since the level of nonspecific label associated with "total" membrane was not significantly greater than that associated with "edge" membranes (Fig. 3).

After 1 hr of incubation in ^{125}I-NGF, the interiors of the cell bodies were barely labeled above background in comparison to the neuritic processes, suggesting that there is not much uptake of ^{125}I-NGF at the level of the cell body. Nevertheless, when the labeling densities for the cellular organelles were analyzed, several of the organelles were labeled well above background. The labeled organelles consisted of lysosomal structures (myelin figures, multivesicular bodies, and degradative vacuoles), small clear vesicles, and some intensely labeled unidentifiable structures (Table I). Of these organelles, the multivesicular bodies had the highest labeling index (L.D. = 21). The nucleus, cytoplasm, and Golgi apparatus were not labeled above background; for example, there was only 1 grain associated with the nucleus. The labeling density for the most highly labeled organelles, as well as the absolute level of labeling, was greatly reduced when the cultures had been incubated in excess unlabeled NGF (data not shown).

Since, in mass neuronal cultures, it is possible that ^{125}I-NGF could enter the cell body either directly or via retrograde transport from the neurites, we examined the distribution of grains in a culture system in which all of the cellular label had been transported from the axons retrogradely.

Retrograde transport of ^{125}I-NGF in compartmentalized cultures

In most compartmentalized cultures, many of the neurons extend axons into both of the side chambers (Campenot, 1977). ^{125}I-NGF can be added to one such side chamber at low concentrations and the appearance of radioactivity into cellular material in the central compartment and into the opposite side chamber can be determined. The time course of the appearance of radioactivity in the two compartments is shown in Figure 4. Radioactivity appears in the central chamber consistent with an axoplasmic transport rate of about 3 mm/hr. After approximately 8 to 10 hr at 36°C, an apparent steady state level of accumulation was attained which was maintained for at least another 24 hr of continued incubation. Incubating cultures with 20 μg/ml of colchicine blocked the appearance of radioactivity in the cen-

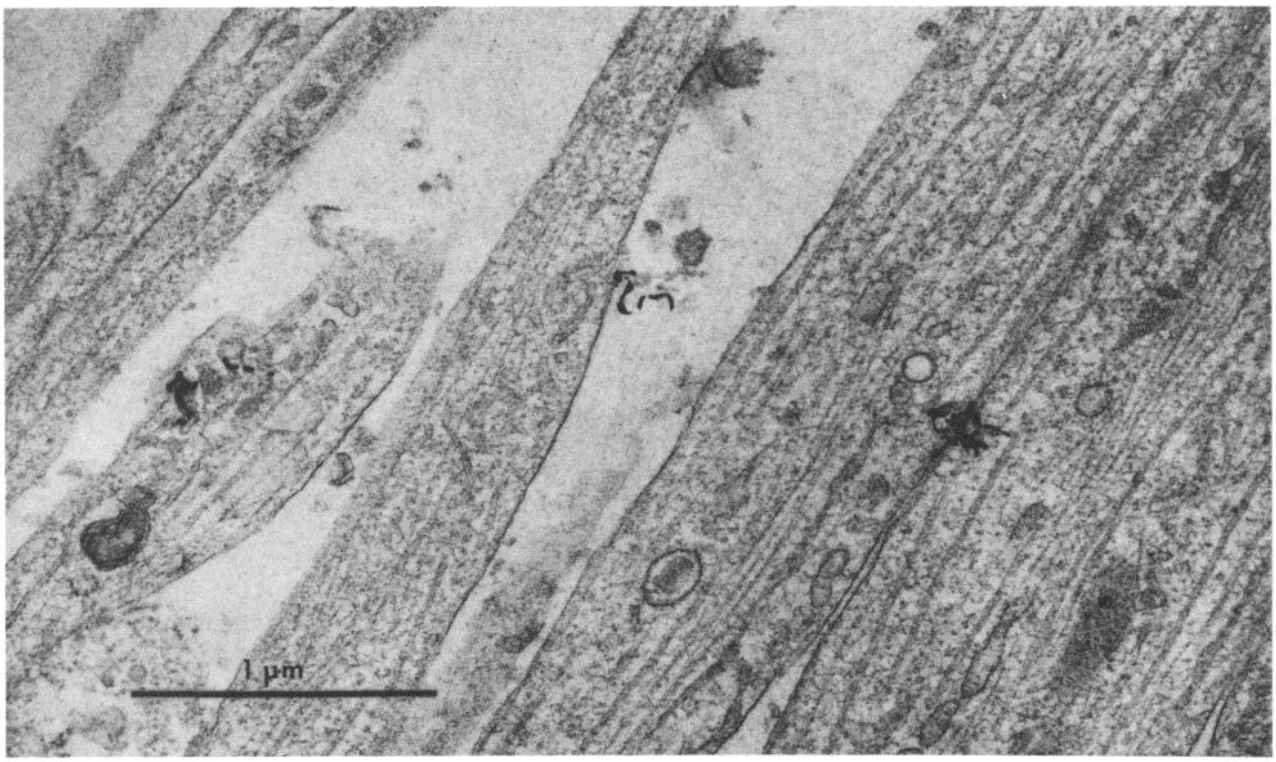

Figure 2. Surface binding along neuronal processes. Electron microscopic autoradiogram of a bundle of processes from a 10-d culture of sympathetic neurons. Micrographs similar to this one were used to calculate the amount of surface binding. Magnification × 39,000.

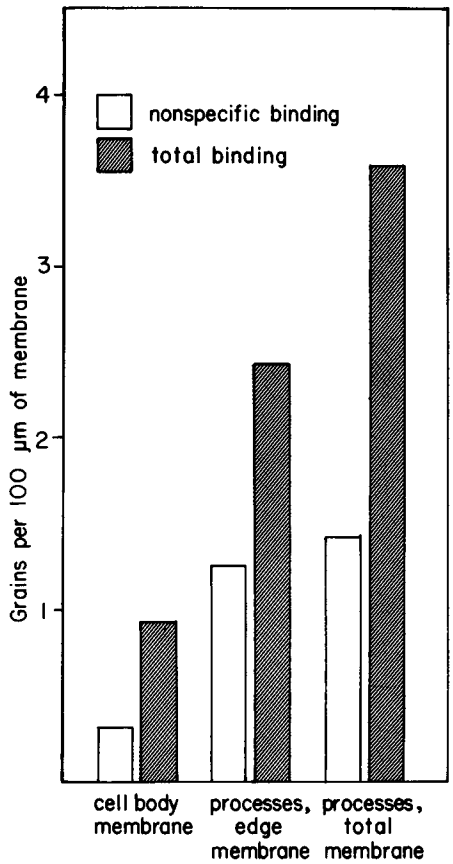

Figure 3. Surface binding on sympathetic neurons. Mass cultures were exposed for 1 hr to 30 ng/ml of ^{125}I-NGF (total binding) or to 30 ng/ml of ^{125}I-NGF in the presence of a 100-fold excess of native NGF (nonspecific binding). To calculate the grain density for cell body membrane or "edge" membrane by electron microscopic autoradiography, only grains lying on or directly outside of the cell membrane (within 100 nm) were counted. When processes were organized in tightly packed fascicles, the value for "edge" membrane was derived by counting the grains associated with the cell membranes at the outside edge of the fascicles and relating that figure to the amount of such membrane. To derive the value for "total" membrane, all of the grains overlying the fascicle were related to the total amount of membrane in the fascicle. These data have been corrected for autoradiographic background.

tral chamber which supports the notion that label arrives at the cell bodies via an axoplasmic transport system.

Although there is some leakage of ^{125}I-NGF across the sealed chamber divider, the cell body-associated radioactivity is not due to leakage of ^{125}I-NGF and direct uptake by the cell bodies, since the addition to the cell body-containing central compartment of a large excess of unlabeled NGF has no effect at all on the appearance of label in the cell bodies. In addition, after about 8 to 10 hr of continued incubation, increased levels of radioactivity begin to appear in the medium bathing the cell bodies. Incubating cultures with colchicine prevents the appearance of additional radioactivity in the central compartment. The radioactive material in the medium most probably represents labeled NGF that has been taken up, retrogradely transported, metabolized, and then released. The characteristics of the released material have not been determined.

The appearance of radioactivity in the neuronal cell bodies is due to a specific interaction with saturable receptors. Incubating axonal endings with ^{125}I-NGF in the presence of excess unlabeled NGF reduced the amount of cell body-associated radioactivity by >90% (Fig. 4). Thus, ^{125}I-NGF uptake is not due to bulk liquid pinocytosis but presumably represents receptor-mediated endocytosis. In addition, excess insulin or EGF had no effect on the retrograde transport of ^{125}I-NGF (data not shown). Analysis of the labeled material accumulated in neuronal cell bodies after 8 hr of incubation indicates that a large fraction (50 to 70%) of the label is TCA precipitable and migrates upon SDS-gel electrophoresis as one band with the appropriate molecular weight of authentic NGF. Furthermore, although the ^{125}I-NGF appeared in the cell bodies within a few hours, none of this radioactivity migrated further from the cell bodies, in the orthograde direction, into distal axons located in the opposite side chamber (Fig. 4) even after 34 hr of continuous incubation. The retrograde transport of ^{125}I-NGF could be detected using concentrations of ^{125}I-NGF as low as 0.5 ng/ml (2×10^{-11} M) and, upon measuring the amount of label accumulated in cell bodies after 8 hr,

TABLE I

Labeled organelles in the cell body after incubation of mass cultures with ^{125}I-NGF for 1 hr

Organelle	Fractional Area	^{125}I-NGF	
		Percent Grains[a]	Labeling Density[b]
Nucleus	7.4	0.9	0.1
Cytoplasm	59.6	28.6	0.5
Rough endoplasmic reticulum	6.0	3.7	0.6
Smooth endoplasmic reticulum	5.3	9.2	1.7
Golgi apparatus	2.2	0.9	0.4
Vesicles (dense core)	0.09	0.9	10.2
Vesicles (small, clear)	1.1	5.5	5.0
Mitochondria	10.2	8.3	0.81
Lysosomes	4.47	13.8[c]	3.1
Multivesicular bodies	0.35	7.4[c]	21.1
Cell membrane	2.9	17.6	6.1
X[d]	0.6	3.7[c]	6.1[d]

[a] Total number of grains = 108.

[b] Labeling density (L.D.) for each organelle is obtained by dividing the percentage of grains assigned to that organelle by the fractional area of that organelle. In a situation where label is concentrated within one class of organelles and that organelle is assigned a large fraction of the grains, other organelles will have a decreased fraction of the total grains as compared to a totally random distribution. In some cases, therefore, organelles will have a L.D. of less than 1.

[c] Included many clumps of grains: each aggregate was counted as 1 grain, and thus, the labeling density is a minimal estimate.

[d] Unidentifiable profiles; some were unidentifiable because clumps of grains obscured the underlying structure.

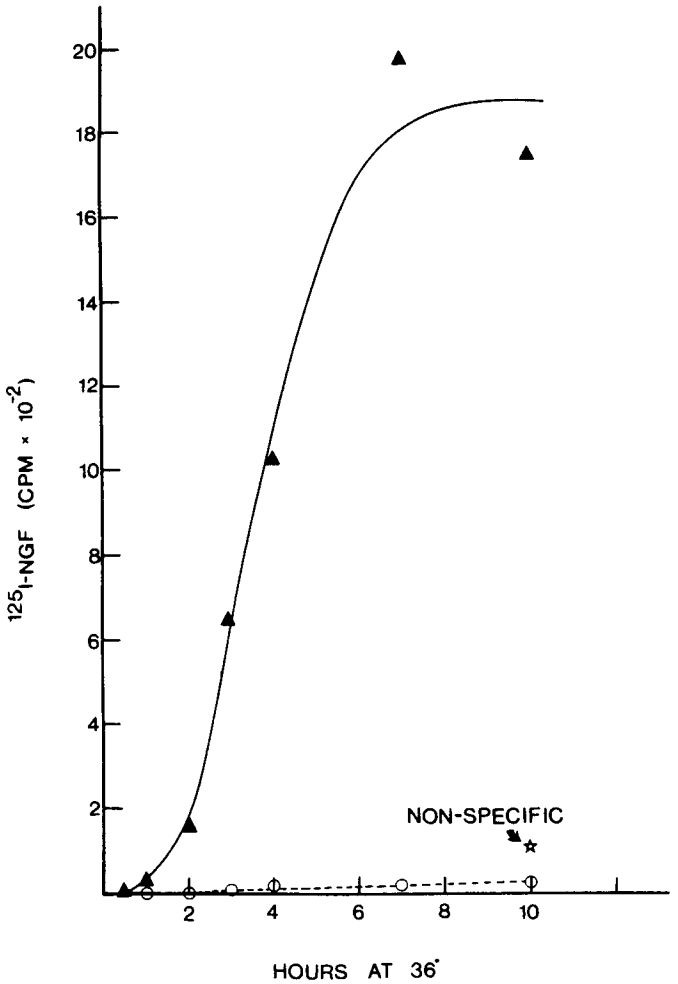

Figure 4. Time course of retrograde transport at 36°C. ^{125}I-NGF (50 ng/ml) was added to one side chamber of compartmentalized sympathetic neuron cultures. At the times indicated, the cell bodies in the central compartment (▲) and the nerve endings in the opposite chamber (○) were harvested and the radioactivity was measured. Cultures incubated with ^{125}I-NGF together with excess unlabeled NGF had a greatly reduced level of cell body-associated radioactivity (*star*).

appeared to saturate at a concentration of about 100 ng/ml (4×10^{-9} M). The nonspecific accumulation in the presence of excess unlabeled NGF varied linearly with the concentration of ^{125}I-NGF (data not shown). At the higher concentrations of ^{125}I-NGF, the leakage across the barrier becomes appreciable with respect to the retrograde transport and precludes an accurate determination of the saturation point.

Electron microscope autoradiography of retrogradely accumulated ^{125}I-NGF

^{125}I-NGF, at 2×10^{-9} M, was added to one side chamber of a compartmentalized culture and incubated for 8 hr at 36°C. At this point, the culture was rinsed, fixed, and processed for electron microscope autoradiography. Biochemical analysis of sister cultures, incubated in the same manner, indicated that 90% of the label in the central compartment was specific, i.e., was displaced if excess unlabeled NGF was added to the side chamber containing the ^{125}I-NGF.

High resolution autoradiography demonstrated many grains localized over the cell bodies (Fig. 5), allowing a quantitative analysis of the subcellular distribution of label. The results from such an analysis are listed in Table II, and the labeling densities of the various organelles, related to the percentage of total grains attributable to each organelle, are depicted in Figure 6. The labeling analysis indicates that the highest concentration of grains appeared over lysosomal organelles, including lysosomes, secondary lysosomes, residual bodies, and multivesicular bodies. As in the case of mass cultures (Table I), the most highly labeled organelles are the multivesicular bodies (Table II), with a labeling density of about 17. The total number of grains associated with the multivesicular bodies (7%) was less than in the other lysosomal organelles, but multivesicular bodies occupied only 0.4% of the cell body cross-section. Dense core vesicles and small (50-nm-diameter), clear vesicles were marginally, but persistently, labeled. In contrast, we found no evidence that any nuclear structures were differentially labeled; this included measurements of the nuclear membrane (Table II). Overall, the nuclei accounted for 9.4% of the grains, with a labeling density of 0.47. This value is probably not significantly different from the nuclear labeling density of 0.12 seen in mass cultures after 1 hr, since the latter figure was the result of only 1 measured grain and, thus, subject to significant variation (Table I). It is clear that grains were not accumulated over the cell nucleus (Fig. 5). In addition, there were very few coated vesicles in the cell bodies, and no grains were associated with the few coated vesicles that were analyzed.

Discussion

The apparent affinity constant determined for primary cultures of rat sympathetic neurons ($K_D = 2$ to 5×10^{-9} M) correlates well with previous studies on the survival and growth requirements of rat sympathetic neurons allowed to develop in culture (Chun and Patterson, 1977a, b, c) and the cell surface binding characteristics of rat PC12 cells (Herrup and Thoenen, 1979; Calissano and Shelanski, 1980). Rat sympathetic neurons require 100 ng/ml of NGF (4×10^{-9} M) for optimal survival and growth, while PC12 cells bind NGF with an affinity of 3×10^{-9} M. On the other hand, embryonic chick sensory and sympathetic nerve cells exhibit two classes of NGF receptor (Sutter et al., 1979; Olender and Stach, 1980) differentiated by a 50- to 100-fold difference in affinity constants. The higher affinity receptors are less abundant but exhibit tighter binding in that dissociation is much less rapid ($t_{1/2} \cong 10$ min) than with the lower affinity receptors. ^{125}I-NGF bound to cultured rat sympathetic neurons shows a similar slow rate of dissociation (E. Hawrot, unpublished observations) and, in this respect, the observed binding to mature rat neurons, although of lower affinity, is similar to the high affinity receptors of chick nerve cells. With the limited amount of cellular material in the primary rat sympathetic cultures, it would be difficult to detect reliably another class of higher affinity receptors if such receptors constituted less than

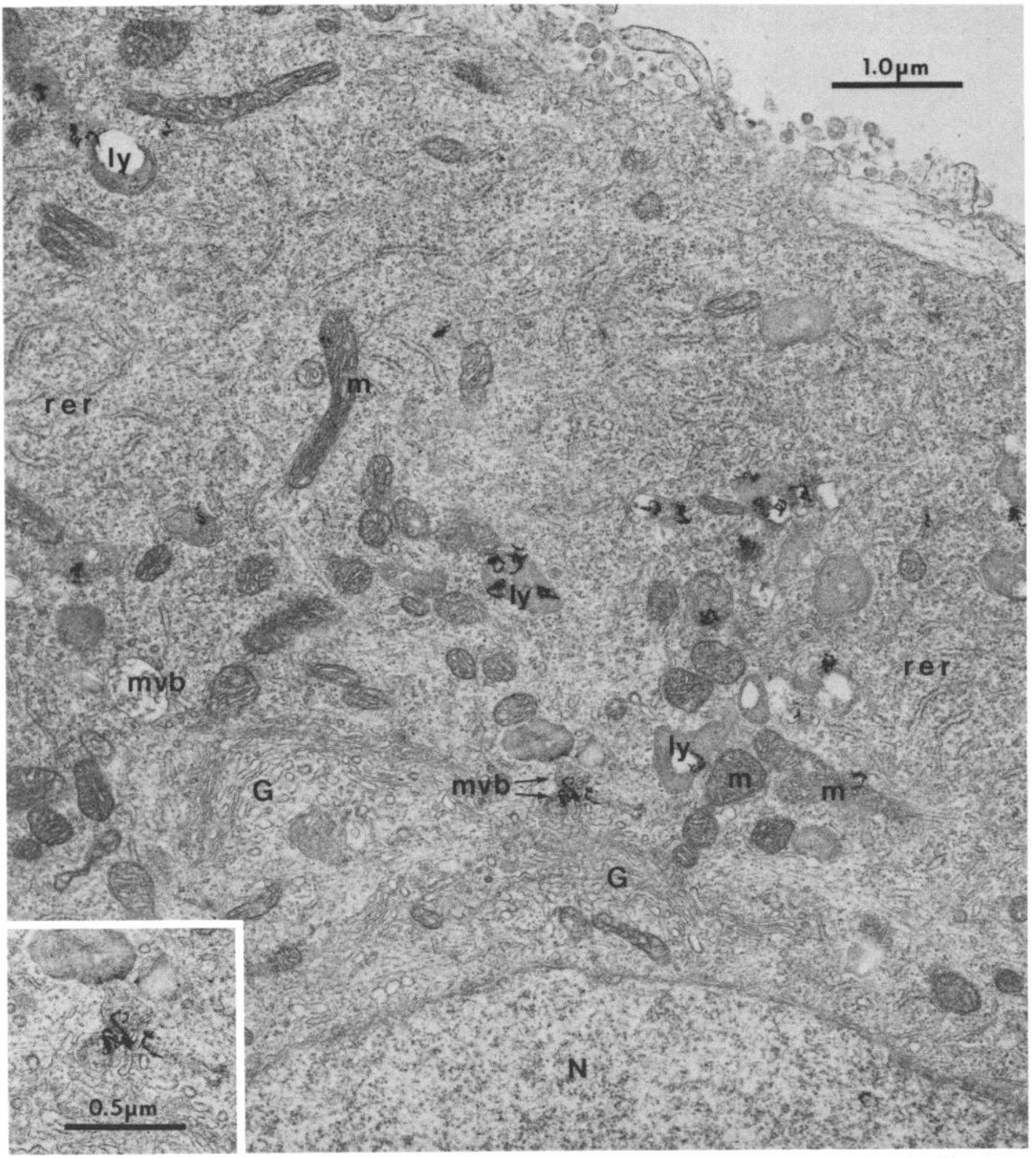

Figure 5. Cell body labeling after retrograde transport. Electron microscopic autoradiogram of a cell body following 8 hr of retrograde transport of ^{125}I-NGF from the peripheral processes (see text). *G*, Golgi apparatus; *ly*, lysosome; *m*, mitochondrion; *mvb*, multivesicular body; *N*, nucleus; *rer*, rough endoplasmic reticulum. Magnification × 21,000. *Inset*, Detail of multivesicular body. Magnification × 38,000.

10% of the major class of receptors; thus, we cannot rule out the presence of a minor class of additional binding sites of higher affinity. Because of the dissociation characteristics of the major binding sites and the correlation with the biological dose-response curve, we feel that the observed binding sites most probably mediate the long term biological effects of NGF in our system.

The distribution of grains over sections of mass cultures that had been incubated with ^{125}I-NGF for 1 hr indicated that there were twice as many binding sites per unit area of neuritic plasma membrane as there were per unit area of cell body plasma membrane (Fig. 3). Since the incubation was carried out at 36°C and with a concentration of NGF well within the biologically effective range, this distribution of receptors would appear to reflect the localization of binding sites under normal

growth-maintaining conditions. Studies using lectin conjugates as cell surface probes of cultured sympathetic neurons also have suggested a difference in the cell body versus neurite density of various lectin binding sites (Schwab and Landis, 1981). The present study is consistent with the binding and uptake of a fluorescent conjugate of NGF (Levi et al., 1980) and the distribution of immunoperoxidase staining over cultured chick and mouse sympathetic neurons (Kim et al., 1979). In the latter study, the staining product was distributed evenly along perikarya, fibers, and growth cones, but a 2-fold variation in staining intensity would be difficult to discriminate with immunochemistry. We have not yet quantitated the density of grains over growth cones, primarily because of the low number of grains and the relative scarcity of these structures in thin sections.

After 1 hr, the mass cultures appeared to have internalized considerable ^{125}I-NGF, since bundles of axons had twice as many grains over them as would be expected from surface binding alone (Fig. 3). This finding is consistent with temperature shift experiments, indicating that, after 2 hr at 37°C, most of the ^{125}I-NGF previously bound to the surface of sympathetic neurons at 1°C had become internalized as determined by morphological measurements (E. Hawrot and P. Claude, unpublished observations).

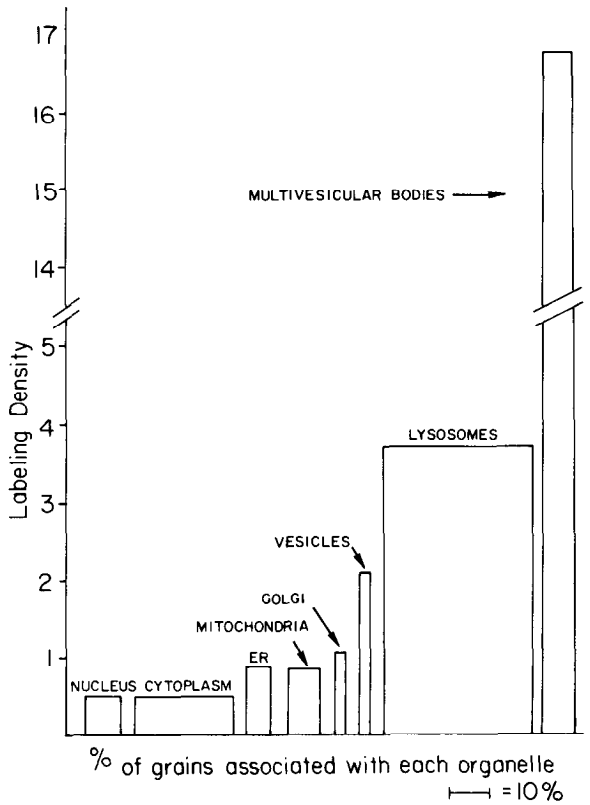

Figure 6. Subcellular distribution after retrograde transport. The extent of labeling of intracellular compartments was determined following 8 hr of retrograde transport of ^{125}I-NGF from the peripheral processes. The *height of each bar* in the histogram indicates the labeling density (percentage of total grains divided by percentage of total area) of each organelle. The *width of each bar* indicates the proportion of total grains associated with each organelle. The greatest number of grains are associated with the lysosomes, while the multivesicular bodies have the highest labeling density. These data have not been corrected for autoradiographic background.

TABLE II

Labeled organelles in the cell body after 8 hr retrograde transport of ^{125}I-NGF

The major portion of the grains analyzed in this experiment were due to specific uptake and transport of ^{125}I-NGF. In parallel cultures, the specific label in the central well containing the cell bodies amounted to 9,000 ± 698 cpm per culture, while the nonspecific label was 895 ± 171 cpm per culture.

Organelle	Fractional Area	Percent Grains[a]	Labeling Density[b]
Nucleoplasm	19.0	8.6	0.4
Nucleolus	0.6	0.5	0.8
Nuclear membrane	0.2	0.3	1.5
Cytoplasm	49.7	24.5	0.5
Rough endoplasmic reticulum	3.9	3.2	0.8
Smooth endoplasmic reticulum	3.5	3.0	0.8
Golgi apparatus	2.3	2.4	1.0
Vesicles (dense core)	0.2	0.8	4.0
Vesicles (small, clear)	1.0	1.8	1.8
Mitochondria	9.7	8.4	0.86
Lysosomes	10.0	37.8[c]	3.8
Multivesicular bodies	0.4	6.7[c]	16.8
X[d]	0.2	1.2[c]	6.0[d]

[a] Total number of grains = 592.

[b] Calculated as in Table I.

[c] Included many clumps of grains; each aggregate was counted as 1 grain, producing an underestimate of the labeling density.

[d] Unidentifiable profiles; some were unidentifiable because clumps of grains obscured the underlying structure.

Specific, high affinity retrograde transport of ^{125}I-NGF could be readily demonstrated in compartmentalized cultures (Fig. 4) and the results were consistent with *in vivo* studies of retrograde transport from the adult rat anterior chamber of the eye (Hendry et al., 1974; Paravicini et al., 1975; Stoeckel and Thoenen, 1975; Schwab, 1977; Schwab and Thoenen, 1977; Johnson et al., 1978). The advantage of the culture system is that the concentration of ^{125}I-NGF, or other additions, can be carefully controlled and continuously maintained. The retrograde transport of ^{125}I-NGF into the cell bodies and no further in the orthograde direction into distal axons (Fig. 4) is consistent with the demonstrated local requirement of neurites for NGF (Campenot, 1977) and the report that, *in vivo*, ^{125}I-NGF is transported retrogradely by embryonic sensory neurons to the dorsal root ganglion but no further (Brunso-Bechtold and Hamburger, 1979).

As has been observed with several other internalized macromolecules (Bergeron et al., 1979; Gorden et al., 1978; McKanna et al., 1979), we found internalized NGF primarily in membrane-limited organelles. After 8 hr of retrograde transport, the distribution of grains over organelles in the cell body was quite similar to that seen

after 1 hr in mass culture, although the total accumulation of label was greater. Furthermore, in preliminary examinations of cultures that had retrogradely transported ^{125}I-NGF for 5 or 24 hr, similar grain distributions were seen (P. Claude, E. Hawrot, D. A. Dunis, and R. B. Campenot, unpublished observations). Thus, the 8-hr experiment appears to represent the steady state distribution of ^{125}I-NGF in the cell body. We found NGF particularly concentrated in secondary lysosomes and multivesicular bodies. This observation is consistent with two recent studies demonstrating that the majority of organelles moving toward neuronal cell bodies via retrograde axonal transport are membranous structures resembling lysosomes or residual bodies (Smith, 1980; Tsukita and Ishikawa, 1980). A similar intracellular distribution of labeled hormone has been observed with ^{125}I-insulin internalized by rat hepatocytes (Carpentier et al., 1979) and EGF internalized by A-431 human epithelioid carcinoma cells (McKanna et al., 1979). It is presently unclear whether the association of polypeptide hormones with multivesicular bodies and lysosomes is simply due to the degradation of the hormone, and presumably also the hormone receptor (Carpenter and Cohen, 1979), or whether lysosomal structures are involved in mediating the intracellular effects of the hormones (Szego, 1974; Pastan and Willingham, 1981). On the other hand, electron microscopic autoradiography of human lymphocytes incubated with ^{125}I-insulin (Goldfine et al., 1978) indicated that some of the internalized insulin was accumulated on the nuclear membrane and in the endoplasmic reticulum rather than in lysosomal structures. EGF also has been suggested to accumulate in the nucleus of GH-3 cells (Johnson et al., 1980). The differences in these observations may be related to the different cell types used for various studies.

A number of biochemical and light microscopic studies have been interpreted to suggest either that NGF becomes associated with perinuclear and intranuclear structures (Yankner and Shooter, 1979; Marchisio et al., 1980, 1981) or that NGF can bind to nuclear receptors (Andres et al., 1977; Yankner and Shooter, 1979). None of these studies included a high resolution analysis of the morphological distribution of labeled NGF in intact cells. Using electron microscopic autoradiography, we were able to quantitate the distribution of specific marker over the various organelles and we found no evidence for a significant accumulation of label over the nucleus or the nuclear membrane (Table II). In fact, there were only 2 grains out of a total of 592 that were associated with the nuclear membrane (Table II). The lack of significant nuclear labeling was observed consistently whether the cultures were incubated for 1, 5, 8, or 24 hr in ^{125}I-NGF (P. Claude, E. Hawrot, D. A. Dunis, and R. B. Campenot, unpublished observations). Although after 8 hr of retrograde transport, 9% of the grains were associated with the nucleus, this level of labeling did not represent a marked increase over background and mitochondria accounted for a similar percentage of grains (Table II). If the radioactivity in the nucleus or the nuclear membrane had been even 5 times more concentrated, it would have been easily demonstrable, since we were able to detect accumulated label in the lysosomes and the multivesicular bodies and since other workers were able to localize ^{125}I-insulin to the nuclear membrane of human lymphocytes using a similar technique (Goldfine et al., 1978).

Our findings contrast markedly with the report that, in PC12 cells, 63% of the total cell-bound NGF was in the nucleus after 17 hr incubation (Yankner and Shooter, 1979). In the PC12 study, nuclei were isolated after Triton X-100 disruption of plasma membranes. In the case of insulin, it has been suggested that the binding of labeled ligand to isolated nuclei can be misleading, since contaminating membrane fragments often are removed incompletely from nuclear fractions (Sikstrom et al., 1978). Without direct morphological analysis of labeled hormone in intact cells, it is difficult to rule out the possibility of contamination of subcellular fractions.

The lack of significant nuclear labeling in the present study does not rule out the possibility that some minor fraction of the internalized NGF migrates to the nucleus and that this NGF is important for long term biological effects. Our results do, however, clearly show that, in cultured rat sympathetic neurons dependent on NGF for survival and development, ^{125}I-NGF binds specifically to the plasma membrane surface, is internalized and retrogradely transported, and accumulates in the cell body. Label does not, however, appear to accumulate at any point on the nuclear membrane or in the nucleus. If NGF does act directly on the nucleus through a specific nuclear receptor, this action is at a much more subtle level than can be detected by looking at the total distribution of labeled NGF within responsive neurons.

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