

Inhibitors of Protein Synthesis and RNA Synthesis Prevent Neuronal Death Caused by Nerve Growth Factor Deprivation

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Abstract. We have developed an experimental paradigm to study the mechanism by which nerve growth factor (NGF) allows the survival of sympathetic neurons. Dissociated sympathetic neurons from embryonic day-21 rats were grown in vitro for 7 d in the presence of NGF. Neurons were then deprived of trophic support by adding anti-NGF antiserum, causing them to die between 24 and 48 h later. Ultrastructural changes included disruption of neurites, followed by cell body changes characterized by an accumulation of lipid droplets, changes in the nuclear membrane, and dilation of the rough endoplasmic reticulum. No primary alterations of mitochondria or lysosomes were observed. The death of NGF-deprived neurons was characterized biochemically by assessing [³⁵S]methionine incorporation into TCA precipitable protein and by measuring the release of the cytosolic enzyme

adenylate kinase into the culture medium. Methionine incorporation began to decrease ~18 h post-deprivation and was maximally depressed by 36 h. Adenylate kinase began to appear in the culture medium ~30 h after deprivation, reaching a maximum by 54 h. The death of NGF-deprived neurons was entirely prevented by inhibiting protein or RNA synthesis. Cycloheximide, puromycin, anisomycin, actinomycin-D, and dichlorobenzimidazole riboside all prevented neuronal death subsequent to NGF deprivation as assessed by the above morphologic and biochemical criteria. The fact that sympathetic neurons must synthesize protein and RNA to die when deprived of NGF indicates that NGF, and presumably other neurotrophic factors, maintains neuronal survival by suppressing an endogenous, active death program.

THERE are many examples of natural cell death which occur during normal vertebrate development (Glücksmann, 1951). Cell death plays an essential role in shaping and refining many tissues during ontogeny as well as in the adult state. Natural cell death is particularly common in the development of the nervous system (Hamburger and Levi-Montalcini, 1949; Oppenheim, 1981), where an average of about half of all neurons produced during embryogenesis normally die before adulthood (Cowan et al., 1984; Oppenheim, 1985). Natural cell death in the developing nervous system provides a mechanism whereby a neuronal target may determine the extent of its innervation. It is generally believed that neurons depend on, and compete for, neurotrophic factors released in limited amounts by their targets (Hamburger and Oppenheim, 1982). Those which acquire sufficient neurotrophic factor survive, while neurons deprived of adequate trophic support die.

Nerve growth factor (NGF)¹ is the best characterized

neurotrophic factor (for reviews see Greene and Shooter, 1980; Thoenen and Barde, 1980). NGF is a polypeptide synthesized and released continually in minute quantities by the targets of sympathetic and neural crest-derived sensory neurons. It binds to specific receptors on neuronal processes, whereupon both NGF (Hendry et al., 1974) and its receptor (Johnson et al., 1987) are transported retrogradely to the cell body. The physiologic significance of NGF in determining neuronal survival has been demonstrated by experiments in which NGF is either supplemented or removed in vivo. Exogenously administered NGF decreases cell death during development (Hendry and Campbell, 1976; Hamburger et al., 1981) or after axotomy (Hendry and Campbell, 1976; Yip et al., 1984). Conversely, greater numbers of sympathetic and sensory neurons die if endogenous NGF is removed by administering antisera to the factor (Levi-Montalcini and Booker, 1960) or by making the animal autoimmune to NGF (Johnson et al., 1980). The mechanism by which NGF allows the survival of dependent neurons is poorly understood (Bothwell, 1982; Yanker and Shooter, 1982; Bradshaw et al., 1985).

Two general schemes might account for the death of neurons when trophic support becomes insufficient. One possibility is that neurons require trophic factor for sustained

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1. *Abbreviations used in this paper:* AK, adenylate kinase; NGF, nerve growth factor.

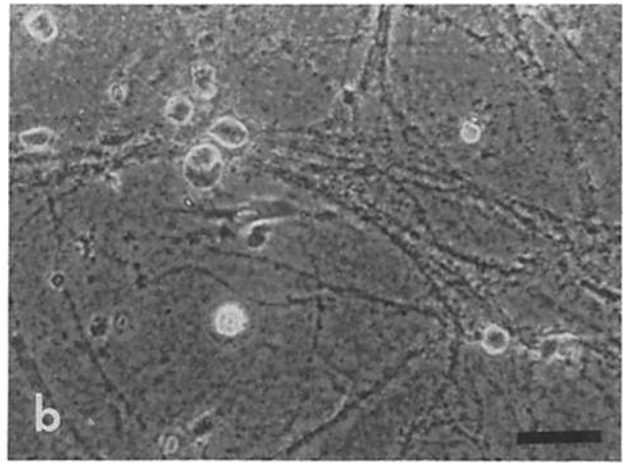
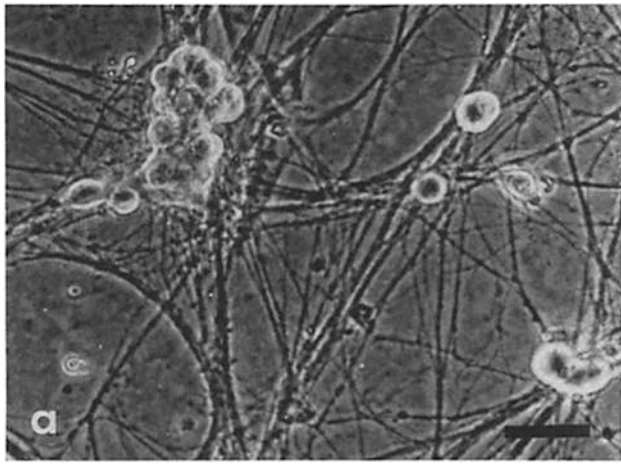


Figure 1. Dissociated sympathetic neurons in vitro. Both *a* and *b* were grown in the presence of NGF for 1 w followed by 2 d in the continued presence (*a*) or absence (*b*) of NGF. The NGF-deprived neurons have died. Bar, 50 μ m.

metabolic activity, i.e., trophic factors “support life.” This hypothesis predicts that neurons would atrophy and degenerate passively when deprived of trophic factor. Alternatively, death caused by trophic factor deprivation may be a metabolically active process, i.e., the role of trophic factors may be to repress an active suicide response and thereby “suppress death.” In the present study we have addressed this question with an in vitro model of NGF deprivation. Cultures of sympathetic neurons were established in the presence of NGF and then acutely deprived of trophic support. This experimental paradigm mimics the physiologic situation encountered by neurons during development or after axotomy, when trophic support becomes insufficient and neurons die.

The mechanism of neuronal death after NGF deprivation was investigated by inhibiting various classes of biosynthetic reactions. We found that the death of NGF-deprived sympathetic neurons in our system could be prevented entirely with inhibitors of protein or RNA synthesis. If NGF supported “life,” one would have expected these drugs to hasten neuronal death since the inhibition of RNA and protein synthesis in itself is ultimately lethal. Because these inhibitors actually prevented death, these results indicate that trophic support of sympathetic neurons suppresses an active suicide response which requires the continued synthesis of RNA and protein to mediate cell death.

A preliminary report of this work has appeared in abstract form (Martin et al., 1987).

Materials and Methods

Cell Culture

Primary dissociated cultures of sympathetic neurons were prepared from the superior cervical ganglia of embryonic-day-21 rats by the method of Johnson and Argiro (1983) as modified by DiStefano et al. (1985). Cells

were typically plated on collagen at a density of $\sim 20,000$ cells/well (0.75 ganglia/well) in 24-well tissue culture plates (Costar Data Packaging Corp., Cambridge, MA). Cultures were grown for 7 d in culture medium (0.6 ml/well) which consisted of 90% Eagle's minimal essential medium (Gibco, Grand Island, NY), 10% FCS (Armor Biochemicals, Kankakee, IL), 20 μ M fluorodeoxyuridine and 20 μ M uridine to kill nonneuronal cells, and 50 ng/ml 2.5S NGF (prepared by the method of Bocchini and Angeletti, 1969).

Phase-Contrast Micrographs

Micrographs of neurons in vitro were taken with a Nikon Diaphot microscope equipped with a 20 \times phase-contrast objective. A Nikon F-2 35 mm camera was fitted to this scope and micrographs were taken while using a green interference filter and Kodak Tri-X film, ASA 400.

Electron Micrographs

Neurons were plated on collagen-coated 15-mm Aclar (33C, 5-mil; Applied Chemical, Morristown, NJ) mini-dishes and cultured as above. Upon completion of the experiment, cultures were fixed for 4 h in 3% glutaraldehyde in 100 mM phosphate buffer, pH 7.3, containing 0.45 mM Ca^{++} . Subsequently, cultures were postfixed in buffered OsO_4 , dehydrated in graded alcohols, and embedded in Spurr's medium. Thin sections were cut parallel to the bottom of the culture dish and stained with uranyl acetate and lead citrate. Sections were examined with a Philips 200 electron microscope.

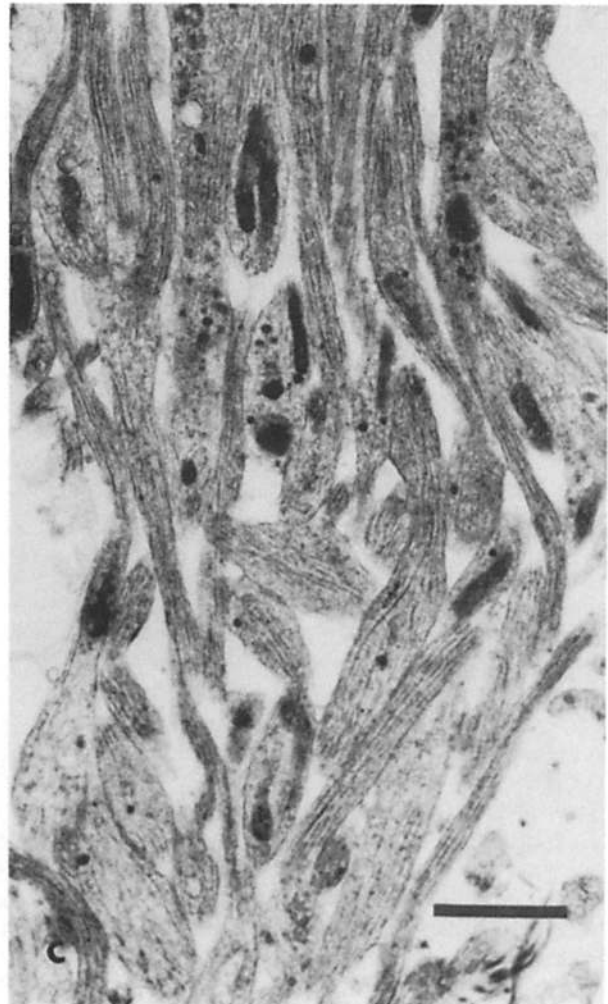
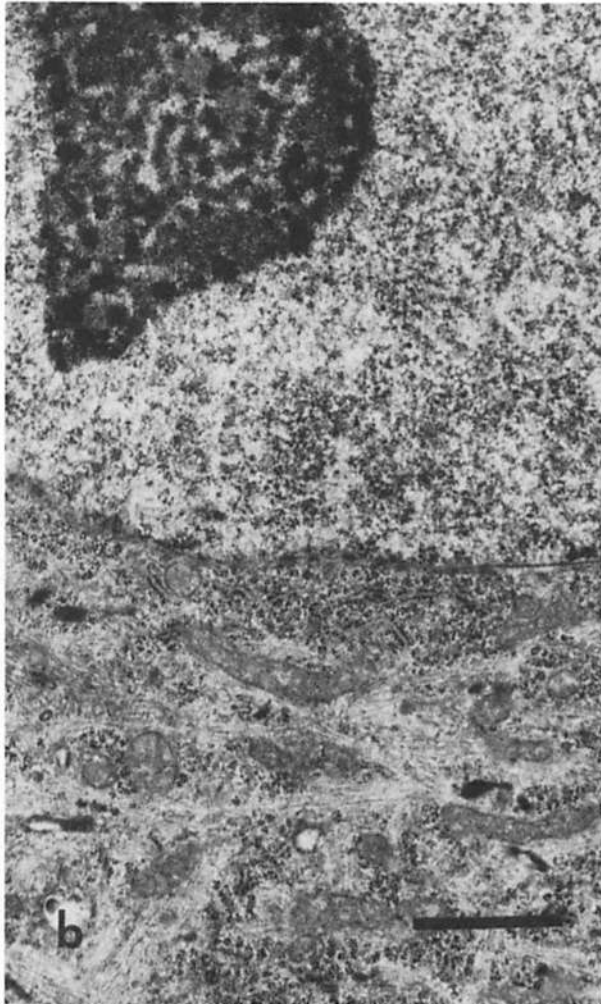
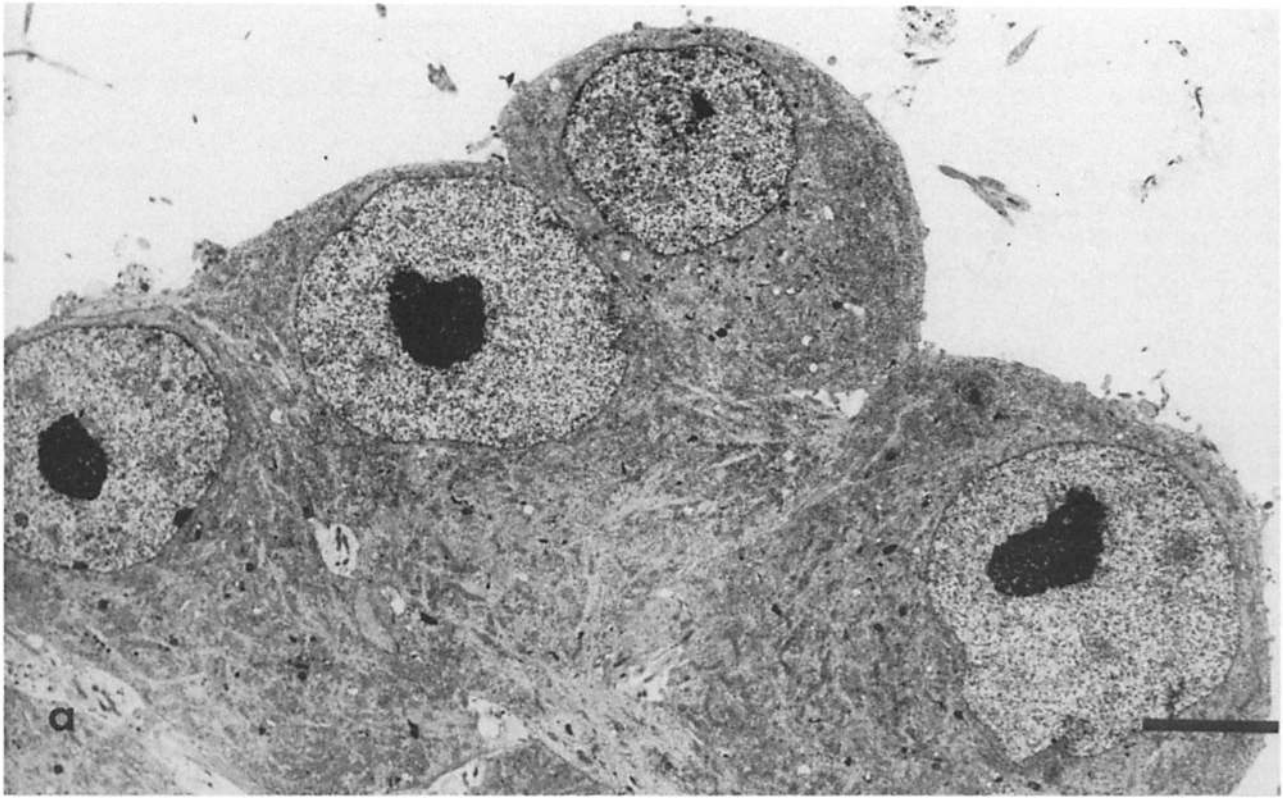
Methionine Incorporation Assay

Each culture well received 33 μ Ci/ml [^{35}S]methionine (New England Nuclear, Boston, MA) in normal medium for 10–20 h. TCA precipitable counts were assessed by the method of DiStefano et al. (1985). Data were corrected by subtracting the counts nonspecifically bound to collagen-coated wells lacking cells and were expressed as percentages relative to [^{35}S]methionine incorporated by control (untreated) neurons on the same plate.

Adenylate Kinase Assay

As cell membranes become disrupted, enzymes which are normally retained intracellularly leach into the culture medium. For example, lactate dehydrogenase has commonly been measured in culture medium as an indicator of cell death. Enzymes will differ in their use as indicators of plasma membrane disruption in different culture systems because they are present

Figure 2. Ultrastructural appearance of dissociated rat superior cervical ganglia neurons maintained in tissue culture for 1 w in the continuous presence of NGF. (*a*) Cultured neurons, often aggregated in small clusters, contained large spherical nuclei with delicate chromatin patterns and single prominent nucleoli, surrounded by large amounts of dense cytoplasm. (*b*) At higher magnification the perikaryal cytoplasm was rich in rough endoplasmic reticulum and polysomes, and contained scattered mitochondria and dense bodies. The nucleoplasm was dispersed with little heterochromatin and surrounded a nucleolus which had a classical granular and fibrous substructure. (*c*) Neurons were typically enmeshed in a feltwork of large numbers of neurites. Bars (*a*) 5 μ m; (*b* and *c*) 1 μ m.



in differing amounts in various cells and in the serum which is normally included in their culture medium. We, therefore, sought an enzymatic activity that was present at low levels in the normal culture medium, but was released in large amounts by neurons as their plasma membranes ruptured.

Four easily measured cytoplasmic enzymes of high cellular activity were tested as markers of cell damage sufficient to cause significant release: lactate dehydrogenase, glucosephosphate isomerase, creatine kinase, and adenylate kinase (AK)(EC 2.7.4.3). The last was chosen because the ratio between the activity released and the activity of the culture medium (because of its serum content) was by far the most favorable. For example, although the glucosephosphate isomerase activity of the cultured neurons was twice that of AK, and the activity released by NGF deprivation was fourfold greater, the baseline isomerase activity in the culture medium alone was 100 times that of AK.

Samples were prepared for the AK assay as follows. Medium was drawn off the cells in a given well and set aside (the "initial sample") for AK assay (see below). This medium was replaced with an equal volume of medium containing 0.1% Triton X-100 which entirely disrupted any intact plasma membrane. After 30 min the Triton medium was removed. Samples could be frozen at -70°C until the time of assay without loss of activity.

A 50 μl aliquot of the sample (corresponding to ~ 1700 cells) was added to 1 ml of the assay reagent in a 10×75 -mm fluorometer tube. The reagent consisted of 50 mM imidazole-HCl buffer, pH 7.0, containing 0.5 mM ADP, 1 mM glucose, 2 mM MgCl_2 , 100 μM NADP^+ , 0.02% BSA, 2 $\mu\text{g/ml}$ of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and 10 $\mu\text{g/ml}$ yeast hexokinase. Readings were started after 2 or 3 min with a sensitivity setting equivalent to 15 μM NADPH full scale. Readings were made until at least 5 μM NADPH had been formed (usually 10–20 min). Assays were conducted with batches of 10 samples added and read at 20-s intervals. Corrections were made for the AK content of culture medium from parallel collagen-coated wells lacking cells. Control experiments showed that 0.1% Triton X-100 had no effect on AK activity.

The AK activity of the initial sample was expressed as a percentage of the sum of the released (initial sample) and Triton-extracted activity. This value thus represents the fraction of total available intracellular AK which was released during the experiment.

Antiserum to NGF

Because NGF sticks to glass, plastic, and collagen, the mere substitution of NGF-free medium fails to deprive totally the neurons of residual bound factor in these culture conditions. To effect rapid and total NGF deprivation, polyclonal antiserum against NGF was added to the culture medium at a final concentration of 1%. This antiserum was produced by guinea pigs immunized with mouse-NGF as described by Rich et al. (1984). It was heat inactivated at 56°C for 30 min to destroy complement and had a titer of 4,000–8,000 against mouse-NGF in the embryonic chick DRG explant assay (Fenton, 1970) with minor modifications (Gorin and Johnson, 1979). Normal medium with 1% antiserum demonstrated no toxicity to PC-12 cells or African green monkey kidney (Vero) cells after 48 h, as assessed morphologically and by total methionine incorporation. Sympathetic neurons grown in the presence of 1% non-immune guinea pig serum for 48 h were indistinguishable from control neurons.

Reagents

Except as noted above, all reagents were purchased from the Sigma Chemical Company (St. Louis, MO).

Results

Light Microscopy

Dissociated sympathetic neurons were plated on collagen in the presence of NGF and the antimetabolic fluorodeoxyuridine.

Within 24 h of plating, the neurons began to extend neuritic processes which fasciculated and covered the dish by 5–6 d. The neuronal cell bodies tended to aggregate in clusters and were phase-bright, with prominent nuclei and nucleoli (Fig. 1 *a*). Nonneuronal cells (mostly Schwann cells and fibroblasts) were killed by the fluorodeoxyuridine between days 3–5 so that after 7 d >95% of the cells in culture were neurons. The fluorodeoxyuridine did not appear to affect the neurons adversely and remained in the culture medium throughout each experiment. Although neuronal cultures remained healthy for longer than 2 mo, all experiments were conducted on 7–8-d-old cultures because the NGF-dependency of sympathetic neurons in vitro has been reported to decrease with time in culture (Lazarus et al., 1976). We have confirmed this observation, and have noted that the decreased NGF dependence begins between 3 and 4 w in culture.

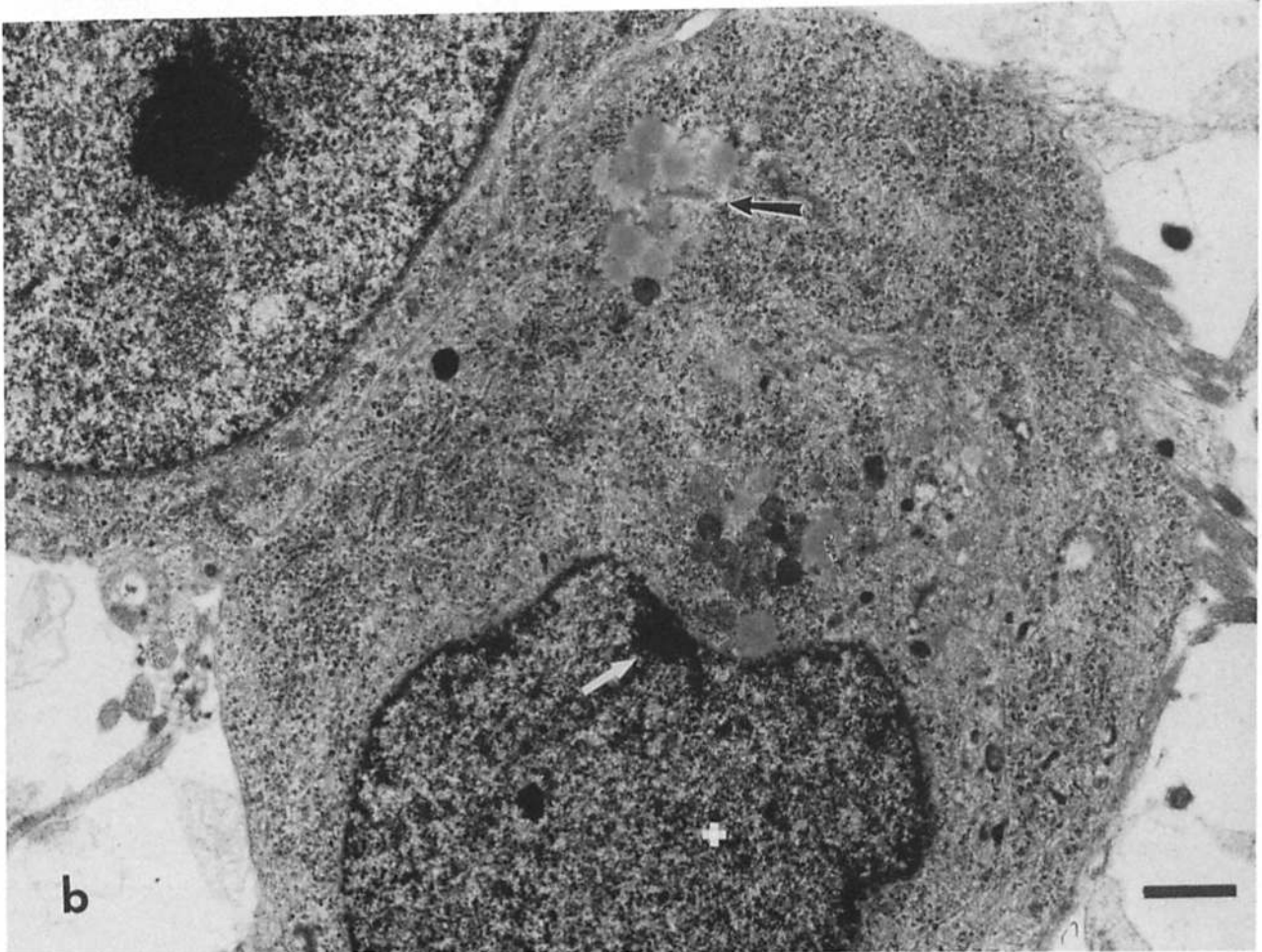
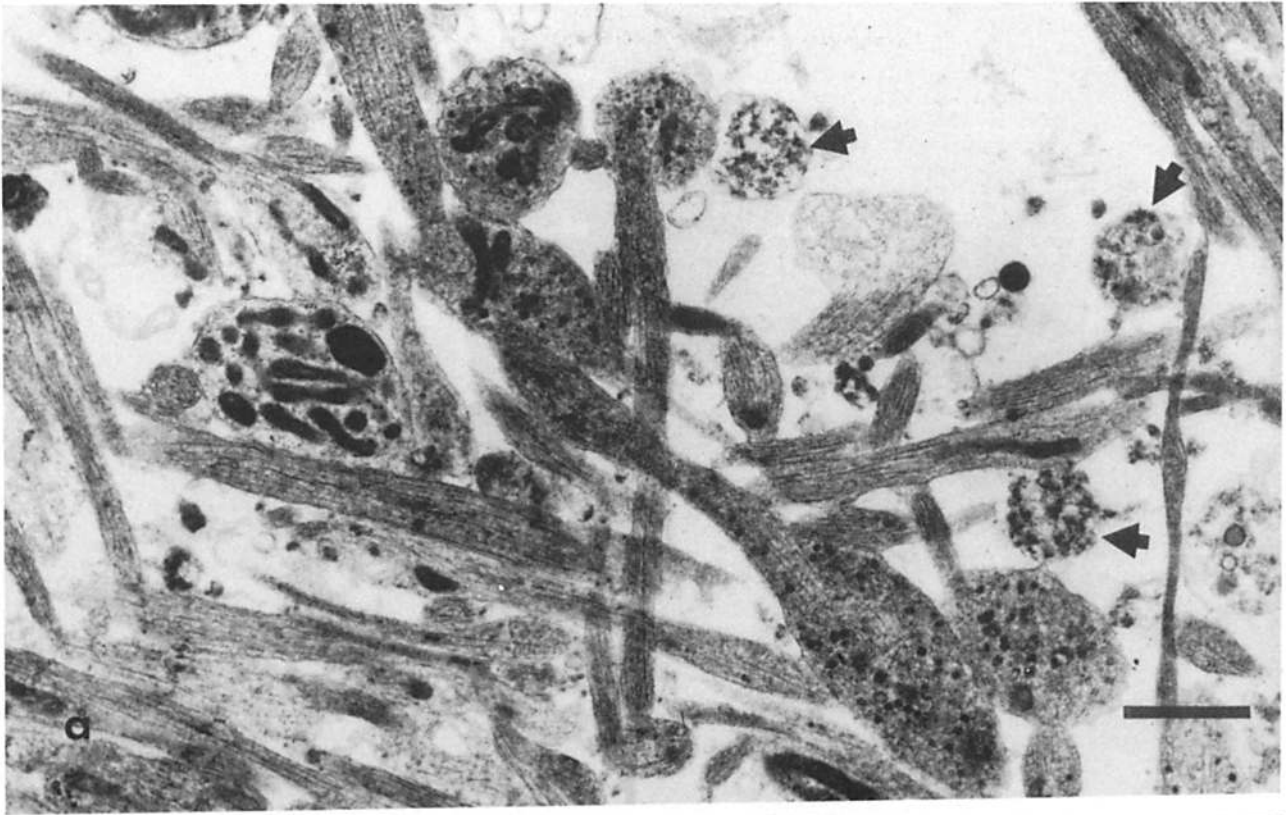
NGF was removed from 7-d-old cultures by adding anti-NGF to the culture medium. The first changes appeared 18–24 h after NGF deprivation when the neurites became thinner and disrupted in places, leaving behind bits of neuritic debris. By 30 h some cell bodies were smaller and phase-dark. There were distinctly fewer recognizable neurons on the dish at 36 h, although no debris was seen floating in the culture medium. The intact cells that were observed at this time were frequently alone, surrounded by scattered, phase-dark debris (Fig. 1 *b*). Neuronal demise appeared to be rapid, affecting individual neurons at slightly different times between 24 and 48 h post-deprivation. By 48 h only a few phase bright neurons could be found, isolated amid the remnants of cell bodies and neuritic debris. This debris remained attached to the culture dish for several days before lifting up and floating into the medium.

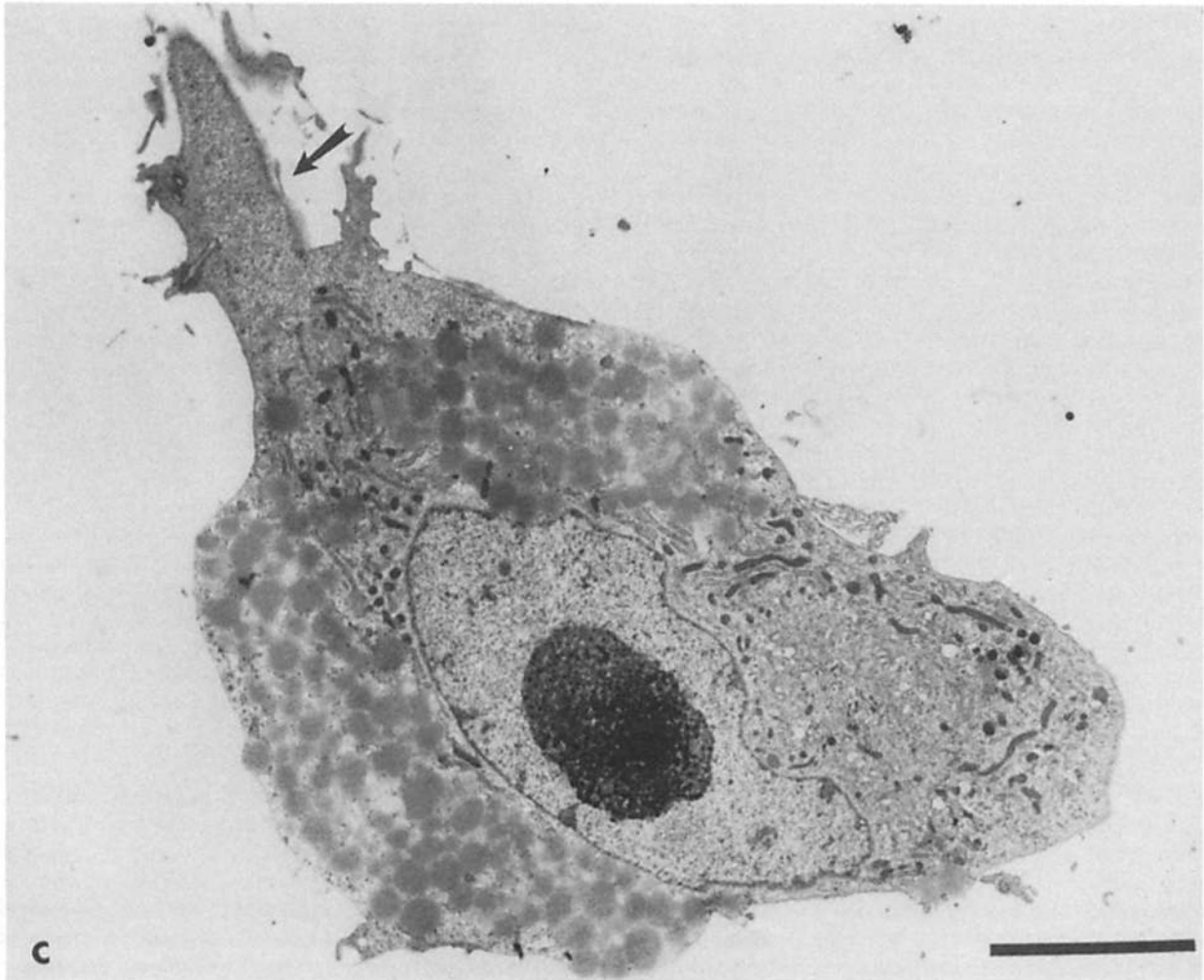
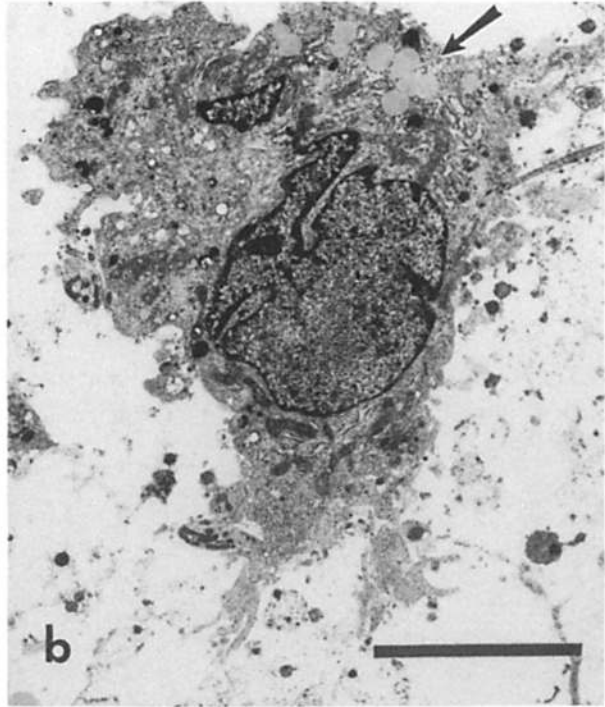
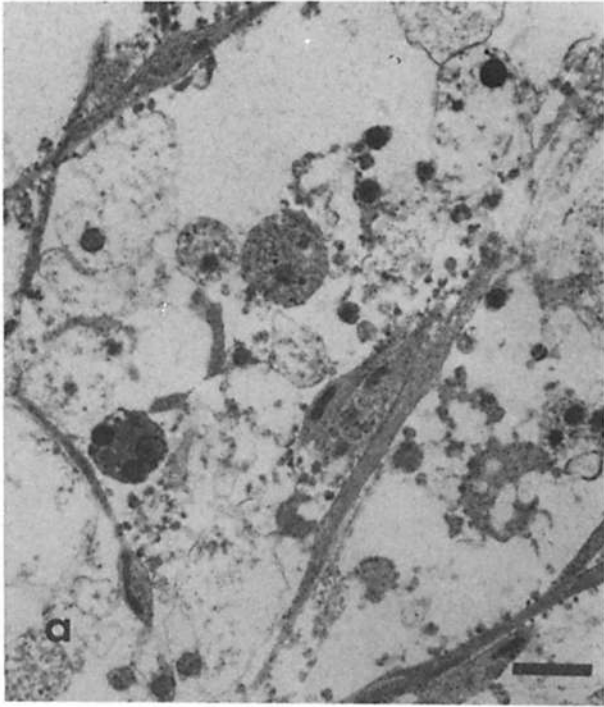
To demonstrate that the debris present at 48 h represented dead neurons, rather than viable atrophic neurons, the cultures were washed thoroughly and refed medium containing NGF (500 ng/ml). No changes in the cultures were observable over the next several days and the debris began to lift off the culture dish as it had before. Bioassay of the medium at the conclusion of the experiment demonstrated an appropriate NGF activity, indicating that residual antiserum had been completely washed out.

Ultrastructure

Sympathetic neurons were grown for 7 d in the presence of NGF and then prepared for electron microscopy after various periods of NGF deprivation. Untreated neurons frequently clustered in small groups and exhibited a normal ultrastructural appearance (Fig. 2, *a–c*). Cultured neurons had large vesicular nuclei with finely dispersed chromatin, large prominent nucleoli, cytoplasm filled with polysomes and rough endoplasmic reticulum, a complement of threadlike mitochondria (Fig. 2 *b*) and occasionally one or two lipid droplets. The interneuronal neuropil was composed of large

Figure 3. Ultrastructure of 1-w-old sympathetic neurons and neuropil deprived of NGF for 18–24 h in vitro. (*a*) At 18 h, neurites, containing normal and degenerating organelles, were in various stages of axonal dilation, degeneration, and disintegration (*arrows*). (*b*) The earliest alterations in sympathetic neuronal perikarya (24 h) consisted of a diffuse increased density of the nucleoplasm (+, compare with more normal appearance of nucleus at the upper left margin of the micrograph), development of small heterochromatic patches (*white arrow*), and apparent nuclear shrinkage resulting in irregularity of the nuclear perimeter. The cytoplasm contained increased numbers of lipid droplets (*black arrow*) with little apparent alteration of the rough endoplasmic reticulum, mitochondria, or cytoskeleton. Bars, 1 μm .





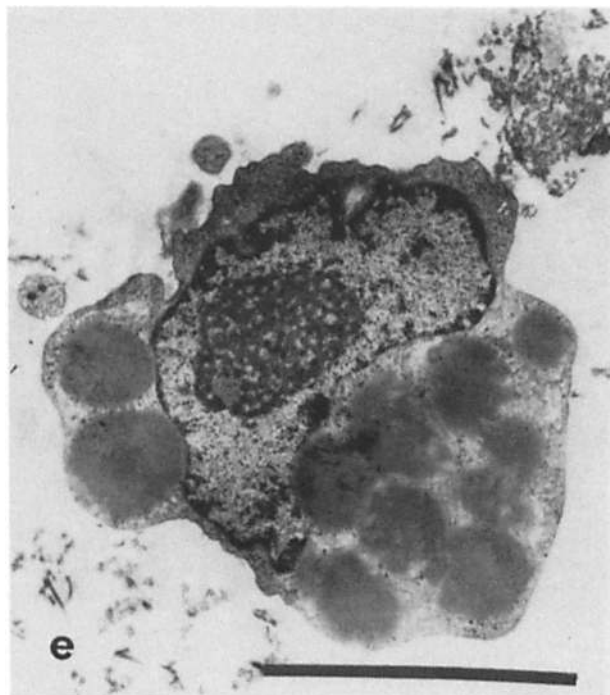
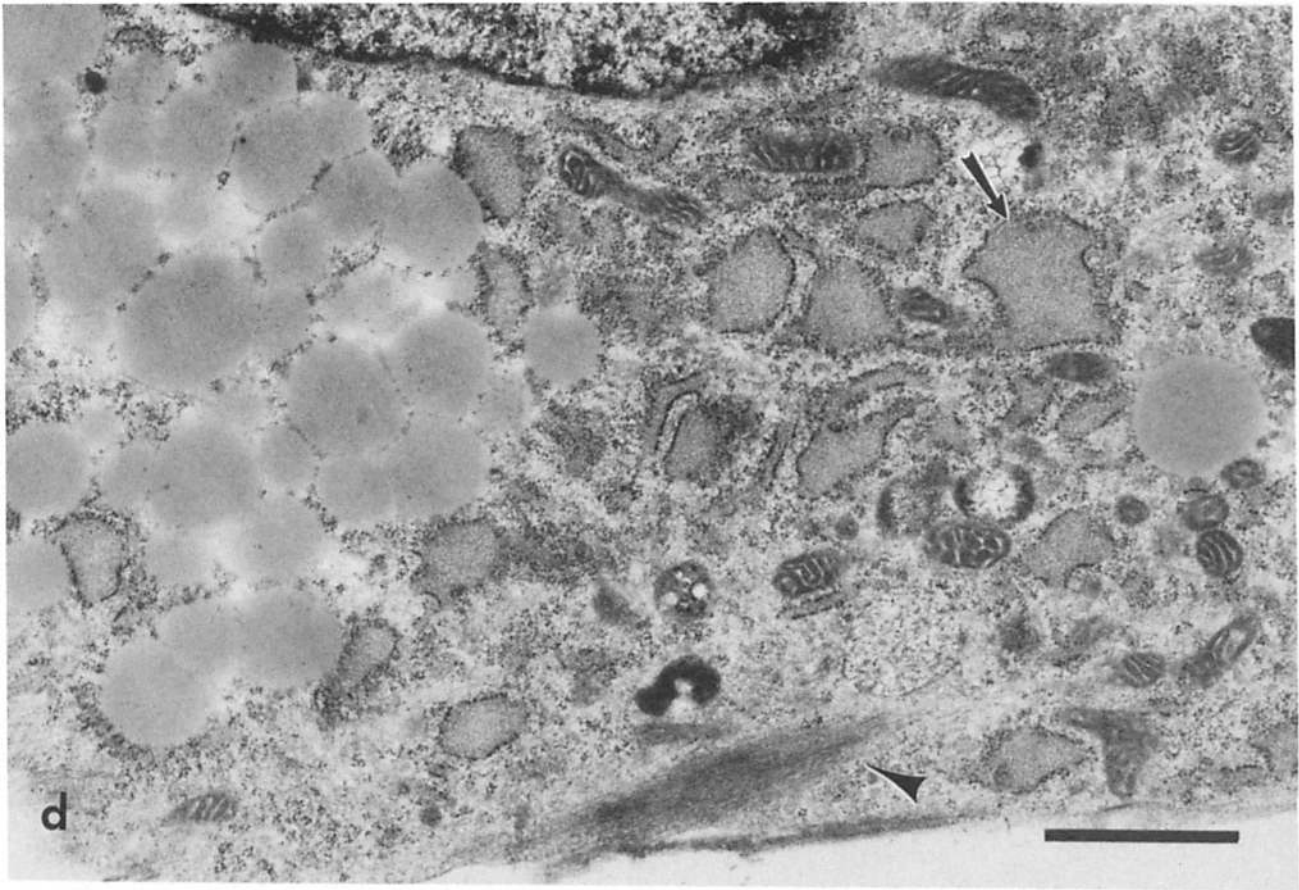


Figure 4. Degenerative alteration in sympathetic neurons after 30–48 h of NGF deprivation in vitro. (a) Active axonal degeneration involved the majority of neuritic profiles, which resulted in large amounts of extracellular debris. (b) Neuron with marked nuclear distortion contained decreased amounts of cytoplasm and scattered lipid droplets (*arrow*). (c) A neuron containing numerous lipid droplets had only a few large blunt neuritic processes (*arrow*). Few, if any, axonal termini ended on its perikaryon. Despite the considerable neuronal cytoplasmic pathology, the nuclear and nucleolar structures showed little worsening compared to earlier times. (d) Normal lipid droplets lacked a limiting membrane and showed little substructure. In addition, the rough endoplasmic reticulum showed foci of dilatation by dense intraluminal contents (*arrow*). Scattered patches of neurofilamentous material (*arrowhead*) were admixed. (e) Neuronal cytoplasm was occasionally markedly diminished, resulting in impressive neuronal atrophy. Bars: (a and d) 1 μm ; (b, c, and e) 5 μm .

numbers of small, normal appearing neurites with a prominent cytoskeleton (Fig. 2 c).

The first detectable ultrastructural alteration in cultures subjected to NGF deprivation occurred 12–18 h after the addition of anti-NGF and consisted of increased numbers

of abnormal or actively degenerating neurites (Fig. 3 a). The ultrastructural appearance of the axonopathy was represented by a continuum of degenerative alterations, ranging from swollen axons containing numerous intra-axonal multivesicular bodies or dense degenerating organelles to frank

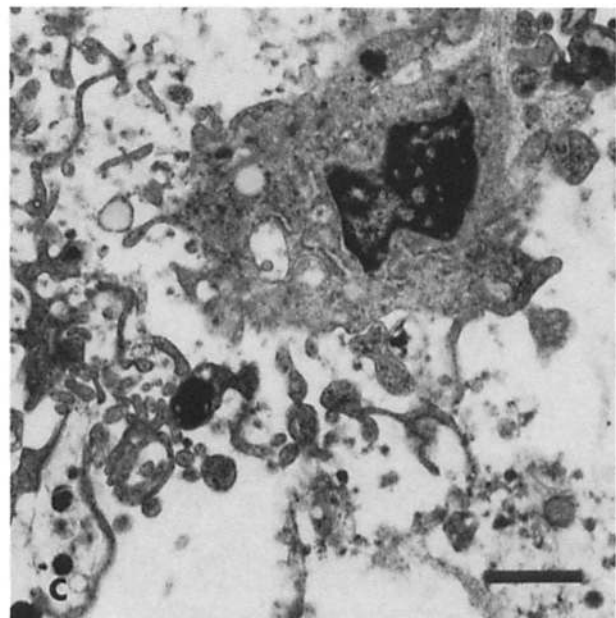
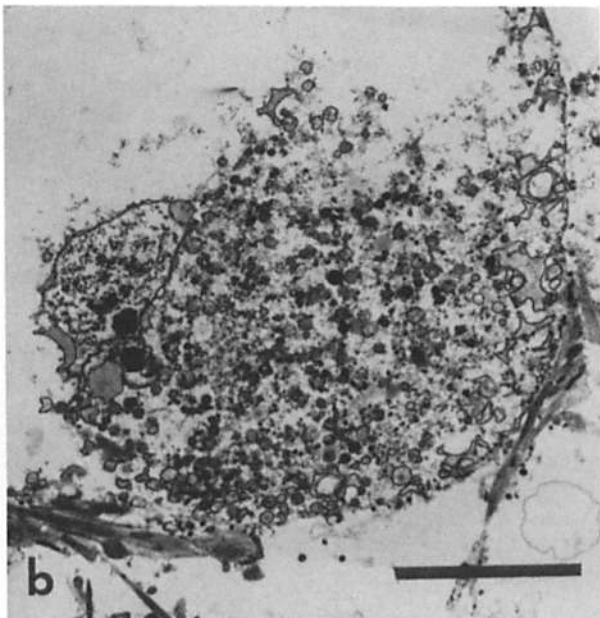
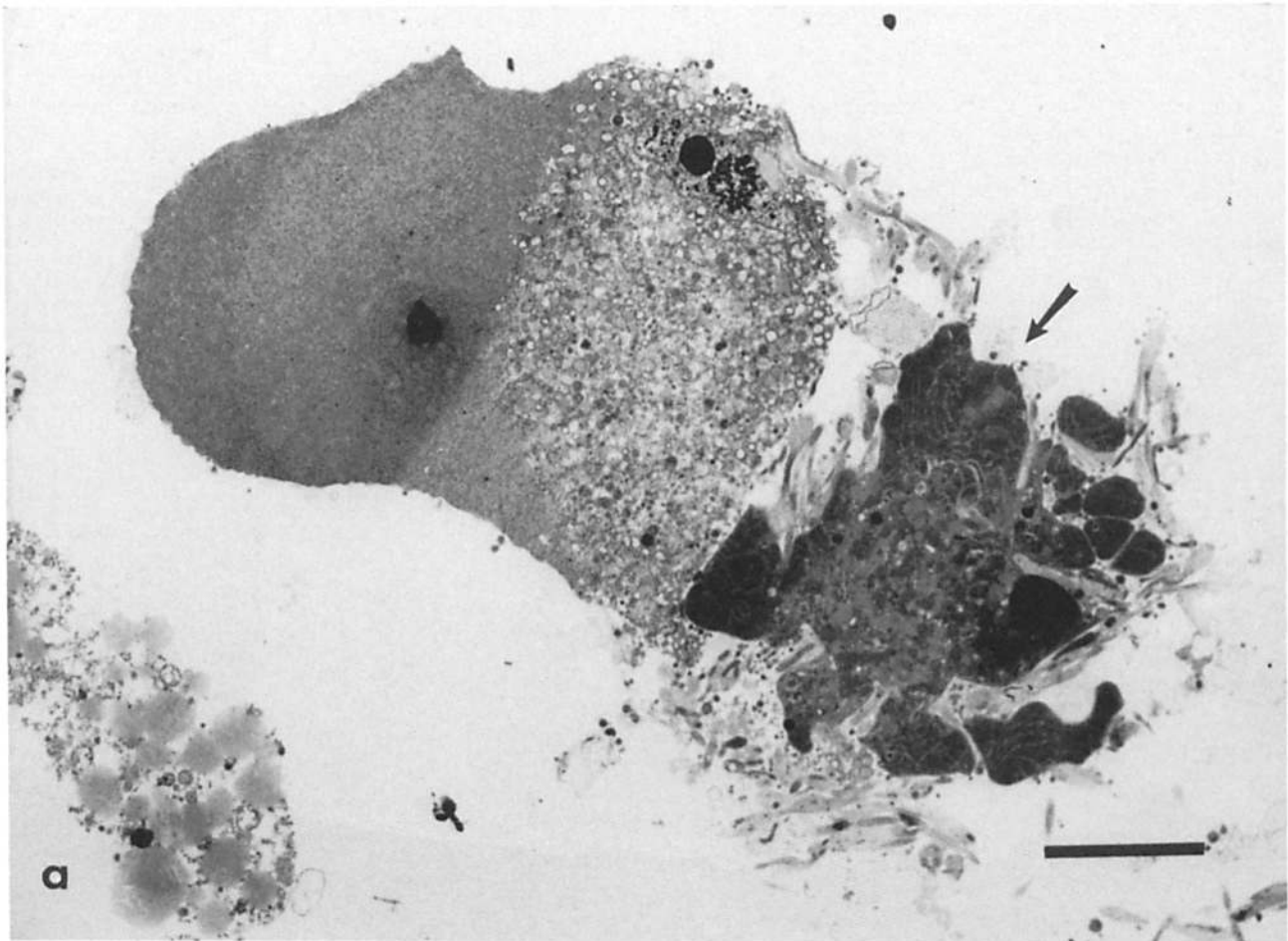


Figure 5. Final stages of degeneration of neurons after 36–48 h of NGF deprivation in vitro. A group of neurons exhibited different patterns of degeneration. The center neuron of *a* is vacuolated with disintegration of normal subcellular elements and loss of the integrity of the nuclear and plasma membranes. This pattern, the most common ultrastructural appearance of degenerating neurons in this study, is shown again in *b*. A second pattern (*a*, arrow; and *c*) was characterized by a marked increase in nuclear and cytoplasmic density with protrusion and eventual pinching off of membrane-bound blebs containing cytoplasmic constituents and, occasionally, nuclear fragments. Bars: (*a* and *b*) 5 μm ; (*c*) 1 μm .

loss of axonal integrity resulting in the release of axonal debris into the culture medium. Although many degenerating axons were adjacent to neurons, degeneration did not involve axonal termini exclusively. There was no clear evidence of structural alterations involving the neuronal perikarya at the earliest time of distinct axonopathy (roughly 12–18 h); however, subtle changes in the perikarya began to develop 18–24 h after NGF deprivation. The earliest neuronal cell body alterations (Fig. 3 *b*) consisted of (*a*) slight shrinkage of the nucleus, which resulted in an irregular nuclear perimeter, diffuse increase in the density of chromatin and development of small patches of heterochromatin; and, (*b*) appearance of small lipid droplets, occurring singly or in small groups in the perikaryal cytoplasm. At this time polysomes and rough endoplasmic reticulum, mitochondria, and neuronal cytoskeletal elements were well preserved.

Most neuronal perikarya began to develop significant ultrastructural alterations between 24 and 30 h. The degree of axonal degeneration increased markedly (Fig. 4 *a*) with prominent axonal dissolution. Few normal neurites remained. Neurons developed marked nuclear irregularity and apparent shrinkage (Fig. 4 *b*) without the development of large discrete nuclear heterochromatin aggregates. Surviving neurons gave rise to few neuritic processes which may represent the residua of preferential pruning of axonal processes at earlier times. Increased numbers of lipid inclusions were found in the neuronal cell body, which occasionally resulted in the marked degree of lipid accumulation seen in a few neurons (Fig. 4 *c*). Accumulated lipid droplets typically lacked substructure and a limiting membrane (Fig. 4 *d*). Marked accumulation of lipid droplets displaced normal cytoplasmic organelles, especially rough endoplasmic reticulum, the total amount of which appeared diminished in comparison with controls. In addition, the cisternae of the rough endoplasmic reticulum were often dilated by a dense granular material (Fig. 4 *d*). The majority of ribosomes remained attached to rough endoplasmic reticulum or represented cytoplasmic polysomes. A few bundles of neurofilaments were also encountered in the perikaryal cytoplasm (Fig. 4 *d*). Neurons became atrophic, sometimes to an extreme degree (Fig. 4 *e*).

The final phases of neuronal degeneration began substan-

tively at 30 h and were advanced at later times. Several patterns of degeneration were observed (Fig. 5). The first and most common degenerative pattern was the formation of swollen clear vacuoles admixed with degenerating organelles in lucent perikaryal cytoplasm, which eventually resulted in the dissolution of the nuclear and plasma membranes and the liberation of intracellular contents into the culture medium (Fig. 5, *a* and *b*). Another degenerative pattern, although infrequent, was characterized by the condensation of nucleoplasm and cytoplasm forming dense cytoplasmic protrusions of the neuronal plasmalemma containing fragments of cytoplasmic and nuclear debris (Fig. 5, *a* and *c*). It was difficult to assign a precise longitudinal sequence of morphologic events associated with neuronal death because at later times all stages of death were observed. Neuronal perikarya showed no morphologic evidence of autophagy.

Biochemical Profile of Cell Death

Sympathetic neurons were grown in the presence of NGF for 7 d. NGF deprivation was then induced by adding anti-NGF to sequential cultures over the next 72 h at various intervals. Thus, by 10 d after dissection, cultures were available which had been deprived of NGF from 0 to 72 h. The amount of the cytosolic enzyme, AK, released into the culture medium was then determined as a percentage of the total amount releasable (as described in the Materials and Methods section). Fig. 6 shows that AK began to appear in the culture medium after ~30 h of NGF deprivation and reached a maximal value by 54 h. Because AK is normally retained within the cytoplasm, its appearance in culture medium indicates disruption of neuronal plasma membranes. The time frame of AK release into the medium correlates with the ultrastructural observation of cell body disintegration. Media from the cultures prepared for ultrastructural observation were assayed for released AK and found to follow the same time course of increase as that shown in Fig. 6 (data not shown). This experiment was repeated three times with nearly identical results.

Analogous to the above experiment, 1-w-old cultures were deprived of NGF for various periods of time and then metabolically labeled by adding [³⁵S]methionine to the culture

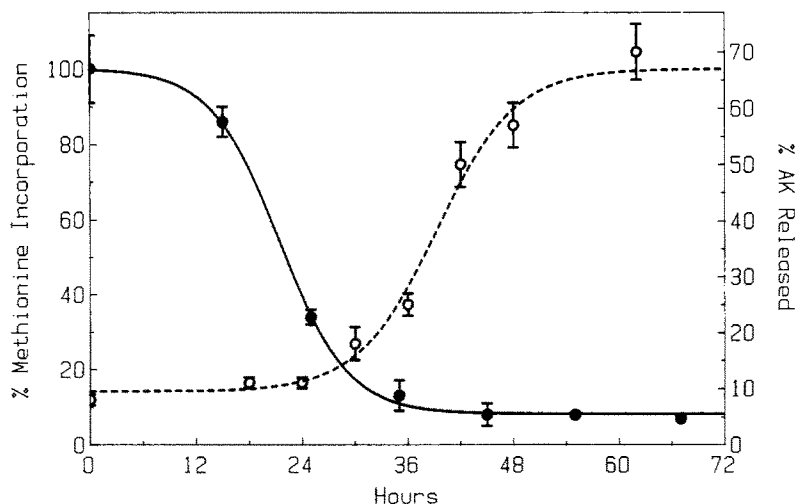


Figure 6. Decreased methionine incorporation (●—●) and release of the cytosolic enzyme, AK, into the culture medium (○—○) by sympathetic neurons as a function of the duration of NGF deprivation. Neurons were grown for 1 w in the presence of NGF and then deprived of NGF at time 0 hours. Cultures were fed [³⁵S]methionine 5 h before the time at which each incorporation data point is plotted and assessed for TCA-precipitable counts 5 h after each point, allowing a total incorporation period of 10 h. Released AK is expressed as the percentage of total activity. Each point represents the mean ±SD of triplicate cultures.

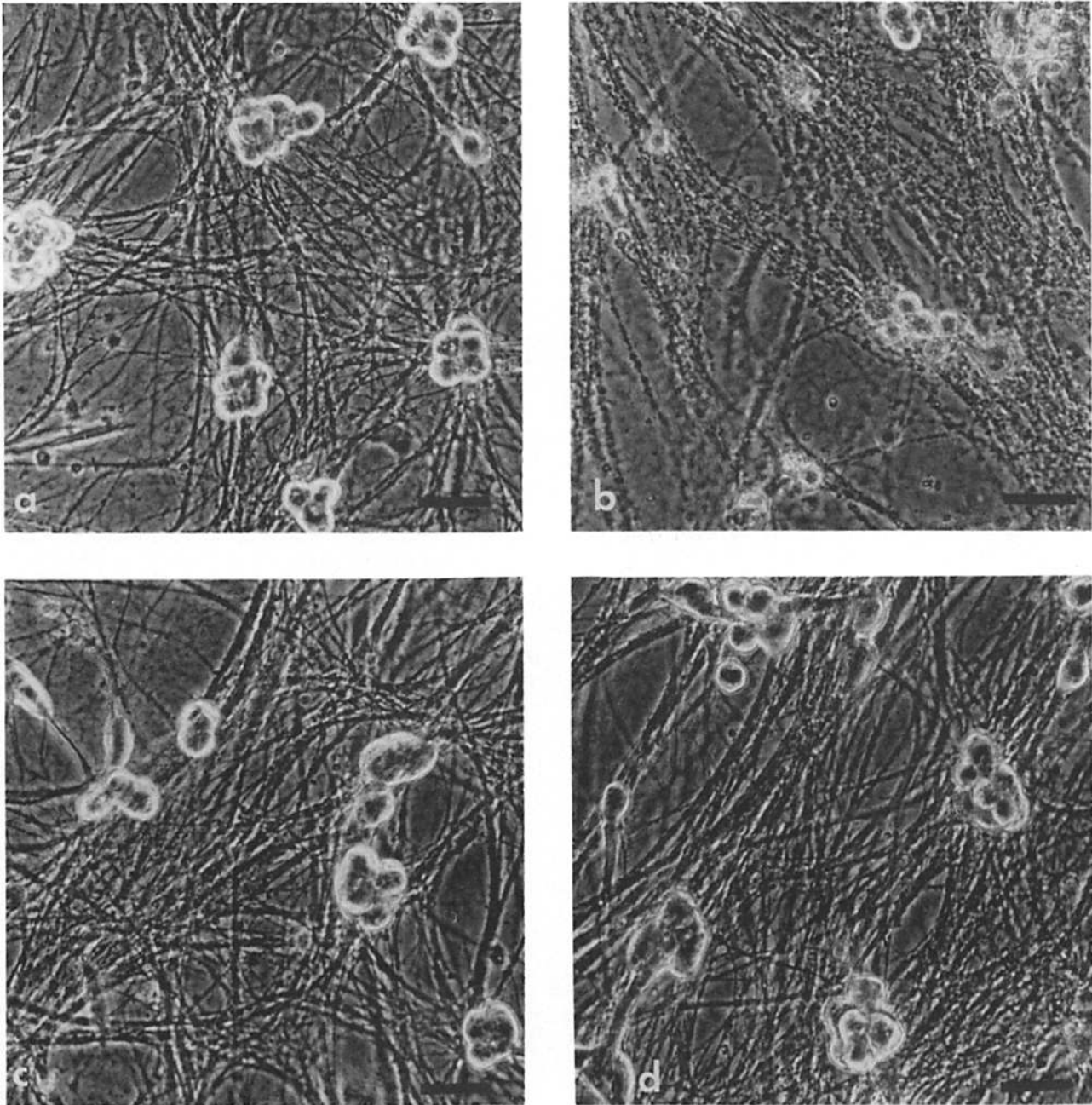


Figure 7. Effect of protein synthesis inhibition on neurons deprived of NGF. 1-w-old sympathetic neurons were treated for 48 h as follows: (a) Control neurons; NGF present (50 ng/ml), no cycloheximide; (b) NGF-deprived neurons, no cycloheximide; (c) NGF present, with cycloheximide (1 µg/ml); (d) NGF-deprived neurons with cycloheximide. NGF deprivation, which normally causes the death of sympathetic neurons, fails to kill in the presence of cycloheximide. Bars, 50 µm.

medium for 10 h. The amount of [³⁵S]methionine incorporated into TCA-precipitable protein was expressed as a percentage of that incorporated by sister cultures not deprived of NGF. This method assesses the net accumulation of new protein during the labeling period, but can not distinguish between decreased protein synthesis or, alternatively, increased protein degradation. Methionine incorporation began to decrease after ~18 h of NGF deprivation and was maximally depressed by 36 h (Fig. 6). The decreased methionine incorporation preceded the release of AK by ~12 h.

This experiment was repeated twice with nearly identical results.

Cycloheximide Prevents Death

We sought to determine whether the demise of NGF-deprived neurons represented passive atrophy or, alternatively, an active "suicide" process. We reasoned that if neurons degenerated passively when deprived of NGF, the inhibition of biosynthetic reactions should, if anything, hasten their de-

struction. In contrast, if neuronal degeneration subsequent to NGF deprivation were active, such a process might be slowed or blocked by the inhibition of macromolecular biosynthesis. Cycloheximide, an inhibitor of eukaryotic protein synthesis, decreased methionine incorporation in our system half-maximally at 0.1 $\mu\text{g/ml}$ and entirely at 1 $\mu\text{g/ml}$ (data not shown). The effect of cycloheximide was reversible: 3 d after removing cycloheximide from the culture medium, methionine incorporation returned to control values. Complete inhibition of protein synthesis with 1 $\mu\text{g/ml}$ cycloheximide did not begin to show significant adverse effects until 4–5 d. Therefore, it was possible to inhibit protein synthesis for 48 h and then rescue the neurons by washing out the cycloheximide.

Neurons were grown for one week in the presence of NGF and then cultured for 48 h with either NGF (Fig. 7 *a*) or anti-NGF (Fig. 7 *b*). In contrast to control neurons (Fig. 7 *a*), those deprived of NGF for 48 h (Fig. 7 *b*) were phase dark and degenerated. Figs. 7, *c* and *d* show neurons treated identically to those in Fig. 7 *a* and 7 *b*, respectively, except that cycloheximide (1 $\mu\text{g/ml}$) was included in the culture medium. Although this concentration of cycloheximide completely inhibited protein synthesis, it did not affect the morphology of the cells significantly; after 48 h of inhibited protein synthesis (Fig. 7 *c*) the neurons were still phase bright with thick neurites. Fig. 7 *d* shows NGF-deprived neurons which received 1 $\mu\text{g/ml}$ cycloheximide over the 48-h period of deprivation. These neurons were alive, with clusters of phase-bright cell bodies and intact, continuous neurites. No degenerated neuronal debris was present. Comparing Fig. 7 *d* with *b* demonstrates the dramatic effect that inhibition of protein synthesis had in preventing neuronal death after NGF deprivation.

The saving effect of cycloheximide was quantified by measuring the release of AK into the culture medium. Fig. 8 demonstrates the percentage of total AK released over a 48-h period in which neurons were treated with cycloheximide and/or anti-NGF. Comparing Fig. 8, bars 1 and 2, reveals that 48 h of NGF deprivation caused more than a 20-fold increase of AK released into the culture medium. In the presence of cycloheximide, however, (Fig. 8, bars 3 and 4) 48 h of NGF deprivation resulted in little more than a twofold increase of AK release. This experiment was repeated three times with nearly identical results.

Since the inhibition of protein synthesis by cycloheximide was reversible, the saving effect of cycloheximide could be quantified with the methionine incorporation assay. Cultures were grown for 1 w in the presence of NGF and then treated with cycloheximide and/or anti-NGF for 48 h. All cultures were then washed thoroughly and fed cycloheximide-free medium containing NGF (500 ng/ml). Three days later the neurons were metabolically labeled for 16 h with [^{35}S]methionine. Fig. 9, bar 1 illustrates the amount of [^{35}S]methionine incorporated into TCA-precipitable protein as a percentage of that incorporated by neurons which had never been deprived of NGF nor exposed to cycloheximide. Fig. 9, bar 2 demonstrates that 48 h of NGF deprivation, despite subsequent replacement of NGF, results in total neuronal death. The very small amount of methionine incorporation observed in these cultures (Fig. 9, bar 2) is probably due to non-neuronal cells (which are not dependent on NGF). The neurons represented by Fig. 9, bar 4 were deprived of NGF exactly as those in Fig. 9, bar 2, but in the presence of cyclo-

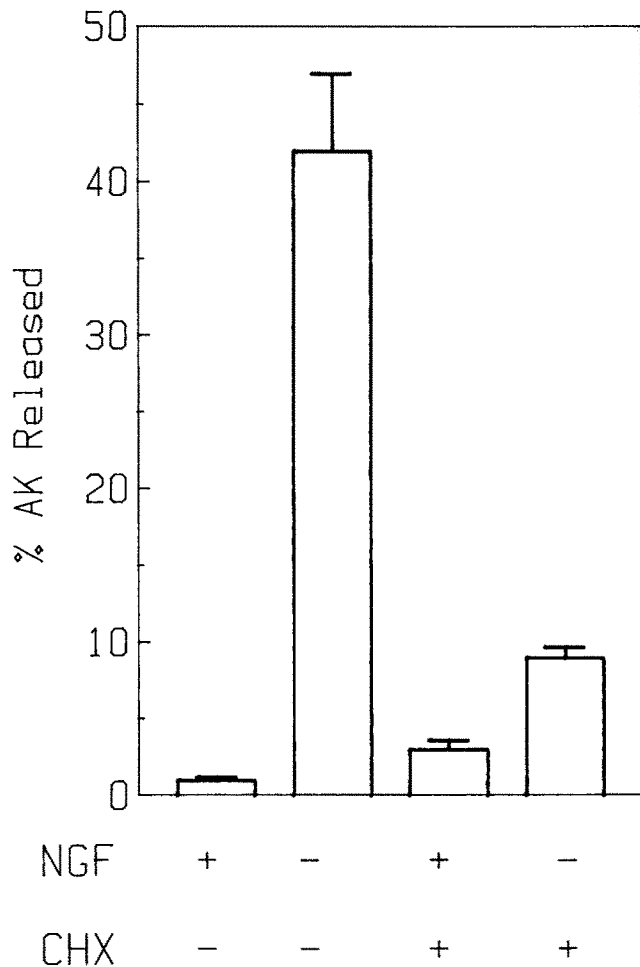


Figure 8. Effect of cycloheximide (1 $\mu\text{g/ml}$) (CHX) on AK release caused by 48 h of NGF deprivation. Neurons were grown in the presence of NGF for 1 w and then treated as in Fig. 7 for 48 h. Each bar represents the mean \pm SD of triplicate cultures.

heximide. The methionine incorporation of these cultures after washing out cycloheximide and replacing NGF was equivalent to cycloheximide-treated cultures which were not deprived of NGF (Fig. 9, bar 3). The inhibition of protein synthesis during the 48-h period of NGF deprivation had totally prevented the neurons from dying, and the saved neurons were viable upon replacement of NGF and removal of cycloheximide. This experiment was repeated three times with nearly identical results.

Puromycin and anisomycin, other inhibitors of protein synthesis, also prevented neurons from dying when deprived of NGF (data not shown). Because these drugs inhibit protein synthesis by mechanisms different from cycloheximide, it is likely that all the drugs prevent the death of NGF-deprived neurons by inhibiting protein synthesis rather than by some other nonspecific property.

Actinomycin-D Prevents Death

Since protein synthesis was required for neurons to die when deprived of NGF, we asked whether the death response used altered protein synthesis from existing mRNA or the synthesis of new proteins from newly transcribed mRNA. The inhibition of RNA synthesis by actinomycin-D (0.1 $\mu\text{g/ml}$) in our

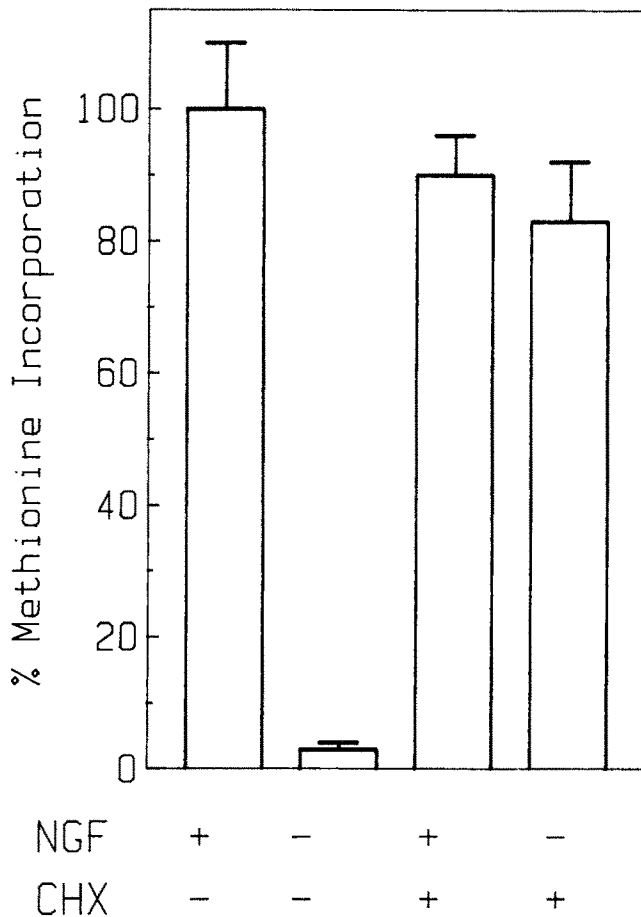


Figure 9. Effect of cycloheximide (1 $\mu\text{g/ml}$) (*CHX*) on viability of neurons deprived of NGF for 48 h. Neurons were grown in the presence of NGF for 1 w and treated as in Fig. 7 for 48 h. Cultures were then washed and fed NGF-containing medium (500 ng/ml) without cycloheximide. After a 3-d recovery period, [^{35}S]methionine was added to the medium for 16 h. TCA-precipitable counts are expressed as a percentage of those incorporated by cultures which were not deprived of NGF. Each bar represents the mean \pm SD of triplicate cultures.

system was consistent with the fact that methionine incorporation gradually decreased over the next 24 h, presumably due to the degradation of mRNA. Neurons remained alive and intact after 48 h of actinomycin-D, an observation which is not surprising in light of the fact that neurons could survive 4–5 d of complete protein synthesis inhibition. Unlike cycloheximide, the effects of actinomycin-D were not reversible: Neurons were never able to incorporate methionine into protein after a 24-h exposure to actinomycin-D, despite extensive washing. Neurons eventually died after 4–5 d of actinomycin-D treatment, at times similar to that seen after cycloheximide treatment.

Week-old neurons were treated with actinomycin-D and/or anti-NGF. After 48 h, the amount of AK released into the medium was measured (Fig. 10). Fig. 10, bars 1 and 2, shows the characteristic increase of released AK indicating disintegration of neuronal plasma membranes caused by NGF deprivation. When RNA synthesis was inhibited (Fig. 10, bars 3 and 4), NGF deprivation did not cause increased AK release, indicating that neurons did not die when deprived

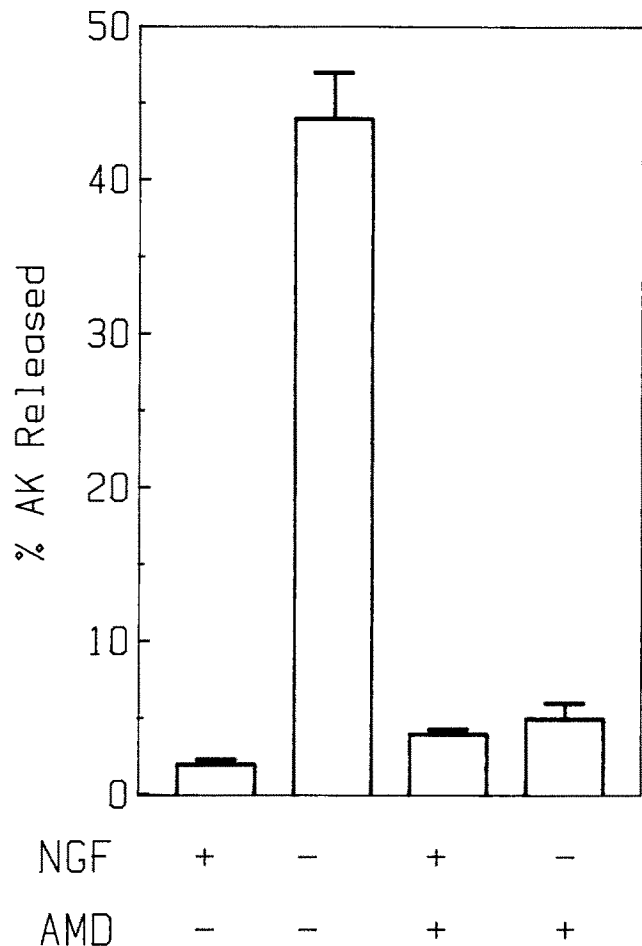


Figure 10. Effect of the RNA synthesis inhibitor, actinomycin-D (0.1 $\mu\text{g/ml}$) (*AMD*), on AK release from neurons deprived of NGF for 48 h. Neurons were grown for one week in the presence of NGF and then for an additional 48 h with or without actinomycin-D in the presence or absence of NGF. Released AK was assayed after this 48-h period. Each bar represents the mean \pm SD of triplicate cultures.

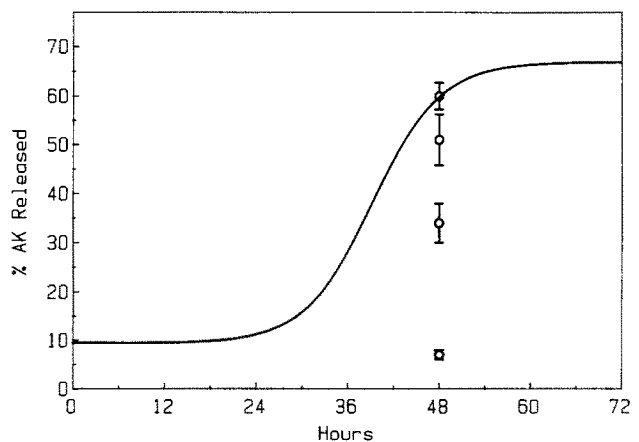


Figure 11. Effect of inhibiting protein synthesis at different times after NGF deprivation. Axes and curve are the same as presented in Fig. 6. The four points plotted, from top to bottom, represent the released AK when cycloheximide was added at 30, 24, 18, and 12 h post-deprivation, respectively. Each point represents the mean \pm SD of triplicate cultures.

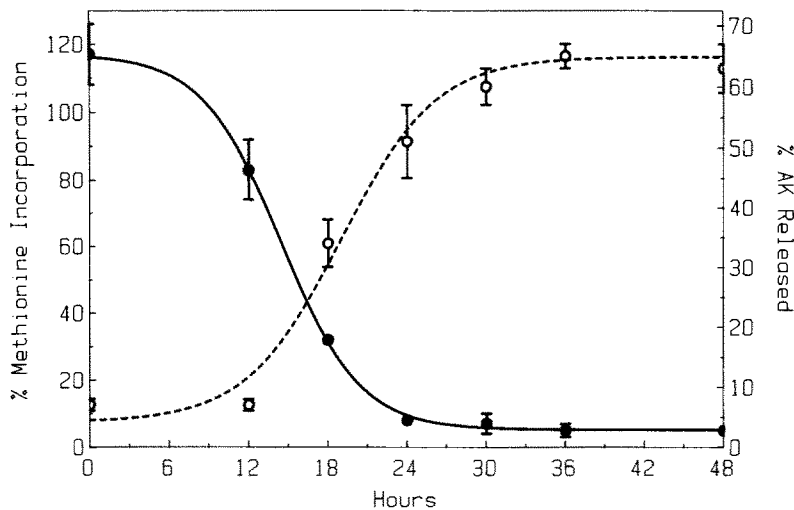


Figure 12. Effect of inhibiting protein synthesis at different times after NGF deprivation. The abscissa represents the time at which cycloheximide was added relative to the initiation of NGF deprivation. Cycloheximide remained in the media, along with anti-NGF, from that time to the end of the experiment, 48 h after NGF deprivation commenced. Released AK was measured at this time (○- -○). Methionine incorporation was assessed in sister cultures after a 3-d recovery (in media with NGF, without cycloheximide) which followed the 48-h period of NGF deprivation (●—●). See text for details. Each point represents the mean \pm SD of triplicate cultures.

of NGF. The synthesis of new RNA was thus required for neuronal death caused by NGF deprivation. This experiment was repeated three times with nearly identical results. Dichlorobenzimidazole riboside (50 μ M), a selective inhibitor of mRNA synthesis, also prevented neuronal death subsequent to NGF deprivation (data not shown).

Inhibitors of Lysosomal Function Do Not Prevent Death

It is possible that the destruction of dying neurons subsequent to NGF deprivation is mediated by lysosomal autophagy, although the ultrastructural results suggest that this is not the case in the cell body because perikaryal phagosomes were not observed as a primary event in the degeneration process. We attempted to prevent neuronal death subsequent to NGF deprivation with various inhibitors of lysosomal proteases which included leupeptin, chloroquine, and PMSF (each from 10^{-6} to 10^{-2} M). None of these agents prevented neuronal death subsequent to NGF deprivation.

Time Course of the Translational Event

We sought to determine how long the addition of cycloheximide could be delayed after NGF deprivation and still prevent neuronal death. Neurons were grown for 1 w in the presence of NGF, and then deprived of NGF as previously described. Cultures received cycloheximide at various times after NGF deprivation. After 48 h of NGF deprivation, the AK released by each culture was measured and is plotted in Fig. 11 with the same axes and curve of Fig. 6 for reference. The points plotted from top to bottom represent the released AK from cultures which received cycloheximide at 30, 24, 18, and 12 h post-deprivation, respectively. These data show that inhibition of protein synthesis within 12 h of NGF deprivation entirely prevented cell death, but after 24 h was much less effective.

We wished to determine more precisely when cycloheximide becomes unable to prevent neuronal death, i.e., when neurons become "committed" to die despite inhibition of protein synthesis. Week-old neuronal cultures were deprived of NGF at time 0 and received cycloheximide at various times thereafter, as represented by the abscissa of Fig. 12. After

48 h the amount of AK released into the culture medium was measured. Thus, for example, the point at 18 hours represents the fraction of total AK released from neurons which were deprived of NGF, treated with cycloheximide 18 h later, and then assayed for AK in the medium 30 h after the addition of cycloheximide. Sister cultures were prepared in the same way to assess saving as determined by the methionine incorporation assay, but instead of assaying AK after 48 h of NGF deprivation these cultures were washed thoroughly and fed medium containing NGF without cycloheximide. After 3 d of recovery, the cultures were labeled with [35 S]methionine for 16 h and assayed for TCA-precipitable counts. Fig. 12 demonstrates that if cycloheximide was added to the cultures within the first 12 h of NGF deprivation, the neurons did not die. Their plasma membranes remained intact as shown by low amounts of released AK and high methionine incorporating capacities. After 24 hours of NGF deprivation, though, addition of cycloheximide to the cultures could no longer prevent cell death, as demonstrated by the maximal AK values and minimal methionine incorporation. A similar time course of saving result was obtained with actinomycin-D (data not shown). Because cycloheximide and actinomycin-D could no longer prevent the death of more than half of the neurons after \sim 18 hours of NGF deprivation, 18 h is the "commitment point"—the duration of NGF deprivation after which neurons will die despite inhibition of RNA or protein synthesis.

Discussion

The Experimental Paradigm

This paper describes an *in vitro* model of the natural death of sympathetic neurons which occurs when trophic support (i.e., NGF) becomes insufficient. The experimental paradigm differs from many previous studies in which NGF was added to cells which respond to NGF but are not dependent upon it for survival (e.g., the PC12 cell line or certain neuroblastomas). At some point during development, neurons become dependent on trophic support from their target such that its removal (via competition, target extirpation, or axotomy) results in neuronal death. The effects of NGF on

naïve cells in stimulating neurite outgrowth may act via mechanisms not directly relevant to the survival promoting role of NGF. Our model approximates the situation encountered by neurons during development or after axotomy, when target-derived trophic support becomes insufficient and neurons die.

This model offers several advantages for studying cell death. Large, homogenous populations of sympathetic neurons can be cultured easily in the presence of NGF. The neurotrophic factor for these neurons (NGF) has been identified and well characterized. A similar experimental paradigm, though, could be used to study other neurotrophic interactions with other classes of neurons. Conducting the experiments *in vitro* allows control over the availability of trophic factor and is amenable to the assessment of cell vitality with biochemical techniques.

Cell Death as an Active Process

Our data demonstrate that RNA and protein synthesis are required for sympathetic neurons to die when deprived of NGF. This indicates that NGF suppresses an activity (or activities) which, if not restricted, causes cell death. If NGF acted primarily to support or stimulate normal cellular processes in a general fashion, then the inhibition of protein and RNA synthesis would be expected to accelerate the demise of neurons upon NGF deprivation. Since neuronal death was prevented by these inhibitors rather than accelerated, the death of NGF-deprived neurons must result from the activity of a new set of proteins or the altered (e.g., increased) activity of existing proteins which are capable of killing the cell in the stereotypic manner described here. We will refer to this new or augmented set of activities collectively as the "death program."

The presence of NGF could act at any of several levels to suppress the death program. The first possibility would be that NGF selectively blocks the transcription of the mRNA for the protein(s) which produces cell death or of mRNAs which are required for the subsequent production of these proteins. In this scheme, NGF represses the synthesis *de novo*, or at greatly increased levels, of the specific death protein(s), or a cascade (see below) of proteins required for cell suicide. Actinomycin-D and cycloheximide thus mimic the survival-promoting effects of NGF by similarly, although nonselectively, inhibiting the synthesis of the mRNA and protein(s) representing the specific death protein(s) or the cascade which produces them. We favor this hypothesis since ample evidence exists of NGF's ability to regulate the level of mRNA synthesis for specific proteins (e.g., Dickson et al., 1986; Tiercy and Shooter, 1986). We would assume similarly that NGF deprivation may be associated with specific mRNA and protein synthesis.

A second possibility is that NGF acts to suppress the activity of a constantly present killer protein(s) directly through some posttranslational modification. For example, NGF might maintain killer proteins in a latent state by phosphorylation. Loss of NGF would allow dephosphorylation and thus activation of the killer protein(s). The ability of NGF to affect post-translational modifications, such as phosphorylation, has been observed in other systems (e.g., Halegoua and Patrick, 1980; Rowland et al., 1987). However, if NGF represses suicide solely by this mechanism (i.e., by acting

through posttranslational modifications) our data require that both the mRNA and the potentially modified protein(s) be labile and rapidly turning over to account for the dramatic protective effects of actinomycin-D and cycloheximide. Thus, after the inhibition of RNA or protein synthesis, insufficient amounts of the mRNA and killer protein(s) would exist to produce the suicide response upon NGF deprivation.

A third, although less likely, possibility is that NGF represses the death program by inhibiting the synthesis of the killer protein(s) from mRNA (i.e., by repressing translation); thus cycloheximide mimics the action of NGF. If this is the case, our data require that the mRNA for the protein(s) must be unstable and rapidly turning over, such that insufficient amounts of the protein would be synthesized if RNA synthesis were inhibited. This general mechanism seems unlikely because few examples exist where a hormone regulates translation in mammalian cells and NGF has never been shown to mediate such regulation. The above hypotheses are not mutually exclusive. NGF may be able to prevent both the synthesis, as well as the activity, of the putative killer protein(s).

We observed that the addition of cycloheximide within 18 h of NGF deprivation prevented cell death. In similar culture conditions, NGF deprivation for as short as 1 h (C. L. Wilcox, personal communication) was sufficient to cause Herpes reactivation from latently infected sympathetic neurons (Wilcox and Johnson, 1987). This suggests that the information signifying NGF deprivation was available at the cell body much sooner than 18 h after NGF deprivation. A possible explanation for the lag between deprivation and commitment is that NGF deprivation might initiate a cascade of regulatory steps, of which only the last stage actually produces the killer protein(s). Thus, adding cycloheximide during the first steps would abort the cascade before the actual killer proteins were synthesized.

Significant ultrastructural changes were not observed in perikarya until 24 h after anti-NGF addition. Thus, the neurons appeared to be committed to die before any observable ultrastructural changes. The fact that the commitment point precedes significant morphological change means that cycloheximide, and actinomycin-D cannot inhibit the actual killing mechanism once it reaches a certain critical point (presumably the synthesis of a sufficient amount of critical protein). This observation also indicates that the killing activities must be relatively stable, once synthesized or activated, because the entire sequence of neuronal killing can then take place in the absence of RNA or protein synthesis. This again argues for the hypothesis that NGF normally suppresses the synthesis of new mRNA which codes for new protein because the alternate hypotheses require the assumption of unstable, short-lived mRNA or protein.

A requirement for protein synthesis has been demonstrated in other cases of natural cell death. The death of glucocorticoid-treated lymphocytes, for example, is prevented by the inhibition of protein synthesis (Cohen and Duke, 1984), as is the death of palatal epithelial cells (Pratt and Greene, 1976) and the resorption of tadpole tails (Tata, 1966). Natural cell death has been extensively studied in invertebrates (Truman, 1984) and in some cases has been found to be dependent on RNA and protein synthesis (Lockshin, 1969; Fahrback and Truman, 1987). In the nematode, genes required for the natural death of a neuron have been

identified (Ellis and Horvitz, 1986). It has, in fact, been argued that an active death program must be present in every cell of all multicellular organisms (Umansky, 1982). Therefore, study of the mechanism of neuronal death in this system may be applicable outside of the nervous system.

Morphology of Death

The significance of lipid droplet formation by neurons during NGF deprivation remains uncertain. The dramatic inclusion of lipid droplets in the cell body reflects altered lipid metabolism or transport. One possibility is that the neuritic membrane accumulates at a rate exceeding degradative capability with the excess amassing as droplets in the neuronal perikarya. The observed depression of protein synthesis at the times of this lipid buildup may result in insufficient amounts of lipoproteins required for lipid export. Alternatively, the droplets may reflect lipid which is newly synthesized in excess of the rate at which it can be used. The latter possibility seems less likely because we observed lipid droplets to accumulate progressively as cells die. One would also not expect lipid biosynthesis to continue unabated at times when protein synthesis has ceased. Abundant lipid droplet formation in NGF-deprived sympathetic neurons has not been observed in vivo (Levi-Montalcini et al., 1969; Angeletti et al., 1971; Schucker, 1972; Pannese, 1976), suggesting that it is not pathognomonic of NGF deprivation. Perhaps the in vivo environment provides interactions which limit neuronal lipid loading, such as hemotogenously derived phagocytic cells which may engulf excess neuronal lipid and other debris.

The fact that nuclei and nucleoli tended to remain normal until late stages of death suggests that they might be participating in RNA synthesis, perhaps directing the final stages of the death program. A minority of degenerating neurons (Fig. 5, a and c) showed an ultrastructural appearance consistent with the form of death known as "apoptosis" (Wyllie, 1980) which has been suggested as a common morphological motif of natural cell death.

It is difficult to interpret a precise longitudinal sequence of ultrastructural events which describes the death of NGF-deprived neurons because at any given time between 24 and 48 h after deprivation, all stages of neuronal death could be observed in at least some of the cells. Our impression is that each neuron undergoes a rapid demise over a period of an hour or so; each member of the population beginning at different times between 18 and 36 h (our unpublished results).

In summary, we have shown that RNA and protein synthesis are necessary for neuronal death caused by NGF deprivation. Several hypotheses can explain the prevention of neuronal death by cycloheximide and actinomycin-D. We favor the hypothesis that NGF represses a cascade of new protein synthesis from new mRNA which eventually results in the synthesis of proteins which kill the cell.

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