after medial preoptic lesions (*3, 7, 8, 14*). However, neither of these factors appears to be responsible for death, since there were no apparent differences in the degree of hyperthermia or hyperactivity between those rats that lived and those that died. Our data do not support the hypothesis that rats with preoptic lesions typically die of pulmonary edema. Autopsies and macroscopic inspection of lung tissue revealed signs of pulmonary edema only in one rat that died 2 hours after the lesion was made. This rat had the classical symptoms including pink frothy fluid exuding from its mouth and nostrils and enlarged lungs (*8, 15*). Although the lesion placement in this animal was indistinguishable from those of the others, lesions that produce pulmonary edema have generally been reported to lie more posteriorly than those produced here (*15*).

Medial preoptic lesions disrupt cardiovascular function, and moderate cold stress lessens this disruption. Although the mechanism of this protection is unclear, our results describe a simple method for keeping rats with large bilateral medial preoptic lesions alive and in good health while greatly impairing their ability to maintain normal body temperatures in the cold.

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9. Hooded rats from Blue Spruce Farms were housed in a colony room maintained at 25° ± 1°C on a 12:12 light-dark cycle, with free access to dry food (Wayne Lab Blox) and water.
10. Rats were injected with atropine sulfate (0.25 ml) and anesthetized with 3.0 to 4.0 ml of Equithesin per kilogram of body weight. Stainless steel insect pins (#00) insulated to within 0.5 mm of their tips were stereotaxically implanted 0.5 mm anterior to bregma, 0.75 mm lateral to the midsaggital suture, and 8.0 mm below the surface of the cortex (horizontal skull). A miniature thermistor (Veco 32A7) was attached to one of the electrodes 4 mm above its tip. Tail temperature was measured with a thermistor (Gulton L1547) implanted under the skin of the tail 5 cm from the base. The EMG and EKG were recorded from two loops of stainless steel wire sutured to the acromiotrapezius muscle (EMG) or to the sides of the rib cage (EKG). All leads were soldered to a connector (ITT Cannon, MDI-15SL1) cemented to the rat's skull.
11. Oxygen consumption was measured with an oxygen analyzer (Applied Electrochemistry, S-3A or Beckman OM-11). Values are reported at standard temperature and pressure.
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Retrograde Axonal Transport of Endogenous Proteins in Sciatic Nerve Demonstrated by Covalent Labeling in vivo

Abstract. *Extracellularly applied* N-*succinimidyl* [*2,3-*^{3}*H*]*propionate was used in vivo to covalently label intra-axonal proteins in the rat sciatic nerve. This technique permitted a unique view of axonal transport of proteins independent of biosynthesis. The proteins detected in slow anterograde transport (1 to 2 millimeters per day) correspond to cytoskeletal proteins described in previous reports. The slowly retrogradely transported component (3 to 6 millimeters per day) was composed primarily of a single protein with a molecular weight of 68,000.*

The movement of cytoplasm and intracellular particles in axons, referred to as axonal transport, is governed by elaborate mechanisms (*1*). The use of pulse-labeling (in which de novo synthesized proteins labeled by radioactive amino acids applied to the neuron perikarya are observed being transported intra-axonally) has provided a wealth of data about anterograde axonal transport (*1, 2*). This approach, however, cannot be applied to retrograde transport, because the distal axons and nerve terminals do not synthesize proteins.

The existence of retrograde transport has been established by direct microscopic visualization of intra-axonal particles (*3*), the use of exogenous macromolecular tracers (*4*), and ligation (*5, 6*). Attempts to use pulse-labeling in combination with the ligation approach has led to the hypothesis that the macromolecules are transported

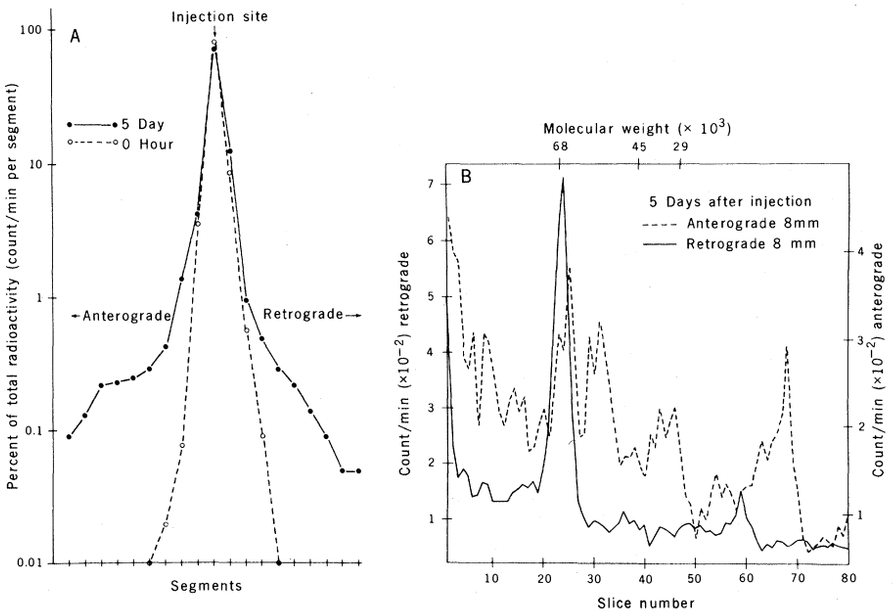

Fig. 1. Labeling of intra-axonal proteins that undergo axonal transport. (A) Distribution of TCA in 3-mm segments of nerve after injection of ^{3}H-*N*-SP into the sciatic nerve. Immediately after injection, virtually all the labeled protein was confined to the segment containing the injection site. At 5 days, significant labeled protein was found in segments greater than 20 mm proximal to and distal from the injection site. (B) Polyacrylamide gel electrophoresis of labeled proteins. Segments were homogenized in 0.1*N* HCl and, after TCA precipitation, were subjected to electrophoresis on 11 percent polyacrylamide gels in SDS. The gels were sliced into 1.5-mm slices, and the radioactivity of each slice was determined by liquid scintillation counting. The anterograde-transported proteins constitute a complex pattern including major peaks and complexes. In contrast, the retrograde transport shows only one major peak.

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similarly in anterograde and retrograde directions (*6*). We now report the use of a new approach in the study of intra-axonal movement of proteins, one well adapted to the study of the retrograde transport of endogenous proteins.

We covalently labeled intra-axonal proteins with radioactive agents (*7*) in a restricted zone of axons of the rat sciatic nerve. In this manner, a localized labeling of all the axoplasmic proteins is produced, independent of de novo protein biosynthesis, so that the bidirectional movement of labeled proteins away from the injected site can be followed. We used *N*-succinimidyl [2,3-^{3}H]propionate (^{3}H-*N*-SP) as the covalent labeling reagent since (i) it dissolves in aqueous solutions and reacts rapidly at physiologic *p*H, (ii) it dissolves in lipids and rapidly permeates all membranes, (iii) it labels intracellular proteins whether they are cytoplasmic or in particles, and (iv) it does not harm the cell's vital functions (*7*, *8*).

Female Sprague-Dawley rats, weighing between 350 and 400 g, were lightly anesthetized with ether. The sciatic nerve was exposed and 1 μl of saline containing 100 mCi of ^{3}H-*N*-SP (specific activity, 50 Ci/mmole, Amersham) was injected into the nerve over a period of 5 minutes, through a 29-gauge stainless steel hypodermic needle attached to a Harvard infusion pump. The animals were allowed to recover from anesthesia and, at invervals after the injection, were killed by decapitation. The nerve was removed from each and divided into 3-mm segments, which were each homogenized in 2 ml of 0.1*N* HCl. Radioactivity was determined from samples through the use of trichloroacetic acid (TCA) precipitation and the conventional filter paper method. Samples were prepared for electrophoresis by TCA precipitation; the precipitated pellets were washed twice with acetone and ether to remove the TCA; and the residue was dissolved in sodium dodecyl sulfate (SDS) and urea containing sample buffer before electrophoresis on 11 percent polyacrylamide gel in SDS with the buffer system described by Neville (*9*).

Immediately after injection, virtually all the labeled proteins were found in the 3-mm segment at the injection site (Fig. 1A). The amount of TCA-precipitable radioactivity on either side fell exponentially to background levels. By 1 day after injection, significant amounts of radioactively labeled proteins were found bidirectionally, substantially outside the initial injection site. A sample taken 5 days after injection is shown in Fig. 1A. Similar observations were made at times ranging from 2 to 45 days after the injection.

The intra-axonal location of the radioactivity from the injection site was determined by autoradiography. At 5 and 10 days after injection, the nerve was removed, fixed in Formalin, embedded in paraffin, sectioned at 7 μm, and processed for autoradiography (*10*). A low-magnification view of a nerve 5 days after injection is shown in Fig. 2A. At the injection site (asterisk), which in this nerve was confined to a distance of less than 1.5 mm, all the elements in the peripheral nerve were labeled. However, in both directions from the injection site, silver grains could be seen in a more defined linear pattern, extending to the most distant regions of the nerve sections (as far as 30 mm). Views of both longitudinal and cross sections at higher magnification (Fig. 2, B and C) show that the silver grains are primarily intra-axonal. Myelin, Schwann cell nuclei, and blood vessels in areas away from the injection site were not significantly labeled. The intra-axonal pattern of labeling was identical in both the retrograde and anterograde direction and at both 5 and 10 days after injection.

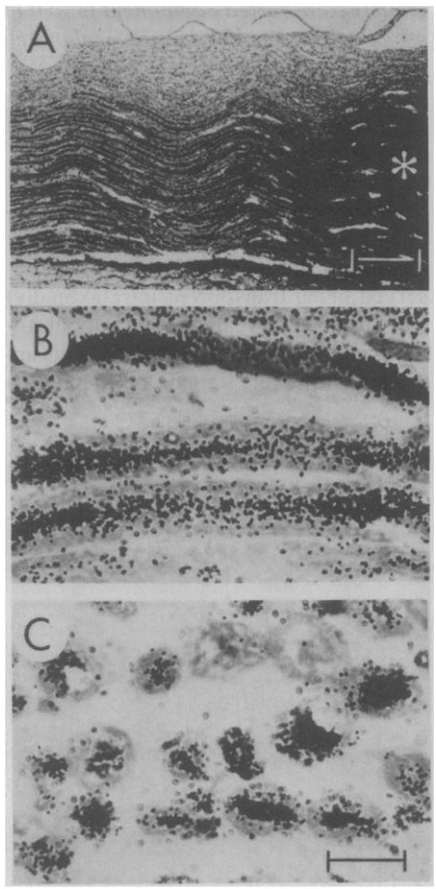

Fig. 2. Autoradiographs of injected nerves 5 days after ^{3}H-*N*-SP labeling. Nerves were fixed in Formalin, and processed for autoradiography. Myelin was stained with toluidine blue. (A) Low-power view. The injection site (asterisk) shows diffuse staining of all tissue components. Extending from the injection site, the silver grains form a linear pattern, consistent with their being restricted to axons. Scale, 100 μm. (B) Longitudinal section 5 days after injection, proximal to injection site. Silver grains in less densely labeled axon are confined to the axonal region, and the myelin is principally unlabeled. Scale as in (C). (C) Cross section 5 days after injection, distal to injection site. The majority of the silver grains are confined to the axons. Scale, 12 μm.

The SDS gel pattern of the transported proteins at 5 days is illustrated in Fig. 1B. The labeled proteins 8 mm in the anterograde direction from the injection site, corresponding to a transport rate of 1 to 2 mm per day, are complex but resemble those previously reported moving at this rate in the rat sciatic nerve (*11*). This pattern differs from the gel pattern of labeled proteins that remained at the injection site (*12*).

In sharp contrast, the nerve segment 8 mm proximal to the injection site (retrograde or toward the cell body) shows a much simpler pattern (Fig. 1B). There is only one major peak, at a molecular weight of about 68,000, and various other minor peaks. Similar patterns (not illustrated) were found in the nerve at positions from 8 to 20 mm proximal to (retrograde from) the injection site. The dramatically different patterns of labeled proteins moving in the anterograde and retrograde directions argues against diffusion as the mode of translocation. Furthermore, the fact that the proteins which moved distally are so similar to those found in the slow component by traditional pulse-labeling (*11*) indicates that normal slow axonal transport mechanisms were not disturbed by our labeling method (*13*).

This finding of a prominent 68,000 peak in the injection site (*12*) which is then transported slowly (3 to 6 mm per day) in the retrograde direction is the most significant observation of this study. A protein of similar size is abundant in neurofilament preparations from sciatic nerve (*14*). Investigators have recently found two distinct proteins with molecular weight 68,000 in that neurofilament preparation, one of which represents the neurofilament subunit; the other, 50 times more abundant, they proposed might be a serum albumin contaminant of the preparation (*15*). The equally abundant 68,000 protein we have observed was transported selectively in the retrograde direction and was located exclusively intra-axonally. It is possible that this intra-axonal protein may be related to serum albumin since serum proteins have recently been detected by immunocytochemical techniques in central neurons whose axons terminate outside the central nervous system (*16*).

We have shown that ^{3}H-*N*-SP when applied extracellularly in vivo can label intracellular proteins with no apparent deleterious effects on cell function. In addition to providing a unique view of axonal transport, ^{3}H-*N*-SP and other related agents (*7*, *8*) can be used as radioactive vital stains to investigate a variety of biological phenomena. This vital stain has the additional unique feature of potentially providing information about the nature of the proteins involved.

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7. Covalent protein labels have traditionally been used to distinguish between membrane proteins that face the cytoplasmic versus the extracellular surface [see K. L. Carraway, *Biochim. Biophys. Acta* **415**, 379 (1975) for a review]. In previous studies, we found that ^{125}I-labeled Bolton-Hunter reagent [*N*-succinimidyl-3-(4-hydroxy, 5-[^{125}I]iodophenyl propionate] effectively labeled all the proteins in isolated squid axoplasm [T. Yoshioka, H. C. Pant, I. Tasaki, J. Baumgold, G. Matsumoto, H. Gainer, *Biochim. Biophys. Acta* **538**, 616 (1978); H. C. Pant, S. Terrakawa, J. Baumgold, I. Tasaki, H. Gainer, *ibid.* **513**, 132 (1978)]. The intra-axonal (axoplasmic) proteins were labeled equally well when the reagent was applied extracellularly to intact axons with no deleterious effects on the action potential mechanism or general cellular vitality (H. C. Pant and H. Gainer, unpublished observations).
8. The ^{3}H-*N*-SP is one of a class of *N*-acylating reagents, which covalently attach to the free α- and ϵ-amino groups in proteins [H. Boyd, I. C. Calder, S. J. Leach, B. Milligan, *Int. J. Pept. Protein Res.* **4**, 109 (1972); S. J. Leach and H. Boyd, *ibid.* **5**, 239 (1973)]. This reagent reacts rapidly with protein substrates (half-time is 2 to 3 minutes) at physiologic *p*H and is soluble in both aqueous and organic solvents. It rapidly penetrates cell membranes and labels proteins bound to cytoplasmic and intracellular membranes equally well. Exposing intact rat posterior pituitaries to ^{3}H-*N*-SP labeled cytoskeletal proteins as well as intragranular neurosecretory proteins (neurophysins) [(D. Fink and H. Gainer, *Brain Res.* **177**, 208 (1979)]. The label spontaneously hydrolyzes to inactive products in aqueous solution in the absence of substrate. This hydrolysis is relatively slow (half-time about 90 minutes), and hence, the ^{3}H-*N*-SP is maintained in a small volume of toluene, which is added to the aqueous solution just before use. The residual toluene in the solution is then blown off by a stream of nitrogen.
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11. The labeling profile of the proteins (Fig. 1B) resembles the slow component of anterograde transport in its molecular weight distribution [R. J. Lasek and P. Hoffman, in *Cell Motility*, R. Goldman, T. Pollard, J. Rosenbaum, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1976), p. 1021; P. Hoffman and R. J. Lasek, *J. Cell Biol.* **66**, 351 (1975)]. The profile at 8 mm contains both slow-component a (Sc_a) and slow-component b (Sc_b) proteins. At later times after injection (for example, 10 days) and longer distances from the injection site, we have been able to resolve the Sc_a component (that is, the neurofilament triplet and tubulin) and the complex Sc_b component separately (D. Fink and H. Gainer, *J. Cell Biol.*, in press).
12. Immediately after injection of ^{3}H-*N*-SP, the injection site contained three prominent labeled proteins with molecular weights of 68,000, 27,000 and 16,000. The 27,000 and 16,000 peaks are identical in size to those of myelin proteins previously described in the rat sciatic nerve [S. Greenfield, S. Brostoff, E. H. Eylar, P. Morrell, *J. Neurochem.* **20**, 1207 (1973); S. Micko and W. Schlaepfer, *ibid.* **30**, 1044 (1978)]. From 2 to 45 days after labeling, the injection site still contained the 27,000 and 16,000 proteins, but the 68,000 protein was absent (D. J. Fink and H. Gainer, *J. Cell. Biol.*, in press) (see Fig. 1B for the location of the 68,000 peak after 5 days).
13. The ^{3}H-*N*-SP labeling procedure is not restricted to the study of slowly transported proteins. We have recently applied this approach to study fast axonal transport in the rat sciatic nerve and hypothalamo-neurophyophysial system.
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Substance P: Does It Produce Analgesia or Hyperalgesia?

Abstract. *In the hot plate test, substance P given intravenously at doses of 5 × 10^{-5} and 5 × 10^{-4} gram per kilogram caused analgesia, while lower doses caused hyperalgesia. The influence of substance P on nociception depended on the individual mouse's sensitivity to pain (control response latency). Analgesia was produced by substance P administered to mice with high sensitivity to thermic stimulation, whereas hyperalgesia occurred in mice whose control latencies were longer than normal. This result is interpreted as an indication that substance P is capable of normalizing responsiveness to pain and could be classified as a regulatory peptide.*

Substance P (SP) produces potent, long-lasting analgesic activity in mice as determined by the hot plate technique (*1*, *2*). Substance P was found to be a long-lasting analgesic also in the tail-flick test of rats to which it had been intracerebrally administered (*3*). Oehme *et al.* (*4*) reported, in contrast, that SP produced hyperalgesia. Frederickson *et al.* (*2*) have partly resolved those conflicting observations by showing that SP has a dual action on nociception; their studies indicated that SP in small doses produced analgesia in mice and that this analgesic effect was blocked by naloxone. With higher doses of SP the analgesic activity was lost and hyperalgesia occurred when the higher doses were given in combination with naloxone. Frederickson *et al.* (*2*) concluded that very small doses of SP probably released endorphins, while higher doses caused direct excitation of neuronal activity in nociceptive pathways. Our experiments suggest that the capability of SP to produce analgesia or hyperalgesia may depend also on the responsiveness of the animals to pain (as indicated by response latency) before the SP is administered.

We tested SP for its action on nociception by means of the hot plate procedure and acetic acid–induced writhing (*4*). ICR standard mice (18 to 22 g) were used. The temperature of the hot plate was controlled thermostatically at 57°C. A plexiglass cylinder (10.0 cm in height, 20.5 cm in inner diameter, and with open top) was used to confine the mice to a restricted area of the plate surface. Substance P was administered intraperitoneally or intravenously. Control mice were given isotonic saline. The time, in seconds, from first contact with the plate to first hind-paw licking was recorded as response latency. Control latencies were estimated 1 day before testing and also 15 minutes before SP treatment. The values 15 minutes before SP

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