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Slow Axonal Transport of Neurofilament Proteins: Impairment by β,β′-Iminodipropionitrile Administration

Abstract. *β,β′-Iminodipropionitrile (IDPN) administration prevented normal slow axonal transport of [^{35}S]methionine- or [^{3}H]leucine-labeled proteins in rat sciatic motor axons. Ultrastructural and electrophoretic studies showed that the neurofilament triplet proteins in particular were retained within the initial 5 millimeters of the axons, resulting in neurofilament-filled axonal swellings. Fast anterograde and retrograde axonal transport were not affected. The IDPN thus selectively impaired slow axonal transport. The neurofibrillary pathology in this model is the result of the defective slow transport of neurofilaments.*

The axon utilizes special systems of cytoplasmic motility to convey materials along its length. These axonal transport systems are generally distinguished, on the basis of direction and rate of movement, into fast, slow, and intermediate anterograde transport (conveying materials away from the cell body) and retrograde transport (carrying materials toward the cell body) (*1*). Neither the mechanisms of transport nor the relationships between these systems are fully defined. A unitary mechanism for all types of transport has been proposed in which the differences in rate are related to the proportion of time that various transported materials are associated with the transport mechanism (*2*). Alternatively, a mechanism for slow transport distinct from that for bidirectional rapid transport has been suggested (*3*).

Identification of selective effects of pharmacologic agents on the various transport systems provides one approach to further studies of the mechanisms and the interrelationships of the axonal transport systems. In this study, we have examined the effects on axonal transport of β,β′-iminodipropionitrile (IDPN). Previous studies (*4*, *5*) have shown that IDPN administration produces large neurofilament-filled swellings in the most proximal portion of the axon. Since neurofilaments are known to be carried by slow transport (*1*, *3*), this pathology suggested that IDPN might have an effect on slow transport. Our results show that IDPN selectively impairs slow axonal transport, without direct effects on fast or retrograde transport. This model is of special interest, since it represents the first disorder in which the pathogenesis of neurofibrillary pathology can be reconstructed.

Slow axonal transport was studied by injecting [^{3}H]leucine or [^{35}S]methionine into the lumbar ventral horns of Sprague-Dawley or Wistar rats (*6*). The animals were returned to their cages, and 1 to 8 weeks later they were killed. The sciatic nerves were rapidly removed and divided into 5-mm segments. These nerve segments were each homogenized manually in a mixture of sodium dodecyl sulfate, urea, and β-mercaptoethanol (*3*) and heated to 100°C for 4 minutes. After centrifugation, only a minute residuum remained undissolved, and essentially all the radioactivity in the segments was solubilized (*3*). To construct curves of the distribution of radioactivity along the nerve, an aliquot of each sample was counted by liquid scintillation techniques, and counts per minute for each segment were plotted against the position of the segment along the nerve (*3*, *6*).

In addition, to determine the pattern of migration of individual slowly transported proteins, portions of the samples were subjected to electrophoresis on polyacrylamide slab gels (*7*); the gels were then impregnated with 2,5-phenyloxazole and dried, and fluorograms were prepared by exposure of Kodak type RP x-ray film to the gels for 2 weeks to 4 months (*7*). [The x-ray film was preexposed to a measured light flash (*8*).] The resulting fluorograms revealed the relative amounts of individual labeled proteins in each segment of nerve.

The IDPN (Eastman Kodak, Rochester, N.Y.) was administered in one of two ways: by intraperitoneal injection of 1 or 2 g/kg, or by sustained exposure to 0.05 percent IDPN in the drinking water only (*9*). Because of the different means of administration, transport studies were performed on animals ranging from 3 to 12 months of age. Age-matched controls, purchased at the same time as experimental animals, were used in all studies.

In 14 normal animals the curves of slow transport were similar to those previously reported (*1*, *3*), with the major slow component peak moving down the nerve at 1.5 to 2 mm/day (in 200-g animals) (Fig. 1a). The fluorograms from these control animals (Fig. 2a) showed the three major groups of labeled proteins described by Hoffman and Lasek (*3*): actin (molecular weight, 46,000); proteins presumptively identified as tubulin (molecular weights, 53,000 and 57,000); and the neurofilament triplet proteins with estimated molecular weights of 68,000, 145,000, and 200,000 (*3*, *10*). In each of 11 normal rats, the rate of actin and tubulin migration ranged from 0.5 to 5 mm/day, with the density of label greatest in segments corresponding to rates of 1.0 to 3.5 mm/day (Fig. 2a). The neurofilament triplet proteins moved together at a more restricted range of rates of 1 to 2.5 mm/day, coinciding with the major slow component peak (Fig. 2a).

Similar studies were performed with rats injected with IDPN. In these studies IDPN was given either 1 to 2 days before or 1 to 2 days after microinjection of the labeled precursor into the spinal cord. Groups of animals were then killed 7, 14, or 21 days after labeling. At all times after labeling, the major slow transport peak failed to migrate beyond the initial 5 to 10 mm of the ventral roots (Fig. 1b). Gel fluorography (21 days after labeling) showed that movement of all the major slow component proteins was abnormal, with the neurofilament triplet proteins being the most strikingly affected (Fig. 2b). Most of the labeled neurofilament triplet proteins were retained in the initial 5 to 10 mm of the roots; only a small proportion were transported beyond this level. Following injection of IDPN, tubu-

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lin and actin transport was abnormally slow (Fig. 2b). The impairment of tubulin and actin transport was most dramatic in 14 animals exposed to IDPN for 2 to 9 months (Figs. 1c and 2c).

These results indicated that the transport of a large proportion of the slow component proteins, synthesized after and shortly before IDPN administration, was arrested within the proximal axon. In a second series of experiments we examined the effect of IDPN administration on slowly transported constituents already well en route down the axon, asking whether they continued to migrate or were arrested in transit. Four experimental animals were injected intraspinally with [^{35}S]methionine; after 5 weeks they were given IDPN (injection schedule). These animals were killed 3 weeks later (8 weeks after labeling). Two control animals were similarly labeled but received no IDPN. The radiometric curves and fluorograms showed that IDPN administration stopped the movement of a large proportion of the slow component at a position corresponding to that expected after 4 to 5 weeks of slow transport of label away from the spinal cord. Only a small proportion of neurofilament triplet proteins moved beyond this region; compared to the controls, a substantial amount of tubulin and actin was also retained in this region. Thus all along the axon IDPN administration arrested transport of a large proportion of the slow component. One implication of this finding is that sustained exposure to IDPN can be used to study the effects on the axon of a failure to renew axoplasmic constituents normally.

To determine whether IDPN selectively impaired slow transport or affected all modes of transport, fast and retrograde transport were next examined. Fast transport was studied by injecting the lumbar ventral horns with [^{35}S]methionine, then removing the nerves 4 to 5 hours later and plotting the distribution of radioactivity (*6*). Sciatic nerve bed temperature was continuously regulated to 37°C throughout these studies (*5*). In normal animals the front moved 392 ± 28 mm/day. In nine nerves from animals given IDPN (injection) 2 days before the transport studies, the rate was 396 ± 38 mm/day and the conformation of the curves was normal. As a measure of the normality of retrograde transport, the accumulation of intramuscularly injected ^{125}I-labeled tetanus toxin distal to a sciatic nerve ligature was studied. No difference was found between control animals and those with long-term exposure to IDPN (*11*).

On the basis of these results we can construct a model to explain the neurofibrillary pathology caused by IDPN intoxication. The slowly transported column of axoplasm is in large part halted in its transport down the axon by IDPN administration. The neurofilament proteins continue to be synthesized in the nerve cell body and enter the proximal axon. Since they cannot be transported more distally, there is an excessive accumulation within the proximal axon. The pathologic consequence of this transport defect is the formation of massive neurofilament-filled proximal axonal swellings. As initially described by Chou and Hartmann (*4*) and confirmed by our own histologic and ultrastructural studies, these swellings are detectable within a few days after injection of IDPN and progressively enlarge over the next month to become spherical swellings up to 100 μm in diameter. The swellings are filled with normal-appearing 10-nm neurofilaments arranged in whorled fascicles; microtubules, retaining their usual longitudinal orientation, extend through these swellings. The swellings extend into the initial 5 mm of the ventral root, but never

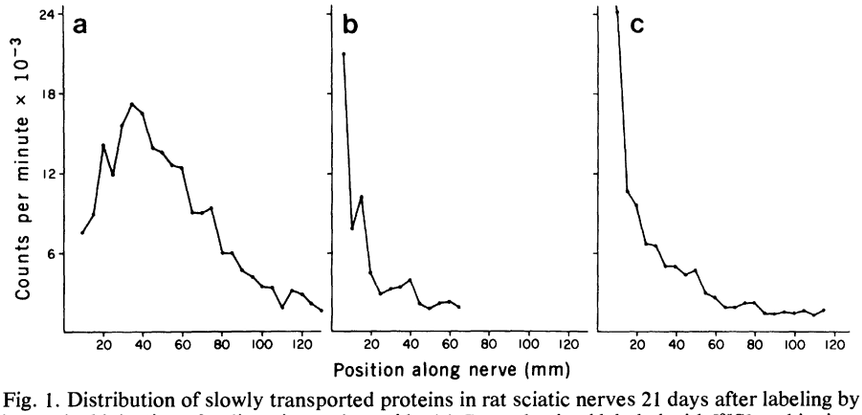

Fig. 1. Distribution of slowly transported proteins in rat sciatic nerves 21 days after labeling by intraspinal injection of radioactive amino acids. (a) Control animal labeled with [^{35}S]methionine. (b) Experimental animal that received IDPN (2 g/kg) intraperitoneally 1 day after labeling with [^{35}S]methionine. (c) Experimental animal that received 0.05 percent IDPN in drinking water for 9 months and was labeled with [^{3}H]leucine. Fluorograms from these nerves are shown in Fig. 2. The curves were obtained by plotting the counts per minute in sequential 5-mm nerve segments against the position of the segments along the nerve, with the origin of the L5 ventral root from the spinal cord taken as 0 mm. The major slow component peak [20 to 50 mm along the nerve in (a)] is retained in the initial nerve segments in the IDPN-intoxicated animals, indicating that proteins synthesized after (b) as well as before (c) IDPN administration failed to be transported.

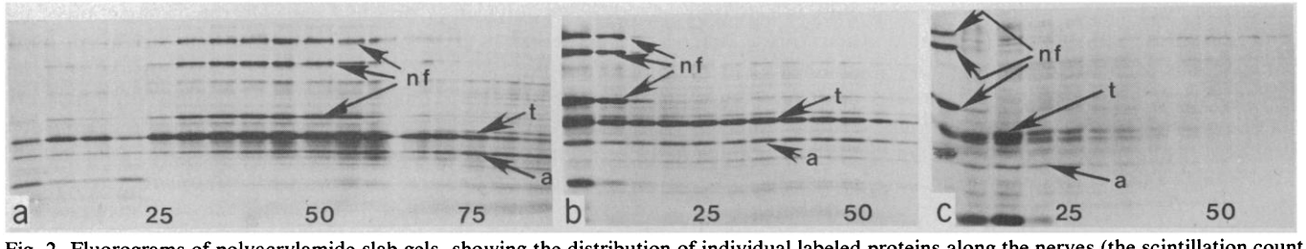

Fig. 2. Fluorograms of polyacrylamide slab gels, showing the distribution of individual labeled proteins along the nerves (the scintillation count curves for these nerves are shown in Fig. 1). Each track represents a 5-mm nerve segment, with the most proximal segment at the far left; the numbers (25, 50, or 75 mm) identify the distance of the segments along the nerve. (a) Control animal 21 days after labeling with [^{35}S]methionine; 5 to 17.5 percent gradient gel; exposure, 6 weeks. The neurofilament triplet proteins (*nf*) (68,000, 145,000, and 200,000 daltons) are present primarily in tracks corresponding to 25 to 50 mm (that is, they have moved 1 to 2.5 mm/day). Tubulin (*t*) and actin (*a*) are present over a wider spectrum of velocities (0.5 to 5 mm/day). (b) Experimental animal 21 days after intraspinal injection of [^{35}S]methionine and 20 days after injection of IDPN; 5 to 17.5 percent gradient gel; exposure, 6 weeks. The neurofilament triplet proteins are in large part retained in the initial two segments; transport of tubulin and actin is impaired as well, compared to control. (c) Experimental animal given IDPN in drinking water, 21 days after labeling with [^{3}H]leucine; 7.5 percent gel; exposure, 4 months. (In the first track an artifact in the x-ray film is present between the tubulin and actin bands.) The marked impairment in slow transport is apparent.

more distally. During this process, the nerve cell bodies appear normal.

An attractive hypothesis to explain the impairment of axonal transport produced by IDPN is a direct toxic effect on some element of the slow transport mechanism. Alternatively, IDPN could produce a postsynthetic alteration in the axonal cytoskeleton, rendering it incapable of transport. Such an alteration could affect neurofilaments primarily, with secondary effects on tubulin and actin transport. In this regard it may be instructive to study the effect of IDPN on slow transport in neurons containing relatively few neurofilaments, asking if transport of tubulin and actin is impaired. Our data can exclude two considerations: first, since slowly transported constituents synthesized before (as well as after) IDPN administration are arrested, the transport defect cannot be due to intrinsic abnormalities of the proteins acquired during their synthesis. Second, since the impairment of slow transport can be detected shortly after injection of IDPN, it cannot be due to preexisting structural pathology of the axon.

The neurofibrillary pathology in IDPN intoxication appears to result directly from the abnormality in neurofilament transport. Focal accumulation of neurofilaments is a prominent pathologic feature of a number of other clinical and experimental disorders. Neurofilamentous swellings in proximal axons, similar to those induced by IDPN, have been found in human amyotrophic lateral sclerosis (*12*) and in hereditary canine spinal muscular atrophy in Brittany spaniels (*13*). Neurofilamentous swellings in the distal axon are found in a variety of "dying back" neuropathies, including a childhood neuropathy in man and the human and experimental toxic neuropathies induced by hexacarbons, acrylamide, and carbon disulfide [for a review, see (*14*)]. While the mechanisms underlying these changes may vary, IDPN intoxication represents the first model in which the pathogenesis of neurofibrillary changes can be reconstructed, and raises the possibility of abnormalities in slow axonal transport in other disorders.

Finally, this pathologic process provides information pertaining to the biology of axonal transport. First, the ultrastructural and biochemical correlation between the accumulation of neurofilaments in the proximal axon and accumulation of the 68,000-, 145,000-, and 200,000-dalton proteins in the same region strongly supports the identification of these proteins as components of neurofilaments. This identification has been based on inference from previous axonal transport studies (*3*) and (in the case of the 68,000-dalton protein) by ultrastructural and immunological approaches (*10*). Second, our observation that IDPN selectively impairs the transport of the neurofilament proteins, as well as actin and tubulin, lends support to the concept that these cytoskeletal proteins share a common transport system that is separate from that of fast and retrograde axonal transport.

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10. The association of the 68,000-dalton proteins with neurofilaments has been confirmed by immunologic and electron microscopy studies [W. W. Schlaepfer, *J. Cell Biol.* **74**, 226 (1977); ——— and R. G. Lynch, *ibid.*, p. 241]. Whether the higher molecular weight proteins are integral components of the neurofilament or are neurofilament-associated proteins remains to be determined.
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Temperature Sensitivity: A Cell Character Determined by Obligate Endosymbionts in Amoebas

Abstract. *A strain of* Amoeba proteus *has lost its ability to survive at temperatures above 26°C as a result of becoming dependent on endosymbiotic bacteria that are psychrophile-like. The observed temperature sensitivity develops in fewer than 200 host cell generations (18 months of culture) after the host cells are experimentally infected with the symbionts.*

We previously reported a case of newly established bacterial endosymbiosis in amoebas, where initially harmful bacterial parasites changed to stable symbionts that are now required by the hosts for their survival (*1*). The establishment of the mutually dependent symbiosis took several years during which the harmful effect of infection gradually diminished and the hosts became dependent on the symbionts. The hosts' dependence was first demonstrated by transplanting the nuclei of symbiotic amoebas into enucleated cytoplasm of the nonsymbiotic counterparts (*1*). Such nucleocytoplasmic hybrids were nonviable unless live bacteria obtained from symbiotic amoebas were transferred into the hybrid cells. Later, the hosts' dependence was also demonstrated by removing the symbionts with chloramphenicol (*2*) or trimethoprim (*3*). Symbiotic amoebas died after losing their symbionts at concentrations of these drugs which were harmless to nonsymbiotic amoebas. The strains of symbiotic and

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