CLINICAL AND EXPERIMENTAL STUDIES OF DISTAL AXONOPATHY—A FREQUENT FORM OF BRAIN AND NERVE DAMAGE PRODUCED BY ENVIRONMENTAL CHEMICAL HAZARDS*

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INTRODUCTION

This presentation will summarize our clinical and pathological studies of the nervous system degeneration (distal dying-back axonopathy) in man and experimental animals produced by acrylamide monomer and certain hydrocarbon compounds (hexacarbons). "Distal axonopathy" is a term recently introduced to describe those diseases which are expressed as symmetrical, distal, axonal degeneration occurring concurrently in the peripheral nervous system (PNS) and in selected tracts of the central nervous system (CNS).[1] Most of the humans and experimental animals with these conditions initially appear to have peripheral neuropathy. The human distal axonopathies include: (1) many of the naturally occurring, genetically determined, system disorders (spino-cerebellar degenerations), (2) certain nutritional disorders (thiamine deficiency), (3) uremic neuropathy, (4) the neuropathies associated with some malignancies (multiple myeloma), and (5) the toxic neuropathies induced by industrial chemicals (acrylamide, *n*-hexane, cresyl phosphate, arsenic, carbon disulfide), and pharmaceutical agents (nitrofuradantoin, isoniazid). Indeed, it now seems likely that distal axonopathy constitutes one of the fundamental and most common histopathological reactions of the mammallian nervous system to exogenous toxins.[2]

In 1972 we began our experimental animal studies of "dying-back" peripheral nerve degeneration produced by acrylamide.[3] At that time, the dying-back hypothesis was a generally accepted concept in neuropathology: in brief, the idea suggested that a toxic derangement of neuronal metabolism would cause the nerve cell body to withdraw metabolic support from its axonal process so that degeneration would commence in the nerve terminal and, as neuronal metabolism became progressively impaired axonal degeneration would move in a seriate, retrograde (dying-back) fashion towards the cell body. If the intoxication was stopped it was assumed that axonal regeneration would commence and the animal (or human) would eventually recover. Neurons with the longest and largest diameter axons were thought to be most vulnerable to dying-back degeneration since they supported the greatest metabolic load.

The scope of our investigations broadened considerably in succeeding years leading to: (1) a reappraisal of the dying-back hypothesis, (2) a recognition of

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irreversible, subclinical and clinical effects of distal axonopathies on the human central nervous system, (3) a morphological rationale for the wide variety of previously enigmatic clinical phenomena in the human toxic neuropathies and, (4) evidence of the usefulness of neuropathology in the screening of chemicals for neurotoxicity.

Experimental Studies of the PNS

Acrylamide

Our initial studies of acrylamide-induced PNS axonal degeneration were based on the commonly-held assumption that dying-back nerve fiber degeneration would begin in the nerve terminals and proceed in a seriate fashion up the fibers of the longest nerve (sciatic). Accordingly, we focused on the hindpaw of the cat where we examined the spatio-temporal pattern of axon degeneration in sensory terminals supplying pacinian corpuscles,[4] the equatorial zone of nearby muscle spindles, and adjacent motor nerve terminals supplying extra-fusal muscle fibers. Cats were daily injected with 10 mg/kg of an aqueous solution of acrylamide and tissue was obtained at stages of intoxication. Animals developed an unsteady, staggering gait and head tremor after two weeks. After 28 days the animals were unable to stand but did not display foot drop. Animals underwent whole body perfusion with glutaraldehyde; tissue was embedded in epoxy resin and examined by light and electron microscopy. Histopathological examination revealed that the most sensitive structure was the 7–11 μm axon of the pacinian corpuscle which began to degenerate shortly before the larger, 11–20 μm axon supplying annulospiral muscle spindle terminals. Both sensory endings were more vulnerable than the juxtaposed motor nerve terminals. Both Pacinian corpuscle terminals in the forepaw began to degenerate at the same time as those in the hindpaw, despite the one-third greater length of axons supplying the hindpaw. In addition, by exploring axonal regions proximal to the nerve terminals of pacinian corpuscles and muscle spindles, it was apparent that degeneration here sometimes preceded onset of terminal degeneration. Thus, these studies with acrylamide directly challenged two of the basic tenets of the dying-back hypothesis, namely that axonal length and diameter dictated vulnerability and that degeneration began at the axon terminal before proceeding proximally.[3]

Hexacarbons

The hexacarbon studies were undertaken after completion of the initial acrylamide investigation in order to evaluate further the dying-back process in the PNS. The decision to use hexacarbon intoxication as an experimental model was dictated by several factors: one was the report that workers with prolonged exposure to *n*-hexane[5] or methyl *n*-butyl ketone (MBK)[6] developed a symmetrical, distal, dying-back neuropathy; a second was the discovery that the water-soluble and readily administered compound 2,5-hexanedione was the probable neurotoxic metabolite of both *n*-hexane and MBK,[7-8] and a third was the histopathological observation, in the hexacarbon neuropathies, of paranodal giant axonal swellings caused by accumulations of large numbers of 10 nm neurofilaments.[9] The swollen axons were easily visible in teased fiber preparations with the light microscope (Figure 1). This technical advantage allowed us to address the issue of the site of the initial histopathological events in the dying-back degeneration evoked by hexacarbons in experimental animals. Our *in vivo* studies with the hexacarbons (MBK, *n*-hexane and 2,5-hexanedione) were conducted in cats and rats, and employed various routes of administration depending on the physical properties of the compound.[8,10-15] Intoxicated cats and rats lost weight, then

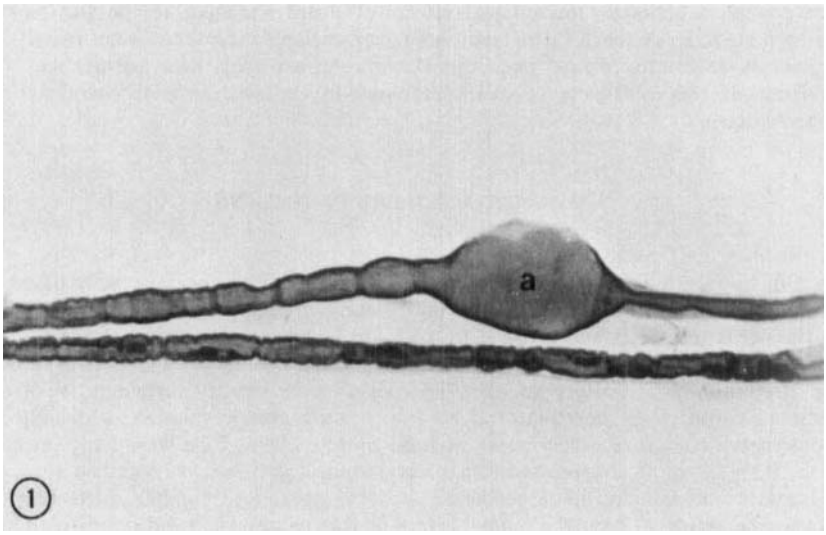

FIGURE 1. Single myelinated fibers dissected from posterior tibial nerve of a rat intoxicated with *n*-hexane by inhalation. Note the focal internodal axonal swelling (*a*) in the top fiber. Fibers were teased in epoxy resin.

developed an unsteady gait from hindlimb weakness, followed by hindlimb foot drop (FIGURE 2). With continued intoxication, forelimb distal weakness appeared and, after very prolonged intoxication, animals became quadriparetic and were unable to sit or stand. At no stage did they display truncal ataxia or head tremor, clinical

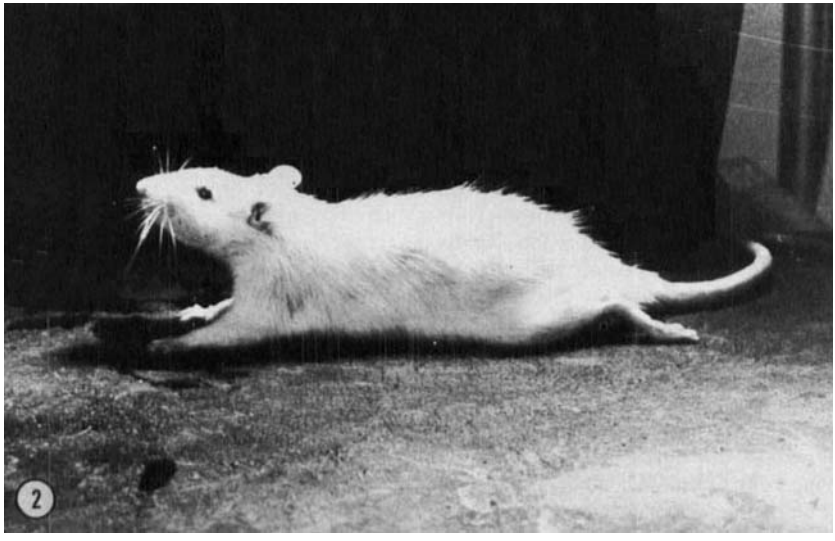

FIGURE 2. Rat with severe hindlimb weakness and foot-drop after 130 days of inhalation of *n*-hexane.

features which had been prominent in cats intoxicated with acrylamide. Histopathological studies, performed after whole body perfusion and the same tissue processing as for acrylamide animals, revealed that the large myelinated fibers of the tibial nerve branches to the calf muscles displayed paranodal giant axonal degeneration (FIGURE 3) before similar changes were evident in the more distal plantar nerve fibers in the hindpaw (FIGURE 4). The nerves to the calf muscles contain the largest diameter myelinated axons in the hindlimb, and their special sensitivity to hexacarbons suggested that axon diameter might play a major role in determining vulnerability. The importance of axonal length was also apparent because the hindlimbs containing long nerves, were consistently involved before forelimbs, supplied with shorter nerves. Surprisingly, regenerating fibers were also seen scattered among the degenerating axons during the course of intoxication. Another important observation in the

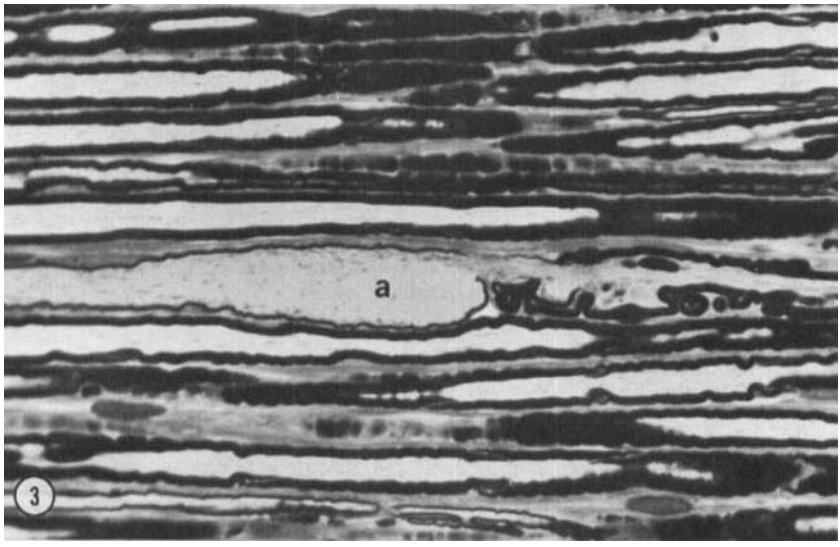

FIGURE 3. Longitudinal section of tibial nerve of a rat intoxicated with 2,5-hexanedione. Note the paranodal giant axonal swelling (*a*) associated with an abnormally thin myelin sheath. One micrometer epoxy section stained with toluidine blue, ×480.

hexacarbon studies was that degeneration did not begin in the terminal and travel steadily up the nerve fiber, but rather began in a *multifocal* pattern on the proximal sides of multiple nodes of Ranvier in distal nerve fibers. This process was analyzed further in single living PNS fibers undergoing an analogous pattern of distal axonal degeneration in organotypic CNS-PNS-muscle combination tissue cultures exposed to 2,5-hexanedione dissolved in the nutrient fluid. It was found that giant axonal swellings developed on the proximal side of the node of Ranvier at the very end of the parent fiber, but above the thinner diameter terminal branches supplying the muscle fibers.[1] These *in vivo* and *in vitro* findings confirmed our earlier observations in acrylamide animals, namely, that the axonal degeneration in dying-back neuropathies did not begin at the terminal and progress towards the cell body in a seriate fashion, as the term "dying-back" had implied. The controversy concerning the pathobiology of neurotoxic axonal degeneration has been recently reviewed.[16]

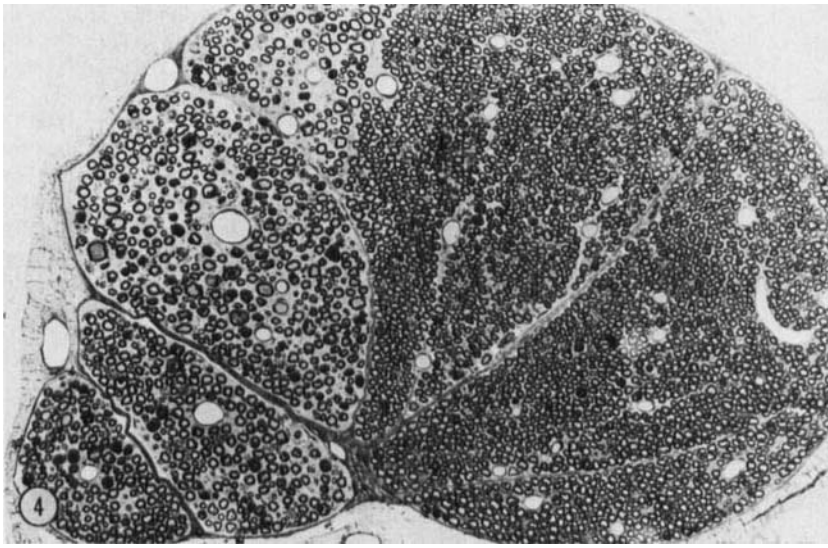

FIGURE 4. Transverse section of the tibial nerve taken from the upper popliteal fossa of a rat intoxicated with 2,5-hexanedione. The large, normal-appearing fascicles on the right constitute the main body of the tibial nerve bound for the foot. The small fascicles on the left, bound for the calf muscles, contain swollen axons, some fiber loss, and variable endoneurial edema. One micrometer epoxy section stained with toluidine blue, ×40.

We therefore concluded that "dying-back" was an inaccurate and misleading term to describe the pathological process, and suggested that the designation "central-peripheral distal axonopathy" was more appropriate.[1] The need for this somewhat cumbersome term was emphasized by the fact that for many clinicians and neuropathologists, the appellation "dying-back" had become synonymous with *peripheral* neuropathy, despite the demonstration by Cavanagh[17] and others that varying degrees of CNS degeneration sometimes accompanied the peripheral changes. The need to give equal emphasis to the central and peripheral nervous system became self-evident when we demonstrated widespread distal axonal degeneration in the CNS occurring concurrently with similar changes in the PNS.

EXPERIMENTAL STUDIES OF THE CNS

Intoxication with acrylamide and hexacarbons

In contrast to the PNS changes, the distribution of axonal accumulations of neurofilaments produced by these two compounds appeared identical.[13] A striking and consistent finding in the hexacarbon neuropathies was the development of axonal degeneration in certain areas of the central nervous system at the same time changes were first noted in the most vulnerable areas of the PNS. In general, these changes were found in the distal regions of long spinal nerve tracts and, later, in shorter CNS pathways. As in the PNS, tractal degeneration appeared to spread toward the neuron cell body with continued intoxication. The distribution of the changes in *asymptomatic*, hexacarbon-intoxicated animals showed that the rostral regions of long, ascending sensory tracts (gracile fasiculus, dorsal spinocerebellar) and caudal regions of long, descending motor pathways (corticospinal) were first affected. The sensory pathways

were more sensitive than the descending fibers, and the gracile nucleus (FIGURE 5) and mossy fibers and white matter of the anterior cerebellar vermis were the first CNS structures to display axonal swellings (usually concurrent with the first enlarged axons observed in the most vulnerable PNS fibers, the tibial nerve branches to the calf muscles). In animals with more prolonged intoxication and *moderate* clinical impairment these same areas displayed an advanced stage of axonal degeneration accompanied by breakdown of the myelin sheath and a mild astrocytic proliferation. At this time, axonal swellings were present in more proximal regions of these tracts (as the axonopathy progressed towards their cells of origin) as well as in the preterminal and terminal axons scattered in the spinal gray matter from levels C_4 through S_3. In cats with very *prolonged intoxication* and severe weakness in all extremities, axonal swellings were present in the distal optic tract, superior colliculus, lateral geniculate body and mammillary bodies. In these animals, the original sites of CNS axonal swelling displayed marked and permanent axonal loss with glial replacement (FIGURE 6). These studies confirmed many ideas about the dying-back process that had been drawn from earlier reports of the spatio-temporal pattern in the PNS. The distal regions of long and large CNS fibers were susceptible to degeneration and axon length appeared an important factor in determining vulnerability; this was suggested by the observation that the gracile tracts began to degenerate before the shorter cuneate tracts. Since the teasing technique was not practicable in the CNS, it was difficult to evaluate critically the evolution of disease as it affected single CNS fibers, although it can be stated that there was no reason to believe that the process differed markedly from that previously found in the PNS.

Among the important observations in the CNS studies relevant to the environmental health hazard—the theme of this symposium—was the axonal degeneration in the lateral geniculate and mammillary bodies.[14] From these findings it is possible that

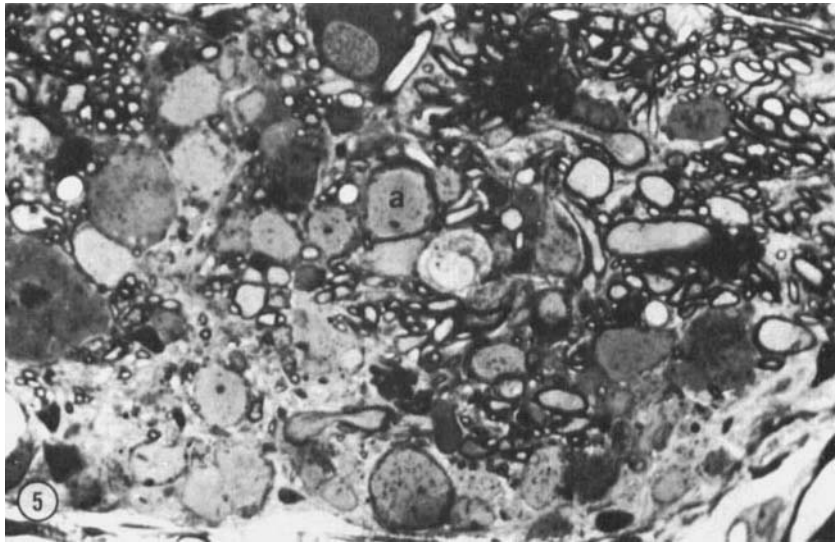

FIGURE 5. Transverse section from the most rostral gracile tract of a rat with early 2, 5-hexanedione axonopathy. Swollen axons (*a*) are prominent. Epoxy section stained with toluidine blue, ×112.

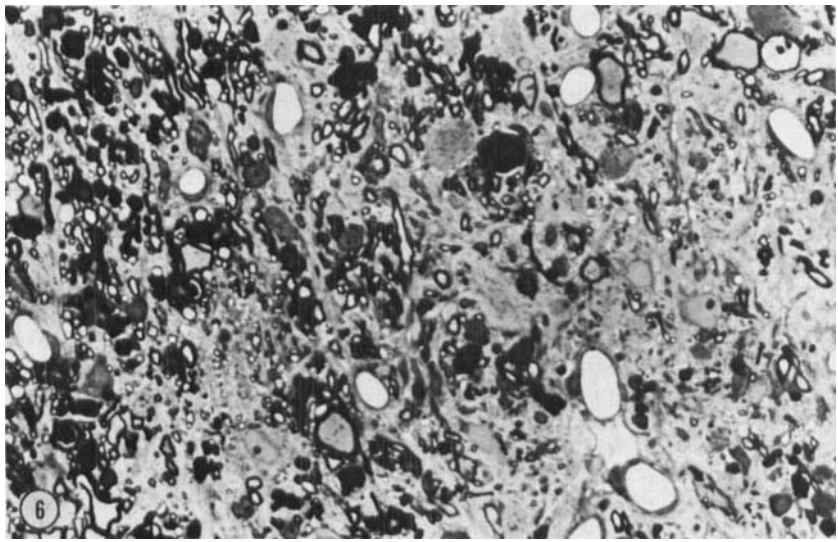

FIGURE 6. Transverse section from the rostral gracile tract of a cat in the late stage of 2, 5-hexanedione axonopathy. There is profound fiber loss, scattered myelin debris and moderate gliosis. Few axonal swellings are present, in contrast to Figure 5, ×104.

prolonged, low-level industrial and environmental exposure to *n*-hexane, acrylamide or other environmentally prominent compounds that evoke distal axonopathy (carbon disulfide, cresyl phosphate) may provoke subtle changes in areas of the nervous system which may be vital to memory and vision. This is of great significance to the general public since, as neuronal function and number decline in the course of the normal aging process, individuals exposed to such compounds might experience premature or accelerated deterioration in vision or mentation.

Recovery from hexacarbon intoxication

Several of the cats with very prolonged intoxication were allowed to recover for periods of up to two years. These animals gradually regained weight, became mobile and, after a year's recovery, appeared to have regained strength in all extremities. However, on attempting to walk, all animals manifested stiff-legged, unsteady hindlimb movements that resulted in a swaying motion of the pelvis. Attempts at running resulted in a reeling movement and the animals fell frequently to either side. Histopathological examination revealed that the gracile nuclei consistently displayed the most severe changes in these recovered animals, with a striking axonal loss, diffuse gliosis and loss of neurons. Many of the remaining neurons were undergoing trans-synaptic degeneration and appeared shrunken with dark-staining cytoplasm. In the lateral columns of the lumbar spinal cord there was moderate axonal loss accompanied by mild gliosis. The anterior horn cells and other lumbar neurons were unremarkable. There was also severe fiber loss in the ventral-medial tracts of the thoracic spinal cord. Foci of gliosis and fiber loss were prominent in the white matter of the anterior vermis of the cerebellum. The optic tracts, lateral geniculate body and mammillary bodies appeared unremarkable in every recovered animal. In the PNS of the recovered animals there was mild fiber loss in distal nerve twigs, compensated for by many

clusters of small-diameter, regenerating, myelinated axons. Muscle spindle primary endings and neuromuscular junction axon terminals appeared normal in gastrocnemius and hindfoot lumbrical muscles. Based on these data we concluded that (1) the degeneration in the corticospinal tracts and cerebellar vermis probably accounted for the residual spastic-ataxic gait in these animals, (2) the reinnervation of neuromuscular junction terminals correlated with the recovery of distal strength and muscle bulk, (3) the trans-synaptic degeneration of neurons in the gracile nucleus would eventually result in axonal loss in the medial lemniscus in a "chain degeneration" pattern.

The presence of this latter phenomenon in a toxic axonopathy strengthens the proposed relationship of these conditions to the human spino-cerebellar degenerations, where trans-synaptic chain degeneration has been suggested as responsible for fiber loss in CNS tracts not primarily involved in the disease process. The apparent, total disappearance of axon swellings, without axonal loss, in the visual nuclei and mammillary bodies, raises the exciting possibility that recovery from the initial stages of CNS axonal damage may occur. It is possible that the early axonal change, preterminal accumulation of neurofilaments and swelling (FIGURE 7), either may occur without irreversible disruption of the synaptic architecture and the axon can reconstitute itself; or that there was disruption of the synapse due to axonal degeneration, yet the axon was able to sprout and reestablish connection with the target neuron once intoxication had ceased.

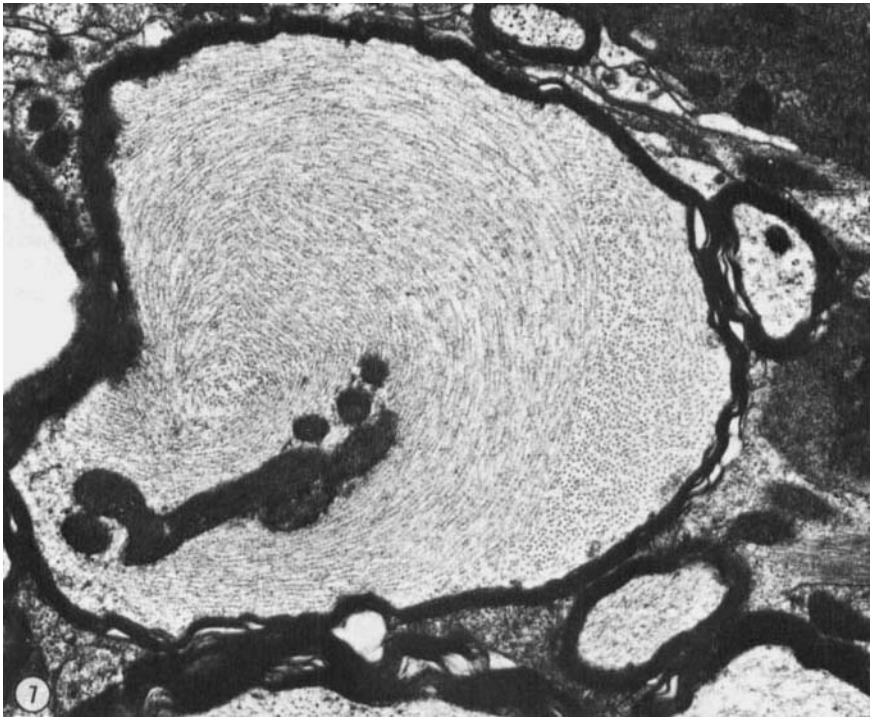

FIGURE 7. Transverse section of a CNS myelinated fiber in the anterior cerebellar vermis of a rat intoxicated with acrylamide. The axon is swollen and contains a massive, abnormal accumulation of 10 nm neurofilaments, ×11,100.

Studies of Humans with Acrylamide and Hexacarbon Intoxication

Acrylamide

We have studied seven patients with varying degrees of nervous system impairment following exposure to acrylamide monomer. The following presentation is typical of one of the moderately involved individuals:

This 45-year-old male was hired as a grouter and had had no previous exposure to acrylamide. He received daily high level skin exposure to acrylamide monomer, the principal component of the grouting compound. After three months he complained of fatigue and a seven pound weight loss, and also noticed that his hands were red and his skin peeling. By four months, he felt slightly unstable while walking and would occasionally lose balance when turning corners. Numbness of the feet and an aching pain in the calves developed by five months. He continued to work for another two weeks until his hands became numb, and his gait so unsteady that he was accused of drinking on the job. He had no urinary bladder dysfunction or impotence.

His palms were red, moist and peeling upon examination. Vital signs and general physical examination were normal. Neurological examination revealed no abnormalitites of cranial nerves or mental status. His speech was unremarkable and no nystagmus was present. Strength in the proximal upper extremities was normal but there was weakness, without wasting, of the intrinsic muscles of the hands and weakness of the wrist extensors (4/5 MRC scale). Neck, trunk, abdominal and proximal lower extremity muscles were of normal strength. There was weakness of dorsi-flexion at the ankles (4/5 MRC scale). The tendon reflexes in the arms were sluggish, and there was loss of knee and ankle jerks, and absent plantar responses. A mild loss of pin-prick sense was evident below the wrist and knees (glove-and-stocking distribution). Position sense was normal throughout. There was a striking loss of vibration sense (256 cps) over ankle, knee and wrist. Coordinated movements in the upper extremities were unaffected. There was no past-pointing or loss of check of rapid movement, but truncal ataxia was evident. Heel-to-knee-to-shin maneuvers were unsteady and poorly performed. His gait was broad-based and, on sudden turns, he reached for support. He was unable to stand with his feet together and eyes open without falling to either side. Motor nerve conduction in the medial and ulnar nerves was 55 meters per second (normal), and 35 meters per second (mild slowing) in the common peroneal nerve. Lumbar puncture revealed clear, acellular fluid with a normal protein value.

Six months later, motor and sensory examinations were normal except for moderate impairment of vibration sensation in the lower extremities. The tendon reflexes were two throughout except for absent Achilles reflex. His gait was narrow-based without staggering except when requested to make sudden turns. He could stand with the feet together and Romberg's sign was not present.

The patient illustrates many of the cardinal features usually associated with a mild-moderate degree of toxic axonal neuropathy: insidious onset, gradual progression, symmetrical distal (stocking-glove) motor and sensory loss, normal CSF protein and mild impairment in motor nerve conduction. Unusual features of this illness included the truncal and gait ataxia, and the severe loss of vibration sensation. Early, severe vibration sense loss, disproportionate to impairment of other sensory modalities or weakness, and early gait ataxia have been consistent features in our experience with human acrylamide intoxication and have been observed by others.[18–19] In extreme cases of acrylamide neurotoxicity, other clinical findings have been prominent, including transient impaired vision (especially depth perception) and impairment of recent memory without loss of other cognitive functions. Upon recovery, in the extreme cases, we have observed truncal ataxia, lower extremity spasticity, and residual recent memory loss.

Hexacarbon

We have studied 13 humans with *n*-hexane and two individuals with MBK intoxication. As with acrylamide, some of the cases had a limited exposure to the hexacarbon and only a mild distal, symmetrical, peripheral neuropathy from which they made a complete recovery. Three of these cases have been published elsewhere.[5] The following presentation is typical of one of the severely impaired individuals who was exposed to prolonged, high levels of *n*-hexane:

This 22-year-old female worked long hours in a small, poorly ventilated factory. She handled rags saturated with a solution containing *n*-hexane and inhaled large amounts of *n*-hexane vapor for two years before developing anorexia, weight loss, and a cramping sensation in the hands. Two weeks later she noticed cramping in the calves and an unsteady gait that was improved by the use of high-heeled boots. Two months later her legs felt much weaker and she noted a sensation of numbness of the toes. These symptoms steadily increased in intensity over the next two months until she was unable to walk to work, when she was admitted to a hospital. Neurological examination revealed no abnormalities of cranial nerves or mental status. There was diffuse, symmetrical, distal, flaccid weakness of all extremities. The intrinsic muscles of the hands and dorsiflexors of the feet were 2/5 MRC scale. Proximal limb muscles were 3/5 MRC scale, and abdominal muscles were 3/5 MRC scale. The vital capacity was normal. No tendon reflexes could be elicited. There was a moderate-to-severe loss of pin sensation in a stocking-glove distribution, with only a slight dimunition in position and vibration sense. After four months in this state she gradually began to improve, was discharged from the hospital, and steadily regained strength over the next 18 months. Since that time she noticed no further improvement of strength and began to walk again, but in an abnormal fashion. Neurological examination, two years after discharge from the hospital, revealed that she walked with a slow, stiff-legged, waddling gait, could stand on a narrow base and Romberg's sign was not present. Rapid alternating movements of the upper extremity were performed well, as were finger-to-nose movements. Distal and proximal upper extremity muscles were 4/5 MRC scale, distal lower extremity muscles were 4/5 MRC scale. Many large, proximal lower extremity muscle groups were 4/5 MRC scale (hamstrings, glutei, quadriceps). She was unable to rise from a chair without using her hands for support, and unable to sit up from a supine position without rolling to one side and using her upper extremities for assistance. There was a moderate dimunition of pin-prick and touch sensation below the ankle and wrist, but position and vibration sensation was normal. The tendon reflexes were 2 in the upper extremities and 3 in the lower extremities with bilateral Babinski signs present. There was increased resistance to rapid movement of the lower extremities with a spastic catch.

This patient also demonstrated many of the features usually associated with toxic axonal neuropathies. However, she developed a neuropathy of unusual severity with quadriparesis and near total paralysis of the distal extremities because of the prolonged, high-level exposure to the hexacarbon toxicant. The motor impairment was far more severe than the sensory loss. This has been a consistant feature, along with an early loss of the Achilles reflex, in the majority of our cases of *n*-hexane neuropathy. In our experience, other individuals with prolonged high-level exposure to *n*-hexane have had an even more devastating clinical course than illustrated in this case, eg. several have been quadriplegic for months, two have required transient respiratory assistance, two have been left with visual impairment, and one adolescent has experienced a general decline in initiative and intellectual ability. Several of our cases with extreme degrees of *n*-hexane distal axonopathy have, upon recovery, demonstrated lower extremity spasticity, but none has been incontinent and, in contrast to the acrylamide cases, none has had residual truncal ataxia. There has been a report of several

individuals with optic atrophy[20] and one case report of lower extremity hyperreflexia and a spastic catch following recovery from *n*-hexane intoxication.[9]

Clinical-Pathological Correlation in Human Neurotoxic Distal Axonopathy

A meaningful neuropathologic study of humans with toxic distal axonopathy is an extraordinarily difficult undertaking both because of technical limitations and because the disease moves in space with time. Therefore, in our elucidation of the clinical-pathological correlation in the human toxic distal axonopathies, we have depended heavily on morphological data obtained from animal experiments. Before presenting our rendition of this unusual veterinary exegesis of human clinical data to diverse medical and scientific audience, whose expertise is largely in areas other than neuropathology or neurology; it may be helpful to present a brief exposition of the limitations imposed by conventional neuropathological techniques in the study of humans with this disease process.

Conventional Neuropathology Limitations in the Study of Human Distal Axonopathy

Although distal axonopathy is one of the most common forms of human neurotoxic reaction, these diseases have largely remained clinical and pathological enigmas in man. It is extraordinarily difficult to perform significant morphological studies in living patients with distal axonopathy when one is limited to sampling small, isolated segments of accessible distal sensory peripheral nerves (usually the sural at the ankle). Although much can be learned from an individual nerve biopsy, if it is fortuitously performed in a zone of active degeneration and immediately fixed in glutaraldehyde and processed for teasing and EM; even under the best of circumstances, enough tissue can rarely be obtained for an analysis of the dynamic events taking place at various levels of the same fiber. Often the nerve biopsy is performed in an area where the initial degenerative events have long since taken place and moved more proximally, leaving a picture of end-stage axonal and myelin breakdown common to many disorders of nerve and of little diagnostic or academic value. An equally frustrating and confusing experience is to obtain a normal sural nerve biopsy in a patient in the early stages of toxic neuropathy. We had just such an experience with the patients reported in our initial study of human *n*-hexane neuropathy, whose sural nerve biopsies were normal.[5] Had we not detected pathological changes in nerve twigs in anterior tibial muscle biopsies in these individuals, it would have been impossible to identify any nerve fiber degeneration. In summary, it may be stated that to the diagnostic pathologist, the PNS in human distal axonopathy presents a difficult challenge because of the regional variations in pathology. Further, the PNS in human distal axonopathy presents a striking contrast to other organs such as the liver, where disease processes (cirrhosis, hepatitis) are often more diffuse and a diagnosis can be obtained by a needle biopsy performed in a readily accessible area.

A problem of greater magnitude is an analysis of the post-mortem CNS changes of individuals who die following toxic distal axonopathies. Fortunately, these are rarely lethal diseases and, except for cases of TOCP intoxication, there are very few well-documented histopathological studies of the human CNS in these conditions.[21] Since most deaths in the course of distal axonopathy occur at the late stages of the

disease or, more commonly, the individual dies many years after recovery; it is very difficult to learn much about the evolution of the process from the available human post-mortem material. Other limitations imposed by conventional post-mortem analysis are: the PNS and CNS structures vulnerable to distal axonopathy are rarely examined in a systematic fashion, the tissue is not fixed until many hours post-mortem, tissue is immersed in formaldehyde and then a light microscope analysis performed on paraffin-embedded tissue. To elucidate the limitations of conventional techniques we performed an experiment designed to compare the degree of CNS histopathological change that could be detected after whole body perfusion with glutaraldehyde and epoxy resin embedding to that found with formaldehyde-fixed tissue embedded and stained in paraffin. Four rats with severe neurological impairment from identical degrees of hexacarbon intoxication were used for this study. Two were perfused with glutaraldehyde, the tissue embedded in epoxy resin and one micron sections studied with the light microscope, and two were sacrificed by pentobarbital overdose and the nervous system promptly placed in formaldehyde, paraffin-embedded and stained with conventional neuropathological techniques. The perfused rats demonstrated abundant degeneration in the spinal cord, gracile nucleus, cerebellum, optic tracts, superior colliculus and mammillary bodies, in contrast to the formaldehyde-fixed tissue in which the only detectable abnormality was moderate axonal and myelin destruction in the gracile nucleus. Therefore, to perform a meaningful clinical-pathological correlation in the human distal axonopathies we have been obliged to extrapolate from findings in the relevant animal models discussed in the previous sections, and prudently relate these data to the clinical phenomena in humans with distal axonopathy.

Clinical-Pathological Correlation of Human and Experimental Neurotoxic Distal Axonopathy

(1) The early loss of Achilles reflex in human acrylamide neuropathy correlates with the demonstration of early axonal change in the primary terminals on muscle spindle fibers. A recent physiological study by Lowndes *et al.* which showed diminished ankle jerk and dynamic response to stretch of the soleus muscle spindle in acrylamide-intoxicated cats strongly supports this thesis.[22]

(2) The consistent early, severe vibratory sense loss in the acrylamide-exposed individuals correlates with the exquisite vulnerability of the feline pacinian corpuscle in acrylamide intoxication.

(3) The early loss of Achilles reflexes and onset of foot-drop in the *n*-hexane exposed individuals correlates with extreme vulnerability of large diameter tibial and peroneal nerve branches to calf muscles and anterior compartment muscles respectively.

(4) The prominent truncal and gait ataxia, a striking accompaniment of human acrylamide neuropathy reflects either (or both) the vulnerability of mossy fiber axons in the cerebellar vermis or the peripheral muscle spindle afferents as in (1).

(5) The lower extremity spasticity that emerges on recovery from *n*-hexane and acrylamide neuropathy correlates with the degeneration in the motor pathways in the lumbar spinal cord in the intoxicated cats.

(6) Permanent visual impairment, or transient visual loss, was occasionally observed in our severely impaired humans with high level, prolonged *n*-hexane and acrylamide intoxication, and has been erroneously referred to as "optic neuritis" in previous reports of human *n*-hexane intoxication.[20] The demonstration of axonal degeneration in the distal optic tract and lateral geniculate bodies of the hexacarbon

and acrylamide intoxicated animals provides a sound histological basis for these phenomena.

Two additional observations are relevant to the problem of human clinical manifestations of toxic distal axonopathies. One is: the common belief that all toxic axonal neuropathies present an *identical* stereotyped, distal, symmetrical stocking-glove sensory and motor loss is erroneous; consistent patterns of sensory modality loss and degree of motor impairment occur among these conditions, and these patterns correlate well with the experimental histopathology. Familiarity with the clinical profile of a given neurotoxin may, in the future, provide diagnostic clues to physicians evaluating such patients. It is possible that as more axonal neurotoxins are identified that other, more striking, deviations from this stereotyped picture will emerge.

The second observation is nuclear to the theme of this symposium: namely that the clinical manifestations of CNS degeneration in many toxic distal axonopathies are clinically obscured by the weakness and sensory loss due to the peripheral nerve changes. Hence, clinicians misleading refer to these conditions to as "toxic *neuropathies*." However, when the patient is removed from the toxic environment, the peripheral nerves commonly undergo near complete regeneration while the manifestations of CNS damage (especially spasticity) concurrently emerge. The importance of the unrecognized subclinical CNS changes that may occur in the visual system and the mammillary bodies of individuals with "toxic neuropathies" have already been discussed.

The Advantages of Experimental Neuropathology in Screening Potential Environmental Neurotoxins

The word *neurotoxin* conveys a variety of meanings. To the behaviorist, neurotoxins produce abnormal patterns of behavior, to the physiologist, a discrete functional impairment; to the pharmacologist, a metabolic alteration; to the neurologist, a clinical syndrome; and to the neuropathologist and neurochemist respectively, structural and biochemical alterations of the central and peripheral nervous systems. Each specialist advocates the techniques of his own discipline as a means of screening substances for potential neurotoxicity. This results in a bewildering variety of possible approaches, each of seemingly equal value to the outsider whether the individual is responsible to a regulatory agency, industry, labor or simply is an environmentally concerned citizen. Part of this confusion arises from the practice of using the word *neurotoxin* to describe compounds with widely differing pathophysiological actions. For example, the term includes the biological neurotoxins such as venoms, curare, tetanus and diphtheria toxins; pharmacological agents such as tranquilizers, narcotics, anesthetics, stimulants, antidepressants, hallucinogens and antimicrobials, and environmental pollutants such as heavy metals, pesticides, solvents, and a variety of other industrial chemicals. There is a paucity of information on the neurotoxic effects of most compounds and this lack of data has retarded the development of a systematic classification of neurotoxins. Once the basic mechanisms of neurotoxic injury have been determined and the vulnerable metabolic pathways elucidated, then, ideally, a substance could be submitted to a battery of *in vitro* physiological or biochemical tests that would determine its neurotoxic potency. Unfortunately, science is a long way from achieving that goal, and one is forced to rely on time-consuming, often inaccurate and expensive bioassays that employ varying combinations of behavioral, morphological and pharmacological techniques. The choice of technique is made even more complicated since many neurotoxins will produce an acute reversible pharmacological reaction as an immediate effect of high exposure and a structural, often

irreversible damage after chronic low-level exposure. For example, *n*-hexane produces a pharmacological and reversible CNS narcosis in rats at levels of 30,000 ppm administered for 60 minutes. By contrast, several hundred ppm administered continuously over a period of many weeks will precipitate a central-peripheral distal axonopathy. Another example is provided by the cresyl phosphates: after acute dosing, these compounds produce a striking, reversible neuromuscular blockade because of their anticholinesterase properties. However, the well-known delayed neurotoxic effect produced by these compounds is entirely unrelated to inactivation of this enzyme. The delayed neurotoxic effect is, in fact, a distal axonopathy characterized by striking pathological changes in the spinal cord tracts and peripheral nerves.[17] Another peril of bioassay screening, that of species variation, is well illustrated by the cresyl phosphates. It is almost impossible to produce distal axonopathy in a rat with triorthocresyl phosphate, except after very prolonged exposure, whereas the cat will develop axonal degeneration that can be detected within days of a single injection.

A current popular approach in screening a compound for potential neurotoxicity is the study of behavioral alteration (often utilizing operant conditioning) following exposure of an animal to the suspected neurotoxin. This method of testing is currently under consideration by many federal regulatory agencies, academic toxicology centers and commercial laboratories. These techniques are of value in assessing substances whose neurotoxic properties exert a purely pharmacological effect on the nervous system.

It has been our experience with both experimental acrylamide and hexacarbon intoxication that it is possible to detect discrete changes in the nerve fiber axons of the vulnerable tibial nerve branches before any change in locomotion appears. These histopathological observations have been made possible by utilizing either whole body glutaraldehyde perfusion, or by simply biopsying the vulnerable PNS tissue followed by glutaraldehyde fixation, further processing and eventually embedding the tissue in epoxy resin. Such methods, although originally developed 20 years ago for electron microscopy, readily permit the observation of minute pathological changes, can be entirely performed by unsupervised technicians, are routine in most academic centers, and, most important, yield an enormous gain in sensitivity over the conventional histological techniques employed by most commercial and industrial testing facilities. Other major advantages are that the perfusion process optimally fixes all organs for sensitive light microscope assay, and that the same tissues are immediately available for electron microscope examination, should this be necessary. A demonstration of the tremendous advantage of this simple technique over conventional autopsy immersion fixation was described in the previous section, where conventional histopathological techniques failed to reveal advanced degrees of axonal pathology in the CNS of intoxicated animals. The use of outmoded conventional morphological techniques is so pervasive and well-entrenched, that the U.S. Environmental Protection Agency has recommended that the "demyelination" assay for neurotoxicity in fowl be continued in the testing of pesticides for neurotoxic properties.[23] We question the wisdom of this decision for several reasons: neurotoxicity screening tests relevant to man should optimally be conducted in mammals, and the histopathological procedure employed is not capable of detecting the early primary axonal changes which result in the myelin breakdown; thus the name "demyelination" is a misnomer for this type of reaction.

In addition to the distal axonopathies, the subject of this paper, these sensitive, perfusion-based techniques can be readily applied to other types of pathology produced by neurotoxins, such as those primarily affecting myelin (myelinopathies), the neuron (neuronopathies) or blood vessels (the vasculopathies). More detailed information on the general aspects of these entities is available in recent reviews.[2, 24]

Several points require emphasis in designing neurotoxicity tests for the detection

of neurotoxic diseases such as distal axonopathies. Firstly, it should be understood that the nervous system changes are a response to systemic intoxication irrespective of the route of administration of the toxic compound. The causation of axonal damage is not understood, but is generally assumed to result from one or more discrete metabolic neuronal lesions produced by the neurotoxic agent.[16] Secondly, there is a steep dose-response curve for a neurotoxic chemical which produces structural change. Thus, once the appropriate species, level, and duration of intoxication have been established, virtually 100 percent of animals will display nervous system damage. A third principle underlying neurotoxicity testing is the need for sub-acute or chronic testing protocols if they are to have environmental significance.

It has not been determined with certainty that morphological assays are more sensitive than behavioral testing for detecting the earliest certain signs of neurotoxicity in the distal axonopathies. Since morphologically and biochemically biased centers such as the Albert Einstein Neurotoxicology Unit have only employed gross observations of behavior in intoxicated animals, and centers with strong investment in behavioral testing, such as the National Institute of Environmental Health Sciences, have depended heavily on behavioral tests for neurotoxicity sensitivity there has not been an opportunity to assess the relative of the two techniques. Therefore our Neurotoxicology Unit and the N.I.E.H.S. have recently begun a collaborative study of behavioral analysis and a simultaneous morphological assay of the nervous system at various stages of intoxication in acrylamide-treated rats. From this long overdue collaborative effort, there should emerge a clear idea of the relative uses and merits of these techniques in detecting evidence of neurotoxicity in the distal axonopathies.

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