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STUDIES ON THE MECHANISM OF ACTION OF 2,5-HEXANEDIONE NEUROTOXICITY

The Ohio State University

Ph.D.

1979

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STUDIES ON THE MECHANISM OF
ACTION OF 2,5-HEXANEDIONE NEUROTOXICITY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Joseph Price Nachtman, B.S., M.S.

* * * * * *

The Ohio State University
1979

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J. Bianchine
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D. Wiechers

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Adviser
Department of Pharmacology
I would like to thank my advisor, Dr. Daniel Couri, whose example and guidance encouraged me to return to graduate school. Dr. Couri was always helpful and allowed me the opportunity to pursue many avenues of approach in my research projects. His enthusiasm will serve as a model for my professional life.

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Presented at the 1978 American Industrial Hygiene Conference, Los
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Drug Metabolism - Drs. D. Couri and D. Feller
Radioisotope Methodology - Drs. D. Feller and L. Malspeis
Immunology - Dr. R. Lang
Physiology - Dr. S. Strauch
Biochemical Neuropathology - Drs. A. Yates and L. Horrocks
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TABLE OF CONTENTS

ACKNOWLEDGMENTS ii
VITA iii
LIST OF TABLES viii
LIST OF FIGURES ix
LIST OF ABBREVIATIONS AND SYMBOLS x
LIST OF REAGENTS xii

INTRODUCTION
A. General Properties of Hexacarbons 1
B. Toxicity of Methyl Butyl Ketone and Metabolites 2
C. Axoplasmic Transport 6
D. Microtubule Structure and Function 9
E. Biochemical Bases of Peripheral Nerve Pathology 12
F. Membrane Microviscosity Studies 16
G. Electrical Properties of Nerve Cell Membranes 20
H. Electromyography of Peripheral Neuropathy 21

STATEMENT OF PURPOSE 24

METHODS
A. Animal Model 26
B. Colchicine Binding Assay 26
C. Separation of Microtubule Monomer from Polymer 27
D. Characterization of Microtubule Binding Protein 30
E. Polyacrylamide Gel Electrophoresis 32
F. Viscosity Measurements 33
G. Membrane
   1. Myelin Preparation 34
   2. Red Blood Cell Ghost Preparation 37
H. Protein Assay 38
I. Phospholipid Methods 39
   1. Extraction and Purification
   2. Isolation of Lipid Classes
   3. Cholesterol Method
   4. Cerebroside Method
   5. Phospholipase A Assay
   6. Gottfried Phosphorous and Cerenkov counting of $^{32}$P
J. Nerve Conduction Velocity of Rat Sciatic Nerve 46

RESULTS
A. Microtubules and HDO Toxicity 50
B. Microviscosity Data for HDO Neuropathy 56
C. Effects of Nerve Section on Myelin Microviscosity 61
D. Fluorescence Polarization of Red Cell Ghosts 64
E. Hexanedione Effects on Recorded EMG 67
F. Acute Effects of HDO on Electromyography 75
G. Recovery of Nerve Conduction Velocity from Chronic HDO Treatment 81
H. Oxygen Uptake of Sciatic Nerve 86

DISCUSSION
A. Microtubules and HDO Toxicity 94
B. Microviscosity Data for HDO Neuropathy 95
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thin Layer Chromatography of Lipids</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>Distribution of Polymeric Versus Monomeric Tubulin in Rat Sciatic Nerve and Brain Treated with 2,5-Hexanedione</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>Colchicine Binding of Fractions of 0.5% Sciatic Nerve to Control Homogenate</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>Oxygen Uptake of Sciatic Nerve</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td>Phospholipase A Activity in Normal and HDO Treated Rats</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>Sciatic Nerve Phospholipid Content</td>
<td>90</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
</tr>
<tr>
<td>4</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>57</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
</tr>
<tr>
<td>7</td>
<td>62</td>
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<td>8</td>
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<td>13</td>
<td>78</td>
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<tr>
<td>14</td>
<td>82</td>
</tr>
<tr>
<td>15</td>
<td>84</td>
</tr>
<tr>
<td>16</td>
<td>92</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>ACH</td>
<td>Acetyl choline</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>Deuterium oxide</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>Diethyl amino ethyl cellulose</td>
</tr>
<tr>
<td>Distal NCV</td>
<td>Average Sciatic Nerve Conduction; velocity between popliteal space and plantar tendon</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPH</td>
<td>1,6-Diphenyl-1,3,5-hexatriene</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyogram</td>
</tr>
<tr>
<td>FAP</td>
<td>Fast Axoplasmic Transport</td>
</tr>
<tr>
<td>GM$_2$</td>
<td>Tay Sachs ganglioside</td>
</tr>
<tr>
<td>HDO</td>
<td>2,5-Hexanedione</td>
</tr>
<tr>
<td>I$_{II}$</td>
<td>Fluorescent Intensity with Polarization parallel to incident beam.</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>I</td>
<td>Fluorescent Intensity with Polarization perpendicular to incident beam</td>
</tr>
<tr>
<td>K_a</td>
<td>Association constant</td>
</tr>
<tr>
<td>ln</td>
<td>natural logarithm</td>
</tr>
<tr>
<td>MBK</td>
<td>Methyl n-Butyl Ketone</td>
</tr>
<tr>
<td>MEK</td>
<td>Methyl Ethyl Ketone</td>
</tr>
<tr>
<td>MTS</td>
<td>Microtubule stabilizing buffer</td>
</tr>
<tr>
<td>NCV</td>
<td>Nerve Conduction Velocity</td>
</tr>
<tr>
<td>NIOSH</td>
<td>National Institute for Occupational Safety and Health</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical Density</td>
</tr>
<tr>
<td>Overall NCV</td>
<td>Average Nerve Conduction Velocity between the Sciatic Notch and Plantaris Tendon</td>
</tr>
<tr>
<td>P</td>
<td>Phosphorous; Fluorescence Polarization</td>
</tr>
<tr>
<td>PLA</td>
<td>Total Phospholipase A</td>
</tr>
<tr>
<td>POPC</td>
<td>1-Palmitoyl-2-oleoyl-phosphatidyl choline</td>
</tr>
<tr>
<td>PPM</td>
<td>Parts per million</td>
</tr>
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## LIST OF REAGENTS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>Bovine Serum Albumin</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Cerebroside</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Cholesterol 3-oleate</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Colchicine</td>
<td>Aldrich Chemical Company</td>
</tr>
<tr>
<td>$^3$H-Colchicine</td>
<td>New England Nuclear</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide</td>
<td>J.T. Baker Chemical Company</td>
</tr>
<tr>
<td>1,6-Diphenyl 1,3,5-hexatriene</td>
<td>Aldrich Chemical Company</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>Mallinckrodt Chemical Works</td>
</tr>
<tr>
<td>Guanosine 5'-Triphosphate</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>2,5-Hexanedione</td>
<td>Aldrich Chemical Company</td>
</tr>
<tr>
<td>Lecithin</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>$^{14}$C-Lecithin (methyl-choline)</td>
<td>New England Nuclear</td>
</tr>
<tr>
<td>Lysolecithin</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Methyl n-Butyl Ketone</td>
<td>K &amp; K Fine Chemicals</td>
</tr>
<tr>
<td>Orcinol Monohydrate</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>$^{32}$P-H$_3$PO$_4$ - Carrier Free</td>
<td>New England Nuclear</td>
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<td>Perylene</td>
<td>Aldrich Chemical Company</td>
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<td>Phospholipase A$_2$ - Crotalus Atrox</td>
<td>P-L Biochemicals</td>
</tr>
<tr>
<td>Sepacryl Superfine S-200</td>
<td>Pharmacia Fine Chemicals</td>
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<tr>
<td>Silical Gel, 100 mesh</td>
<td>Mallinckrodt Chemical Works</td>
</tr>
<tr>
<td>Chemical</td>
<td>Supplier</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>Sigma Chemical Company</td>
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<tr>
<td>Sodium Pentobarbital, injectable</td>
<td>Abbott Labs</td>
</tr>
<tr>
<td>TEMED</td>
<td>Eastman Kodak</td>
</tr>
<tr>
<td>Thrift Solve</td>
<td>Kew Scientific</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>J.T. Baker Company</td>
</tr>
<tr>
<td>Tris HCl</td>
<td>Sigma Chemical Company</td>
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</table>
INTRODUCTION

A. General Properties of Hexacarbons Producing Peripheral Neuropathy

n-Hexane is produced from catalytic cracking, hydrocracking, and thermal cracking of crude oil. These processes use high temperatures and pressures to cleave carbon-carbon bonds of crude petroleum, producing shorter chain hydrocarbons. Fractional distillation of the products separates the alkanes into groups by volatility: pentane, heptane and octane co-condense with hexane. Production of hexane in the United States was $1.6 \times 10^8$ kg in 1974 (United States International Trade Commission, 1974). Hexane is used industrially and commercially as an ink, adhesive and paint solvent.

Methyl-n-butyl ketone (MBK) is produced from a catalyzed reaction of acetic acid and ethylene under pressure followed by distillation to purify the products. MBK is an excellent solvent for oils, nitrocellulose, resins, waxes and fats and lacquers. Its physical properties differ from hexane as MBK is less volatile: vapor pressure 3.8 vs 150 torr, boiling point 128 vs 69 °C.

2,5-Hexanediene (HDO) is a metabolite of both hexane and MBK. HDO is used to a limited extent industrially as a solvent and chemical intermediate for organic synthesis. The vapor pressure is 1.6 torr, suggesting that there is little hazard from inhalation. No case of human neuropathy
from inhalation of HD\textsuperscript{2}O has been reported, although HD\textsuperscript{3}O in drinking water produced neurotoxicity (Abdel-Rahman, et al., 1978; Spencer et al., 1978).

The National Institute for Occupational Health (NIOSH) estimated that 2.5 million workers are exposed to n-hexane. Occupations at risk include lacquer and paint application, resin and rubber, synthesis, fuel handling from refinery to delivery stages and plastic laminating operation.

B. Toxicity of Methyl Butyl Ketone and Metabolites

Hexane and methyl n-butyl ketone (MBK) have been shown to cause peripheral neuropathy not distinguishable from hexane neuropathy (Allen et al., 1975). In a study of 86 cases of peripheral neuropathy from a fabric coating plant, 11 were afflicted with both motor and sensory impairment, 38 showed sensory paresthesias, and 37 were clinically asymptomatic but had preclinical electromyographic changes.

Incidence of these cases was highest for members of the printing shop (21.5\% of employees). Printing operators, having greatest contact with the inks and solvents had an incidence of 36.1\%, followed by pan washers (28.6\%). No symptoms were noted before December, 1972, indicating the disease to be of recent origin. In August, 1972, MBK replaced methyl isobutyl ketone as a printing ink solvent at this fabric coating plant.

Airborne concentrations inhaled by printers averaged about 9 PPM MBK and 331 PPM MEK (methyl ethyl ketone). Other similar plants producing similar products but not using the solvent MBK showed no neurotoxicity among their employees.
Chief clinical signs and symptoms of MBK toxicity are as follows:

gradual onset of sensory loss, predominately distal proceeding to proximal regions. In more severe cases, motor involvement occurs with sparing of proprioception and deep tendon reflexes (large fibers spared, A group). Progression of symptoms persists in spite of cessation of exposure. However, gradual recovery may follow.

Electron microscopic examination of single teased nerves shows a paranodal denudation of myelin with focal internodal myelin loss. Axonal swellings with highly dense masses of microfilaments appear, Schwann cells remain intact. Yamamura found hexane to be a neurotoxic chemical to workers. Two workers using hexane as a glue solvent in the manufacture of sandals became quadriplegic. A survey revealed 93 cases of polyneuropathy, all of which were associated with sandal gluing at home. Gas chromatographic analysis of the rubber paste solvent used in these cases found 70% n-hexane with approximately 25% toluene. Environmental concentrations of hexane varied widely, from 500 to 2,500 PPM. Skin absorption was not determined but cited as an additional route of exposure. Among the 93 workers in Yamamura's study, 53 experienced sensory polyneuropathy and 32 had sensorimotor polyneuropathy. Of this latter group, 8 workers showed muscle atrophy upon examination.

Yamada, 1972, studied two plants where hexane was used and noted 17 workers showing signs of polyneuropathy. This was manifested by fatigue and loss of appetite initially followed by distal sensory paresthesia and difficulty in walking. Three months after cessation of exposure, the progression of disease was stopped and gradual recovery was noted over the next two years.
The first report of hexane neuropathy in the United States occurred when Herskowitz et al., 1971, investigated polyneuropathy symptoms among cabinet finishing workers. Air analysis determined an average concentration of 650 PPM hexane with peak concentrations reaching 1,300 PPM. Electron microscopy of anterior tibialis muscle biopsy showed two types of axonal changes:

1. Increased neurofibrils and abnormal membraneous structures
2. Clumping and degeneration of mitochondria within the axon with many onion bulb structures.

In an electrophysiological study, Truhaut et al., 1973, exposed Wistar rats to hexane (2,000 PPM) and heptane (1,500 PPM) one to six months. These exposures reflected occupational contact: five hours/day, five days/week. Technical grade hexane was used and consisted of 15% n-hexane and other isomers of 6 and 7 carbon alkanes. Sciatic and saphenous nerves were removed after 1, 2, and 5 months of exposure. Electrophysiological analysis showed a decreased nerve conduction velocity and excitability with an increased refractory period which progressively worsened.

In rats inhaling MBK, sciatic nerve morphological changes were examined and correlated with counts of microfilaments per square micron of axonal area (Saida et al., 1976). These authors noted that the primary lesion is an increase in neurofilament density, followed by paranodal swelling of myelin. This swelling appeared to be secondary to axonal swelling rather than a primary myelin pathology. Unmyelinated axons were affected but to a much lesser extent. Schwann cells appeared active with increased rough endoplasmic reticulum and Golgi apparatus. "Inpouching"
or reversal of myelin curvature also appeared. Microtubule counts were not diminished as reported by earlier investigators, but became clustered close to periphery of the axon cross section. This may have caused counting errors in earlier experiments (Couri et al., 1974).

A study of time course of neuropathy showed that clinical paralysis of hind limbs of rats exposed to MBK coincided with sharp increases in the number of inpouchings, denuded fibers, and giant (swollen) axons per unit cross section of nerve. On the basis of this data, the authors attributed the observed muscle weakness to axonal atrophy. Cell bodies in dorsal and ventral roots and motor end plates remained normal up to the time of paralysis onset. No central chromatolysis was observed.

Another approach to the study of axonopathy is fast axoplasmic transport. Using radioactive amino acids it is possible to determine the transport of proteins from the cell body to axonal terminal. If fast axoplasmic transport is measured in rats intoxicated with MBK, it is possible to detect a slowing of flow rate in treated with respect to control (Sahenk et al., 1977). Furthermore, these authors established that animals could be grouped by symptoms: MBK exposed but normal, moderate or severe paralysis. Axoplasmic flow rate decreased as symptoms worsened: control, 405 mm/day; exposed but unimpaired, 360; partially paralyzed, 297; severely paralyzed, 265. Thus, a decrease in axoplasmic flow rate occurs before observable motor deficit.

Many investigators have noted that MBK, hexane and 2,5-hexanedione produce very similar neuropathic conditions (Abdel-Rahman et al., 1978, Spencer and Schaumberg, 1977).
C. Axoplasmic Transport

Because axoplasmic transport is attenuated by MBK exposure, a consideration of factors affecting this property is indicated. Axonal proteins arise largely from the cell body ( > 90%), with mitochondrial protein synthesis and Schwann cells contributing small amounts (Barondes, 1976; Lasek et al., 1974). Protein synthesis studies indicate that up to 90% of the proteins in the nerve are synthesized in the cell body and are transported through the axoplasm to the terminal synapse. Consequently, protein content of synapses and distal portions of peripheral nerve axons is dependent upon an axoplasmic transport system.

Axoplasmic transport is classified by its rate and direction: i.e., fast and slow orthograde and retrograde flow. The slow and retrograde flow rates are approximately 1-2 mm per day, the fast component much greater at 410 mm per day (Ochs, 1972). Metabolic insults (such as azide, dinitrophenol and anoxia) were found by Ochs to block axoplasmic transport at the site of insult. Systemic injection of radioactive precursors by earlier investigators measured only the slow flow rate because the label was introduced into the axon over a long time scale. Ochs (op. cit.) injected $^3$H-leucine directly into the dorsal root ganglion to provide a high specific activity amino acid pool of the cell body. This fast flow rate is constant regardless of fiber type, myelinated or unmyelinated, age or sex of animal, or species. Fast axoplasmic flow (FAP) is also seen with lipid glycoprotein precursors such as glucosamine or fucose.
FAP is dependent upon energy metabolism. Metabolic inhibitors applied locally or systemically to the axon will block the flow rate: examples of this inhibition include anoxia, cold, cyanide, azide and oxidative-phosphorylation uncouplers. This effect is not due to inhibition of protein synthesis because local anoxia of a distal segment of axon effectively "dams" the labelled protein proximal to the anoxia with no effect on radioactivity incorporated.

If transported proteins are placed on sodium dodecyl sulfate gel slabs for electrophoresis and autoradiography, many molecular weights are seen ranging from 50,000 to 4000,000. If the initial "wave" amplitude is measured at 2 hours, a decrease in wave amplitude and spread is seen (Ochs, 1972). This implies that fast flow is composed of particulate matter, lipid, glycoprotein and protein, which is diluted when it is transported down the axon. In contrast, Lasek et al., 1974 have shown that slow transport is a discrete motion in which the radioactivity pulse does not diminish in amplitude or peak area, indicating that the label is transported as an intact unit down the axon.

Previous studies have shown that MBK neuropathy will produce a reduction in fast axoplasmic flow rate (Sahenk et al., 1977). This implies that an axonal trophic deficiency is responsible for the disease since vinca alkaloids block flow and also produce peripheral neuropathy. Chronic vincristine therapy of leukemia patients can result in a dying-back degeneration of peripheral nerves (Casey et al., 1973). Light microscope examination of these nerves revealed the presence of fibrillary masses with an absence of axoplasmic flow in vivo.
It is concluded that an interruption of fast axoplasmic flow is related to peripheral axonal neuropathy; whether it is a cause or effect remains to be seen. Neurotoxins affecting primarily myelin and axons as a secondary target do not change fast flow. Ranish and Dettborn, 1976, found no effects on fast axoplasmic transport in paroxon neuropathy.

The relationship between axoplasmic transport and nerve function is not fully understood. It would appear that an interruption of axoplasmic flow would be enough to account for nerve degeneration, since the cell body makes 90% of all protein used by the cell. There are other agents which can block axoplasmic flow in vitro, such as vinca alkaloids.

Vinca alkaloids are drugs which are extracts of the periwinkle plant. These drugs are useful in cancer chemotherapy because they can bind to the mitotic spindle and prevent its motion. Thus, chromosomes of dividing cells are locked in metaphase and die because the chromosomes cannot be used to synthesize protein. Vinca alkaloids are toxic to rapidly dividing cells such as tumor cells, gastrointestinal mucosa cells, and lymphatic tissues. Surprisingly, vinca alkaloids are also toxic to non-dividing nerve cells. Patients on long term chemotherapy for leukemia suffer from a degeneration of peripheral nerves. Histological preparations from nerve biopsies of these patients resemble MBK toxicity: a distal-proximal disease of axons, increased neurofilamentous masses, and decreased nerve conduction velocity. A study of Casey et al., 1973, examined vincristine therapy patients and determined that the amplitude of the compound muscle action potential decreased with time of treatment and nerve conduction velocity of affected limbs also decreased.
D. Microtubule Structure and Function

Microtubules are ubiquitous organelles associated with motile processes in the cell. All mammalian cells contain microtubules which are required for basic transport within and without the cell: chromosome separation with the mitotic spindle, ciliary movement as seen in hair cells of epithelium, secretion of granular packets of insulin by the \( \beta \) -cells of the pancreas, and pseudopod extension by phagocytic cells.

Microtubules are composed of building blocks or monomeric units called tubulin. This monomer has a molecular weight of approximately 110,000 daltons and is composed of two non-identical subunits, \( \alpha \) and \( \beta \) (Bryan, 1974). Only chemical means can separate these subunits. Acetylation of highly purified tubulin monomer can separate \( \alpha \) and \( \beta \) chains on polyacrylamide gel electrophoresis.

Tubulin will assemble or disassemble into microtubules depending upon the external environment. Cold (4 °C) favors disaggregation as does excessive heat. Chemical influences on microtubule assembly or polymerization have been studied. There are some chemicals which favor monomeric form, others which favor polymeric tubulin. Treatment of microtubule preparations containing no monomer with cAMP, D\(_2\)O or hexylene glycol will favor a stabilization of the microtubule structures. Treatment with colchicine will depolymerize the assembly into its tubulin units (Wilson et al, 1974).

Examination of the microtubule by electron microscopy indicates that the monomeric units assemble in a cylinder whose width is 250 nm. One turn around the cylinder is lined with 13 subunits which form a large, helical array. This basic structure is identical in microtubules prepared
sources as diverse as sea urchin egg and chick brain. Amino acid sequences of the N-terminal from these two organisms do not differ, indicating that tubulin is a highly conservative protein.

Colchicine has been found to bind to tubulin prepared from chick brain with time dependent kinetics. The initial binding capacity, assayed by two hour incubation of chick embryo brain tubulin with $^3$H-acetyl colchicine, is found to decay with first-order kinetics and a half-life of four hours. pH extremes (above 10.5 or below 4.5) rapidly and irreversibly destroy the colchicine binding activity. However, with the pH range (4.5 - 10.5) and a salt concentration (5 mM to 500 mM NaCl), initial binding and strength does not vary, although half-time is maximum at pH = 7. This ion independence implies that the nature of binding does not require or involve polar groups. Colchicine binding quantitatively estimates the ability of a tubulin preparation to polymerize.

The dynamics of tubulin-drug binding are complex. Colchicine and vinblastine do not displace each other not compete for the same binding site. The association constant for colchicine ($K_a$) is $2.0 \times 10^6$ liters per mole, with one binding site per molecule. The association of colchicine with tubulin has been explained as a two step reaction (Garland, 1978). Garland calculated the first association rate as follows:

$$P + C \xrightarrow{k_1} (PC) \xleftarrow{k_{-1}} \xrightarrow{k_2} (PC)$$

Here $k_1$ represents the formation rate of a fast, easily dissociable complex, $k_2$ is the association rate for a slowly formed but tightly bound complex ($k_2 = 2 \times 10^3$ s$^{-1}$, $k_{-2} = 5 \times 10^{-6}$ s$^{-1}$). The formation of colchicine
tubulin complex is temperature dependent: very slow at 0° C and reaching a maximum rate at 37° C before dropping with T >40° C.

High protein concentration also increased the stability of binding (length of half-life decreased). This may be due to increased tubulin-tubulin interaction in vitro since initial colchicine binding capacity remains unaffected. This "stabilizing effect" is also mimicked by the addition of vincristine (5.4 x 10^-4 M). Vincristine does not increase the total binding (as extrapolated back to zero time) but instead stabilizes the binding capacity of the tubulin complex. This stability is not complete because binding capacity ultimately decays after six hours.

Heavy water (D2O) will lead to aggregation of microtubules; earlier observations have shown that fast axoplasmic flow rate is reversibly inhibited by 90% D2O (Anderson et al., 1972). These authors used an in vitro system which contained separate chambers for sciatic nerve ganglion, axon and end plate of gastrocnemius. The nerve was ligated at the distal end of the middle compartment to collect all transported radioactivity over an 18 hours period. Ninety % and 50% D2O allowed 15% and 55% of control radioactivity to be collected. This inhibition is reversible if D2O was excluded from the ganglion compartment and placed in the middle compartment so protein synthesis in the cell body was unaffected.

To summarize this section, the relationship between microtubular protein properties and physiological role is complex. Conditions which dissociate microtubules (cold, anoxia, colchicine) as well as those which aggregate them (vinca alkaloids, D2O, hexylene glycol) are capable of blocking axoplasmic transport. It is an attractive hypothesis that toxic
axonal neuropathies are the result of inhibition of axoplasmic transport with a depletion of trophic protein from the axonal terminal and innervated muscle. Since colchicine binding by tubulin parallels the ability of tubulin to polymerize, the binding of $^3$H-colchicine was used to mark changes in tubulin properties induced by hexanidine treatment.

E. Biochemical Bases of Peripheral Nerve Pathology

Nervous tissue has complicated metabolism due to varied cell types and complexity of function. Perhaps the best understood diseases biochemically are inborn errors of metabolism. Deletion of an essential degradative enzyme will cause accumulation of its substrate in many tissues. An example of this is Tay-Sachs disease in which lysosomes lack hexoseaminidase A. In brain, the lysosomes accumulated GM$_2$, a complex glycosphingolipid. Both lipids and proteins accumulate in lysosomes as membraneous cytoplasmic bodies which eventually cause cell death. The brain is affected to such a degree that infants with the disease will die between 3 and 4 years of age. The biochemical basis of this disease is clear in that a specific chemical accumulates which can be traced to an enzyme deficiency.

In contrast to Tay-Sachs and other genetic disorders are toxic neuropathies, which have more subtle causes and effects. HDO induced neuropathy does not feature any striking accumulation of material, such as GM$_2$ ganglioside. Peripheral nerve as a tissue presents some unique properties, compared to brain tissue:

1. Peripheral nerve contains a large amount of collagen which prevents easy homogenization
2. Paired sciatic nerves from a rat weigh only 100 mg whereas the brain weight will exceed 1 gram.

3. The rate of oxidative metabolism in sciatic nerve is 10% that of brain white matter (Majno and Karnovsky, 1958)²³.

4. Peripheral nerve cells have their cell bodies clustered in one locus, i.e., dorsal root ganglion. Cell body, axon, and synaptic terminal are separable on a gross level. CNS neurons are intertwined in layers of other neurons with many cell types. It is very difficult to separate a single population of cell type from brain.

Because myelin makes up half the dry weight of white matter, much attention has been directed at its metabolism, structure and chemical constituents. Initial studies of peripheral nerve myelin indicated that it was metabolically inert (Adams et al., 1963). Radioactive tracer measurement of uptake and degradation of myelin specific lipids and proteins gave slow turnover rates.

More recent studies have shown that myelin of both central and peripheral nervous system is metabolically dynamic. Using precursors of phosphatidyl choline (lecithin), Abdel-Latif and Smith, 1970, showed variable half lives of $^3$H, $^{14}$C and $^{32}$P: $^3$H-choline, 11.7 days; $^{14}$C-glycerol, 2.9 days and $^{32}$P-orthophosphate, 18.6 days. (All data from rat brain). Their results suggest that various portions of the lecithin molecule turnover at different rates. Phosphate and choline were extensively recycled, presumably because they were derived from dietary sources whereas glycerol had the shortest half-life.

As summarized by Benjamins and Smith, 1977, the greater part of studies indicate that proteolipid protein and basic protein of myelin are
stable, whereas higher molecular weight proteins show a half-life of 1-2 months. Cholesterol and galactolipid are stable but phosphatidyl inositol and lecithin have a half-life of less than 40 days.

Enzymes involved in the metabolism and pathology of myelin have been studied extensively in Wallerian degeneration. Webster, 1973 sectioned one sciatic nerve of rats and followed the time course of hydration and phospholipase activity. He prepared 1-(3H) stearoyl phosphatidyl choline and 2-(14C)-linoleoyl phosphatidyl choline in order to simultaneously measure phospholipase A₁ and A₂. Taking a constant anatomical unit, he found a 91% increase in weight at 14 days. Both phospholipase A₁ and A₂ were elevated with maximal values occurring 15 days (A₁) and 21 days (A₂) after nerve section. Webster's data is consistent with the study of Berry et al., 1964 which found lyssolecithin in cat nerve one week after section. Earlier studies by Webster and Thompson, 1962, examined human and rat brains for lyssolecithin but yielded no difference between control human brain and multiple sclerosis plaques. These authors employed a 6 gram column of silicic acid and step gradients of chloroform: methanol: water followed by paper chromatography. Lyssolecithin thus appears to be present in sectioned nerve and normal rat liver but not MS plaques or normal brain.

A more consistent biochemical marker for myelin breakdown or synthesis is the presence of cholesterol-fatty acid ester. Adams and Davison, 1959 and 1960 found large amounts of esterified cholesterol in chick and human fetal brain. Fetal esterified cholesterol was at a maximum of 49% of total cord cholesterol before birth before dropping to zero 7 weeks after birth. Esters in the corpus callosum peaked after birth (24% at 8
weeks.) Adams and Davison also examined tissues histologically and found that the cord peak of cholesterol esters correlated with histological appearance of myelin sheaths. These authors further examined human adult corpus callosum and spinal cord. At this stage, myelin formation is complete; of 7 brains tested, only one contained a slight amount of cholesterol ester. Adams and Davison concluded that esterified cholesterol occurs only in myelinogenesis or regeneration of myelin.

In a comprehensive study of Wallerian degeneration, Mezei, 1970 showed an increase in cholesterol esters over control hen sciatic nerve. This increase occurred at 8, 16, and 32 days after section and amounted to 172%, 489% and 449% of paired control values. Protein content per nerve did not decrease until 32 days. RNA/DNA ratios increased 42% at 8 and 16 days indicating paralleling Schwann cell proliferation. Total DNA and RNA each increased over control at 8, 16 and 32 days.

Using $^{3}$H-cholesteryl palmitate, Mezei determined cholesterol esterase activity. This enzyme decreases as degeneration progresses. Mezei's data indicates that Wallerian degeneration is characterized by cell division or infiltration, increased cholesterol ester, loss of free cholesterol and decreased cholesterol esterase.

Cholesterol ester formation has been associated with a variety of myelin disorders: Wallerian degeneration, subacute sclerosing panencephalitis (Svennerholm et al., 1970), Schilder's Disease (Suzuki et al., 1979) and other immunological demyelinating diseases. Cholesterol has been proposed as a fatty acid carrier involved with myelin synthesis. Developing brain has a sharp increase in cholesterol esters which coincides with
myelin maturation (Eto and Suzuki, 1972). Once esterified, cholesterol has a much greater mobility in acidic ether thin layer chromatography (Rf increases from 0.30 to 0.95). Free fatty acids are damaging to membranes and have a polar carboxyl group which presents a barrier to insertion in a lipid bilayer. Cholesterol ester is postulated to be a carrier for fatty acids in the synthesis and repair of myelin. Eto and Suzuki, 1972, determined that cholesterol-fatty acyl-transferases increase with onset of myelination and remain at high levels through its completion.

F. Membrane Microviscosity Studies

Nervous tissue is made up of a variety of membraneous structures: glial cell (myelin), endoplasmic reticulum, nuclear, mitochondrial membranes. In white matter, myelin alone accounts for 50% of the dry tissue weight. Because structure and function of nerve is closely associated with myelin, a study of membrane properties was undertaken.

There are many physical methods used to study membrane structure: x-ray diffraction, differential scanning calorimetry, spectral methods, and electrical measurements. In this study, fluorescence polarization was chosen as a way of monitoring changes in membrane structure.

The movement of a sphere subjected to random collisions is called Brownian motion. This was first described as the "random walk" problem (Perrin, 1926). The motion of the sphere or ellipse can be understood in terms of a fluorescent molecule. For these studies, 1,6-diphenyl-1,3,5-hexatriene (DPH) was chosen because it has a relatively long fluorescent lifetime (11 nanoseconds) and because it fluoresces in lipid bilayers.
The principle of fluorescence polarization has been derived rigorously (Weber, 1953). Plane polarized light is allowed to fall on the fluorescent molecule, DPH. DPH will absorb at its characteristic wavelength, an electron changing its energy from the ground state to an excited state. Only those electrons with proper electric dipole moments will be able to absorb the photon. After losing a certain amount of energy to rotational or vibrational sources the molecule will re-radiate the photon at a lower energy (higher wavelength). The time intervals between absorption and emission is the fluorescent lifetime.

If DPH undergoes no collisions over this fluorescent lifetime, then the polarization of the emitted photon will also be vertical. However, suppose half the molecules collide with other molecules in the surrounding medium. The vertical polarization will be changed by some angle \( \alpha \). Since these collisions are due to random motion, \( \alpha \) will be randomized.

Two variables have been defined to describe the polarization of this system:

\[
P = \frac{I_{ll} - I_1}{I_{ll} - I_1} \quad (1), \quad r = \frac{I_{ll} - I_1}{I_{ll} - 2I_1} \quad (2)
\]

\( P \) is the degree of fluorescence polarization, \( I_{ll} \) is the intensity of fluorescent light emitted with polarization parallel to the incident beam, and \( I_1 \) the intensity of fluorescence polarization perpendicular to the incident beam. If DPH is rigid and cannot undergo any collisions, \( I_1 = 0 \) and \( P = 1 \). If DPH is a highly fluid environment (low viscosity), the \( I_{ll} = I_1 \) and \( P = 0 \). The variable \( r \) is defined as the fluorescence anisotropy but has theoretical limits of +1 to -1.
In practice, fluorescence polarization is studied using either steady state conditions or transient decay techniques. Steady state can be achieved by bombarding the probe with a continuous beam of linearly polarized light and measuring the fluorescence polarization. Standard spectrofluorometers, such as supplied Aminco-Bowman, can be used.

Perrin studied Brownian motion and applied these equations to fluorescence (Shinitzky and Barneholz, 1978). The general form of his equation in steady state is:

\[
\frac{1}{P} - \frac{1}{P_0} = 1 - \frac{1}{P_0} \left( 1 + \frac{3}{\tau_r} \right)
\]  

(3)

Where \( P_0 \) is the polarization at infinite viscosity \( \tau_r \) is the mean harmonic rotational relaxation time. A plot of \( 1/P \) versus \( T/\eta \) is commonly used to calculate viscosity from polarization data. \( P \), the polarization, and \( T \), absolute temperature, are measured, and is determined for a sample of known viscosity (Chen and Bowman, 1965).

Viscosity of aliphatic hydrocarbons varies with temperature in accordance with the following empirical relation (Shinitzky and Inbar, 1978).

\[
\eta = A \ e^{\frac{\Delta E}{RT}}
\]  

(4)

\( E \) is the flow activation energy, \( R \) is the gas constant and \( A \) is a constant of proportionality. Thus a plot of \( \log_{10} \eta \) versus \( 1/T \) would be a straight line of slope \( \frac{\Delta E}{R} \) (Arrhenius Plot). This relationship holds for isotropic simple liquids and solution of miscible liquids, such
as glycerol-water. Any deviation from linearity represents an effect of non-monogeneous attractive forces within the liquid.

Most membrane preparations have a single slope, linear Arrhenius plot: red cell ghosts, single and multiple lamellar liposomes of known lipid content, synaptosomal membranes (Lentz, et al., 1976; Shinitzky and Inbar, 1976). Intact cells microviscosity has been studied using DPH (Berlin, 1975). Berlin found that polymorphonuclear cell (PMN) preparations were able to incorporate DPH into their membranes and still phagocytize oil droplets with lectin treatment. This phagocytosis was inhibited by colchicine and had linear Arrhenius plot. Comparing the curves obtained with no treatment lectin alone, lectin plus colchicine alone and colchicine alone. Berlin found that only the lectin treatment differed from control. This treatment was the only one in which the PMN cells engulfed the oil drops and featured a decrease in viscosity suggesting a fluidization of the cell membrane. Lectin plus colchicine had no difference from control or colchicine alone. Berlin claimed that this study showed that the lipid bilayer became more fluid as a result of its interaction with lectins. Also, colchicine does not exert its anti-phagocytotic action by a direct membrane effect (e.g., stiffening the lipid).

Phase transitions and fluidity using fluorescent probes of synthetic phospholipid bilayers, single and multilamellar, have been studied by Lentz et al., 1976. A phase transition was defined as any discontinuity in the slope of Arrhenius plot. In studies of single lipid, no such transitions were found except for 1-palmitoyl-2-oleoyl-phosphatidyl choline
(POPC) in multilayered liposomes. This transition temperature was measured at $8^\circ\text{C}$ which compares well with differential scanning calorimetry value, $43^\circ\text{C}$. (op den Kamp, et al., 1975).

Lentz also showed that unsaturated fatty acids produced a decrease in viscosity above the transition temperature. On the other hand, cholesterol has been found to increase viscosity when its membrane concentration was increased (Shinitzky and Inbar, 1976). Microviscosity measurements using fluorescent probes can give much information about membrane properties and membrane function.

G. Electrical Properties of Nerve Cell Membranes

A unique property of nerve and muscle membranes is the existence of a potential difference and the excitability of the cell to outside currents. This potential difference arises because (1) the membrane is selectively more permeable to $K^+$ then $Na^+$; (2) $Na^+$ concentration is actively maintained by an energy requiring pump; (3) organic anions cannot diffuse out of the cell. The distribution of ions across the cell membrane is governed by the Nernst equation (Goodgold and Eberstein, 1977). Potassium ion, being the more permeable ion across the membrane, maintains the resting potential at $-90$ millivolts.

Nerve axons and muscle fibers also have cable properties which allow propagation of currents to spread throughout the membrane. If the resting potential is changed slightly by a stimulating electrode, there is a potential difference which decreases exponentially with distance from the point of stimulation. This is a local reponse in that it does not propagate appreciably beyond the stimulus point. Once this threshold limit is
reached, the membrane conductance to sodium increases dramatically. Since the predicted Nernst potential of sodium is approximately +66 mV, the influx of sodium raises the membrane potential to +20. This generates an action potential which is propagated down the axon without attenuation.

The velocity of nerve impulse propagation was a fundamental unanswered question since electric currents are propagated in conductors and electrolytes at the speed of light. In 1850, von Helmholtz measured the conduction velocity in human sciatic nerve and found that it was neither infinite nor the speed of light but 120 m/sec or about 1/2 the speed of sound. This value is for fastest myelinated fibers. There are a number of determinants of fiber velocity: presence of myelin, fiber diameter, and functional role of the nerve in question. Proprioception and motor nerves are the fastest since reflexes and muscle movements are limited by the conduction time. Myelinated fibers are faster than unmyelinated ones and large diameter fibers faster than smaller ones. The presence of a myelin sheath around the axon limits depolarization of the membrane to gaps in the sheath. In this way, the action potential jumps from gap to gap without having to depolarize the whole membrane in between gaps propagated by saltatory conduction instead of local currents as in unmyelinated nerve. Myelin loss through biochemical or immunological degradation necessarily results in a decreased conduction velocity.
H. Electromyography of Peripheral Neuropathy

A sensitive diagnostic test of nerve toxicity in mammals is the electromyogram (EMG). This term is used to mean both a study of muscle electrical activity and the conduction velocity of motor nerves (a general text of electromyography is Goodgold and Eberstein, 1977).

Toxic neuropathy has been studied in shoe workers exposed to hexane and other paraffinic hydrocarbons (Perticoni and Cianchetti, 1976). A total of 86 afflicted workers were examined every 3 months or until EMG data was normal. Among the parameters considered were maximal motor nerve conduction velocity (NCV) of the peronal nerve, minimal motor NCV, distal latency, and existence of polyphasic insertional potentials. Patients were grouped by maximal motor NCV into 3 categories: Group I, NCV <35 m/sec; Group II, 35 <NCV < 44 m/sec; and Group III, normal NCV. Clinical impairment followed NCV classification as Group I patients all had severe motor deficit to point of flaccid quadriplegia, Group II only mild motor deficit predominately in distal muscles, and Group III had sensory symptoms (numbness and pain). The authors attributed the EMG findings to a dying back axonal disease, although they had no histological data.

In a study of MBK effects on peripheral nerve (Johnson et al., 1977) sciatic-tibial motor NCV was found to differ from control at 2 months in rats exposed to 1000 ppm MBK. Ulnar NCV was measured for the 1000 ppm rats and found to differ from controls at 4 months. Monkeys exposed to the same levels developed a sciatic-tibial NCV deficit at 6 months. Despite
removal from exposure, NCV was depressed for 2 months and did not return to pre-exposure value until 5 months later.

EMG changes are known to precede clinical observation of myopathy (Goodgold and Eberstein, 1977; Wiechers, 1977). These changes are positive sharp waves and fibrillation potentials and are recorded from resting muscle using a teflon coated monopolar electrode.

Positive sharp waves are diphasic waves recorded in muscle which have an initially rapid positive deflection followed by a slow negative decay back to baseline. This is the first sign of denervation and is thought to reflect an increased irritability of the muscle to mechanical injury.

Fibrillation potentials occur once the muscle is denervated and supersensitive to acetyl choline (ACH). Blockade of ACH release with botulinum toxin produced both fibrillations and supersensitivity. The exact origin is unknown as local anesthetic blockade of innervation will produce muscle paralysis but no fibrillations.

Nerve conduction velocity decreases as demyelination of nerve axons progresses (Truhaut et al., 1973). In the case of MBK neuropathy, this change was observed after appearance of fibrillations and positive waves, presumably because loss of a few axons can produce denervated muscle without affecting the observed NCV (Hurd, 1975).
The microscopic pathology of HDO neuropathy was one of axonal degeneration. The prominent features were a distal-proximal progression of disease, swelling of the large, myelinated fibers near the nodes of Ranvier, and myelin thinning secondary to axonal degeneration. Because 10 nm neurofilaments proliferate and microtubules redistribute in the axon, a study of microtubular properties was undertaken. Using both $^3$H-colchicine and gel electrophoresis as markers for tubulin, the subcellular distribution, half life of colchicine binding, and recovery were studied in chronically treated hexanedione rats.

The next area to be investigated was a physical property of membranes microviscosity. Using fluorescence polarization techniques, the lipid phase of nerve homogenates and myelin preparations from HDO intoxicated animals was studied as a function of temperature. Because the temperature behavior revealed a change in the treatment groups over control, another model of axonal degeneration was used to compare microviscosity effects. The time course of nerve section is well understood biochemically and histologically, so this offers a useful model of comparing the effects of nerve degeneration on membrane viscosity. To determine whether chronic treatment produces membrane changes in general, red blood cell ghosts were prepared from HDO treated animals.
Because membrane microviscosity was different in the 0.1% HDO-treated group, an otherwise normal group, electrophysiologic studies of the HDO neuropathy was undertaken. This allowed a quantitative description of the onset and recovery of NCV and latency parameters in this neuropathy.

Although this disease is thought to be primarily axonal, with myelin effects secondary, a study of lipid metabolism and oxygen uptake was undertaken. It was hoped that the metabolic basis for HDO toxicity would be elucidated by this series of experiments.
METHODS

A. Animal Model of Neurotoxicity

Male, Wistar rats 160-200 g were used for this study. Animals were maintained on 0, 0.1, and 0.5% 2,5-hexanedione (HDO) in drinking water (v/v). Preliminary studies showed that HDO in drinking water produces the same neuropathy as that seen with 2-hexanone or hexane inhalation. (Spencer and Shaumberg, 1977). Animals which drank 0.5% (v/v) HDO lost weight with respect to controls before onset of observable muscle weakness and wasting. After 2 months of drinking 0.5% HDO water, rats weighed only 60% of controls. These results in the rat are similar to those in guinea pig. Abdel-Rahman et al., 1978, found a decreased body weight, CNS toxicity (pupillomotor activity) and severe paralysis of hind limbs in guinea pigs treated with 0.5% hexanedione in drinking water.

Sciatic nerves were dissected by the methods of Yates et al., 1976. A single nerve unit was removed; the upper limit was the obturator internus and lower limit the insertion of the achilles tendon onto the calcaneus.

B. Colchicine Binding Assay

$^3$H-colchicine was obtained from New England Nuclear (Boston, Massachusetts), specific activity 10 Ci/mmol. Radiochemical purity was verified by thin layer chromatography (System used: Methanol, 200 ml; 58% ammonium
hydroxide, 3 ml; on silica gel G). Binding assays were carried out on aliquots of brain or sciatic nerve fractions according to the method of Piperleers et al., 1977. Instead of using dextran coated activated charcoal, micro-chromatography columns of DEAE-sepharose were used. This column consisted of a disposable pasteur pipette (0.5 x 8 cm) fitted with a glass wool plug, to which 400 ul of the gel suspension was applied and washed with 2 ml TS buffer (0.25 M sucrose, 0.01 M phosphate, 0.5 M MgCl$_2$, 0.5 mM GTP, pH = 7.0). The reaction mixture consisted of 50 ul of the solution to be assayed and 10 ul $^3$H-colchicine (ring C, $^3$H-methoxy) made up to 65 uM. These were pipetted in a 12 x 75 mm test tube, mixed by vortex, and stoppered. The tubes were placed in a Dubnoff Incubator (Precision Scientific) and agitated for 60 min at 37° C. The tubulin-colchicine complex which adhered to the anion exchanger was washed with at least 6 ml of TS buffer. The radioactive drug-protein complex was eluted directly into a scintillation vial with 0.5 M NaCl (2 ml) and 15 ml dioxane-PPO-POPOP cocktail. Radioactivity was determined in a Packard 3255 Liquid-Scintillation Spectrometer. Gel filtration chromatography using a Sephacryl S-200 column verified the molecular weight of tubulin; the tubulin absorbance peak at 280 nm coincided with the radioactivity peak.

C. Separation of Microtubule Polymer from Monomer

Piperleers et al., 1977 showed that these forms can be separated by high speed centrifugation. The separation of monomeric from polymeric tubulin was carried out as shown in Fig. 1. Nerve or brain was homogenized in a teflon-glass homogenizer using 50% glycerol, 5% DMSO, 0.5 mM GTP, 0.5 mM MgCl$_2$, 0.5 mM EGTA in 10 mM phosphate at room temperature, pH = 7.00 (MTS). This was centrifuged at 105 x g, for 45 min. at 20° C. The
FIGURE 1

This is the method used for the separation of tubulin forms derived from either brain or sciatic nerve. SUP I contains monomeric tubulin, SUP II microtubules and PPT membrane bound tubulin.
Homogenize Brain/Sciatic Nerve
2ml MTS Buffer per g wet tissue

SPIN $10^5 g$ 25°C 45'

SUP I

Pellet

Resuspend in Equal Volume of TS Buffer

SPIN $10^5 g$ 4°C 30'

SUP II

PPT

FIGURE 1
supernatant (SUP I) contains monomeric tubulin, the pellet resuspended in the TS buffer, centrifuged $10^5 \times g$, for 30 min., at $4^\circ C$ to disaggregate the microtubules. The supernatant (SUP I) and pellet (PPT) both contain colchicine binding activity which co-eluted with tubulin on gel filtration (Sephacryl S-200). Repeated washes and centrifugation did not remove colchicine binding activity from the pellet. Membrane bound tubulin has been shown to have different properties from soluble tubulin. (Nath and Flavin, 1978)

D. Characterization of Microtubulin Binding Protein

In order to determine that the binding $^3H$-colchicine was binding to tubulin a preparative gel filtration was used. Column material was Sephacryl Superfine S-200 (Pharmacia Fine Chemicals Co., Piscataway, N.J.). The column was packed at $4^\circ C$ in 10 mM phosphate, 10 mM MgCl$_2$ buffer, pH = 7.0 (Piperleers et al., 1977). The gel was suspended in 50 mM phosphate buffer and degassed under vacuum. A slurry was made by gentle mixing and poured over a column to avoid bubble formation. A Pharmacia P-3 peristaltic pump was installed to pack the column with gravity at a flow rate of 1.6 ml per minute. After 1 hr of packing a flow adapter was inserted, care taken to avoid the introduction of air bubbles. The column was run for 30 minutes against gravity to insure even packing and final adjustments on the flow adapted made. Final column size was 1.5 x 30 cm. Blue dextran elution fixed a typical void volume of 18 ml. Maximum sample volume was 2.0 ml at a flow rate of 1.0 ml/min collected in 2 minute fractions. Presence of proteins in the eluate was monitored by ISCO Model UA-5 Absorbance Monitor set to 280 nm. Fractions were collected and protein concentration determined according to Bradford, 1976.
To verify that tubulin is the compound binding colchicine, a binding incubation was carried out using 0.5 uCi $^3$H-colchicine and brain SUP I. After one hour of incubation, the reaction mixture was placed on the Sephacryl S-200 and eluted as previously stated. Using protein standards of known molecular weights on the column, the protein-colchicine complex was determined to elute in a fraction corresponding to approximately 105,000 daltons. A second peak of radioactivity was free colchicine; no protein was detected in these fractions.

A unique property of tubulin is its ability to polymerize at 37° C and depolymerize at 0° C (Bryan, 1974). Rat brain or sciatic nerve TS homogenate was incubated at 0° C for 30 min to depolymerize microtubules. This was spun at 100,000 x g to remove membranes, nuclear and mitochondrial fractions. To this supernatant was added an equal volume of glycerol, MgCl$_2$, EGTA and GTP to make MTS buffer concentrations. After incubation for 45' at 37° C, centrifugation at 100,000 g was carried out. The pellet thus obtained was resuspended in TS buffer, placed on Sephacryl S-200 column, and the fraction corresponding to colchicine binding protein placed on gel electrophoresis (Section E). Upon staining and destaining 90% of the absorbance was found under one band of molecular weight 105,000 daltons. Two small peaks were seen in the region of high molecular weight, approximately 220,000 daltons. This compares reasonably well with the tubulin monomer molecular weight 120,000 determined by Eipper, 1972. Identical results were obtained for microtubular protein obtained from either brain or sciatic nerve, although brain yielded more protein.
E. Polyacrylamide Gel Electrophoresis

A more accurate means of determining molecular weight utilized polyacrylamide gel electrophoresis. Disc gel rods were cast according to Davis, 1964. Solution A consisted of 48 ml 1 N HCl, 36.6 g Tris, and 0.23 ml N, N, N', N'-tetramethylethylene diamine (TEMED, Eastman Kodak 8718) and enough distilled water to make 100 ml, pH = 8.9. Solution C was made up of 28.0 g acrylamide, 0.735 g bis-acrylamide and enough water to 100 ml. Two parts of Solution C, one part Solution A and three parts 0.14% ammonium persulfate (W/V), 2% sodium dodecyl sulfate (SDS). This solution was kept on a stirring plate until cast into 5 x 75 mm glass tubes for polymerization. The polymerizing solution was introduced by allowing it to run down the sides of the glass rods, avoiding bubble formation. Glass tubes were precoated with Photo-Flo 200 before use (Eastman Kodak, Rochester, N.Y.). Polymerization required approximately 45 minutes.

Protein samples for analysis were mixed in 12 x 75 mm test tubes with one drop of bromphenol blue, 50 ul 40% sucrose, 10 ug α-chymotrypsinogen internal standard. Samples were applied to rods placed in a Pharmacia buffer (6.0 g/L Tris, 28.8 g/L glycine, 10 g SDS/L. pH = 8.3). Sample gels were run at a current of 4 mg/gel and took approximately 60 minutes to complete.

Gels were carefully removed from the tubes and placed in test tubes containing staining fixing solution. This solution was 1 gram Naphthol Blue-black per 100 ml 7% acetic acid. After one hour of staining, the
staining solution was decanted and gels placed in a Pharmacia GD-4 destainer. Electrophoretic destaining was carried out in 7% (v/v) glacial acetic acid.

Quantitation of protein was accomplished by using an ISCO gel scanner, 0.1 absorbance full scale, 580 nm filter. Peak area of absorbance plotted against known protein standards gave linear results from 0-50 ug bovine serum albumin.

F. Viscosity Measurements

Lipid bilayers have a number of measurable physical properties, e.g. viscosity, dielectric constant and spectral absorbance. Of particular interest is the use of fluorescent molecules, such as diphenyl hexatriene and perylene, for probing membrane structure and fluidity. As developed by Shinitzky et al., 1971, these molecules can absorb a photon of polarized light and after a certain interval, the molecule will fluoresce. The emitted photon will have a new polarization dependent upon the collisions it undergoes within its lifetime (hence the viscosity of the medium surrounding it). $P$ was calculated by measuring fluorescence intensity. Parallel to incident beam ($I_{\perp}$) and intensity perpendicular ($I$).

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\perp} - I_{\parallel}}$$

(5)

Figure 2 shows a typical plot of $1/P$ versus $T/\eta$. To derive this curve, Ostwald viscometers were calibrated with glycerol-water solutions of known viscosity. From these values, the viscosity versus temperature
was determined for mineral oil. Using this data, a standard curve of \( \ln \eta \) versus \( T^{-1} \) is generated. Polarization of known concentrations of fluorescent molecules in mineral oil was then determined for a range of temperatures. Since the viscosity of mineral oil is known, \( P \) and \( T \) are measured, a standard curve of \( 1/P \) versus \( T/\eta \) can be obtained (Fig. 2). In this example, the fluorescent molecule employed is diphenyl-hexatriene (DPH). Thus, for a sample containing membranes the probe will provide an index of the membrane microviscosity.

Mineral oil or some type of hydrocarbon must be used since probes employed here will not fluoresce in aqueous or glycerol solutions. Presence of a hydrophobic environment of any kind is essential: e.g., biological membrane, synthetic micelle, or hydrophobic core of protein. Fluorescence measurements were made with an American Instruments Co. Aminco Bowman Spectrophotofluorimeter fitted with polarizing filters according to Chen and Bowman, 1965.

G. Membrane Preparations

1. Myelin Preparation

Myelin was prepared from the peripheral nervous system (PNS) by the method of Adams et al., (1968): Sciatic nerve was dissected and stripped of fat and connective tissue. The nerves were weighed and immersed in 0.7 M glycine-0.05 M triethylamine buffer (pH = 6.0) for two hours 4° C. This softens the collagen matrix and facilitates homogenization. After incubation the nerves are rinsed well with distilled water and placed in 0.32 M sucrose. A ground glass mortar and pestle is used
FIGURE 2

This is a standard curve relating temperature viscosity ratio to inverse polarization.

$$P = \frac{(I_{\downarrow} - I_{\perp})}{(I_{\downarrow} + I_{\perp})}$$ where $I_{\downarrow}$ ($I_{\perp}$) represents the fluorescence polarization intensity in relationship to the incident beam polarization.
\[ \frac{1}{P} = 5.1435 \frac{T}{\eta} + 1.8618 \]

**FIGURE 2**
to grind the nerves; the homogenate is layered over 0.8 M sucrose and centrifuged in a Beckman L5-75 swinging bucket for 3 hours at 23,000 x g at 4° C. In this system, myelin settles at the interface, adipose floats and nuclei and mitochondria pellet at the bottom.

Recovery of myelin was greatest when nerves were minced and incubated in glycine buffer: 66% of whole homogenate cholesterol and 76% cerebrosides were recovered in the myelin interface. The myelin interface contained an increased concentration of cholesterol and cerebroside over homogenate, expressed as umol/mg protein, but very little succinic dehydrogenase (SDH). The chief advantage of this method is that myelin and mitochondria are well separated. Adams also reported that specific activity leucine aminopeptidase, an axonal membrane enzyme was increased 63% in myelin over homogenate value, indicating that axonal membrane follows myelin with approximately the same enrichment over homogenate.

Peripheral nerve as tissue is more difficult to study than brain or liver because it yields less weight per animal and because its collagen matrix prevents easy homogenization.

2. Red Cell Ghost Preparation (Hanrahan and Ekholm, 1974)

This method prepares red blood cell ghosts (RCG) by osmotic lysis followed by several rinse steps. Two buffers were used, isotonic Tris buffer (310 imOsm, 0.172 M Tris, pH = 7.6) and hypotonic Tris buffer (20 imOsm Tris, pH = 7.6) which is prepared by a dilution of isotonic Tris. Tris-HCl was obtained from Sigma Chemical Co., St. Louis, MO.
Up to 10 ml of blood was collected from a rat by cardiac puncture into heparinized tubes, and centrifuged immediately at 1000 x g for 30 minutes at 4° C. Plasma and buffy coat were removed by suction and the cells gently resuspended in isotonic Tris by inversion and spun at 1000 x g for 30 minutes at 4° C. This washing procedure was repeated twice more and the tubes were kept on ice. The washed cells were then suspended in isotonic Tris to a 50% hematocrit. The cells were dispersed by repeated inversions for 1 minute before lysis.

In a typical preparation, 5 ml aliquots of the final suspension were placed in 50 ml polyethylene tubes. Thirty ml of cold hypotonic Tris were blown into the tubes with a pipette. After standing 5 minutes the tubes were spun at 20,000 x g for 40 minutes at 4° C. The hemolyzed red cells sedimented but the interface between the supernatant and packed ghosts was difficult to see. The supernatant was decanted and any membrane adhering to the bottom of the tube dislodged. This procedure was repeated until the membranes were colorless. At least four washes were performed in all cases.

H. Protein Assay

The protein content of brain or sciatic nerve homogenates and fractions was determined by Coomassie Brilliant Blue G binding extinction (Bradford, 1976). A dye reaction mixture was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 (Sigma Chem. Co., St. Louis, MO.) in 50 ml methanol. The reference erroneously says ethanol. One hundred ml 85% (w/v) phosphoric acid was added and sufficient water to make up one liter. A typical sample is assayed in duplicate 0.5 ml aliquots to
which 2.5 ml of Coomassie reagent was added to make 3 ml total volume. This was vortexed and absorbance determined at 595 nm. Standards of 0-50 ug bovine serum albumin (Sigma) were prepared for each run in the same buffer as unknowns.

The color developed to maximum absorbance within 5 minutes and remained stable up to one hour. This method was chosen over others because it is quick and very sensitive. Bradford claims that buffer interferences from K+, Mg$^{2+}$, EDTA and Tris seen in the Lowry procedure do not arise. A sample containing 50 ug of protein produced O.D.$_{595}$ = 0.8. A typical standard curve has the following formula: $\text{ug protein} = 324.4 \times (\text{O.D.}_{595}) + 2.33$ ($r = 0.986$). This relation is linear up to but not greater than 50 ug protein per assay.

I. Phospholipid Methods

1. Extraction and Purification

This scheme for the analysis of lipids employed the Folch extract. (Folch Pi, et al., 1957). In this method, 19 ml extraction chloroform: methanol mixture (2:1, v/v) was added to each ml of homogenate (or gram tissue) and filtered to separate. The non-lipid residue contains globular proteins, glycogen, nucleic acids, and other water soluble compounds. To the filtrate was added 0.2 volumes of water which was allowed to separate. The Folch Lower Phase contains neutral and polar lipids, and proteolipid protein. The Folch Upper Phase contains gangliosides and other water soluble molecules. A mixture of chloroform: methanol: water (200:100:75) was prepared and allowed to separate. The upper phase of this was used to
wash the Folch Lower Phase extract of neutral tissue. These washes (3 times, 1 ml per wash) were either saved or removed by suction (Albers et. al., 1972).

Silicic acid column chromatography was used to separate classes of Folch Lower Phase extracts. Samples were dried under nitrogen or vacuum at 50° C, reconstituted in CHCl₃, and applied to 1.0 g columns of silicic acid (100 Mesh, Mallinckrodt, Chemical Works, St. Louis, MO.). The total Folch lower phase was applied to the silicic acid column which was washed with 10 ml CHCl₃ to collect Neutral Lipids, then 10 ml each CHCl₃: CH₃OH = 2:1 and 1:2, then 10 ml CH₃OH. All methanol containing extracts were combined to yield a Polar Lipid fraction. Extracts of 4 rat sciatic nerves (< 200 mg tissue) or 1 brain (1 g tissue) were found to separate cleanly into neutral and polar lipid fractions with no overlap. Elution of each lipid species from the column was determined by thin layer chromatography.

2. Isolation of Lipid Classes

Two systems were used to separate neutral and phospholipids. Neutral lipids were separated on Silica Gel G plates (Merck, Inc) by one-dimensional development in a solvent system of petroleum ether: diethylether: glacial acetic acid = 135: 12: 0.75. Polar lipids were spotted on separate plates and run in a solvent system of CHCl₃: CH₃OH: 58% NH₄OH = 100:50:10. Relative migration of each compound was recorded and standards were run on each plate containing unknowns. In terms of tissue weight equivalents, 200 mg of sciatic nerve and 50 mg brain wet weight were spotted on the plate. Spots corresponding to phosphatidyl-choline (lecithin), lysolecithin, sphingomyelin, cholesterol and cholesterol esters were identified by
TABLE 1

Thin Layer Chromatography of Lipids

<table>
<thead>
<tr>
<th>Compound</th>
<th>System*</th>
<th>Rf**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebroside</td>
<td>I</td>
<td>0.85</td>
</tr>
<tr>
<td>Lecithin</td>
<td>I</td>
<td>0.42</td>
</tr>
<tr>
<td>Lysolecithin</td>
<td>I</td>
<td>0.25</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>I</td>
<td>0.33</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>II</td>
<td>0.15</td>
</tr>
<tr>
<td>Cholesterol ester</td>
<td>II</td>
<td>0.95</td>
</tr>
</tbody>
</table>

* System I: CHCl₃:MeOH:NH₄OH (100:50:10)
  System II: Petroleum Ether: Diethyl Ether
  Glacial Acetic Acid (135:12:0.75)

** Rf = \frac{\text{mm from origin to center of spot}}{\text{mm from origin to solvent front}}
sublimed iodine vapors and scraped for further analysis \textit{(vide infra)}. Standards of phospholipids and polar lipids were obtained from Sigma Chemical Co., St. Louis, MO: phosphatidyl choline, cerebroside, cholesterol, cholesterol oleate, lysophosphatidyl choline and sphingomyelin.

3. Cholesterol Method

Cholesterol content of TLC scraping was determined using a \( \text{FeCl}_3 - \ \text{H}_2\text{SO}_4 \) oxidation reaction (Zlatkis et al., 1953). Stock \( \text{FeCl}_3 \) solution was prepared by 1 gram \( \text{FeCl}_3 \) in 10 ml glacial acetic acid. Working \( \text{FeCl}_3 \) solution is a dilution of 2 ml stock solution to 200 ml final volume with concentrated sulfuric acid.

Cholesterol standards were prepared in CHCl\(_3\) to a concentration of 1 ug/ul. These were stored in brown bottles at \(-11^\circ\) C until use. Standards ranging from 50 to 500 ug were pipetted into 16 x 125 mm test tubes and dried under \( \text{N}_2 \). Because silica gel did not change color development or blank values, TLC samples were treated the same as standards. Glacial acetic acid (3 ml) was added to tubes followed by 100 ul distilled water, and then the tubes were mixed. Three ml of \( \text{FeCl}_3 \) working reagent were added to each tube. Silica gel was sedimented by centrifugation at 1000 x g for 10 minutes. The supernatant was decanted into 1 cm glass or plastic cuvettes and the optical density at 565 nm was determined in a Beckman Model 26 spectrophotometer.


Cerebroside levels were determined by measuring galactose from thin layer gel scrapings. Orcinol reagent was prepared by adding 100 mg
orcinol monohydrate (Sigma) to 140 ml concentrated sulfuric acid. Once dissolved, distilled water was slowly added to 200 ml final volume.

In a typical assay, a constant area of unknown or cerebroside-standard containing silica gel was scraped into a test tube. The samples were cooled on ice for 5 minutes. Three milliliters of orcinol reagent were added, tubes vortexed and incubated for 20 minutes at 80°C. Reaction was stopped by placing the tubes on ice for 5 minutes. Tubes were vortexed, centrifuged for 10 minutes at 1000 x g to sediment silica gel, and decanted into plastic cuvettes. A standard curve was constructed by plotting absorbance at 505 nm versus amount spotted. This method gave linear 2 results for 0-100 ug cerebroside per 4 cm silica gel. Blank silica gel was scraped and treated as unknown. The blank gel was analyzed and its supernatant used in the reference cell. Compared to a reagent blank, silica gel gave an absorbance of 0.09.

5. Phospholipase A Method

The following is a modification of Webster's method (Webster, 1973). HDO treated and control rats were sacrificed by decapitation and sciatic nerves dissected as previously described. Nerves from the same animal were quickly frozen in liquid nitrogen with a cooled mortar and pestle. The crushed powder was transferred to a ground glass homogenizer with tight fitting plunger and distilled water added. Homogenates were diluted to a final concentration of 20 mg wet weight tissue per ml water.
The substrate solution was prepared by evaporating 2.5 umol and 10,000 DPM $^{14}$C-lecithin in each 12 x 75 mm test tube. The substrate contained $^{14}$C-methyl-choline. To each tube was added 0.5 ml buffer, 0.1 M Na acetate - 0.9 % NaCl containing 3 mg Na taurocholate per 0.5 ml. Buffer pH was adjusted to 5.0. Webster's method differs because he used a $^3$H-fatty acid in the 1-position, and a $^{14}$C-fatty acid in the 2-position. By measuring $^3$H and $^{14}$C simultaneously, he was able to determine phospholipase A$_1$ and A$_2$ separately. The method used here measures total phospholipase A = A$_1$ + A$_2$. Enzyme activity was measured by $^{14}$C-lysolecithin released.

Incubations were carried out for 1 hour at 37° C. The reaction was stopped by addition of 10 ml CHCl$_3$:CH$_3$OH (2:1) and extracted with the same solvent. Samples were dried under N$_2$ at 50° C, reconstituted with CHCl$_3$:CH$_3$OH and aliquots subjected to Polar Lipid thin layer chromatography system. Both lysolecithin and lecithin spots were scraped and placed in 15 ml Thrift Solve (Kew Scientific). Enzyme activity was calculated as nanomoles lysolecithin per hour per 10 mg wet nerve. Results were compared to control sciatic nerve, substrate without nerve addition, and snake venom phospholipase A$_2$. (P & L Biochemicals, Inc. - Crotalus atrox venom). Supplied stated specific activity range was 300-400 units per mg protein. This standard gave a value of 350 units per mg protein in acetate NaCl buffer system. The substrate contained less than 2% total radioactivity under the lecithin spot.

6. Phosphate Assay (Gottfried, 1967)

This method was used to detect organic and inorganic phosphate in aqueous, organic solvent and silica gel carriers. Samples containing up
to 1.0 umol phosphate were placed in dry, acid washed 25 x 250 mm Pyrex test tubes to which was added 2 ml 10 N \( \text{H}_2\text{SO}_4 \) and 3 glass beads. These tubes were placed in a 250° C heating block for 25 minutes, after which 2 drops of 30% \( \text{H}_2\text{O}_2 \) were added. After 3 minutes, the tubes were checked for a clear or white residue. If an oily black residue formed because too much lipid was present, more \( \text{H}_2\text{O}_2 \) was added until the residue turned white. Tubes were removed from the heating block and an amount of 16.7% \( \text{Na}_2\text{SO}_3 \) was added to equal the drops of \( \text{H}_2\text{O}_2 \) used. Tubes were filled with distilled water to approximately 25 ml, then 5 ml of 0.5% (w/v) ammonium molybdate and 5 ml 0.025% (w/v) hydrazine sulfate are added. The tubes were vortexed and placed in a boiling water bath for 10 minutes. Then the tubes were removed from heat and placed in an ice water bath until cool. The volume in each tube was adjusted to 50 ml with distilled water and 7 ml n-butanol added. After vortexing twice, the absorbance of the butanol layer was determined in a Beckman model 26 UV-VIS at a wave length of 795 nm. A standard containing 0.5 umole P produced an optical density of 0.030 ± 0.0037 (mean ± S. Dev.). This method is linear up to 1.0 umole P if dilutions of the butanol layer are made in reagent butanol to bring them to 7 ml.

Pulse chase experiments using \( ^{32}\text{P} \) as orthophosphate required a scheme for Cerenkov counting. Because the phosphate digest leaves an intense blue color in butanol, liquid scintillation techniques would be hampered by both chemical and color quench. Preparation of samples was accomplished by a modification of an existing method (St. C. Palmer, 1969). Butanol containing \( ^{32}\text{P} \) from Gottfried analysis was placed in 16 x 125 mm test tubes. Then 1.5 ml 10 N NaOH and 0.5 ml 30% \( \text{H}_2\text{O}_2 \) were added and
tubes vortexed. The tubes were placed in a 100° C bath for 20 minutes or until all the blue color was bleached. Upon removal the tubes were allowed to cool. A slight greenish tinge sometimes remained due to the reagents. This was removed by addition of 0.5 ml % M sodium arsenite. If a white precipitate formed, tubes were heated in running hot water until it dissolved. Since the aqueous (lower) layer is alkaline, the $^{32}$P will be extracted quantitatively into the lower phase. The butanol layer was removed by suction and total volume made up to 12 ml. This was decanted into a scintillation vial with a polyethylene sealed cap since foil caps leach color. Counting efficiency was 41% using a wide open window (gain 2.0) of a Packard 3255 Liquid Scintillation Spectrometer. Using a known amount of $^{32}$P and wide open window, gain settings were varied until maximum efficiency was obtained.

J. Nerve Conduction Velocity of Rat Sciatic Nerve

A recording of the rat electromyograph (EMG) was accomplished by inserting a bare, stainless steel electrode through the center of the abductor digiti quinti muscle of the foot of a rat anesthetized with 40-50 mg/kg sodium pentobarbital, i.p. A reference bare electrode was inserted subcutaneously at a more distal site of the foot. This muscle receives its nerve supply from the lateral plantar nerve which arises from the sciatic tibial nerves. Anatomical loci were verified by dissection using a rat atlas of anatomy as a guide (Green, 1935). Electrodes were inserted into exposed muscle and stimulus sites to insure accuracy of technique. The electromyogram was displayed on a Model J Clinical Electromyograph (TECA Corp., White Plains, N.Y.). Voltage scale used was 5 mV/cm, time scale 2 msec/cm. Hard copy record was obtained using a Dumont Laboratories
A typical normal electromyogram with definition of terms: stimulus artifact, distal latency, nerve conduction time and nerve conduction velocity.
A TYPICAL NORMAL ELECTROMYOGRAM WITH DEFINITION OF TERMS.

Stimulus Artifact

Plantar Stimulus Point No. 1
Sciatic Notch
Stimulus Point No. 3

$\Delta_1 =$ Distal Latency
$\Delta_2 =$ Nerve Conduction Time Between Stimulus Points No. 1 and No. 3

$$NCV = \frac{\text{Distance Between Point 1 & 3 in mm}}{\Delta_2} \text{ (m/sec.)}$$

FIGURE 3
Oscilloscope camera (Clifton, N.J.), Type 453, Film type 084 Polaroid pack (3000 ASA rating). EMG electrodes used for stimulating were 80 microns in diameter with teflon insulation up to the tip.

Three sites of stimulation were chosen: sciatic notch, popliteal space, and plantaris tendon. The same EMG was recorded in each case, with the time delay between points of constant phase equaling the conduction time between the stimulated regions of the nerve (See Fig. 3). Knowing the length of nerve, dividing this by the time delay equals the maximal nerve conduction velocity (NCV). The largest fibers have the fastest rate of conduction. Thus, the time delay to firing was measured by drawing a line through the linear portion of the muscle action potential. The interval between the intersection of this line and the t-axis and the stimulus artifact defines the latency (milliseconds) of the muscle from a given stimulation point (Figure 3). Left and right quantities were averaged to give one value per animal.

To minimize sources of error, an electrode holder was designed to standardize the distance between stimulating electrodes. This holder was made out of 1/4" lucite plastic with holes drilled 32 and 35 mm apart and also served to keep the electrodes at right angles to skin. Landmarks were verified by touch since the sciatic notch, the head of fibula and the plantaris tendon can be felt through the skin. Using oscilloscope tracings, the following parameters were calculated: Distal Latency, Plantar-Popliteal NCV (Distal NCV), Popliteal-Sciatic Notch NCV (Proximal NCV), and Plantar-Popliteal NCV (Overall NCV). Distal NCV and Proximal NCV changes were measured separately to record the progression of nerve degeneration.
RESULTS

A. Microtubules and HDO Toxicity

The specific activity of $^3$H-colchicine binding for monomer-polymer forms of tubulin is summarized in Table 1. Comparing sciatic nerve obtained from rats drinking 0.5% HDO in water with controls, it appears that the treatment increased binding by a factor of 2 in two compartments: SUP I and PPT. A fraction which represents microtubules, SUP II, is unaffected by treatment. Brain showed dramatic changes in these fractions with treatment: 1.26 to 0.38 in SUP I, 2.31 to 0.97 in SUP II, and 0.06 to 0.49 in PPT (all in nmole/mg protein).

Soluble protein content decreased to about 80% of control values in both treated sciatic nerve and brain, although tissue wet/dry weight ratios remained constant. This decrease in protein content would account for the increase in specific activity of binding in sciatic nerve from 0.5% treated over controls, but this does not explain the decrease in brain values. The data in Table 2 illustrate the recovery of subcellular distribution of microtubular protein from sciatic nerve preparations. Treatment of a rat with 0.5% hexanedione in drinking water for 2 months did not change either the total binding (124 pmol/fraction) or the subcellular distribution. The sum of binding activity from treated fractions was not statistically different from control or treated homogenate.
Table 2

Distribution of Polymeric vs. Monomeric Tubulin in Rat Sciatic Nerve and Brain Treated with 2,5-Hexanedione*

<table>
<thead>
<tr>
<th></th>
<th>CONTROL**</th>
<th></th>
<th>0.5% Treated**</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. Nerve</td>
<td>Brain</td>
<td>S. Nerve</td>
<td>Brain</td>
</tr>
<tr>
<td>SUP I</td>
<td>.023</td>
<td>1.26</td>
<td>.042</td>
<td>.38</td>
</tr>
<tr>
<td>SUP II</td>
<td>.14</td>
<td>2.31</td>
<td>.12</td>
<td>.97</td>
</tr>
<tr>
<td>PPT</td>
<td>.11</td>
<td>.06</td>
<td>.21</td>
<td>.49</td>
</tr>
<tr>
<td>TOTAL</td>
<td>.273</td>
<td>3.63</td>
<td>.372</td>
<td>1.84</td>
</tr>
</tbody>
</table>

* Data is expressed as nmoles Colchicine bound per fraction per mg protein.

** Standard Deviation is approximately 10% mean in all cases.
TABLE 3

Colchicine Binding of Fractions of 0.5% Sciatic Nerve to Control Homogenate

<table>
<thead>
<tr>
<th>CONTROL</th>
<th>0.5% Treated</th>
<th>pmol measured/fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2541 Homogenate</td>
<td>—</td>
<td>124</td>
</tr>
<tr>
<td>—</td>
<td>2541 Homogenate</td>
<td>103</td>
</tr>
<tr>
<td>—</td>
<td>2541 SUP I</td>
<td>23</td>
</tr>
<tr>
<td>—</td>
<td>2541 SUP II</td>
<td>38</td>
</tr>
<tr>
<td>—</td>
<td>2541 PPT</td>
<td>65</td>
</tr>
</tbody>
</table>
In other experiments, homogenate subfractions were reconstituted in an effort to demonstrate any possible alteration in binding activity related to subfraction influences. In each case, the sum of activities measured separately was equal to the activity of pooled fractions. Therefore, no evidence of inhibitory or facilitory alterations in \(^3\)H-colchicine binding was apparent from HDO treatment. Furthermore, direct addition of HDO (0.1 - 10 mM) did not affect binding activity. Deuterium oxide and vincristine are each capable of promoting microtubule assembly and block fast axoplasmic transport and stabilize colchicine binding (See Introduction). Therefore, the half life of colchicine binding in vitro should indicate whether any vincristine-like activity is present.

To test this hypothesis, sciatic nerve homogenates were prepared from animals treated with HDO for two months (see Methods). At 2 months of 0.5% HDO treatment, rats showed moderate to severe clinical paralysis, body weight decreased to 60% of controls. Those drinking 0.1% HDO water were not visibly different from controls in either clinical impairment or body weight. Homogenates were incubated with \(^3\)H-colchicine at 37° C. Aliquots were removed 0, 1, 3 and 5 hours after excision. \(^3\)H-colchicine binding was measured in quadruplicate on pooled nerves: 2 nerves per animal, 2 animals per group. Results of this experiment are shown in Figure 4. No difference between the three groups is apparent. Conditions which delay binding decay also stabilize microtubule assembly (Borisy et al., 1974). The data suggests the time decay of colchicine binding is independent of treatment. The half life of 4.3 hours for control compares well with 5 hours observed by Bamburg et al., 1973.
FIGURE 4

Time decay of colchicine binding measured in rat sciatic nerve homogenates. Rats were treated for 260 days with 0.1% and 0.5% HDO in drinking water. Sciatic nerves were excised and homogenized in TS buffer incubated at 37°C. Aliquots were removed at 0, 1, 3, and 5 hour time intervals after excision.
FIGURE 4

SCIATIC NERVE

DPM Colchicine Bound x 10^3

0 1 2 3 5 Hr.

□ — □ Control
○ — ○ 0.5%
● — — 0.1%
B. Microviscosity Data for HDO Neuropathy

The effect of HDO on sciatic nerve homogenate microviscosity is illustrated by Figure 5. The outstanding feature of this data is the biphasic nature of \( \eta \) dependence upon temperature. Using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe of the lipid bilayer, a high viscosity component is observed in control for temperatures, \( T < 20^\circ C \) (293°K). Analysis of control data suggests the \( \eta \) versus \( T \) variation may be (1) error at low \( T \), (2) a hyperbolic dependence, or (3) two straight lines in \( \log_{10} \eta \) vs. \( T^{-1} \). However, fitting one line through all points gave a correlation coefficient \( r = 0.89 \). If two lines are drawn, one including points such that \( T > 20^\circ C \) and the other \( T < 20^\circ C \), the correlation coefficients are \( r_1 = 0.998 \) and \( r_2 = 0.997 \), respectively. Repeated experiments gave the same curve with biphasic slope. Data obtained for nerves from 0.1 and 0.5% HDO treated nerves show a monophasic dependence with lower microviscosity values with the 0.5% HDO. The 0.1% HDO treated rats represent a subtoxic exposure since they show neither symptoms of neuropathy nor weight loss. The microviscosity profile demonstrated not only markedly lower microviscosity values point for point, but an absence of the lower temperature curve phase transition.

Figure 5 depicts myelin microviscosity/temperature curve by Arrhenius plot. Note that 0.5% values all lie along a straight line giving a constant slope, whereas control myelin again yields a biphasic dependence with transition temperature, \( T = 20^\circ C \),
Arrhenius plot of sciatic nerve homogenates from control, 0.1% and 0.5% 2,5-hexanedione treated animals (2 months). Diphenyl-hexatriene was added in 1.5 µl acetone to a final concentration of 1.0 µM.
FIGURE 5
Arrhenius plots of sciatic nerve myelin prepared from control 0.5% HDO treated animals. The time course of treatment was greater than 2.5 months. DPH was added in acetone solvent to a final concentration of 1 µM.
FIGURE 6

MYELIN PREPARATION

- Control
- 0.5% HDO

\[ n \text{ Centipoise} \]

\[ {^\circ}\text{K}^{-1} \times 10^{-3} \]
C. Effects of Nerve Section on Myelin Microviscosity

Because of the dose dependent effects of hexanedione on viscosity of homogenate or myelin from sciatic nerve, an axonal neuropathy model was chosen. Wallerian degeneration was employed because it is the best characterized and understood axonal neuropathy. The biochemical, morphological and electrophysiological indices of nerve section have been extensively studied (Mezei, 1970; Webster, 1973; Bradley, 1974).

Rat sciatic nerves were section and removed 2, 6, 10 and 20 days later. The results of myelin membrane microviscosity measurements are shown in Figure 7. Nerve section produces a rapid loss of myelin partially due to phospholipase A activation (Webster, 1973) and myelin itself becomes phagocytized and degraded beginning 48 hours after section (Bradley, 1974). To compare these effects with that of HDO treatment, a group of animals was kept on 0.5% HDO with myelin microviscosity measurements taken after 20 days of treatment. The curve shows no effects different from control on membrane microviscosity of 20 days HDO treatment.

In this study, demyelination produced by a model axonal neuropathy, nerve section, displays a monophasic dependence of viscosity versus temperature. This is the same type of dependence as shown by 2 months treatment of rats in vivo with 0.1 or 0.5% HDO. However, treatment with 0.5% HDO for 20 days did not eliminate the biphasic curve seen with longer treatment.
FIGURE 7

Arrhenius plot of Wallerian degenerated sciatic nerve. Data is shown for 2, 6, 10 and 20 days post section, as is data from unsectioned nerve treated in vivo with 0.5% HDO for 20 days.
ARRHENIUS PLOT OF MYELIN VISCOSITY PREPARED FROM SECTIONED RAT NERVE.

FIGURE 7
D. Fluorescence Polarization of Red Blood Cell Ghosts

Red blood cell ghosts were prepared from animals treated for 2 months with HDO according to Hanrahan and Eckholm, 1974. Fluorescence polarization was determined as described in Methods and its reciprocal plotted against inverse temperature in Figure 8. The reason for considering red cell ghosts is two fold:

1. The red cell ghost is a standard natural membrane preparation. Much is known of its lipid and protein content and the antigenic determinants on the cell surface. The viscosity and fluorescence polarization of the red cell ghost have been studied as a function of temperature.

2. If HDO exerts an effect on membranes in general, then red cell ghosts from treated animals would show this effect.

Figure 8 data does indicate an effect of HDO administration in vivo on red cell ghosts. Note that all curves are monophasic, meaning that there are no phase transitions. Ghosts derived from either HDO group display essentially the same slope. Multiple regression analysis of this data revealed that control, 0.1% and 0.5% treatment group were best fit by 3 separate lines. Correlation coefficients for these groups were -0.997, -0.988 and -0.995, respectively. Regression analysis done on the best 2 lines, comparing control with 0.1% and 0.5% treatment taken together gave a poorer fit (r = .950). Although regression analysis works best, the effects on red cell ghosts are not dose dependent, as seen in the
Fluorescence anisotropy of red cell ghosts prepared from animals on HDO at specified levels for 2 months. Inverse polarization is the ordinate, inverse temperature the abcissa.
FLUORESCENT POLARIZATION OF RED BLOOD CELL GHOSTS FROM CHRONIC HEXANEDIONE TREATED ANIMALS.

- ○ 0.5%
- □ 0.1%
- ■ Control

FIGURE 8
nerve homogenates. Either 0.1% or 0.5% HDO treatment seems to produce the same curve, each different from control but not from each other.

When preparing the red cell ghosts, a red tint was noted in the 0.5% preparation which was not detected in the other samples. Optical spectra of control, 0.1%, and 0.5% groups are presented in Figure 9. There are weak absorbance peaks at 540 and 576 nm which correspond to oxyhemoglobin. These peaks were not washed out by repeated centrifugation (See Methods). This result suggests that ghosts derived from the 0.5% treated animals retain some hemoglobin. The absorbance change at 576 nm is only 0.04 O.D., which does not interfere with the fluorescent probe, as it absorbs at 250 nm and emits at 350 nm.

E. Hexanedione Effects on Recorded EMG

Oscilloscope tracings of muscle action potential recordings are shown in Figure 10. The top tracing is an EMG of a normal abductor digiti quinti muscle of the foot. The time base here is 2 cm/msec, 5 msec full scale, the vertical axis 5 mV/cm. The same EMG is recorded in all three traces, the top evoked by sciatic notch stimulation, the middle by popliteal space, and the bottom by plantaris tendon stimulation. The time of the muscle action potential is characterized by its latency, 1.31 msec, and short delay between stimulus points. Distal NCV is 57.9 m/sec and Proximal NCV is 58.1 m/sec. The bottom photograph is the same animal's nerve muscle unit after three weeks of ingestion of 0.5% hexanadione in drinking water. The time base is lengthened to 2 msec/cm but the vertical axis scale remains 5 mV/cm. In this case, the distal latency is increased
FIGURE 9

Optical spectra of red cell ghosts showing oxyhemoglobin peaks at 540 and 576 nm. Animals were treated with HDO for 2 months.
OPTICAL SCAN OF RED BLOOD CELL GHOSTS FROM CHRONIC HEXANEDIONE TREATED ANIMALS.

FIGURE 9
FIGURE 10

These are typical electromyograms of 0.5% treated animals. Each tracing in a superposition of at least five supramaximal stimuli applied to the sciatic nerve, recorded from the abductor digiti quinti muscle of the foot. The tracing with the shortest latency was elicited by plantar stimuli, the next shortest by popliteal stimuli and the longest latency by sciatic notch stimuli. The top set of tracings is a pretreatment rat, and the bottom set, 3 weeks of 0.5% HDO in drinking water.
<table>
<thead>
<tr>
<th>LATENCY (msec.)</th>
<th>NCV (m/sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distal</td>
</tr>
<tr>
<td>1.27</td>
<td>51.7</td>
</tr>
<tr>
<td>2.25</td>
<td>35.3</td>
</tr>
</tbody>
</table>

FIGURE 10
FIGURE 11

Evoked electromyogram of a rat treated with 0.5% hexanediolone for 18 weeks. Each tracing is a superposition of at least five supramaximal stimuli applied to the sciatic-tibial-plantar nerve as discussed in Figure 10.
18 WEEKS

<table>
<thead>
<tr>
<th>LATENCY msec.</th>
<th>NCV m/sec.</th>
<th>Distal</th>
<th>Proximal</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.80</td>
<td>25.3</td>
<td>33.8</td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 11
to 2.15 msec. Distal NCV decreased from a pretreatment value of 57.9 m/sec to 35.3 m/sec. Proximal NCV also decreased from 58.1 to 45.0 m/sec.

Because this technique involves stimulating a nerve and recording the muscle action potential, there is always a segment of nerve between the stimulus point and the muscle. The conduction path is from the axon to the neuromuscular junction, chemical transmission across the junction, then muscle action potential propagation to the active recording electrode. From the plantar stimulus point to the insertion of the recording electrode is a distance of approximately 10 mm. If one assumes the NCV along this section of nerve is the same as the Distal NCV, then the increased delay time in msec is equal to 10 mm ÷ NCV (m/sec). Looking at the 3 week Distal NCV, 37.8 m/sec, the increase in conduction time is 0.26 msec. Since the distal latency changes by 0.8 msec, it is apparent that there are additional factors involved in the distal latency increase than just slowed NCV. Neuromuscular junction and muscle conduction factors must also be affected. Figure 11 depicts the EMG obtained from an animal which had been drinking hexanedione for 4.5 months. The time base is 1 msec/cm, the vertical scale 5 mV/cm. The EMG itself is different from the others in that the peak to peak amplitude is much lower (3.5 mV versus 20 mV). The neuromuscular unit is so extensively degenerated that the beginning or end of the EMG is difficult to define. Latency is increased to 2.51 msec compared with the two week value of 2.15 msec and control, 1.31 msec. Proximal and Distal NCV values were 24.9 m/sec and 23.8 m/sec respectively. The 4.5 month animal's EMG shows extremely dispersed waves, called polyphasic waves. Both normal and three week EMG tracings still show good synchrony of fiber firing because stimulation evokes similar waves.
F. Acute Effects of Hexanedione Treatment on Electromyography

The nerve conduction velocity (NCV) of sciatic-tibial nerves was used to measure changes in neuromuscular function produced by 0.5% HDO in drinking water. This data is presented in Figures 12 and 13. Nerve conduction changes were measured weekly on four rats, the distal (Plantar-popliteal) and proximal (Popliteal-sciatic notch) NCV's determined until significantly depressed compared to pretreatment values. After three weeks of treatment the rats were placed on tap water alone and allowed to recover. Although the proximal NCV was depressed at week 1 and 2, the distal NCV showed more striking decreases, dropping from a control value of 54 m/sec to 37 m/sec at 2 weeks of treatment. This remained depressed through to week 4 when the rats were returned to normal tap water.

No improvement in distal NCV was seen until two weeks of recovery. At this point, proximal NCV returned to 49.7 m/sec which is not significantly different from control, but different from 3 week values, P <.005. Proximal NCV decreased early but recovered quickly to control value. These results indicate that there is a rapid onset of NCV decrease, evident in both proximal and distal sections of the leg. Removal of exposure to HDO does not immediately reverse the distal NCV depression. This result agrees with clinical observation of humans exposed to hexacarbons who remain impaired after cessation of exposure (Shirab et al., 1974).

The distal latency is defined as the delay in milliseconds from the end of the stimulus artifact to the rapid rise of the abductor digiti quinti muscle action potential. The stimulus point is the plantaris tendon, chosen to minimize nerve conduction time. Results of Figure 13 indicated
Nerve conduction velocity changes with HDO treatment. Four rats were maintained on 0.5% HDO in drinking water and were evaluated weekly for Plantar-Popliteal (Distal) NCV, Popliteal-Sciatic Notch (Proximal) NCV, and Plantar-Sciatic Notch (Overall) NCV. After 3 weeks animals were placed on tap water to measure recovery of NCV parameters. Results of left leg and right leg were averaged to give one value per animal for each NCV parameter.
EFFECT OF HDO ON NCV

- Distal NCV
- Proximal NCV
- Overall NCV

* NS = Not Significant

** = Significantly different from Control p < 0.005; Dunnett's t-test

FIGURE 12
FIGURE 13

Distal Latency and body weight changes with HDO treatment. These parameters were measured on the same 4 animals as Figure 12. After 3 weeks of drinking 0.5% HDO water, the animals were placed on tap water to measure recovery of Distal Latency and body weight.
EFFECT OF HDO ON DISTAL LATENCY AND BODY WEIGHT.

** = p<.01
*** = p<.005
Dunnett's t-test

FIGURE 13
that there was an immediate rise in latency (1.31 to 2.1 msec) followed by
a decrease to 1.62 msec which is still elevated above control (P < .005).
Body weight also followed this trend as the animals do not regain pre-
treatment weights within the 2 week recovery period.

Analysis of variance (ANOVA) for repeated measures and Dunnetts' t-test were performed on data obtained in Figures 12 and 13. ANOVA showed
that body weight, distal latency, distal NCV and proximal NCV means for
posttreatment times were different from control (P < .05, .01, .005, and
.05, respectively).

Dunnetts' t-test analysis of treatment means (Winer, 1971) determined that mean body weights were below control, P < .01 and distal latency
time was elevated over pretreatment mean, P < .005. Distal NCV was not
affected significantly 1 week post treatment but decreased at weeks 2 -5
(P < .005), then recovered to control velocity at week 5, two weeks after
HDO was withdrawn. Proximal NCV was affected at week 1 and 2, P < .005
then recovered to control velocity through treatment and recovery, weeks
3 - 5.

Fibrillations and sharp positive waves were observed in the gastro-
cnnemius muscle at the 3 week period, and positive waves were visible at
week 2 before the distal NCV drop. This correlates well with the observa-
tion of Wiechers, 1977, that positive waves occur as early as 18 hours
after nerve section. Clinical electromyographers have determined these
parameters to occur before onset of neuromuscular disease (Goodgold and
Eberstein, 1977).
In summary, the EMG parameters (distal latency and NCV), describe the time course of neuropathy consistent with the clinical picture of the disease: distal-proximal progression of degeneration with a delayed recovery of limb use.

G. Recovery of EMG from Chronic Hexanedione Treatment

Animals treated with 0.1 and 0.5% HDO for 4.5 months were placed on normal tap water to examine recovery. NCV measurements were taken at 0, 2.5, 5.0 and 7.5 weeks of recovery. 0.1% treated animals were not different from pair fed controls in body weight, distal NCV and latency. No fibrillations or sharp positive waves were seen in 0.1% or control group, indicating no loss of axons.

Of the four rats in the 0.5% treated group, two died one week after removal of HDO water. Although it could not be determined that HOD toxicity was responsible for the toxicity, these two dead animals had no evocable EMG and could not walk with their hind limbs. These animals could not use their hind limbs but had to drag them behind while crawling with front limbs. NCV data from these animals were not used. Figure 14 illustrates the recovery of 0.5% animals. Distal NCV increases at a rate of approximately 3 m/sec per week of recovery. The proximal NCV was not as low as the distal NCV and the former recovered more rapidly as it reached 50 m/sec by 5 weeks while the distal NCV was only 31 m/sec. Full recovery was not seen at 7.5 weeks.

Fibrillations were measured in the gastrocnemius of all 0.5% animals and filled the oscilloscope with waves. This is the highest rating clinically (+++++) and indicates that even at 7.5 weeks post treatment many
Recovery of distal nerve conduction velocity in rats treated for 4.5 months with 0.5% HDO. The Plantar-Popliteal (Distal) NCV was measured in each leg of an animal, then averaged to give one reading per animal.
RECOVERY OF NCV AFTER HDO TREATMENT.

○ 0.5%
● 0.1%
□ Control

FIGURE 14

Distal NCV (m/sec.)

Weeks Recovery

0 2.5 5.0 7.5
FIGURE 15

Recovery of Distal Latency after HDO treatment for 4.5 months. Rats were treated for 4.5 months with 0.5% HDO water, then placed on tap water at 0 time to check recovery.
RECOVERY OF DISTAL LATENCY AFTER HDO TREATMENT.

○ 0.5%
● 0.1%
□ Control

FIGURE 15
muscles are still denervated. No fibrillations were detected in 0.1% or control animals indicating no axonal loss. The shape of the evoked EMG from 0.5% animals is irregular and shows polyphasic waves in contrast to either control or 0.1% animals (Figure 11).

Distal latency of 0.5% tended to show recovery, changing from 0 recovery week value of 3.11 m/sec to 2.44 and 2.28 m/sec at 2.5 and 5.0 weeks. The 5 week and 7.5 week values were still elevated, however; therefore recovery was not complete (Figure 15).

H. Oxygen Uptake Results

These studies showed a decrease in $O_2$ uptake of nerves from 0.1% to 0.5% hexanedione treated animals (Table 4). Values for 0.1% and 0.5% HDO treated animals are not statistically different from each other but do differ from control ($P < .005$ and $P < .01$, respectively). Control oxygen uptake was 6.5, 0.1% group was 3.94 and 0.5% group was 4.30 (all $\mu l O_2/hr-100 \text{ mg tissue}$).

Bryant et al., 1977, noted that HDO and other diketones are uncouplers of oxidative phosphorylation. It is recognized that neuronal integrity is dependent upon energy supply. Low doses of metabolic poisons such as cyanide and carbon monoxide can cause peripheral and central neuropathy.

I. Lipid Metabolism Effects

Because more than 50% of nerve dry weight is lipid, studies were carried out to detect changes in lipid metabolism either as a primary or secondary effect.
Table 5 lists results of total phospholipase A (PLA) for control, 0.1%, and 0.5% HDO treated rats. Control sciatic nerve PLA was 0.40 μmol lecithin/hr-10 mg wet tissue. Treated groups exhibited lower activity, 0.25 and 0.30 μmol/hr-10 mg but this was not statistically significant. Brain PLA determinations gave higher results for 0.1% group but 0.5% and control were not different. These measurements were obtained by using two animals per group, pooling each animal's sciatic nerves.

Phospholipid determinations were carried out on control and treated nerves, as shown in Table 6. Lecithin concentrations were unchanged through treatments. Sphingomyelin was elevated in the 0.5% treated group at 8 weeks but this is not statistically significant. Lysolecithin was found to be twice control and this value is significant to P< 0.05. Unfortunately there are other lipids which have the same relative migration as lysolecithin, so it cannot be unequivocally stated to be an increase in lysolecithin. Contamination from phosphatidylinositol and phosphatidyl serine would have to be excluded by another step of purification.

A pulse chase experiments was conducted with $^{32}$P-$\text{H}_3\text{PO}_4$. Rats drinking HDO or tap water for 2 months were injected with 0.26 mCi $^{32}$P via the intraperitoneal route. Brain and sciatic nerves were removed at 4, 8 and 12 days post injection. Phospholipids were extracted and purified as stated in Methods. Lecithin was chosen as the phospholipid to be studied, since it is plentiful in sciatic nerve and data exists in reference to its half life (Abdel-Latif and Smith, 1970).
### TABLE 4

Oxygen Uptake of Sciatic Nerve

<table>
<thead>
<tr>
<th>GROUP</th>
<th>O₂ UPTAKE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Tissue Wt. Mg)</td>
<td>ul/100 mg. Tissue</td>
</tr>
<tr>
<td></td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>Control (95)</td>
<td>6.46 ± 0.58</td>
</tr>
<tr>
<td>0.1% Hexanedione (105)</td>
<td>3.94 ± 0.38 *</td>
</tr>
<tr>
<td>0.5% Hexanedione (95)</td>
<td>4.30 ± 0.96 **</td>
</tr>
</tbody>
</table>

₀² Uptake Values Represent Data of Two Separate Experiments with Quadruplicate Determinations Per Group.

* Statistically Different from Control, P < .005
** Statistically Different from Control, P < .01
### TABLE 5

**Phospholipase A in Normal and Hexanedione Treated Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (S.D.)</th>
<th>$t_{stat}$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sciatic Nerv.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>.40 (.016)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1%</td>
<td>.25 (0.82)</td>
<td>-1.92</td>
<td>n.s.</td>
</tr>
<tr>
<td>0.5%</td>
<td>.30 (.030)</td>
<td>-0.671</td>
<td>n.s.</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>.31 (0.68)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1%</td>
<td>.49 (.075)</td>
<td>4.24</td>
<td>$P &lt; .025$</td>
</tr>
<tr>
<td>0.5%</td>
<td>.29 (.070)</td>
<td>0.529</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Data is expressed as umol lecithin hydrolyzed per hour per 10 mg wet tissue.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Group</th>
<th>Average</th>
<th>t-stat</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μmol/nerve (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>Control</td>
<td>.38 (.23)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.1%</td>
<td>.41 (.18)</td>
<td>.21</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>.80 (.23)</td>
<td>2.63</td>
<td>-</td>
</tr>
<tr>
<td>Lecithin</td>
<td>Control</td>
<td>.89 (.16)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.1%</td>
<td>.97 (.18)</td>
<td>.664</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>1.00 (.14)</td>
<td>1.05</td>
<td>-</td>
</tr>
<tr>
<td>Lysolecithin</td>
<td>Control</td>
<td>.50 (.06)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.1%</td>
<td>.51 (.07)</td>
<td>.22</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>1.06 (.11)</td>
<td>8.85</td>
<td>P &lt;0.05</td>
</tr>
</tbody>
</table>

*The t-stat is calculated for treated groups as compared to controls.*
Results of this experiment are shown in Figure 15. Data is expressed as $^{32}$P-DPM in lecithin per nerve. Note that at 4 days post injection, the amounts of $^{32}$P incorporated into control, 0.1% and 0.5% groups are constant. However, at 8 and 12 days, the 0.5% group shows higher activity than either control or 0.1% groups. These results are difficult to interpret because synthesis or degradation of lecithin could contribute to the difference.
FIGURE 16

Incorporation of $^{32}$P in sciatic nerve phosphatidyl choline. Rats were placed on 0, 0.1 and 0.5% HDO in drinking water for 8 weeks, then injected i.p. with 0.26 mCi $^{32}$P as orthophosphate at day 0. Two animals from each treatment group were sacrificed 4, 8 and 12 days after injection. Lecithin was isolated and $^{32}$P radioactivity determined as previously described.
INCORPORATION OF $^{32}$P INTO SCIATIC NERVE PHOSPHATIDYL CHOLINE

- ○ 0.5%
- ● 0.1%
- □ Control

![Graph showing DPM $^{32}$P/Nerve Unit against Days with significance levels marked with * for p<0.05 and ** for p<0.01.](image)

**FIGURE 16**
DISCUSSION

A. Microtubules and HDO Toxicity

The relationship between axoplasmic flow and microtubules is complex. Although agents binding microtubular proteins will inhibit axonal transport, McClure et al., 1976, showed that transport continues in the absence of visible microtubules. These authors used an in vitro preparation to measure fast axonal transport in sciatic nerve. This system allowed the middle length of nerve to be superfused with 40 mM Ca$^{2+}$ producing microtubular disaggregation but no effect on flow. The authors postulate that mitotic inhibitor blockade of axoplasmic flow involves either tubulin properties dissociated from microtubules or another site of action. Colchicine binding activities reported here are in accord with these findings in that there are no alterations in microtubular binding activity associated with HDO treatment.

An explanation for the decreased fast axoplasmic flow in MBK neuropathy is offered by the work of Griffin et al., 1977. Intoxicating rats with 2,5-hexanediol for 3 weeks, they injected L-5 ventral horns with $^3$H-leucine. Animals were perfused with glutaraldehyde 18 hours after injection and sciatic nerves analyzed by electron microscope autoradiography.
The paranodal swellings contained increased grain densities. Grains were also associated with mitochondria and smooth endoplasmic reticulum. Because these swellings contained microfilaments, the authors stated that transported proteins are "diverted into swellings and do not move as quickly toward the axon terminal". Swellings were largely found outside the microtubular structures, indicating a lack of relationship between fast axoplasmic transport from microtubules. Yates et al., 1976, examined sciatic nerve from developing rabbits with respect to chemical constituents and morphological changes. They determined that the best unit to consider is a constant anatomical length. In degenerative or developmental conditions, water content and protein content will vary. Table 2, which expresses $^3$H-calcine binding per nerve, indicates no difference between control and HDO treated animals. Table 2 shows some variation in that the total protein content of the nerve was decreased with 0.5% HDO group. This resulted in a higher specific activity of binding with no increase in activity per nerve, when corrected for decreased protein content.

B. Microviscosity Data

Plotting $\log_{10}$ viscosity versus $1/T$ using values from a homogeneous substance such as glycerol-water solutions yields a straight line. Hence the existence of a change in slope of this curve implies that a phase transition is taking place. Gel phase transition temperature is defined as that temperature at which a bulk property varies discontinuously with temperature. Such transitions are first order; that exhibited in Figure 5 is a second order transition because the viscosity is continuous but its derivative is not. Ferromagnetism is an example of this transition phenomenon in which the molecular field of nearby spins causes the
spontaneous magnetization to disappear at the Curie temperature, 310° K (Wannier, 1966). Other discontinuous properties are heat capacity and electrical polarization. Animals treated with 0.1% and 0.5% HDO do not show this phase transition which occurs at about 20° C in control sciatic nerve. Using brain-derived myelin analyzed by electron spin resonance techniques, Viret and Letterier, 1976, investigated temperature effects on the order parameter, S. This is defined as an average over all molecules and is a measure of the distribution of spin label molecular axes with respect to the membrane normal axis. An Arrhenius plot of S in myelin yielded a biphasic slope with discontinuity at 14.5° C in physiologic solution, normal in calcium content. This shifted to a higher value, 19.5° C, in 10⁻³ M calcium. The label employed was a nitrooxide attached to the C-5 of stearic acid.

Lentz et al., 1976, used single and multilamellar vesicles of known lecithin content to determine DPH-detectable changes in viscosity with temperature. Single lamellar vesicles of distearoyl lecithin produced no phase transition; however, 1-palmitoyl-2-oleoyl-lecithin multilamellar vesicles gave a viscosity curve much like Figures 5 and 6 except the transition temperature was 11° C. Myelin preparations are multilamellar structures (Norton, 1977). This result implies that interactions between layers, not within layers, are responsible for the biphasic slope.

The phase transition present in control sciatic nerves and absent in the HDO treated groups (Figure 5) is representative of a quantitative and qualitative alteration of the myelin preparation which could be related to the neuropathology. It is important that the monophasic
profile is obtained in the 0.1% HDO group yet these animals do not show clinical or electrophysiological signs of neuropathy. The 0.5% HDO animals show clinical paralysis and diminished microviscosity as well as a monophasic profile. Therefore, the phase transition is antecedent to the clinical paralysis. This study may provide a basis for analyzing the onset and progression of the peripheral neuropathy.

The HDO effect upon microviscosity does not seem to be a direct effect upon membranes. The Arrhenius plot of control sciatic nerve homogenates was unchanged when determined in the presence of a final concentration of 30 mg% or 300 mg% HDO. HDO has an oil/water partition coefficient of 0.05 (Patty, 1949), and does not enter lipid bilayers readily.

Another fluorescent probe, perylene, produced three superimposable curves for control, 0.1% and 0.5% HDO treatment. Perylene is a flat, aromatic hydrocarbon which has a shorter fluorescent lifetime than diphenylhexatriene and is sensitive in regions of high fluidity. Because it cannot undergo tortional motion but only out-of-plane rotations, its fluorescent polarization changes only with gross membrane perturbations.

C. Microviscosity Changes Following Nerve Section

Wallerian degeneration is one of the best understood axonal neuropathies. It has been characterized extensively in terms of protein, lipid, and nucleic acid metabolism (Mezei, 1970, Domonkos and Heiner, 1968, and Johnson et al., 1949). Loss of myelin marker lipids (free cholesterol and cerebroside) has been reported. (Wood and Dawson, 1974) The time
course of myelin degeneration suggests that maximum dispersion of myelin begins at 48 hours post section. Loss of cholesterol progresses steadily through 15 days post section, cerebroside dropping similarly to 22% control value. Concomitant with a 50% loss of total phospholipid at 15 days is a peak in phospholipases $A_1$ and $A_2$ activities, each approximately 7 times control. Total protein does not decrease until well after 15 days post section.

Microviscosity data for Wallerian Degeneration (Figure 7) indicates a loss of the biphasic nature of control myelin with little difference between 2, 6, 10 or 20 days post section. The slope of the Arrhenius plot is proportional to the energy of activation, $E$, and this slope did increase from 1.00 to 1.56 from day 2 to day 20. In addition to membrane viscosity studies, $\Delta E$ is dependent upon lipid composition, since cholesterol addition to micelles decreased $\Delta E$ but raised viscosity point for point (Shinitzky and Inbar, 1976).

The effects of sectioning or HDO treatment on sciatic nerve viscosity appear to be identical. There is a loss of high viscosity, liquid crystallization at low temperature, with preservation of the low viscosity curve at temperatures above $20^\circ$ C. The addition of cholesterol or unsaturated fatty acids to liposomes will not produce the same effects, as the slope of a monophasic $\log_{10}$ vs $T^{-1}$ changes, yielding a new $\Delta E$ value.

Figure 7 also contains microviscosity data for 20 day treatment with 0.5% HDO. Note that the myelin viscosity has a biphasic curve similar to control, indicating that longer treatment is necessary before observing loss of the biphasic character.
All studies reported here were done with whole sciatic nerve. Three week treatment with 0.5% HDO had no effect on the whole nerve. NCV studies support the idea that the distal half of the nerve is affected more than the proximal half. Thus, it could be that taking the whole nerve masks early changes limited to the distal portion.

D. Phospholipase and Phospholipid Levels in Sciatic Nerve

Disorders of lipid metabolism have been found to occur with several neuropathic conditions (Webster, 1973). Accordingly, general tests were carried to determine gross anomalies of phospholipid metabolism.

Phospholipase A activity, although elevated in Wallerian degeneration, was found to be unaffected in sciatic nerve of rats treated for 2 months with 0.1% and 0.5% HDO (Table 5). Data is expressed in micromoles of lecithin hydrolyzed per 10 mg wet weight tissue per hour. Whole brain values show a slight elevation in 0.1% group but this was not evident in the 0.5% group.

Since lysolecithin and cholesterol esters are present in acutely degenerating nervous tissue, total lipid extracts from chronic HDO treated rats were analyzed. Table 6 data shows that lecithin and sphingomyelin were present in equimolar amounts per nerve, but cholesterol esters were not detected in any group. The spot which has the same Rf as lysolecithin in 0.5% group contained two times the amount of lipid phosphorous in control or 0.1% (P < 0.05, Student's t-test). This one dimensional TLC system does not separate lysolecithin from other polar phosphatides, such as phosphatidyl inositol and phosphatidyl serine. All three of these lipids contain phosphorous and could contribute to this observation.
Lysolecithin is capable of inducing local demyelination, as Berry, 1965, determined by direct injection of this material into sciatic nerve. Only 2-6 micrograms of the material was sufficient to cause complete demyelination of the injection site within 3 hours.

E. Phospholipid Synthesis and Turnover Using $^{32}$P

As mentioned in the Introduction, cerebroside and cholesterol will decrease when nerve is sectioned, phospholipases $A_1$ and $A_2$ are each activated. Measurements of phospholipase $A$ ($A = A_1 + A_2$) failed to find any difference between control or HDO treated animals. Webster and Thompson, 1962, extracted normal and multiple sclerosis plaques to determine lysolecithin content. Necropsy material for analysis was obtained from post-mortem victims of multiple sclerosis (MS) and disease free cadavers (controls). No attempt was made to state severity of lesion or active state of demyelination. These authors could see no elevation in lysolecithin of MS plaques when compared to controls. This result may have occurred because the plaques were inactive or extensively degenerated, in which case there would be little lysolecithin to be found.

The data of Figure 16 indicates that lecithin-$^{32}$P incorporation of all groups is equal at day 4 but the 0.5% group has a higher activity at days 8 and 12, with an apparent peak near 8 days. Davison and Dobbing, 1959, measured $^{32}$P uptake into sciatic nerve following i.p, administration of $\text{H}_3^{32}\text{PO}_4$. Total phospholipid radioactivity of sciatic nerve peaked at approximately 10 days, a value consistent with the 8 day peak in lecithin seen here.
F. HDO Effects upon EMG Parameters

Individual examples of EMG recordings (Figures 10 and 11) suggest a widening of the muscle action potential (MAP) and an increased latency with HDO treatment. With very long treatment the muscle action potential becomes completely dispersed and attenuated in amplitude, reflecting extensive loss of fast motor nerves.

The slowing of Distal NCV, in addition to the polyphasic waves of Figure 10 are consistent with results obtained in human neuropathy (Perticoni and Cianchetti, 1976). These authors reported an increase in Distal Latency and NCV. Patients removed from occupational exposure hexane for up to 16 months had persistent latency increases and polyphasic MAP. Their conclusions were than EMG parameters closely paralleled clinical paralysis: mildly affected workers had a peronal NCV between 35-44 m/sec severely affected (paralyzed) workers NCV was less than 35 m/sec.

The 3 week effects of HDO (Figures 12 and 13) are predominately distal since the latency and NCV are more depressed distally than proximally. These early changes are reversible in part, although latency does not easily recover. Chronic effects on NCV and latency show only partial recovery (Figures 14 and 15).

Using inhalation of 100 or 1000 ppm methyl butyl ketone to induce neuropathy, Johnson et al, 1977, measured sciatic-tibial NCV in rats. Inhalation of 1000 ppm MBK for 6 hours/day, 5 days/week produced a significant NCV drop in rats after 8 weeks of treatment. No measurements of
metabolites were done, so it is difficult to compare this dose with 0.5% HDO in drinking water, but the latter produced effects at 2 weeks, much earlier than MBK inhalation.
SUMMARY

This study was undertaken to investigate the mechanism of action of hexanedione in producing axonopathy of the sciatic nerve. Because histological and axoplasmic transport studies implicated a derangement of microtubules, a study of the effects of HDO on colchicine binding of sciatic nerve and their subcellular fractions was done. There was no discernible change in the $^3$H-colchicine binding in soluble, microtubular or particulate fractions of sciatic nerve, nor was there an increase or decrease in the half-life of colchicine binding.

Myelin breakdown did occur in the course of this neuropathy, so a fluorescent probe technique of membrane viscosity was employed. Homogenates of control nerves exhibited a biphasic Arrhenius plot of viscosity with a slope discontinuity observable at $20^\circ$ C. Comparing treated groups, 0.1% and 0.5% each had monophasic plots. As chronic dosage increased from control to 0.1% to 0.5% HDO, a progressive decrease in membrane viscosity was found. If this measurement is applied to sectioned nerve, similar monophasic curves were obtained. These changes persisted from 2 to 20 days after nerve section. The myelin fraction of control nerve was responsible for the biphasic Arrhenius plot, with the mitochondrial-nuclear pellet and adipose fractions contributing little to the phase transition. Animals treated with 0.1% HDO in drinking water for 2 months did not show body weight loss or decreased

103
nerve conduction velocity. It is therefore evident that membrane microviscosity displays changes which are antecedent to EMG effects with long term treatment.

NCV determinations of 0.1% animals were normal with 4.5 months of treatment. However, 0.5% treated animals were affected within 1-2 weeks, having decreased Distal NCV and body weight, increased Distal Latency. The Distal NCV recovered to control values 2 weeks after withdrawal of the toxicant. Distal Latency decreased but remained above control. In 4.5 months of treatment, Distal NCV recovered somewhat but remained below control, Distal Latency lagged behind as well.

In comparing EMG changes with microviscosity parameters, it would seem that EMG is a more reliable diagnostic criterion for onset of toxicity. Changes in Distal NCV and Latency occur at 2 weeks, while nerve microviscosity still resembles control at 20 days. Even 4.5 months of 0.1% produced no EMG effects, but the viscosity was much different from control. Microviscosity measures a different parameter of myelin structure than the NCV. Since myelin is a highly compact network of concentric lipid bilayers, one would predict it to have a high viscosity. Figure 6 gives a value of 900 centipoise (cP) for myelin at 37° C, which is much higher than viscosity measured in polymorphonuclear lymphocytes, 335 cP, or human red cell ghosts, 180 cP (Feinstein et al., 1975). The decreased myelin viscosity seen with the 0.5% HDO treatment is consistent with axonal swelling and myelin breakdown. The red cell ghost experiment indicates an effect of HDO on the membrane which leads to an increase of inverse polarization and a decreased viscosity. The subtoxic dose of HDO produces no EMG changes but
does affect microviscosity profiles. Therefore, some changes in myelin
viscosity occur which do not alter EMG parameters.

In contrast, the observed NCV decrease comes as a logical extension
of the observed damage to the nodes of Ranview. Loss of saltatory conduc-
tion or increased internodal gap would account for the NCV decrease. The
distal-proximal progression of the toxicity was verified in this study.

Disorders of lipid metabolism do not appear to be involved in the
progressive degeneration of the sciatic nerve induced by hexanedione.
Some changes were found in $^{32}$P turnover and a lysolecithin-like phospho-
lipid appeared elevated, but the overall picture is not one of altered
lipid catabolism.

Based on the results of this study, a mechanism of HDO toxicity can
be postulated: An early effect on the myelin-axon unit causes a deficit
in NCV and Latency. This is followed by a physical change in myelin struc-
ture as measured by microviscosity techniques. It appears that an inves-
tigation of cohesive forces in myelin lamellae would be a key factor in
understanding the morphological and physiological changes in nerve function
and structure.


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