INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand markings or notations which may appear on this reproduction.

1. The sign or “target” for pages apparently lacking from the document photographed is “Missing Page(s)”. If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure you of complete continuity.

2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in “sectioning” the material. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.
RAMSEY, CRAIG CARLISLE

2,5-Hexamidine Induced Axonopathy in the Crayfish, Procambarous Clarkii, Medial Giant Axon

The Ohio State University

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106
2,5-Hexanedione induced Axonopathy

In the Crayfish, Procambarous clarkii, Medial Giant Axon

Dissertation

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

By

Craig Carlisle Ramsey, B.S. in Chem., M.S.

*****

The Ohio State University

1980

Reading Committee:
John J. Curry
Karl Kornacker
Philip B. Hollander

Approved By

[Signature]
Adviser
Division of Sensory Biophysics
DEDICATION

To Debra B Ramsey

who made this work possible

and put it in perspective.
ACKNOWLEDGEMENTS

I would like to thank the many people without whose help this project could neither have been begun nor completed.

In particular, I am grateful to: Joseph P. Nachtman and Mohamed Shawkey Abdel-Rahman whose work provided an impetus for this study; Professor Leo E. Lipitz for the use of his equipment and experience; Professor Joseph Cassim, for many stimulating discussions; Professors John J. Curry and Karl Kornacker for carefully reading this manuscript; Professor Philip B. Hollander, whose unlimited enthusiasm, support, and help is greatly appreciated; and Debra B. Ramsey, for her patience and love.
VITA

January 9, 1953........ Born - Philadelphia, Pennsylvania
1974............... B.S. in Chem., Purdue University, West Lafayette, Indiana
1974 - 1975 ........ Department Fellow, Department of Physiology, The Ohio State University
1975 - 1977 ........ Graduate Teaching Associate, College of Biological Sciences, The Ohio State University
1977 ................. M.S., The Ohio State University, Columbus, Ohio
1977 - 1978 .......... Graduate Research Associate, Division of Sensory Biophysics, The Ohio State University
1978 - 1980 .......... Graduate Research Associate, Department of Pharmacology, The Ohio State University

PUBLICATIONS


PLAN OF STUDY

Neurophysiology Neurophysiology
Membrane Biophysics John J. Curry
Computer Engineering Karl Kornacker
Electropharmacology Kenneth J. Breeding
Philip B. Hollander
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>A. Normal Axons</td>
<td>3</td>
</tr>
<tr>
<td>1. Anatomy</td>
<td>3</td>
</tr>
<tr>
<td>2. Axoplasmic Transport</td>
<td>6</td>
</tr>
<tr>
<td>3. Excitable Membrane</td>
<td>7</td>
</tr>
<tr>
<td>4. Passive Electrical Properties</td>
<td>17</td>
</tr>
<tr>
<td>5. The Propagated Action Potential</td>
<td>21</td>
</tr>
<tr>
<td>B. Diseased Axons</td>
<td>23</td>
</tr>
<tr>
<td>1. Wallerian Degeneration</td>
<td>23</td>
</tr>
<tr>
<td>2. Dying Back Disease</td>
<td>25</td>
</tr>
<tr>
<td>a. Tri-orthocresyl Phosphate</td>
<td>27</td>
</tr>
<tr>
<td>b. Acrylamide</td>
<td>28</td>
</tr>
<tr>
<td>c. Hexacarbons</td>
<td>31</td>
</tr>
<tr>
<td>III. RATIONALE</td>
<td>39</td>
</tr>
<tr>
<td>IV. METHODS</td>
<td>41</td>
</tr>
<tr>
<td>A. Animals</td>
<td>41</td>
</tr>
<tr>
<td>B. Solutions</td>
<td>42</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (cont.)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. Acute IN VITRO Exposure Method</td>
<td>43</td>
</tr>
<tr>
<td>D. Chronic IN VIVO Exposure Methods</td>
<td>43</td>
</tr>
<tr>
<td>E. Quantitative Analysis</td>
<td>47</td>
</tr>
<tr>
<td>F. Tissue Bath</td>
<td>51</td>
</tr>
<tr>
<td>G. Temperature Control</td>
<td>55</td>
</tr>
<tr>
<td>H. Gross Dissection</td>
<td>56</td>
</tr>
<tr>
<td>I. Fine Dissection</td>
<td>60</td>
</tr>
<tr>
<td>J. Stimulation</td>
<td>64</td>
</tr>
<tr>
<td>K. Recording</td>
<td>68</td>
</tr>
<tr>
<td>L. Experimental Protocol</td>
<td>70</td>
</tr>
<tr>
<td>M. Statistical Analysis</td>
<td>73</td>
</tr>
<tr>
<td>V. RESULTS</td>
<td>75</td>
</tr>
<tr>
<td>A. Normal Action Potential Parameters</td>
<td>75</td>
</tr>
<tr>
<td>B. Effect of Temperature</td>
<td>83</td>
</tr>
<tr>
<td>C. Effect of Time</td>
<td>87</td>
</tr>
<tr>
<td>D. Toxicity of HDO to Crayfish</td>
<td>94</td>
</tr>
<tr>
<td>E. Effects of Chronic IN VIVO Exposure to HDO</td>
<td>94</td>
</tr>
<tr>
<td>F. Effect of High Osmolarity Superfusion Medium</td>
<td>108</td>
</tr>
<tr>
<td>G. Effects of Acute IN VITRO Exposure to HDO</td>
<td>113</td>
</tr>
<tr>
<td>VI. DISCUSSION</td>
<td>126</td>
</tr>
<tr>
<td>A. Mechanisms Proposed for Observed Changes</td>
<td>127</td>
</tr>
<tr>
<td>1. Chronic IN VIVO Exposure to HDO</td>
<td>127</td>
</tr>
<tr>
<td>2. Acute IN VITRO Exposure to HDO</td>
<td>128</td>
</tr>
<tr>
<td>B. Conclusions</td>
<td>132</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (cont.)

C. Future Experiments ........................................ 134
VII. SUMMARY .................................................. 137
VIII. APPENDIX .................................................. 139
      A. Temperature Controller ............................... 139
      B. Membrane Potential Amplifier ....................... 145
IX. BIBLIOGRAPHY .............................................. 149
LIST OF TABLES

Table | Page
--- | ---
1. Definition of Action Potential Parameters | 78
2. Normal Action Potential Parameter Values | 80
3. Normal Strength-Duration Parameters | 82
4. Temperature Dependence of Action Potential Parameters | 86
5. Dose Dependent Effects of Chronic IN VIVO Exposure to HDO | 97
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Membrane Model</td>
<td>10</td>
</tr>
<tr>
<td>2.</td>
<td>Cable Model</td>
<td>19</td>
</tr>
<tr>
<td>3.</td>
<td>HDO Concentration Profiles during Chronic IN VIVO Exposure to HDO</td>
<td>46</td>
</tr>
<tr>
<td>4.</td>
<td>Sample Gas Chromatogram and Calibration Curve</td>
<td>49</td>
</tr>
<tr>
<td>5.</td>
<td>Tissue Bath and Superfusion System</td>
<td>53</td>
</tr>
<tr>
<td>6.</td>
<td>Schematic of Crayfish Nerve Cord</td>
<td>62</td>
</tr>
<tr>
<td>7.</td>
<td>Electronic Apparatus for Stimulating and Recording</td>
<td>66</td>
</tr>
<tr>
<td>8.</td>
<td>Schematic of Crayfish Action Potential</td>
<td>77</td>
</tr>
<tr>
<td>9.</td>
<td>Effect of Temperature on Action Potential</td>
<td>85</td>
</tr>
<tr>
<td>10.</td>
<td>Effect of Time on VEL and +DV/DT</td>
<td>89</td>
</tr>
<tr>
<td>11.</td>
<td>Effect of Time on DUR10, DUR50 and -DV/DT</td>
<td>91</td>
</tr>
<tr>
<td>12.</td>
<td>Effect of Time on +DV/DTV and PSP</td>
<td>93</td>
</tr>
<tr>
<td>13.</td>
<td>Dose Dependence of RP and AP</td>
<td>99</td>
</tr>
<tr>
<td>14.</td>
<td>Dose Dependence of VEL</td>
<td>101</td>
</tr>
<tr>
<td>15.</td>
<td>Dose Dependence of +DV/DT and -DV/DT</td>
<td>103</td>
</tr>
<tr>
<td>16.</td>
<td>Dose Dependence of PSP, -DV/DTV, HYP, +DV/DTV</td>
<td>105</td>
</tr>
<tr>
<td>17.</td>
<td>Dose Dependence of DUR100 and HYPT</td>
<td>107</td>
</tr>
<tr>
<td>18.</td>
<td>Effect of High Osmolarity on Action Potential</td>
<td>110</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES (cont.)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>19. Effect of High Osmolarity on Action Potential Parameters</td>
<td>112</td>
</tr>
<tr>
<td>20. Effect of 1.0 gm% HDO on Action Potential</td>
<td>115</td>
</tr>
<tr>
<td>21. Effect of 1.0 gm% HDO on Action Potential Upstroke</td>
<td>117</td>
</tr>
<tr>
<td>22. Effect of 1.0 gm% HDO on +DV/DT, +DV/DTT, -DV/DTV, VEL and APT</td>
<td>120</td>
</tr>
<tr>
<td>23. Effect of 1.0 gm% HDO on HYP, HYPT, PSP, PSPT and DUR100</td>
<td>122</td>
</tr>
<tr>
<td>24. Effect of 1.0 gm% HDO on -DV/DT, -DV/DTT, DUR10, DUR50 and DUR90</td>
<td>124</td>
</tr>
<tr>
<td>25. Schematic of Tempcon 1 Control Module</td>
<td>140</td>
</tr>
<tr>
<td>26. Schematic of Tempcon 1 Power Module</td>
<td>142</td>
</tr>
<tr>
<td>27. Schematic of Membrane Potential Amplifier</td>
<td>146</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

Ca  Calcium ion
Cl  Chloride ion
CNS  Central Nervous System
GC  Gas Chromatography
HDL  2,5-Hexanediol
HDO  2,5-Hexanedione
HDO-VH  van Harreveld’s saline containing 2,5-Hexanediol
HEX  n-Hexane
5H2H  5-Hydroxy 2-Hexanone
HOL  2-Hexanol
K  Potassium ion
MAN-VH  van Harreveld’s saline containing Mannitol
MBK  Methyl n-Butyl Ketone
MEK  Methyl Ethyl Ketone
MIBK  Methyl i-Butyl Ketone
Na  Sodium ion
NCV  whole Nerve Conduction Velocity
NVH  Normal van Harreveld’s saline
PNS  Peripheral Nervous System
Q-10  ratio of process rates at two temperatures 10 apart, i.e. (Rate @ T=20°C / Rate @ T=10°C)
S-D  Strength-Duration
TEA  Tetraethyl Ammonium ion
TOCP  Triorthocresyl Phosphate
TTX  Tetradotoxin
VEL  single fiber conduction Velocity
I. INTRODUCTION

Many disorders of the nervous system produce initial changes in nerve axons, whereas, differences detectable in the cell body may appear as a late feature of the disease or may be completely absent. Hence these disorders are described as axonopathies (Spencer and Schaumberg, 1978a). Axonopathy may be associated with changes in the myelin sheath and Schwann cells. These changes are thought to be effects rather than causes of axonopathy. Disorders such as diptheria are not considered axonopathies because the primary lesion is in the myelin sheath. Amyotrophic lateral sclerosis and Friedreich's ataxia are considered to be axonopathies. Nutritional illness may also result in axonopathy as with the Thiamine (vitamin B1) deficiency characteristic of beri-beri and alcoholic polyneuropathy (Victor, 1975). Numerous chemicals may induce axonopathy. These include therapeutic drugs such as diphenylhydantion (Eisen et.al., 1971), isoniazid (Schlaepfer and Hager, 1964), and the vinca alkaloids (Casey et.al., 1973) or industrial compounds such as triorthocresyl phosphate (TOCP) (Cavanagh, 1964), acrylamide (Fullerton, 1969), n-hexane (HEX) (Herskowitz et.al., 1971) and methyl n-butyl ketone (MBK) (Mendell et.al., 1974). It may be concluded that
further study of axonopathy will contribute to an improved understanding of neurophysiology and lead to the amelioration of human suffering.

This study focused on the axonopathy induced by 2,5-hexanedione (HDO). HDO has been strongly implicated as the active metabolite of HEX and MBK. HEX, MBK and HDO (hexacarbon) intoxication, unlike the other axonopathies mentioned above, results in a substantial decrease in nerve conduction velocity (NCV) (Herskowitz, 1971). The mechanisms by which this reduction in NCV may be caused by HDO will be discussed. Electrophysiological experiments are designed to aid in discriminating among the possible causes of this reduced NCV. The observed electrophysiological changes are discussed in relation to the current understanding of the conducted action potential.
II. LITERATURE REVIEW

A. NORMAL AXONS

1. ANATOMY

An axon is a long slender extension of a nerve cell body. Axons are roughly cylindrical with diameters ranging from less than 1 um (vertebrate C fibers) to greater than 1 mm (invertebrate "giant" axons). The membrane which forms the boundary of the axon is continuous with the cell membrane proximally, and with the bouton terminaux or motor endplate distally. The cytoplasm within the axon contains endoplasmic reticulum, 25-30 nm neurotubules, 10 nm neurofilaments, mitochondria and several classes of membrane bound vesicles. The endoplasmic reticulum seems to compartmentalize the cytoplasm and may provide a continuous network of channels through which materials may be transported along the axon (Droz, 1975). The neurotubules and neurofilaments have been hypothesized to contribute to the structural framework of the axon, and to participate in axoplasmic transport (Ochs, 1972). The mitochondria carry out respiration to supply the axon with high energy compounds such as ATP. The vesicles may contain neurotransmitter substances or other materials.
A nerve fiber is composed of an axon and surrounding glial cells (Berthold, 1978). In the PNS these glia are called Schwann cells while in the CNS they are called oligodenroglia. Myelin is composed of many turns of glial cell membrane wrapped around the axon. Cytoplasm is virtually absent from myelin. Myelin strongly affects the electrical properties of axons as will be discussed below. True myelin is absent in invertebrate nerve fibers (Bullock, 1977). Glial cells appear to police the segment of axon with which they are associated. Glial pseudopoda have been observed to scavenge debris from normal and diseased axons (Spencer and Thomas, 1974). Glia are also suspected to participate in the metabolic support of axons (Singer and Salpeter, 1966). The glial cell and basement membrane form a barrier to the free diffusion of potassium ions and presumably other solutes (Frankenhauser and Hodgkin, 1956).

Nerve fibers are bound together into bundles by a sheath of epithelium and connective tissue to form the named tracts and nerves. This sheath serves to support and protect the nervous tissue by regulating the interchange of materials between the interstitial space of the nervous tissue and the rest of the body (Noback and Demarest, 1975).

The medial giant axon of the crayfish is a large central interneuron which originates in the supraesophageal
ganglion (Gl), deccusates and descends unbranched to the sixth abdominal ganglion (Johnson, 1924). This fiber is a "command" neuron for the "tail flip" escape response to certain stimuli presented to the rostral sense organs (Wiersma, 1947). The medial giant axon forms electrical synapses en passent with axons of motor neurons and other interneurons (Johnson, 1924).

The medial giant axon has been investigated using both the transmission and scanning electron microscope. The ultrastructure of this fiber is similar to that of other nerve fibers, except for the apparent absence of neurofilaments (Fernandez, et al., 1970). The axon is surrounded by glial cells and connective tissue which form a lamellated sheath with glia adjacent to the axon (Hama, 1977). Scanning electron micrographs of freeze fractured giant fibers show the internal structure of axonal and glial membranes. Crayfish medial giant axons and associated glia form longitudinally oriented ridges 0.5 to 1.2 μm long and 0.12 to 0.15 μm wide (Peracchia, 1974). Axonal and glial membranes are closely parallel and separated by 30 to 140 Å at the ridges, which extend into the axoplasm 0.3 μm. The axonal membrane at these sites contains a romboidal array of 80 Å globules. The function of these structures is unknown, but they may be sites of metabolic coupling between axon and glia, sites of cell to cell adhesion, or patches of membrane
specialized for excitability (Peracchia, 1974).

2. AXOPLASMIC TRANSPORT

Most if not all structural components of the vertebrate and invertebrate axon are synthesized in the cell body and conveyed via slow or fast anterograde axoplasmic transport to various sites along the axon (Schwartz, 1979; Meyer, 1973). Slow transport proceeds at 1 - 3 mm/day and is thought to reflect bulk flow of cytoplasm from the cell body (Heslop, 1975; Meyer, 1973). Fast anterograde transport proceeds at a rate of approximately 400 mm/day in vertebrate nerves at 37°C (Ochs, 1972). Proteins, glycoproteins, lipids and possibly some organelles are transported at this rate. Fast transport is critically dependent on local energy supplies as demonstrated by the blockage of fast transport by low temperature and metabolic inhibitors (Sabri and Ochs, 1972; Gross, 1973). Calcium in the extracellular space is also a requirement for fast axoplasmic transport (Ochs, et.al., 1977).

Axoplasmic transport has been observed to have two components in the crayfish medial giant axon. A slow, 1 to 3 mm/day, rate probably reflects the bulk flow of cytoplasm (Meyer, 1973). A fast, 10 mm/day, rate has also been found in these axons at room temperature (Fernandez, et.al., 1970). This rate is comparable to the 400 mm/day rate of
fast transport in mammalian axons at 37 °C when the 2 to 2.3 Q-10 of fast transport is considered (Ochs and Smith, 1971). The finding of fast axoplasmic transport in crayfish medial giant fibers has been interpreted as evidence non supportive of Ochs' hypothesis that fast transport involves neurofilaments, since these structures are absent in this axon (Fernandez, et.al., 1970).

3. EXCITABLE MEMBRANE

The excitable membrane of axons has been found to be remarkably similar in most species studied (Cahalan, 1978). A resting potential in the range of -70 to -90 mv is maintained across the membrane. An action potential with a duration of 0.5 to 2.0 ms and an overshoot of 10 to 50 mv is normally conducted in an all-or-none non-decremental fashion. The voltage sensitive conductance pathways of the squid, lobster, myxocila giant fibers and frog nodes of Ranvier respond similarly to pharmacologic agents. Tetradotoxin (TTX) blocks Na current when applied externally but is ineffective when perfused internally to perfused squid giant axons. Tetraethyl ammonium ions (TEA) block outward K currents and are more effective when applied to the internal surface of the membrane. Tertiary amine anesthetics, such as lidocaine block fast inward Na current in a use dependent manner and act more quickly when perfused
internally. The excitable membrane appears to be asymmetric. Receptors for these agents are not equally accessible from either side of the membrane. See Narahashi (1974) for an excellent review of this extensive subject. The region of the ion "channel" which confers selectivity is thought to be located nearer the outside surface while the "gates", which regulate the conductivity of the "channel" are thought to be nearer the inside surface (Cahalan, 1978). The major differences between the axon membranes of these animals appear to be in the density of the various channels.

The apparent selectivity of the pharmacologic agents reviewed above supports the parallel conductance model for the excitable membrane which Hodgkin and Huxley used for their kinetic analysis of membrane currents recorded from voltage clamped squid giant axons (Hodgkin and Huxley, 1952). A modification of this widely accepted mathematical model of excitable membranes is illustrated in figure 1. The model represents the membrane as a parallel electrical network composed of a fixed capacitance, voltage dependent Na and K conductances, a lumped "leakage" conductance and an active transport current source (Kornacker, 1969).
Parallel conductance model of excitable membrane 
(Kornacker, 1969). See text for explanation of symbols.
MEMBRANE MODEL

FIGURE 1
The current flowing outward through the model membrane is described by equation 1, which may be obtained by applying electric circuit theory to figure 1.

\[ I = C\left(\frac{dV}{dt}\right) + g_{Na}(V-e_{Na}) + g_{K}(V-e_{K}) + g_{L}(V-e_{L}) + J \] (1)

where:
- \( I \) = net membrane current (mA/sqcm)
- \( C \) = membrane capacitance (uf/sqcm)
- \( V \) = membrane voltage (mv)
- \( g_{Na} \) = Na conductance (mS/sqcm)
- \( g_{K} \) = K conductance (mS/sqcm)
- \( g_{L} \) = "Leakage" conductance (mS/sqcm)
- \( e_{Na} \) = Na Nernst potential (mv)
- \( e_{K} \) = K Nernst potential (mv)
- \( e_{L} \) = "Leakage" Nernst potential (mv)
- \( J \) = net active transport current (mA/sqcm)
- \( \frac{dV}{dt} \) = derivative of membrane voltage (V/s)
- \( t \) = time (ms)
When the membrane is at rest, equation 1 may be rearranged to yield equation 2 which describes the steady-state potential of the membrane in the absence of applied currents.

\[ V = \frac{g_{Na}(e_{Na}) + g_{K}(e_{K}) + g_{L}(e_{L}) + J}{g_{Na} + g_{K} + g_{L}} \]

assuming: \( \frac{dV}{dt} \) = 0 and \( I = 0 \)

Equation 2 shows that the steady state membrane potential is described as the conductance weighted average of the Nernst potentials. When the membrane is at rest, this average is dominated by the \( K \) term. Equation 3 is a useful approximation of the \( K \) Nernst potential.

\[ e_{K} = RP \]  
assuming: \( g_{K} \gg g_{Na} \) and \( g_{K} \gg g_{L} \)

\( RP = \) Resting Potential

During the peak of the action potential equation 2 is dominated by the \( Na \) term. Therefore, equation 4 is a useful approximation of the \( Na \) Nernst potential.

\[ e_{Na} = V_{p} \]

where \( V_{p} = \) membrane potential at peak of the action potential
assuming: $g_{Na} \gg g_K$ and $g_{Na} \gg g_L$

During a propagated action potential the voltage dependent ionic conductances undergo cyclic changes which cause the membrane potential to: 1) depolarize, 2) overshoot and 3) repolarize. The net membrane current (equation 1) theoretically remains zero during the action potential. The current needed to discharge and then charge the membrane capacitance is thought to flow through the ionic conductance pathways. During the depolarization phase of the action potential the ionic current is carried primarily by Na (Hodgkin and Huxley, 1952). Hence, the rate at which the membrane potential can change is effectively limited by the magnitude of the Na current. For this reason the maximum depolarization rate is often used as an index of Na current (equation 5).

$$i_{Na} = C(dV/dt) \quad (5)$$

where: $i_{Na} = \text{Na current}$

assuming: $g_{Na} \gg g_K$ and $g_{Na} \gg g_L$

and $I = 0$

The ionic composition of crayfish medial giant axoplasm has been reported when the axon is bathed in van Harreveld's medium at room temperature. The concentrations (in mM) were found to be: $Na=17.9$, $K=247$, and $Cl=18.1$ (Wallin, 1966).
Wallin did not attempt to measure the intraaxonal Ca concentration. This allows the Nernst equilibrium potentials to be estimated as: (in mv @ 15°C) as: eNa=61, eK=-94, and eCl=-64. When the external K concentration was elevated, from 5.4 to 150 mM internal Na decreased 50%, Cl increased 1000%, and K remained unchanged (Wallin, 1967b). The external application of 100 uM ouabain results in progressive increase in axoplasmic Na, decrease in K, no change in Cl and reduction in resting potential (Wallin, 1967a). The effects of ouabain, metabolic inhibitors, and low temperature on the resting potential and steady state current-voltage relation were investigated by Lieberman and Lane (1976), who found that 500 uM ouabain induced a 9.6 +/- 0.9 mv (mean +/- std.err. N=16) depolarization and that treatment with ouabain, low temperature (10 °C), or 0 mM external Na caused a 30 to 50 % decrease in resting membrane resistance. This resting resistance change was absent when axons bathed in Cl free, but otherwise similar solutions were treated with 500 to 1000 uM ouabain or low temperature (Lieberman and Nosek, 1976). The depolarization induced by ouabain was 7.1 +/- 1.0 mv (mean +/- std.err. N=7) in the absence of external Cl.

The dependence of the resting and action potentials of the crayfish medial giant axon on the ionic composition of the external medium has been investigated by Yamagishi and
Grundfest (1971). The relative influence of ions in the bathing solution on the resting potential was found to be: K > Na > Ca > Cl. The overshoot was not affected by removal of K from the external medium. A Ca sensitive afterpotential was found by taking the maximum difference between a straight-line approximation to the repolarization phase of the action potential and the tail of the action potential.

TEA was found to block the K conductance in internally perfused crayfish medial giant axons (Shrager, Macey and Strickholm, 1969). TEA was ineffective when applied externally. TTX blocks Na currents in voltage-clamped crayfish axons (Shrager, 1974) as it does in lobster axons (Narahashi, et al., 1964). Tannic acid perfused internally increased the duration of the action potential and prolonged the conductance increase which occurs during the action potential (Shrager, Macey and Strickholm, 1969). Chemical modification of crayfish axons using a series of protein crosslinking aldehydes resulted in a reduction of action potential amplitude and an increase in duration which paralleled the crosslinking strength of the reagent (Shrager, Strickholm and Macey, 1969).

The biophysical characteristics of crayfish medial giant axon membrane have been studied using voltage-clamp. As in squid, there is a fast inward current carried by Na
which is activated by depolarizing pulses to 50 or more mv (Shrager, 1974). A delayed outward current is carried by K. The Hodgkin-Huxley kinetic model adequately described the observed voltage-clamp currents when the K conductance parameter was raised to the fifth rather than the fourth power (Shrager, 1974). In addition to fast Na inactivation a second slower inactivation has been observed in crayfish and squid axons (Shrager, 1977; Adelman and Palti, 1969a,b). N-ethylmaleimide when perfused inside crayfish axons shifts the steady state slow inactivation vs. V curve 14mv in the hyperpolarizing direction (Shrager, 1977). In other words, it is necessary to hyperpolarize the membrane for several hundred milliseconds by this amount to restore the amplitude of the sodium to its pretreatment level (Shrager, 1977).

Proteolytic enzymes including pronase and trypsin are able to remove or block slow Na inactivation when internally perfused at 0.02 mg/ml (Starkus and Shrager, 1978). Fast inactivation is reduced by perfusion with these enzymes at higher concentrations, however, leakage conductance increases during such enzymatic modification and irreversibly damages the axon (Starkus and Shrager, 1978). Tannic acid was found to block fast inactivation somewhat more selectively than enzymes, but still increased leak conductance (Shrager, personal communication, 1979).
4. PASSIVE ELECTRICAL PROPERTIES

An axon is essentially a tubular membrane separating two electrolyte solutions. The membrane is composed of a lipid bilayer and protein (Robertson, 1961). Because the lipid core of the membrane is a non-polar environment, the membrane has a generally low permeability to ions. Ions may traverse the membrane along specific paths or "channels" which may be composed of proteins (Armstrong, 1973). These anatomical and physiological observations may be modeled using cable theory (Kelvin, 1855). An application of cable theory to an unmyelinated axon is illustrated in figure 2 (Hodgkin and Rushton, 1946). A partial differential equation relating internal resistance ($R_i$), membrane resistance ($R_m$), and membrane capacitance ($C_m$) to the membrane potential ($V$), distance ($x$), and time ($t$) is equation 6.

\[ \lambda \left( \frac{dV}{dx} \right)^2 + \tau \frac{dV}{dt} + V - R_p = 0 \]  \hspace{1cm} (6)

where:  \[ \lambda = \sqrt{\frac{R_m}{R_i}} \]
\[ \tau = R_m C_m \]
Cable model of unmyelinated axon (after Hodgkin and Rushton, 1946). See text for explanation of symbols.
CABLE MODEL

FIGURE 2
TAU is the membrane time constant and LAMBDA is the membrane length constant. TAU describes the time course of potential changes brought about by the application of currents to the cable model. LAMBDA describes the spatial distribution of potential resulting from a point potential disturbance at $x = 0$. LAMBDA (equation 6) may be seen to be directly related to the membrane resistance and inversely related to the internal resistance of the axon. For unmyelinated axons the membrane resistance is inversely proportional to the radius of the axon, while the internal resistance of the axon is inversely proportional to the radius squared. If the specific resistance of axoplasm and membrane may be assumed constant, LAMBDA is largely determined by axon radius.

Myelin is formed of Schwann cell membrane wrapped spirally around the axon. The Schwann cell membrane may be modeled as a resistance and capacitance in parallel (Rushton, 1951). The effect of layering up these membranes is to create an electrical network composed of many parallel R-C elements in series. This series arrangement causes myelin resistance to be proportional and myelin capacitance to be inversely proportional to the number of layers. The myelin resistance and capacitance dominates the cable properties in the internodes of myelinated nerves. LAMBDA is greatly increased and TAU reduced by myelination.
5. THE PROPAGATED ACTION POTENTIAL

An action potential is initiated by depolarizing the membrane beyond the threshold for excitation. The action potential involves a cyclic change in membrane ionic conductances which is repeated as the impulse travels along the axon. During this cycle a regenerative increase in Na conductance (Na activation) allows Na current to flow into the axon. The membrane depolarization resulting from the inward flow of Na ions is spread to adjacent membrane by the cable properties of the axon. When the neighboring membrane is depolarized above the threshold, the cycle is initiated there. The threshold is reached by depolarizing the membrane 15 to 40 mv (D'Arrigo, 1971).

The depolarization of the membrane also brings about both a reduction in Na conductance (Na inactivation) and a slower increase in K conductance (delayed rectification). Impulse conduction is ordinarily unidirectional because the Na conductance remains low and the K conductance becomes high following the upstroke and during the repolarization phase of the action potential (Hodgkin and Huxley, 1952).

The velocity (VEL) of impulse propagation is determined primarily by the rate at which resting membrane may be depolarized above the threshold. The depolarization rate
(+DV/DT) is dependent upon both the cable properties of the axon, and the amplitude of the Na current. If the membrane time constant (equation 6) were to increase, the VEL would be expected to decrease because more time would be required for the Na current to depolarize the membrane. Similarly, if the length constant were decreased, the conduction velocity would be decreased, because a shorter segment of axon would be depolarized by the advancing action potential.

The effect of myelin on the propagation of the action potential is to increase both the extent and rate of depolarization of adjacent resting membrane. Myelin also significantly increases the energy efficiency of impulse transmission by restricting the passage of ionic currents to the nodes of ranvier (Rushton, 1951). The volume efficiency of the nervous system is also increased by myelin since smaller diameter fibers may be used without compromising conduction velocity.

The duration of the action potential is determined by the time course of the conduction changes to Na and K (Hodgkin and Huxley, 1952). If the Na current is not inactivated promptly, the duration will be increased. If the K conductance is reduced or delayed, the duration may also be increased.
B. DISEASED AXONS

1. WALLERIAN DEGENERATION

A particularly well studied axonal pathology is Wallerian degeneration, which occurs following separation of most axons from their cell bodies (Waller, 1850). The axon stump proximal to the separation undergoes a limited degeneration which may involve several internodes. The nerve conduction velocity (NCV) of the proximal axon following nerve section is moderately (10 - 20%) depressed. This NCV decrease may be caused by the observed diminution of axon diameter (Cragg and Thomas, 1961). However, the velocity returns to normal (as does the diameter) when regeneration of the distal segment is completed (Cragg and Thomas, 1961). The distal axon may remain excitable for several days. Impulse conduction continues after synaptic transmission fails (Gilliat and Hjorth, 1972). Normal conduction velocity appears to be maintained until impulse conduction abruptly fails.

Morphological changes are first observed in the terminal axon (Lubińska, 1977). The remainder of the axon distal to the separation disintegrates almost simultaneously shortly thereafter. Myelin breakdown quickly
follows axon disintegration. It appears that the health of vertebrate axons is critically dependent upon the cell body.

The behavior of some invertebrate nerves following separation of axon from cell body is unlike that described by Waller (1850). The portion of crayfish giant motor axons distal to a cut were found not to degenerate or disintegrate within a few days (Hoy, et al., 1967). In fact, the distal segment may remain excitable for 80 days or more. It has been speculated that the distal segment may fuse with the sprouts of the regenerating proximal segment. The functional survival time of medial giant axons following section may extend beyond 120 days (Wine, 1973). During this period the glial cells surrounding the distal segment hypertrophy (Nordlander and Singer, 1972). The hypothesis that materials may be transferred between glia and axon (Lasek, et al., 1974) may explain the continued health of the isolated axon (Hoy, et al., 1969). The capability of axons to survive axotomy is found in other invertebrates, including Locusts and Mantids (Boulton, 1969; Boulton and Rowell, 1969). Not all invertebrates share this ability. Cockroach axons degenerate promptly following lesion (Bodenstein, 1967). It may be concluded that some invertebrate giant fibers, especially the medial giant fibers of the crayfish, are not imminently dependent upon a continuous supply of materials directly from their cell bodys.
2. DYING BACK DISEASE

In contrast to Wallerian degeneration, most axonopathies do not involve a distinct separation of the axon from its cell body or the rapid disintegration of the axon. It is more common for the axon to gradually "die-back" from its terminal towards the cell body. The particularly sensitive nature of the terminal axon segment has long been recognised (Sharkey, 1896). Similarly familiar is the observation that longer tracts tend to show degenerative changes earlier than shorter ones (Gowers, 1886). It should be recalled that even in Wallerian degeneration the terminal axon is the site of the earliest degradation. The remoteness of the terminal axon from the cell body may be the reason for the axon terminal's sensitivity to a wide variety of insults.

The hypothesis that a defect at the level of the cell body could lead to a dying-back neuropathy was eloquently presented by Cavanagh (1964). Cavanagh conjectured that a defect in anabolism at the cell body would lead to a deficiency in the substances needed for the maintenance of the axon. Cavanagh's hypothesis predicts that: 1) long large diameter fibers would be compromised first because they would require more of the scarce substances, and 2)
more proximal portions of any given axon would be in a more favorable position to receive the components which were in short supply.

The possibility also exists for an aberration in local axon processes. This hypothesis, originally espoused by Prineas (1969a, b), to account for the histological pattern observed after triorthocresyl phosphate (TOCP) intoxication is based upon the morphological and biochemical differences between axon and soma. The axon has much greater surface area and volume than does the cell body. So, axons may suffer increased exposure to circulating toxins. Furthermore, most of the cellular catabolism may occur within the axonal and dendritic extensions of the cell while the anabolism may be confined mainly to the cell body. A defect in catabolism would, therefore, be more deleterious in an axon. The changes observed by Prineas (1969a, b) in the cell body could be interpreted as signs of reactive adaptation rather than primary pathology, while the changes observed in the axons were more striking than the changes in the cell body and were unlike those associated with Wallerian degeneration. Wallerian degeneration like changes would be expected if a defect in anabolism at the cell body were responsible for "dying-back" disease as suggested by Cavanagh.

A third site of axon pathology may be the axoplasmic
transport system. The accumulation of neurofilaments within acrylamide intoxicated axons led Prineas (1969b) to speculate that the rate of axoplasmic transport might be slowed by this toxin. If axoplasmic transport were slowed, then the flux of materials moving along the axon might be insufficient to maintain the entire axon. The dying-back pattern could be induced by such a defect.

Several experimental models have been used to study dying back diseases. The three major models include intoxication with TOCP, acrylamide, and the six carbon aliphatic compounds, n-hexane, methyl n-butyl ketone, and 2,5-hexanedione. Each of these toxins produce distinct patterns of morphological change while producing similar clinical symptoms.

a. TRI-ORTHOCRESYL PHOSPHATE

Triorthocresyl phosphate (TOCP) was identified as the agent responsible for an outbreak of polyneuropathy in the United States resulting from drinking extract of ginger in place of alcoholic beverages during the prohibition era (Smith et al., 1930). The symptoms of this intoxication usually began with mild sensory loss followed by motor weakness first in the feet and legs, then in the hands and arms. After exposure to TOCP ceased, recovery was slow and
only partial. For an exhaustive account of this and other TOCP intoxication outbreaks see Cavanagh (1964).

Ultrastructural changes caused by TOCP intoxication were found to precede the appearance of clinical symptoms (Prineas, 1969a). Cell bodies in both the ventral horn motor nuclei and in the dorsal root ganglia contained increased amounts of smooth endoplasmic reticulum. Those in the ventral horns also contained abnormally large amounts of neurofilaments and neurotubules. Later, but prior to clinical signs, abnormal membranous profiles appeared in the axoplasm of intramuscular nerves and preterminal CNS axons. At about the onset of clinical neuropathy dense lamellated bodies were observed in motor endplates and central boutons terminaux. Subsequently severely degenerate axons and degenerating myelin were observed, primarily in the distal portions of the nerves examined.

b. ACRYLAMIDE

Acrylamide has been identified as a neurotoxin in man and animals. The clinical manifestations of acrylamide intoxication may include ataxia, muscle weakness, sluggish reflexes and sensory loss. Ataxia is the most prominent symptom during the early phase of this disease and is masked by the later developing muscle weakness. After exposure to this toxin ends, recovery is delayed but substantial.
Sural nerve biopsies from patients in the recovery phase of acrylamide intoxication showed a reduction in the number of large diameter fibers and a greater incidence of short internodes in larger fibers (Fullerton, 1969). The frequency of segmental demyelination was not different from control biopsies. The observation of fairly mature regenerating fibers and degenerating fibers in the same individuals suggested that regeneration was possible during continued exposure to this toxin.

Electromyographic examination of these acrylamide intoxicated patients revealed slight, but nonsignificant, reduction of the maximum conduction velocity. The muscle action potential however exhibited a greatly increased dispersion. This correlated well with the finding that the distal latencies were variable and in some instances quite increased. Sensory action potentials were not observable or were very small in amplitude (Fullerton, 1969). Single fiber recordings have been obtained from cats intoxicated with acrylamide and confirm that treated fibers conduct at normal velocities except in their terminal portions where conduction failure is common. Often electrical stimulation was effective where physiological stimulation of stretch and cutaneous receptors was not (Sumner and Asbury, 1975). The electrophysiological findings in this neuropathy support the conclusion that functional deficits induced by acrylamide
Intoxication are most prominent in and may be restricted to the terminal portions of axons.

Several ultrastructural studies of acrylamide neuropathy have been reported. Prineas (1969b) found an accumulation of 10 nm neurofilaments within intramuscular terminal nerve fibers and within fibers terminating in the spinal grey matter. Terminal fibers were swollen by these accumulations of neurofilaments. This early finding was followed by the appearance of degenerating mitochondria and accumulations of neurofilaments within axons located in the spinal roots. Later, endplates and boutons terminaux enclosed increased numbers of neurofilaments. Cell bodies in the ventral horns of the spinal cord showed dissociation of ribosones from the rough endoplasmic reticulum. Schaumberg et al. (1974) found that the largest and longest fibers were not invariably the first affected by acrylamide intoxication. And Spencer and Schaumberg, (1977) found a multi-focal distribution of terminal axon degeneration.

These observations require some refinement of the dying back concept. While the impression obtained from light microscopy and whole nerve electromyographic examinations is one of an ascending degeneration, the ultrastructural findings lead to the conclusion that individual fibers may degenerate anywhere along their course. Also some fiber types are more susceptible to degeneration than other
similarly sized fiber types. The probability that a given axon segment will degenerate appears to increase towards the distal extremities of the fiber. Thus, the cellular pattern is not necessarily one of retrograde degeneration even though the population of cells does die-back.

**c. HEXACARBONS**

Exposure to six carbon aliphatic compounds such as n-hexane (HEX) or methyl n-butyl ketone (MBK) induces an intoxication polyneuropathy in man and several laboratory animals. HEX is widely used as an industrial solvent and was considered relatively non-toxic (Fairhill, 1957) on the basis of brief respiratory and dermal exposures (Patty, 1929; Oettel, 1936). However, during the mid-sixties it was recognised that workers who inspired HEX vapor at high concentration (500 - 2000 ppm) or who bathed their hands in HEX containing solvents developed, after several months, an insidious, symetric, sensory-motor polyneuropathy (Yamada, 1964).

Several outbreaks of HEX toxic polyneuropathy have since been reported in Japan (Yamada, 1967; Yamamura, 1969), Italy (Abbriitti, 1976) and the United States (Herskowitz et.al., 1971). HEX has been shown to induce a similar neuropathy in mice (Miyagaki, 1967) and rats (Truhaut et.al., 1973). The use of HEX in various glues has been
associated with polyneuropathy when the glue was sniffed (Gonzalez and Downey, 1972; Matsumura et al., 1972; Asbury, 1974; Shirabe, 1974; and Korobkin et al., 1975). The characteristics of HEX neuropathy, which will be discussed in the following section, are strikingly similar to those of the toxic polyneuropathy caused by another industrial solvent, MBK. MBK was implicated by epidemiological data as the neurotoxin responsible for a recent outbreak of toxic polyneuropathy (Allen, 1975). MBK has been proven experimentally to be a neurotoxin in chicken, cat and rat (Mendell, 1974; Spencer et al., 1975; Abdel-Rahman, 1976).

HEX polyneuropathy has been well characterized clinically. The onset of the disease is generally insidious. The earliest complaints are of slight appetite loss and/or paresthesia of the extremities. Progression of the disease is manifested by the appearance of muscle weakness and reflex disturbances (Yamada, 1967). Muscle atrophy in the extremities may occur in advanced cases (Yamamura, 1969). The above mentioned symptoms were found to have a glove-stocking type of distribution by Yamamura (1969). The symptoms of HEX intoxication may continue to intensify for three months after exposure to the toxin has ceased, whereupon gradual, but incomplete improvement usually occurs over the next 6 - 30 months (Yamada, 1967; Perticoni and Cianchetti, 1976). Laboratory findings are
generally unremarkable (Yamanura, 1969; Herskowitz et al., 1971).

Only one instance of MBK toxic polyneuropathy resulting from industrial exposure has been found to date (Billmaier et al., 1974). The clinical signs of this disease included symmetric, predominantly distal, motor and sensory disturbances (Allen, 1975). Mild cases favored sensory loss in the hands and feet. More serious cases evidenced muscle weakness and atrophy. Laboratory results were not abnormal. The severity of the intoxication was directly correlated with the degree of worker exposure to MBK (Billmaier et al., 1974). Moreover the simultaneous exposure to methyl ethyl ketone (MEK) was suspected to potentiate the toxicity of MBK. This suspicion was confirmed by subsequent experiment (Abdel-Rahman et al., 1976). After removal of the toxin from the workplace, the worker's symptoms continued to worsen, in some cases, up to 3 - 5 months. Thereafter improvements were observed which resulted in partial recovery (Allen, 1975).

Electromyographic and electroneurographic examinations of patients suffering from HEX or MBK neuropathy reveal several characteristic abnormalities. Reduced motor nerve conduction velocity (NCV) has been observed in all reported cases of this disease (Herskowitz, 1971; Yoshida et al., 1974; Allen, 1975). The reduced NCV has been interpreted,
in the literature, as evidence of advanced demyelination (Goodgold and Eberstein, 1977). Fibrillation potentials were recorded from all the examined muscles of patients suffering from hexacarbon intoxication. The presence of fibrillation potentials is thought to be indicative of muscle denervation (Perticoni and Cianchetti, 1976). Sensory action potential amplitude reduction has been reported after exposure to HEX (Yoshida et al., 1974; Korobkin, 1975). This is in harmony with the sensory disturbances observed clinically, and is associated with mild demyelination (Goodgold and Eberstein, 1977). The preponderance of the electrophysiological data supports the occurrence of demyelination in moderate to severe cases and partial denervation in all cases.

Histological examination of nerves removed from intoxicated individuals reveals a striking pattern of changes. Foremost among these, is a pronounced, focal axonal swelling visible with the light microscope in teased and cross-sectional preparations (Yamamura, 1969; Ishii et al., 1972). The axonal swellings have been observed most frequently in the paranodal regions of myelinated fibers but may also be observed in the internode and in unmyelinated fibers (Spencer et al., 1975; Saida et al., 1976; Schaumberg and Spencer, 1976). The swollen portions of axons, when viewed with the electronmicroscope, are filled with large
numbers of 10 nm neurofilaments (Herskowitz et al., 1971). The neurofilament accumulations result from an increase in the total content of neurofilaments per axon not from a simple redistribution of neurofilaments within an axon (Saida et al., 1976). Microfilament proliferation has also been observed in Schwann cells (Powell et al., 1978). Axon swelling is accompanied by myelin slippage (Friede and Martinez, 1970) so that the myelin thickness is reduced, and at nodes of Ranvier, myelin is retracted. Segmental demyelination and remyelination may occur with or without concomitant axonal swelling (Mendell et al., 1974; Powell et al., 1978).

Nerve biopsy of patients in the recovery phase of the illness show a preferential loss of large myelinated fibers which is accompanied by a less pronounced loss of smaller fibers (Yamanura, 1969; Goto et al., 1974; Shirabe, 1974). Degeneration of intramuscular axons appears also to be a late feature of the disease process (Herskowitz et al., 1971; Saida et al., 1976). Small diameter, thinly myelinated fibers have been observed and interpreted as evidence of axon regeneration and remyelination (Herskowitz et al., 1971).

The similarity of HEX and MBK induced polyneuropathy both clinically and chemically led Allen (1975) to speculate that the two illnesses may be linked. This suspicion has
been strengthened by studies of the metabolism of HEX and MBK in animals.

The IN VIVO metabolism and excretion of MBK by rats and guinea pigs was investigated by Abdel-Rahman et al. (1976) who found that MBK was converted to 2-hexanol (HOL) and/or 2,5-hexanedione (HDO). Paradoxically, phenobarbital, a liver microsome enzyme inducer, which was expected to increase the potency of MBK by promoting its transformation to a toxic metabolite, was found to reduce the potency of MBK. This may be because MBK, HOL and HDO were more efficiently excreted after treatment with phenobarbital (Abdel-Rahman et al., 1976). The potentiating effect of simultaneous exposure to MEK observed by Abdel-Rahman et al. (1976) may be explained by the increased blood levels of MBK and HDO, observed following exposure of rats to MBK in combination with MEK, versus their blood levels following exposure to MBK alone. DiVincenzo et al. (1976) reported that guinea pigs metabolize MBK to 5-hydroxy-2-hexanone (5H2H), HDO, HOL and 2,5-hexanediol (HDL).

HEX metabolism has also been investigated. HEX was found to be transformed to 5H2H and HDO (DiVincenzo et al., 1976). Urinary excretion of HEX metabolites by the rat was found to favor 1-hexanol (Dolara et al. 1978). Although free MBK was detected by Dolara et al. (1978) it did not appear to be excreted in a dose dependent fashion as was
free 1-hexanol. No data was reported by Dolara for glucuronide bound MBK while bound 1-hexanol accounted for 70 percent of the total urine 1-hexanol. Abdel-Rahman et al. (1976) found it necessary to hydrolyse urine with beta-glucuronidase in order to detect MBK in rat urine.

Methyl i-butyl ketone (MIBK) was found to be metabolized to 4-methyl-2-pentanol and 4-methyl,4-hydroxy-2-pentanone by DiVincenzo et al. (1976). MIBK has recently been reported to be acutely neurotoxic to man (AuBuchon et al., 1979). MIBK induced neuropathy is characterized by spongiform myelopathy. The axons appear to be compressed by the surrounding myelin sheaths. The clinical and histological manifestations of acute MIBK intoxication are different from those of chronic HEX, MBK or HDO intoxication. Chronic exposure to MIBK has been demonstrated ineffective in producing a neuropathy. (Mallou, 1976)

MEK was found to be metabolized to 2-butanol, 3-hydroxy-2-butanone and 2,3-butanediol (DiVincenzo et al., 1976). MEK is apparently not neurotoxic to a variety of animals (Mendell et al., 1974).

Of the several common metabolites of HEX and MBK, HDO appears to be the effective neurotoxin. Although exposure to HOL will induce a reduction of the guinea pig pupillary
response, as does exposure to HDO, HOL is metabolized to MBK and HDO (Abdel-Rahman et al., 1978). HDO produces histological changes in rats similar to those produced by HEX and MBK (Spencer and Schaumberg, 1978; Powell et al., 1978). Electrophysiological changes in rats intoxicated by HDO are similar to those observed in human HEX and MBK neuropathy (Nachtman, 1979). Furthermore, HDO is not metabolized to HOL (DiVincenzo et al., 1976; Abdel-Rahman et al., 1978; Spencer et al., 1978).

It might be suggested that 5H2H or HDL is the effective neurotoxin, but there is little evidence to support this hypothesis. DiVincenzo et al.'s (1976) finding that HDO accumulates preferentially in guinea pig serum suggests that HDO, not HDL or 5H2H, is the directly neurotoxic substance. It has been reported that HDL is less potent than is HDO in inducing axonopathy in tissue cultured neurons (Spencer et al., 1978). Here again HDL is converted to detectable amounts of HDO. In the same system HDO induces an axonopathy but is not transformed to detectable amounts of any metabolites.
III. RATIONALE

Because a significant decrease in NCV is a sensitive and early indication of polyneuropathy induced by hexacarbon intoxication, it is essential to delineate the cellular electrophysiological properties which contribute to this phenomenon. The reduced NCV may be caused by: 1) Paranodal myelin retraction, which is most prominent in large fibers (Spencer and Schaumberg, 1977); 2) Preferential conduction failure in large fibers (Gilliatt, 1966); 3) Altered membrane electrophysiological characteristics leading to a generalized slowing of conduction in all fibers. The evidence, available in the literature, leaves this question unresolved. The objective of this study, was to determine the contribution of altered membrane properties to HDO induced conduction velocity decrease.

The crayfish medial giant axon was selected for this study because its membrane is electrophysiologically similar to that of other nerves, while its anatomy and physiology differ in two critical respects. The crayfish medial giant fiber is unmyelinated. Therefore, paranodal myelin retraction cannot contribute to any alteration in NCV. The crayfish medial giant axon is devoid of neurofilaments. Therefore, axonal swelling and/or axoplasmic transport
blockage caused by the accumulation of neurofilaments cannot contribute to reduced NCV. If conduction failure and/or velocity decrement in large axons is the mechanism whereby NCV is reduced, the medial giant fibers may be expected to suffer conduction changes during hexacarbon exposure. The medial giant fiber preparation facilitates direct measurement of the transmembrane-potential. Such measurements are difficult to obtain from mammalian fibers because of their small size. Analysis of the resting and action potential parameters of crayfish medial giant fibers, may identify altered membrane properties responsible for NCV reduction.
IV. METHODS

A. ANIMALS

Crayfish, of the species Procanbarous clarkii, were obtained from Dahl Biological Supply Co. Inc (P.O. Box 566, Berkeley, CA 94701). The mortality rate resulting from shipment via AIR MAIL SPECIAL DELIVERY was less than 25%. The animals measured 6.5 - 10.0 cm from the tip of the tail shell (not including the tail fan) to the tip of the head. Most animals were bright red, but some were brownish or greenish in color.

The crayfish were maintained at room temperature in shallow 25 X 48 X 16 cm plastic rat trays filled with tap water to a depth of 10 cm. The water was aerated using at least one air stone per 4 crayfish. The trays were thoroughly rinsed weekly, using only hot water, and refilled with cold water. The crayfish were fed 2 brine shrimp or 1 trout meal pellet per crayfish every 2 - 3 days. Crayfish kept in this way maintained good health for periods exceeding one year. The animals continued to molt and reproduce while living in the laboratory environment. Animals were sacrificed for study throughout the year and the date of the experiment is used to identify the data.
obtained from a particular axon.

B. SOLUTIONS

The composition of the Normal van Harreveld's (NVH) medium used during dissection and recording contained (in mM): NaCl, 205; KCl, 5.4; NaHCO₃, 2.3; MgCl₂, 2.6; and CaCl₂, 13.5 (van Harreveld, 1936). NVH was made up using distilled, deionized, double distilled water, and reagent grade salts. The pH of the resulting solution was 6.9 +/- 0.1 at 20 °C. NVH was stored in a polyethylene container until needed. No deterioration of NVH, stored in this way, was observed over periods as long as two months.

For acute toxicity experiments HDO was dissolved in NVH to obtain HDO-van Harrevelds (HDO-VH) of the desired concentration. HDO was obtained from Aldrich Chemical Company, Inc. Addition of HDO to NVH did not alter the pH of the medium. To control for the possible effects of high osmolarity when using 1.0 gm% HDO-VH, Mannitol replaced HDO mole for mole. In other words, 88 mM Mannitol-VH (MAN-VH) was used. HDO-VH and MAN-VH were made up within 3 hours of the beginning of the test phase of acute toxicity experiments.

The solution used to fill the membrane potential recording electrode contained: 172 mM KCl, 37 mM Potassium
Citrate and Citric acid to pH 7.5 (Shrager, 1974). This solution was filtered through Whatman #1 Qualitative filter paper to remove particulate matter that might clog the electrode. It was necessary to store this solution at 0 to 5 °C in order to retard the growth of bacteria.

C. ACUTE EXPOSURE METHOD

The acute effects of HDO on crayfish giant axons were assessed by exposing the axons directly to the toxin. HDO was added to NVH to obtain a concentration of 0.1 or 1.0 gm% (w/v). HDO-VH was perfused over the nerve cord for 2 hours following a 1 hour control period. A 1 hour wash period followed the test period. The contribution of the relatively high osmolarity (20% higher) of the 1.0 gm% HDO-VH to any effects observed during exposure to this solution was assessed by exposing the cord to an equi-osmolar (88 mM) Mannitol-VH.

D. CHRONIC EXPOSURE METHODS

Chronic exposure of crayfish to HDO was achieved using one of two similar methods. METHOD A) Initially the desired amount of toxin was added to 4 l of tap water in a standard size rat tray which was kept in a hood with the exhaust fan running continuously. The water in the trays was not
aerated, but previous experience in this and other laboratories indicated that no aeration was needed when shallow (\( \leq 5 \) cm) depths were employed (Shrager, personal communication, 1977). The trays were cleaned, as before, and the water and toxin were renewed weekly. Spot samples of the medium were stored covered with parafilm at 0 °C for later quantitative analysis using Gas Chromatography (GC). Method A yielded an approximately constant concentration during the early fall when high humidity may have rendered the evaporative losses of water and toxin relatively equal. However, during late fall and winter the loss of water was quite severe, and resulted in significant increases from the desired concentration (see figure 3). METHOD B) When it was recognised that evaporative loss of water and toxin should be better controlled, a semi-closed exposure system was instituted. HDO was added to 6 l of tap water in a rat tray. A 500 ml portion of this medium was transferred to a standard gas humidifier through which compressed air was bubbled before being routed to the tray. Finally, a second rat tray was inverted and used to cover the rat tray. Samples of the medium were collected daily and stored as before for GC analysis. This method maintained a more constant concentration of the toxin than did Method A (see figure 3).
Concentration profiles during one week of exposure to HDO resulting from the two methods used to intoxicate crayfish. See text for description of Methods A and B.
FIGURE 3

METHOD A

METHOD B

intended

(intended)

(days)

(days)
E. QUANTITATIVE ANALYSIS

Quantitative analysis for the amount of HDO present during chronic exposure studies was performed using Gas Chromatography (GC). The method and conditions employed are similar to those reported by Abdel-Rahman et al. (1976). A Varian Aerograph model 2740 GC equipped with a 1.8m X 2mm stainless steel column packed with 60/80 mesh TENAX-GC (Applied Science Laboratories, State College, PA), and flame ionization detectors was used. The pressure of the required Hydrogen, Air and Nitrogen gases was regulated at 20, 20 and 40 PSI respectively. The flow rates of these gases were 23, 300 and 20 ml/min respectively. The injector, column and detector temperatures were 220, 190 and 165 °C. The detector electronics were set as follows: "Cell Voltage B" = "Fl"; "Range B" = 100 pa/mv; "Atten B" = 64; "Mode" = "B"; and the "Bucking" and "Balance" controls were adjusted to null the output after the detector had stabilized for 30 min. The chart speed was 0.25 cm/min. A sample gas chromatogram is shown in figure 4. A calibration curve was constructed by injecting 4 ul samples prepared using known concentrations of HDO equal to 0.010, 0.025, 0.050, 0.075 and 0.100 gm% mixed 1:1 with 0.050 gm% HDL. Samples were diluted to a nominal concentration of 0.05 gm% using distilled HOH then mixed 1:1 with 0.050 gm% HDL.
FIGURE 4

Sample gas chromatogram and calibration curve used to determine concentration of HDO during chronic exposure studies.
Figure 4
(Peak height ratio)
The ratio of the peak heights of HDO and HDL was used in conjunction with the calibration curve to determine the concentration of HDO in the injected sample. Finally, this result was corrected for any dilution to yield an estimate of the true concentration of HDO to which the crayfish had been exposed.
F. TISSUE BATH

The tissue chamber was a long trough constructed out of clear acrylic plastic and teflon coated aluminum (see figure 5). The metal portion of the chamber provided a path of low thermal resistance for heat transfer between the temperature controller heat sink and the superfusion medium. The plastic bottom allowed illumination from below for transmitted light microscopic (Bausch & Lomb Stereo Zoom) observation of the axon. The clear front wall allowed inspection of the axon from a second orthogonal direction. Two views, at right angles to each other, are necessary for successful cannulation of an axon. A plastic plateau was located near the center of the trough to help bend the nerve cord out of the way of the internal electrode. Physiological saline was allowed to flow from a plastic bottle through PVC tubing, a three arm valve, and a Fisher Porter flow metering valve (model no. 795-609, 1.25 mm bore) to the tissue bath. The fluid level in the reservoir was 50 cm above the level of the bath. The three arm valve selected either normal van Harreveld's (NVH) medium or van Harreveld's medium containing the test agent (AGENT-VH). The metering valve was used to adjust the flow rate to 0.7 ml/min except during solution changes when this valve was opened completely to obtain a flow rate of 25 ml/min.
FIGURE 5

Schematic of tissue bath and superfusion apparatus.
solution reservoirs

metering valve

3-way valve

vacuum assisted drain

HEATSINK

tissue bath

TEFLON COATED ALUMINUM

PLEXIGLAS

TISSUE BATH & PERFUSION SYSTEM

FIGURE 5
The 0.7 ml/min flow rate used here was found to be the optimal rate, which minimized the consumption of medium and maintained the axon in a stable state.

The medium entered the trough at the right hand end through a plastic pipe which accepted luer taper connectors. The medium then flowed over the tissue to the left hand end where it was drawn off by a vacuum assisted drain. This arrangement maintained the desired fluid level over a wide range of flow rates without causing ripples which would interfere with visual observation of the axon. The volume of the tissue bath was 33 ml.
G. TEMPERATURE CONTROL

The temperature of the solution surrounding the axon was maintained at 15.0 +/- 0.5 °C. The tissue chamber was designed to ensure good thermal contact between the bathing solution and a heat sink which was cooled by 50:50:50 Methanol:water pumped by a refrigerated (0 °C) circulation bath (Lauda K-2/R). The temperature of the solution near the recording site was measured using a model 402 thermistor probe and model 43-7B telethermometer (Yellow Springs Inst. Co., Inc.). Regulation was accomplished by a feedback control system (see Appendix A) which sensed the temperature of the medium near its exit from the chamber, and warmed the heat sink when the temperature fell below an adjustable set point. When solution changes were made, a transient (<5 minute) 1-3 °C elevation was sometimes observed. The temperature returned to within 0.5 °C of the set point after no longer than 10 minutes. Data recorded during this transient was not included in any calculations. Control experiments showed that the increased resting potential induced by these transients was significant but was quickly reversible.
II. GROSS DISSECTION:

Tools required

(1) 33 x 48 cm porcelain tray
(1) 23 x 28 cm linoleum tack board
(1) 13 cm blunt end scissors
(1) 10 cm sharp end scissors
(1) iris iridectomy scissors
(1) curved tip forceps
(1) #5 Dumont forcep
(1) 15 cm ruler
(1) beaker half filled with NVH
(1) wash bottle filled with NVH
(4) thumbtacks

All tools were clean, dry, at room temperature and within reach. The legs and claws of the crayfish were cut off as close to the body as possible using the 13 cm scissors. The tail fan of the crayfish was tacked to the linoleum using 3 or 4 thumb tacks. The length from the base of the tail to the tip of the head was recorded. The carapace was slit using the 10 cm scissors. The scissor tip did not penetrate deeply into the body rather it skimmed along just inside the shell. Transverse cuts connecting the
slits were made at the base of the carapace and just behind the eye ridges. The central section was lifted up and cut away from the underlying tissue. Cuts were made down the sides of the carapace just behind the eyes and the sides were cut free from the adhering tissue.

Using the curved tip forceps the cartilage bridge over the esophagus was stretched upward and the esophagus was cut using the 10 cm scissors. The cartilage bridge, stomach, and other entrails were gently removed from the abdominal cavity which was then rinsed with NVH. The salivary glands were cut off near their roots and discarded. The green glands (kidneys) were lifted free and discarded after the membrane covering them was peeled away. The area was washed again with NVH.

The tail shell was removed by slitting lengthwise near the scaloped edge of the tail on each side, cutting the shell away from the muscle, and finally cutting the shell away from the telson.

The gills were cut away and the longitudinal cartilage dividers were cut near the floor of the thorax. These dividers were pulled upward, one side at a time, and the attached muscles were cut as near the floor of the thoracic cavity as possible.

The tail muscle was removed by lifting it up using the
cartilage dividers as a handle, cutting on each side of the midline near the floor of the tail until a segment became free, and finally cutting the nerves which entered the muscle as far from the nerve cord as possible. This sequence was repeated six times, once for each tail segment, until all of the muscle was removed. The abdominal nerve cord and ganglia were plainly visible at this point.

The cord was rinsed with NVH and then was freed in the rostral portion of the cephalothorax. The cartilage cross bridge at the front of the thorax was removed by cutting first along the midline and second at its insertion onto the floor on each side. Care was taken to avoid bruising the circumesophageal connectives which lie deep to the bridge near the midline. The circumesophageal connectives were freed from the esophagus by teasing and cutting the connective tissue membranes which cover the connectives and esophagus. The "brain" was then freed from the "skull" by cutting with the i redectomy scissors the nerve bundles which leave this group of ganglia and innervate the various sense organs of the head.

The cord was rinsed with NVH before proceeding to expose the cord in the thorax. The floor of the abdomen was split down the midline by slitting along the midline with the fine scissors and breaking the two sides outwards. When the cord was thus exposed, the nerves exiting the ganglia, on each
side, were cut using iridectomy scissors.

The cord was rinsed one more time before removing it from the animal. The nerves exiting the final abdominal ganglion were cut, and the ganglion grasped firmly with the fine forceps. While lifting the cord gently, the peripheral nerves were cut segment by segment, proceeding in a rostral direction. When all the nerves had been cut, the cord was lifted free of the exoskeleton and transferred to a beaker of NVH and submerged by squirting with NVH from the wash bottle.

This first process took 20 - 30 minutes to complete.
I. FINE DISSECTION

Tools Required

(1) curved tip forceps
(1) iris iridectomy scissors
(1) ultra fine tip scissors
(2) #5 Dumont forceps
(assorted) glass needles with fire polished tips
of 100 to 200 um diameter

The CNS (see figure 6) of the crayfish was mounted in the tissue bath (see figure 5) by carefully slipping the nerve cord just distal to G-1 under the right hand clip and securing the cord just proximal to an abdominal ganglion under the left hand clip so that there was little tension exerted on the cord. The cord was cleaned between G-2 and G-6 of adhering connective tissue, blood vessels and nerves exiting between ganglia, by gently pulling with #5 forceps and cutting with iridectomy scissors. When the preliminary cleaning was complete it was possible to observe the medial giant axons running near the midline on each side of the cord. If the axons were not visible the preparation was discarded.
FIGURE 6

Schematic drawing of a crayfish nerve cord identifying structures referred to in the text.
Schematic of crayfish nerve cord

Figure 6
Provided the axons were intact, the sheath was removed by slitting the sheath lengthwise on each side of the cord, cutting the ventral sheath transversely at each ganglion and pulling the dorsal sheath free from the cord.

After removing the sheath, the paired ganglia G-3 through G-5 were separated by spreading the connectives apart with #5 forceps and cutting along the midline with treedectomy scissors. The ganglia were then trimmed by pulling them laterally with forceps while cutting with the scissors. Cutting the ganglia too near the giant axons may damage them, which is cause to terminate the procedure.

The tension on the cord was increased so that the cord was straight before the medial giant axon was separated from the adjacent fibers. Two glass probes, with fire polished tips, were used. One probe supported the cord ventrally while the second pierced the cord. The resulting hole was enlarged by gently "sawing" the cord lengthwise. When the separated bundles of fibers obscured the view of the axon, they were cut away. After removing the extraneous fibers the medial giant axon appeared smooth and sharply defined.

This second procedure required from 60 to 90 minutes for completion.
J. STIMULATION

The axon was stimulated using a pair of 150 um diameter teflon insulated silver wires on 530 um centers. The wires were supported in a 22 gauge syringe needle and further insulated with INSL-X lacquer. The ends of the wires and insulation were sanded flat using crocus cloth. This electrode provided a good seal when pressed against the side of the nerve cord. When possible the wires were positioned so as to straddle the axon about 3 mm proximal to the subesophageal ganglion (G-2).

The stimulus was provided by a Bioelectric IS2 B-2.5 (1014) stimulus isolation unit driven by a Tektronix model 161 pulse generator. The stimulating current was measured by recording the differential voltage drop across a 100 Ohm resistor placed in series with the stimulating electrode (see figure 7).

The axon was stimulated once every 4 seconds using a just suprathreshold positive rectangular pulse of 0.12 msec duration. Strength-duration (S-D) relations were determined by selecting a stimulus duration of 0.03, 0.06, 0.12, 0.32, 0.64 msec and adjusting the stimulus amplitude until an action potential was just evoked. The S-D relation was determined for both positive and negative pulse polarities.
FIGURE 7

Stimulating and recording apparatus used to study the crayfish medial giant axon.
Stimulating and Recording Apparatus

Figure 7
Each set of stimulus duration and amplitude measurements was fit to the Lapique equation (equation 7) using a computer program (Lapique, 1907).

\[
\text{STIM} = \frac{\text{RHEO}}{1 - \exp(-t/\text{TAU})}
\]  

(7)

where:  
- \( \text{RHEO} \) = intensity of current for threshold stimulus of infinite duration (uA)  
- \( \text{TAU} \) = time constant for excitation (ms)  
- \( t \) = duration of threshold stimulus (ms)  
- \( \text{STIM} \) = threshold stimulus of duration \( t \) (uA)

The values of \( \text{TAU} \) and \( \text{RHEO} \) resulting from the curve fitting operation are reported.
K. RECORDING

A single recording electrode was used throughout this study. The diameter of this electrode at the tip was 33 um while 10 mm back from the tip the diameter was 66 um. A bright (unplatinized) 0.00075" 90% Pt - 10% Ir wire was contained within the electrode and ended 66 um from the tip. In order to retard the buildup of bacteria (which grow in the citrate containing electrode filling solution) the electrode was stored immersed in filling solution (see Solutions for composition) in a refrigerator. The electrode was usually flushed before each experiment by sucking the filling solution out of the capillary and then forcing it to refill under pressure. The Ag/AgCl half cell-electrode holder (EH-25 2.0 mm, WPI Instruments) was stored dry after rinsing with distilled water because Ag/AgCl half cells stored in the filling solution become unstable. The DC resistance of this electrode was 800 kohm. The offset of this electrode was 4-8 mv. The halfcell was connected via a low capacitance driven shield coaxial cable to an Instrumentation Laboratory model 181A switchbox and model 181 Pico metric impedance matching buffer amplifier. The output of the pica was connected to a 50x amplifier (see appendix) which was used to drive an Esterline-Angus model AW recording ammeter and a Nicolet Explorer III digital
storage oscilloscope (see figure 7). The chart recorder provided a continuous analog record of the electrode signal below 1 Hz. The digital oscilloscope was set to take data 2 msec prior to the end of the stimulus pulse and 8.24 msec thereafter using the "cursor trigger mode" of operation. The membrane potential and stimulus current signals were sampled once every 10 usec after being low pass filtered at 100 kHz by the Nicolet Explorer Oscilloscope. The resulting record was stored on a mini-floppy diskette for later waveform analysis.
L. EXPERIMENTAL PROTOCOL

All times within an experiment are reported in minutes after the beginning of the gross dissection. The gross dissection required 20 to 30 minutes to complete. The total dissection was completed between 90 and 120 minutes.

If at least one axon appeared to be larger than 100 μm throughout the cord between G-2 and G-6, the stimulation electrode was positioned against the cord near that axon and a hole was cut in that axon between G-5 and G-6. Then the electrode was inserted 200-300 μm into the axon, the pressure head (see figure 7) on the filling solution of the recording electrode was lowered from 20 to 2 mm of water, and the electrode was advanced into the axon 1-2 mm. A brief (2-5 minute) observation of the resting potential was sufficient to determine if the leakage of electrode filling solution into the axon was acceptably low. Provided the RP held steady or increased the electrode was slowly advanced until an approximately 10 mm conduction path length (CPL) was obtained. Stimulation of the cord was begun after the electrode had been finally positioned. If the RP was greater than -65 mv and the AP was greater than 100 mv the axon was accepted for further study.

A control period lasting one hour followed successful
dissection and cannulation of each axon. During the control period, records were saved every fifteen minutes for later analysis, and the strength duration (S-D) relation was determined. Preliminary experiments showed that records taken every 15 minutes adequately described the state of the axon.

After the control period of acute exposure experiments, a switch to the test medium was made. The flow rate, after a switch to a new solution, was 25 ml/min for the first 5 minutes of the test period. Thereafter, the flow rate was reduced to 0.7 ml/min. The test period lasted for two hours after the solution switch. Records were saved after the 5 minute solution exchange period and at the regular 15 minute schedule. An S-D relation was determined during each hour of the test period.

Finally, a switch to NVH was made to assess the reversibility of any changes observed during the test period. During this wash period records continued to be saved at 15 minute intervals and a final S-D relation was measured.

In a normal experiment, the control period was extended to 4 hours. No solution changes were made. Records were saved every 15 minutes and a total of 4 S-D relations were determined.
Eighty percent of the preparations yielded acceptable (>100 mV) action potentials. Of these successful dissections and canulations 75% remained acceptable through five hours, and 50% were still acceptable during the sixth hour of the experiment. When axons from intoxicated animals were studied the "normal" protocol was employed.
H. STATISTICAL ANALYSIS

Data were excluded from consideration if the temperature was not within 0.5 of 15.0 °C when the record was taken. This sometimes was the case with records taken within the first 10 minutes after a solution change. In some experiments, the axon "died". Axon death is by definition a resting potential less negative than -60 mv. Under these conditions impulse propagation fails. Records taken under these conditions were not included in any calculations. The usual cause of axon death was a depolarization which appeared to spread from the electrode entry hole to the recording site. If the electrode was advanced 2-3 mm away from the depolarized region an acceptable resting potential was almost always observed and the experiment was continued. Electrode filling solution leakage may have caused axon death in some cases.

Records which met the criteria described above were included in the statistical analysis. First, waveform parameters measured from records (normally 4) taken during each hour of observation were averaged to obtain an estimate of the physiological state of the axon during that hour. Second, The averaged parameters were grouped by treatment and hour of observation. Third, differences between the
control and test groups were sought using Dunnett's t test (Dunnett, 1955). The analysis of the chronic exposure data required relaxation of the restrictions on resting potential and action potential amplitude because it was found that some axons were uniformly depolarized. This uniform depolarization was never observed in any axons in the acute treatment groups and could not be excluded as an artefact.
V. RESULTS

A. NORMAL ACTION POTENTIAL PARAMETERS

A drawing of a typical crayfish medial giant fiber propagated action potential with identification of the 18 parameters used to describe this waveform is shown in figure 8. These parameters were measured using a computer program operating on data recorded by the Nicolet Explorer III digital storage oscilloscope, after it was transferred to a Plessy 1-VB mini-computer. The parameters were operationally defined as described in table 1.

The eighteen parameters could be reliably measured in all axons except HYP, HYPT and DUR100. In those axons which lacked a hyperpolarization phase, HYPT and DUR100 were assigned a value equal to the time at the end of the record. The use of a computer program insured that all records were uniformly treated. And, permitted a very precise measurement of the resting potential through averaging the first 180 data points. A reliable measure of the resting potential is essential for measuring and interpreting the other action potential shape parameters.
FIGURE 8

Schematic drawing of a crayfish edial giant axon action potential with identification of points used during parameter measurement.

a. end of stimulus pulse
b. point of 10 mv depolarization
c. point of maximum depolarization rate
d. peak of action potential
e. duration at 10 % repolarization
f. point of maximum repolarization rate
g. duration at 50 % repolarization
h. point of maximum depolarizing after potential
i. duration at 90 % repolarization
j. point of resting potential crossing
k. point of maximum hyperpolarizing afterpotential
FIGURE 8
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RP</strong></td>
<td>resting potential (mv) = average potential during the period ending 0.2 msec prior to the end of the stimulus pulse.</td>
</tr>
<tr>
<td><strong>CT</strong></td>
<td>conduction time (msec) = interval between the end of stimulus and the time of a &gt;= 10 mv depolarization</td>
</tr>
<tr>
<td><strong>AP</strong></td>
<td>action potential amplitude (mv) = the potential of the peak - RP</td>
</tr>
<tr>
<td><strong>APT</strong></td>
<td>time of the action potential peak (msec) with respect to CT</td>
</tr>
<tr>
<td><strong>PSP</strong></td>
<td>Post Spike Positivity (mv) = the maximum difference between a straight line segment approximation to the repolarization phase and the action potential</td>
</tr>
<tr>
<td><strong>PSPT</strong></td>
<td>time of post spike positivity (msec) with respect to CT</td>
</tr>
<tr>
<td><strong>HYP</strong></td>
<td>hyperpolarization (mv) = amplitude of post spike negativity if any, 0 otherwise</td>
</tr>
<tr>
<td><strong>HYPT</strong></td>
<td>time of hyperpolarization (msec) with respect to CT</td>
</tr>
<tr>
<td><strong>+DV/DT</strong></td>
<td>maximum rate of depolarization (v/s)</td>
</tr>
<tr>
<td><strong>+DV/DTT</strong></td>
<td>time of +DV/DT (msec) with respect to CT</td>
</tr>
<tr>
<td><strong>+DV/DTV</strong></td>
<td>voltage at which +DV/DT occurred (mv)</td>
</tr>
<tr>
<td><strong>-DV/DT</strong></td>
<td>maximum rate of repolarization (v/s)</td>
</tr>
<tr>
<td><strong>-DV/DTT</strong></td>
<td>time of -DV/DT (msec) with respect to CT</td>
</tr>
<tr>
<td><strong>-DV/DTV</strong></td>
<td>voltage at which -DV/DT occurred (mv)</td>
</tr>
<tr>
<td><strong>DUR10</strong></td>
<td>action potential duration at 10% repolarization (msec)</td>
</tr>
<tr>
<td><strong>DUR50</strong></td>
<td>duration at 50% repolarization = interval during which the potential is greater than 50% AP (msec)</td>
</tr>
<tr>
<td><strong>DUR90</strong></td>
<td>duration at 90% repolarization = interval during which the potential is greater than 90% AP (msec)</td>
</tr>
<tr>
<td><strong>DUR100</strong></td>
<td>time of RP crossing (msec) with respect to CT (msec)</td>
</tr>
</tbody>
</table>
The means, standard errors of the means and number of axons for the action potential parameters measured during the first hour of observation (HOUR 3) of axons superfused with NVH are given in table 2. The standard errors of the means of the parameters were less than or equal to 5% of the respective means except those of HYP, NYPT, +DV/DTV, -DV/DTV and DUR100. While most axons had a hyperpolarizing afterpotential, some had a prolonged depolarizing afterpotential which did not cross the resting potential during the record. This may explain the large variability associated with the HYP and DUR100 parameters. The relatively small amplitude of the HYP, +DV/DTV and -DV/DTV parameters renders their signal to noise ratio rather low even though they are measured with comparable precision to RP, AP and PSP.

Although the standard errors of the control period action potential parameters are reasonably small, when the hypothesis of equal means is tested during HOUR 3 using Dunnett's t statistic for the NORMAL versus the MAN-VH, 0.1 gm% HDO-VH and 1.0 gm% HDO-VH treatment groups the hypothesis is rejected (P<0.05) in 25 out of 60 tests. The occurrence of these differences is not remarkable in light of the limited number of observations in each treatment group.
### TABLE 2

**NORMAL CRAYFISH MEDIAL GIANT AXON ACTION POTENTIAL PARAMETERS**

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>UNIT</th>
<th>MEAN</th>
<th>+/-</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP</td>
<td>mv</td>
<td>-74.77</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>mv</td>
<td>124.86</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>APT</td>
<td>ms</td>
<td>0.18</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>PSP</td>
<td>mv</td>
<td>22.16</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>PSP-T</td>
<td>ms</td>
<td>0.54</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HYP</td>
<td>mv</td>
<td>-3.29</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>HYPT</td>
<td>ms</td>
<td>2.39</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>VEL</td>
<td>m/s</td>
<td>12.11</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>ms</td>
<td>0.97</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>CPL</td>
<td>mv</td>
<td>11.67</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>+DV/DT</td>
<td>v/s</td>
<td>1371.58</td>
<td>37.81</td>
<td></td>
</tr>
<tr>
<td>+DV/DTT</td>
<td>ms</td>
<td>0.09</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>+DV/DTV</td>
<td>mv</td>
<td>-5.19</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>-DV/DT</td>
<td>v/s</td>
<td>-431.13</td>
<td>6.51</td>
<td></td>
</tr>
<tr>
<td>-DV/DTT</td>
<td>ms</td>
<td>0.33</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>-DV/DTV</td>
<td>mv</td>
<td>15.14</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>DUR10</td>
<td>ms</td>
<td>0.14</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>DUR50</td>
<td>ms</td>
<td>0.31</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>DUR90</td>
<td>ms</td>
<td>0.61</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>DUR100</td>
<td>ms</td>
<td>2.37</td>
<td>0.70</td>
<td></td>
</tr>
</tbody>
</table>

*number of axons equals 12*

*mean time after dissection begun was 155 min*

*temperature was 15.0 °C*

*see text for explanation of parameters*

*S.E.M. = standard error of the mean*
To allow valid comparisons to be made between these groups each axon will serve as its own control for the following analysis. The percentage change from the third to the fourth, fifth and sixth hours will be used to normalize the data from each axon. Recall that the third hour was the control phase, the fourth and fifth hours were the test phase, and the sixth hour was the wash phase. This transformation is justified by: 1) the lack of differences in the preparation of each axon and 2) the reasonable variability (SEM ≤ 5% of MEAN) of the parameters when all the untreated axons during the CONTROL period are grouped together.

The mean strength - duration parameters found during the third (control) hour of normal experiments are shown in table 3. The S-D parameters were not altered by any of the experimental procedures.
### Table 3

**Strength - Duration Characteristics of Normal Axons**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Mean</th>
<th>+/-</th>
<th>S.E.M.</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>+RHEO</td>
<td>uA</td>
<td>232.2</td>
<td>6.64</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>+TAU</td>
<td>ms</td>
<td>0.20</td>
<td>0.01</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>-RHEO</td>
<td>uA</td>
<td>-14.32</td>
<td>3.46</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>-TAU</td>
<td>ms</td>
<td>0.21</td>
<td>0.02</td>
<td></td>
<td>12</td>
</tr>
</tbody>
</table>

S.E.M. = standard error of the mean

N = number of axons
B. EFFECT OF TEMPERATURE

The influence of temperature on the resting and action potentials of a crayfish medial giant axon is illustrated in figure 9. The four records were obtained at 10, 15, 20 and 25 °C. As the temperature of the axon increases; the resting potential increases in magnitude, the conduction velocity increases, the duration decreases and the afterpotential becomes prolonged and depolarizing. The -84 mv @ 20 °C and -93 mv @ 25 °C RP of this axon is in agreement with the values reported in the literature at room temperature (Wallin, 1967a; Lieberman and Lane, 1976). The Q-10 or temperature coefficient (10-20°C) of the parameters is given in table 4. Inspection of figure 9 reveals that these Q10 values themselves vary with temperature. Notice that RP changes more between 20 and 25 °C than it does between 10 and 15 °C.
FIGURE 9

Effect of temperature on resting and action potentials of a crayfish medial giant axon (090679). The temperature adjacent to the axon at the time that the record was obtained is indicated near the peak of each action potential. See text for further explanation.
Effect of temperature on action potential

Figure 9
TABLE 4

EFFECT OF TEMPERATURE ON
CRAYFISH MEDIAL GIANT AXON PARAMETERS

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>Q10</th>
<th>TEMP. COEFF.</th>
<th>UNIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP</td>
<td>----</td>
<td>-0.62</td>
<td>mv/°C</td>
</tr>
<tr>
<td>AP</td>
<td>----</td>
<td>-0.06</td>
<td>mv/°C</td>
</tr>
<tr>
<td>PSP</td>
<td>----</td>
<td>-0.16</td>
<td>mv/°C</td>
</tr>
<tr>
<td>HYP</td>
<td>----</td>
<td>0.08</td>
<td>mv/°C</td>
</tr>
<tr>
<td>APT</td>
<td>----</td>
<td>-0.01</td>
<td>ms/°C</td>
</tr>
<tr>
<td>PSPT</td>
<td>----</td>
<td>-0.05</td>
<td>ms/°C</td>
</tr>
<tr>
<td>HYP T</td>
<td>----</td>
<td>-0.38</td>
<td>ms/°C</td>
</tr>
<tr>
<td>VEL</td>
<td>-1.40</td>
<td>----</td>
<td></td>
</tr>
<tr>
<td>+DV/DT</td>
<td>1.77</td>
<td>----</td>
<td></td>
</tr>
<tr>
<td>+DV/DTT</td>
<td>----</td>
<td>-0.01</td>
<td>ms/°C</td>
</tr>
<tr>
<td>+DV/DTV</td>
<td>----</td>
<td>-0.16</td>
<td>mv/°C</td>
</tr>
<tr>
<td>-DV/DT</td>
<td>-2.25</td>
<td>----</td>
<td></td>
</tr>
<tr>
<td>-DV/DTT</td>
<td>----</td>
<td>-0.03</td>
<td>ms/°C</td>
</tr>
<tr>
<td>-DV/DTV</td>
<td>----</td>
<td>0.02</td>
<td>mv/°C</td>
</tr>
<tr>
<td>DUR 10</td>
<td>----</td>
<td>-0.01</td>
<td>ms/°C</td>
</tr>
<tr>
<td>DUR 50</td>
<td>----</td>
<td>-0.03</td>
<td>ms/°C</td>
</tr>
<tr>
<td>DUR 90</td>
<td>----</td>
<td>-0.05</td>
<td>ms/°C</td>
</tr>
<tr>
<td>DUR 100</td>
<td>----</td>
<td>-0.05</td>
<td>ms/°C</td>
</tr>
</tbody>
</table>

The 10 and 20 °C action potentials illustrated in figure 9 were used to obtain the values reported in this table.
C. EFFECT OF TIME

The medial giant axon preparation is robust. However, it does suffer a gradual decline during the course of an experiment. Dunett's t statistics were computed to test the hypothesis that the fourth, fifth and sixth hour means were equal to the third hour mean of the three normal axons. No parameters were significantly (P<0.05) different from hour 3 during the fourth hour. Only DUR10 differed from hour 3 to hour 5. While during the sixth hour, PSP, VEL, +DV/DTV, -DV/DT and DUR50 were different from hour 3. Figures 10, 11 and 12 illustrate the mean percentage changes from CONTROL for those parameters which changed significantly during NORMAL experiments. It is interesting to note that the +DV/DT parameter did not monotonically decline as did the VEL parameter (see figure 10). The similarity in the magnitude of the changes in -DV/DT, DUR10 and DUR50 (see figure 11) suggests that the duration increase may be related to a slowing of the repolarization rather than the depolarization. The meaning of the changes in PSP and +DV/DTV (see figure 12) is obscure.
FIGURE 10

The normal changes in VEL and +DV/DT during the 4 hours of recording are illustrated. The time is indicated in hours after the beginning of dissection. The change is expressed as mean percentage change from hour 3 to hours 4, 5 and 6. This mean change was computed from the data obtained from three normal axons each of which served as its own control.
VEL AND +DV/DT VS TIME

(hours)

△ VEL

● +DV/DT

* P<0.05

Figure 10
FIGURE 11

The normal changes in DUR10, DUR50 and -DV/DT during the 4 hours of recording are illustrated. The time is indicated in hours after the beginning of dissection. The change is expressed as mean percentage change from hour 3 to hours 4, 5 and 6. This mean change was computed from the data obtained from three normal axons each of which served as its own control.
DUR10, DUR50, -DV/DT VS TIME

* P<0.05

FIGURE 11
FIGURE 12

The normal changes in +DV/DTV and PSP during the 4 hours of recording are illustrated. The time is indicated in hours after the beginning of dissection. The change is expressed as mean percentage change from hour 3 to hours 4, 5 and 6. This mean change was computed from the data obtained from three normal axons each of which served as its own control.
+DV/DTV VS TIME

*P<0.05

PSP VS TIME

FIGURE 12
D. TOXICITY OF HDO TO CRAYFISH

Data describing the toxicity of HDO to crayfish was obtained incidental to the chronic exposure experiments. The crayfish were exposed simultaneously via dermal, respiratory and oral routes. No crayfish died during the first 24 hours of exposure to HDO, hence, doses up to 0.50 gm% are not acutely toxic to crayfish. Sub-acute toxicity was observed during continued exposure to HDO at doses in excess of 0.15 gm%. The average survival time in 0.35 or 0.50 gm% HDO was 10 days. These figures do not include those animals which were sacrificed for electrophysiological study. Therefore, the reported survival times are likely to be too short.

E. EFFECTS OF CHRONIC IN VIVO EXPOSURE TO HDO

The sub-acute exposure study resulted in 7 acceptable axons for which accurate measurements of the dose and cumulative dose were obtained. The hour 3 action potential parameters of the sub-acutely exposed axons and those of the untreated axon (032579) which most closely corresponded to the average of the untreated axons during the CONTROL period, were analysed using the method of linear regression.
The independent variables which were tested included dose, log dose, cumulative dose and log cumulative dose. The student's t statistic was used to test the hypothesis that the slope of the regression line was equal 0. Table 5 shows the parameters for which significant regressions were obtained. Some parameters had a significantly nonzero slope (P<0.05) for regression against more than one independent variable. In these instances regression versus dose always yielded a better fit than did regression versus log dose, cumulative dose or log cumulative dose. This statistical finding is in harmony with the observation that crayfish axons examined at the end of a 7 day exposure cycle were always more strongly affected than were axons examined at the beginning of the exposure cycle. The concentration of HDO rose during the cycle and was restored to the intended value when the aquaria were cleaned. It is also consistent with the observation that a paralyzed crayfish (0.50 gm% 9 days) became free of paralysis after 1 day in a HDO free aquarium. The working hypothesis is that the effect of sub-acute environmental exposure to HDO, on the medial giant axon quickly (approximately 1 day) equilibrates with the prevailing concentration of HDO. This unexpected and interesting finding warrants further study. The rapid equilibration of effect may be related to the route of exposure to HDO used in this study. Excretion of the untransformed toxin would have only returned it to the
aquarium where it would continue to have access to the crayfish. An attractive mechanism is that HDO in the aquarium equilibrates with HDO in the interstitial space of the crayfish.

Significant (nonzero $P<0.05$) slopes were obtained for 11 parameters (see table 5). Graphs of the significant regressions are shown in figures 13, 14, 15, 16 and 17. All of the voltage parameters were progressively altered by increasing the dose of HDO. Only two time parameters, DUR100 and HYPT, were so affected (see figure 17). These parameters are undefined when there is no hyperpolarization following an action potential. RP and AP both decreased in magnitude (see figure 13). The changes in VEL (see figure 14), $+/−$ DV/DT (see figure 15), and PSP, $−$DV/DTV, HYP and $−$DV/DTV (see figure 16) may be related to the smaller amplitude of the action potential.
### TABLE 5
**DOSE DEPENDENT EFFECTS OF SUB-ACUTE EXPOSURE TO HDO**

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>UNIT</th>
<th>INTERCEPT</th>
<th>STD. ERR.</th>
<th>SLOPE</th>
<th>P &lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP</td>
<td>mv</td>
<td>-72.70</td>
<td>0.06</td>
<td>2.38</td>
<td>0.001</td>
</tr>
<tr>
<td>AP</td>
<td>mv</td>
<td>120.45</td>
<td>0.06</td>
<td>-9.09</td>
<td>0.001</td>
</tr>
<tr>
<td>PSP</td>
<td>mv</td>
<td>21.66</td>
<td>0.06</td>
<td>-2.19</td>
<td>0.001</td>
</tr>
<tr>
<td>HYP</td>
<td>mv</td>
<td>-4.41</td>
<td>0.06</td>
<td>0.82</td>
<td>0.05</td>
</tr>
<tr>
<td>VEL</td>
<td>m/s</td>
<td>11.72</td>
<td>0.06</td>
<td>-2.95</td>
<td>0.001</td>
</tr>
<tr>
<td>+DV/DT</td>
<td>v/s</td>
<td>1251.49</td>
<td>0.06</td>
<td>-176.29</td>
<td>0.001</td>
</tr>
<tr>
<td>+DV/DTV</td>
<td>mv</td>
<td>-5.26</td>
<td>0.26</td>
<td>0.69</td>
<td>0.001</td>
</tr>
<tr>
<td>-DV/DT</td>
<td>v/s</td>
<td>-421.47</td>
<td>0.06</td>
<td>51.97</td>
<td>0.001</td>
</tr>
<tr>
<td>-DV/DTV</td>
<td>mv</td>
<td>12.26</td>
<td>0.01</td>
<td>-0.27</td>
<td>0.01</td>
</tr>
<tr>
<td>DUR100</td>
<td>ms</td>
<td>1.42</td>
<td>0.06</td>
<td>1.37</td>
<td>0.01</td>
</tr>
</tbody>
</table>

The slope reported in this table is in units of gm % HDO. The regression was done on the data from 8 axons. Regression .vs. dose was always more significant than .vs. log dose, cumulative dose or log cumulative dose.
FIGURE 13

Dependence of AP and RP on the concentration of HDO in the aquarium on the day of sacrifice. Data was obtained from 8 axons. The axons were removed from crayfish which had been exposed to 0.000, 0.062, 0.072, 0.333, 0.430, 0.720, 0.970 or 1.480 gm % HDO on the day of sacrifice. See table 5 for slope, intercept and significance of linear regression line.
AP AND RP VS DOSE

Figure 13
Dependence of VEL on the concentration of HDO in the aquarium on the day of sacrifice. The axons were removed from crayfish which had been exposed to 0.000, 0.062, 0.072, 0.333, 0.430, 0.720, 0.970 or 1.480 gm % HDO on the day of sacrifice. See table 5 for slope, intercept and significance of linear regression line.
VELOCITY VS DOSE

Figure 14
Dependence of $+\frac{DV}{DT}$ and $-\frac{DV}{DT}$ on the concentration of HDO in the aquarium on the day of sacrifice. The axons were removed from crayfish which had been exposed to 0.000, 0.062, 0.072, 0.333, 0.430, 0.720, 0.970 or 1.480 gm % HDO on the day of sacrifice. See table 5 for slope, intercept and significance of linear regression line.
$+\frac{DV}{DT}, -\frac{DV}{DT}$ VS DOSE

Figure 15
Dependence of PSP, \(-DV/DTV\), HYP and \(+DV/DTV\) on the concentration of HDO in the aquarium on the day of sacrifice. The axons were removed from crayfish which had been exposed to 0.000, 0.062, 0.072, 0.333, 0.430, 0.720, 0.970 or 1.480 gm % HDO on the day of sacrifice. See table 5 for slope, intercept and significance of linear regression line.
Figure 16

PSP, -DV/DTV, HYP, +DV/DTV VS DOSE

Figure 16
FIGURE 17

Dependence of DUR100 and HYPT on the concentration of HDO in the aquarium on the day of sacrifice. The axons were removed from crayfish which had been exposed to 0.000, 0.062, 0.072, 0.333, 0.430, 0.720, 0.970 or 1.480 gm % HDO on the day of sacrifice. See table 5 for slope, intercept and significance of linear regression line.
HYPT AND DUR100 VS DOSE

Figure 17
F. EFFECT OF HIGH OSMOLARITY SUPERFUSION MEDIUM

The effects of a 20% increase in the osmotic strength of the superfusion solution were measured using 88 mM MAN-VH. These three experiments were required as controls for the 1.0 gm% HDO experiments to be reported later. Figure 18 shows two superimposed action potentials from an axon which was treated with MAN-VH. The smaller action potential was recorded just prior to the switch to MAN-VH superfusion. The broader action potential was recorded after 2 hours of superfusion with MAN-VH. The conduction times and upstrokes of the action potentials are superimposable. The peak is higher and later after treatment. The repolarization phase is slower and the hyperpolarization is absent following MAN-VH superfusion for 2 hours. A family of significant (P<0.05) differences (see figure 19) between the parameters of NVH and MAN-VH superfused axons was observed. The repolarization rate and PSPT of MAN-VH axons decreased 15 and 17% respectively during the fourth hour when compared to normal axons. During the fifth hour -DV/DT and PSPT continued to diverge from their normal values, and -DV/DTT, DUR10 and DUR50 became significantly different from normal. During the sixth hour some recovery occurred, but the above mentioned parameters remained different from normal.
Two superimposed action potentials recorded from the same axon (032680) are shown. The normal action potential (labeled NVH) was recorded at the end of hour 3 just prior to the introduction of 88 mM MAN-VH. The action potential labeled MAN-VH was recorded after 2 hours of superfusion with 88 mM MAN-VH.
Effect of 2 hour MAN-VH superfusion on action potential

FIGURE 18
FIGURE 19

Effects of MAN-VH superfusion on action potential parameters. See table 1 and figure 8 for definition of parameters. Parameters not shown did not significantly differ (P>0.05, Dunnett's t test) from normal. Both the NVH and MAN-VH groups were comprised of three axons. The ordinate is expressed as the difference in percentage change from hour 3 between the NVH and MAN-VH groups. Significant differences (P<0.05, Dunnett's t test) are indicated by *.
Figure 19

( hours )

* P < 0.05
G. IN VITRO EFFECTS OF HDO

The effect of 2 hour IN VITRO exposure to HDO on medial giant axons was investigated using 0.1 and 1.0 gm% HDO-VH. The normal group (3 axons) served as the control for the 0.1 gm% HDO group (3 axons). No differences (P<0.05) were found between the mean percentage changes from hour 3 of NVH and 0.1 gm% HDO-VH superfused axons.

The MAN-VH treated group served as the control for the effects of HDO at high concentration on crayfish medial giant axon action potentials. Use of the MAN-VH group as control allows the differences due to specific interactions between HDO and the axon to be separated from those due to the high osmolarity of the 1.0 gm% HDO-VH. The changes in the action potential following 2 hours of superfusion with 1.0 gm% HDO-VH are illustrated in figure 20. Two action potentials, one preceding and one following HDO are shown. The action potential is broadened and the after potential becomes depolarizing after treatment. In addition, the rising phase is slowed. This is shown more clearly in figure 21 where the two action potentials of figure 20 were expanded and translated until they crossed at the point of maximum depolarization rate.
Two superimposed action potentials recorded from the same axon (091479) are shown. The action potential labeled NVH was recorded at the end of hour 3 just prior to the introduction of 1.0 gm% HDO-VH. The action potential labeled HDO-VH was recorded after 2 hours of superfusion with 1 gm% HDO-VH.
Effect of 1.0gm% HDO-VH superfusion on action potential
 FIGURE 21

The action potentials shown in figure 20 have been translated until they intersect at the point of maximum depolarization rate and the time axis has been expanded 4 times.
Effect of 1.0gm% HDO-VH superfusion on action potential
Many differences were found between the MAN-VH and 1.0 gm% HDO-VH groups (3 axons each) (see figures 22, 23 and 24). During the fourth hour the primary changes were a 20% decrease in \( +\Delta V/\Delta T \) (\( P<0.01 \)), a 29% increase in PSP (\( P<0.01 \)) and a 111% decrease in HYP (\( P<0.05 \)) (see figures 22 and 23). Concomitant alterations in VEL, APT, \( +\Delta V/\Delta T \), PSPT and DUR90 may have been caused by the reduced rate of depolarization (see figures 22, 23 and 24). The increased PSP may also have contributed to the delayed PSPT and DUR90.

It should be recalled that \( +\Delta V/\Delta T \), VEL and PSP were not significantly affected by MAN-VH treatment. These changes are therefore peculiar to 1.0 gm% HDO treatment.

During the fifth hour the above mentioned parameters continued to diverge and the rate of repolarization became decreased by 19%. The significantly reduced \( -\Delta V/\Delta T \) (\( P<0.01 \)) was accompanied by significant increases in \( +\Delta V/\Delta T \), \( -\Delta V/\Delta T \), DUR10, DUR50 and DUR90 (see figure 24).

During the sixth hour most of the altered parameters recovered. Only APT, HYP, \( +\Delta V/\Delta T \) and \( +\Delta V/\Delta TV \) remained significantly different (\( P<0.05 \)) from their MAN-VH values (see figures 22 and 23). HYP continued to diverge. APT, \( +\Delta V/\Delta T \) and \( +\Delta V/\Delta TV \) showed some recovery but continued to be different from their MAN-VH sixth hour values.
Effect of 1.0 gml% HDO-VH superfusion on $+\Delta V/\Delta T$, $+\Delta V/\Delta T V$, $-\Delta V/\Delta T V$, VEL and APT. The effect is expressed as the difference in the percentage change from hour 3 between the MAN-VH and 1.0 gml% HDO-VH groups (3 axons each). Significant differences ($P<0.05$, Dunnett's t test) are indicated by *.
Figure 22: Bar graphs showing % change in +DV/DT, VEL, +DV/DTT, +DV/DTV, and APT over 4, 5, and 6 hours. Significant changes indicated by asterisk (*) and P<0.05.
FIGURE 23

Effect of 1.0 gm% HDO-VH superfusion on HYP, HYPT, PSP, PSPT and DUR100. The effect is expressed as the difference in the percentage change from hour 3 between the MAN-VH and 1.0 gm% HDO-VH groups (3 axons each). Significant differences (P<0.05, Dunnett's t test) are indicated by *.
Figure 23
FIGURE 24

Effect of 1.0 gm% HDO-VH superfusion on -DV/DT, -DV/DTT, DUR10, DUR50 and DUR90. The effect is expressed as the difference in the percentage change from hour 3 between the MAN-VH and 1.0 gm% HDO-VH groups (3 axons each). Significant differences (P<0.05, Dunnett's t test) are indicated by *.
Figure 24
DUR100 recovered to such an extent that it resembled DUR100 during a normal sixth hour ($P > 0.05$) rather than during a MAN-VH sixth hour ($P < 0.05$) (see figure 23). This suggests that HDO does not irreversibly alter the membrane under the conditions employed here.
VI. DISCUSSION

The anatomical and physiological properties of the crayfish medial giant axons measured during this study are similar to those reported by others (Yamagishi and Grundfest, 1971; Wallin, 1968; Shrager, Strickholm and Nacey, 1969; Lieberman and Lane, 1976). When crayfish were exposed to HDO in their aquaria at doses of 0.00 to 1.48 gm% for periods of up to 18 days, dose dependent decreases in the resting potential (2.4 mv/gm%), action potential amplitude (9.1 mv/gm%) and conduction velocity (2.95 m/s/gm%) were observed (see figures 13, 14, 15, 16 and 17). Crayfish axons superfused with 20% hyperosmotic physiological saline (mannitol addition) developed during the fifth hour a 15 to 17 % reduction in the rate of repolarization and a depolarizing afterpotential, which was only partly reversible. Axons superfused with 0.1 gm% HDO were not significantly different from normal axons. Axons superfused with physiological saline containing 1.0 gm% HDO developed when compared to MAN-VH superfused axons a 30% reduction in the rate of depolarization, a 24% reduction in conduction velocity, a 20% decrease in the rate of repolarization, a 28% increase in the post spike positivity, and a depolarizing afterpotential.
A. MECHANISMS PROPOSED FOR OBSERVED CHANGES

1. CHRONIC EXPOSURE TO HDO

The dose dependent decrease in the amplitude of all the voltage parameters contrasts sharply with the lack of dose dependent change in most of the the duration parameters of the action potential. This finding suggests that chronic environmental exposure to HDO may not alter the kinetics of the membrane conductance changes which give rise to the action potential. The reduced resting potential may be caused by: 1) a reduced K equilibrium potential, eK 2) a reduced resting K conductance, gK(r) 3) an increased "leakage" conductance, gL or 4) a reduced net active transport current, J (see equation 2). Decreased resting potential was not observed during 2 hour IN VITRO exposure to HDO. Therefore, it is unlikely that either gK(r), gL or J are directly altered by HDO since these characteristics would be expected to respond rapidly to HDO. The effects of pharmacologic agents which are thought to act directly on the membrane are clearly recognisable within ten minutes of their introduction (Narahashi, 1974). A reduction in eK might result from a defect in energy metabolism. The decline of the K concentration gradient would be expected to require a rather long time since axons maintained at low temperature (where Na/K ATPase activity is depressed) run
down very slowly (Shrager, 1974). The reduced action potential amplitude may be attributed in part to the decreased resting potential. Two types of Na inactivation, fast and slow, have been identified in crayfish medial giant axons (Starkus and Shrager, 1978). Both of these would be increased by a tonic depolarization, thereby reducing the availability of the Na conductance system. A reduction of the maximum Na conductance would likely have been reflected in a reduced +DV/DT during the acute exposure experiments if HDO acted in this mechanism. A reduction in the sodium equilibrium potential, eNa, may have contributed to the reduced action potential amplitude. A reduction in eNa would be expected if active transport activity were curtailed because of decreased availability of ATP. The other dose dependent parameters appear to be secondary effects to the reduced resting potential and action potential amplitude. The effects of chronic environmental HDO exposure on medial giant axons are reversed after approximately one day.

2. ACUTE EXPOSURE TO HDO

The primary effect of high osmotic strength physiological saline was a poorly reversible decrease in the rate of repolarization. This led to an increase in the duration of the action potential. The rate of
repolarization is determined by the net outward membrane current. In normal crayfish axons, this current is carried primarily by K ions (Shrager, 1974). It is likely, therefore, that K delayed rectification may be reduced and/or delayed. Alternatively, if Na inactivation were slowed, the initial Na current would become prolonged and the net outward current would be reduced. It is impossible to distinguish between these alternatives without measurements of membrane impedance during the action potential. The method of voltage-clamp could be used in future experiments to delineate the membrane mechanism responsible for the prolongation of the action potential under conditions of high osmotic strength superfusion.

The lack of significant differences between axons superfused with NVH and 0.1 gm% HDO-VH leads to two conclusions. First, it demonstrates that the experimental procedure used to introduce a test solution had no effect on the axons studied here. Second, it suggests that HDO may not directly affect the excitable membrane at physiological levels of HDO, if the sensitivity of nerve membrane to HDO is the same in the crayfish and rodents. The blood levels of HDO in chronically hexacarbon intoxicated rats and guinea pigs have been reported to be 2.5 to 3.1 mg% in plasma (Abdel-Rahman et al., 1976; Abdel-Rahman et al., 1978). It is likely that the concentration of HDO in nervous tissue is
not higher than that reported in plasma since HDO is highly water soluble and would not accumulate in membranes. Therefore, 0.1 gm% HDO-VH is 50 times more concentrated than physiological levels of HDO. Direct effects of TTX and TEA on excitable membranes usually occur within a few minutes (Narashahi et al., 1964; Tasaki and Hagiwara, 1957). Since the 0.1 gm% concentration of HDO and the 2 hour duration of exposure greatly exceeded the conditions under which one might reasonably expect HDO to directly modify membrane processes it may be concluded that HDO does not directly interact with the external membrane surface under physiological conditions. More time might be required if an HDO receptor were located on the internal surface of the membrane.

Axons were exposed to 1.0 gm% HDO-VH to determine if HDO had any direct effects on the resting and action potentials. 1.0 gm% is near the solubility limit for HDO in van Harreveld's medium. The effects of 1.0 gm% HDO were different from the effects of osmotically equivalent mannitol (88 mM). The major difference was that the maximum rate of depolarization was reduced by 1.0 gm% HDO superfusion. This might result from a decrease in the amount of Na entering the cell during the upstroke of the action potential. The amplitude of the resting and action potentials were not significantly reduced by 1.0 gm% HDO.
This suggests that the Na and K equilibrium potentials were not grossly altered by 1.0 gm% HDO. The decreased rate of rise may be related to a change in the kinetics of Na activation. The 25% decrease in conduction velocity correlated well with the 30% decrease in +DV/DT. The maximum rate of repolarization was decreased 19% with respect to the mannitol group and 37% with respect to the normal group. The reasons for this change were discussed above in relation to MAN-VH treatment. The post spike positivity increased during superfusion with 1.0 gm% HDO-VH. This potential is thought to be due to an inward flow of Ca ions (Yamagishi & Grundfest, 1971). If this is true, Ca influx into axons may be promoted by HDO. The hyperpolarizing after potential dissappeared during 1.0 gm% HDO-VH superfusion and was replaced with a longlasting depolarizing after potential. This may reflect a long lasting Na conductance elevation (incomplete inactivation) or possibly the accumulation of K in the Frankenhaeuser and Huxley space adjacent to the axon.
B. CONCLUSIONS

The crayfish medial giant axon provides a unique opportunity to study the electrophysiological effects of HDO because this preparation is both unmyelinated and free of neurofilaments. In this light, the chronic exposure study reported here provides a critical test of the hypothesis that paranodal demyelination is responsible for the reduction of nerve conduction velocity associated with this toxin. The development of a dose dependent decrease in conduction velocity suggests that myelin may not be required for NCV decrease. This finding is consistent with the report of Morgan-Hughes (1968), who found no significant changes in NCV during the latent period of Guinea Pig experimental diphtheritic neuropathy when paranodal demyelination was widespread. This conclusion is further supported by the finding of Koles and Rasminsky (1972) that one ninth of the internodal myelin could be removed adjacent to a node without affecting the internodal conduction time in a mathematical model of myelinated nerve.

The failure to observe axonal swelling in chronically HDO exposed crayfish suggests that the hypothesis which contends that neurofilament accumulations mechanically distend axons is correct (Asbury et al., 1974). However,
the finding of conduction velocity decrement in the absence of neurofilamentous axonal swelling indicates that swelling per se may not be necessary for conduction velocity decrement.

The dose dependent decreases in medial giant axon resting and action potential amplitudes of chronically exposed crayfish are suggestive of reduced Na and K concentration gradients. These gradients might decline as a result of a reduction in Na/K ATPase activity. Since no change in RP or AP was detected during the IN VITRO experiments HD0 probably does not directly interfere with the "pump". "Pump" activity would be reduced if axonal energy metabolism were disrupted by chronic exposure of crayfish to HD0. Disruption of axonal energy metabolism by HD0 is indicated by the reduced IN VITRO oxygen uptake of rat sciatic nerves removed from HD0 intoxicated rats (Nachtman, 1979). A preliminary report suggests that HD0 may inhibit metabolic enzymes (Spencer and Schaumberg, 1978).

The lack of differences between normal axons and axons exposed to 0.1 gm% HD0 IN VITRO suggests that HD0 may not act directly on the membrane at physiological concentrations of this toxin. If the failure to observe any changes during 0.1 gm% HD0 IN VITRO exposure were due simply to the limited duration of the observation period, one might expect the
changes to develop more quickly during 1.0 gm% HDO exposure. The finding that 1.0 gm% HDO exposure IN VITRO results in qualitatively different effects than does chronic IN VIVO exposure, strengthens the conclusion that HDO may act indirectly, possibly through reduced energy supply, on the excitable membrane.

C. FUTURE EXPERIMENTS

While understanding of the mechanism by which HDO acts to reduce conduction velocity in crayfish medial giant axons has been increased by the study reported here, some questions remain unanswered. These questions form the impetus for additional study.

The assumption that the membranes of myelinated vertebrate and unmyelinated invertebrate nerve fibers respond similarly to HDO should be verified. A promising experimental test might involve chronically exposing frogs to HDO and then recording from isolated nodes of Ranvier. Provided that the assumption is correct, a reduced RP would be found in nerves isolated from chronically exposed frogs and would be absent in acutely exposed and normal nerves. To support the comparison of the effects of chronic HDO exposure on crayfish and mammals it would be desirable to measure the plasma levels of HDO in crayfish. Such a study could form the beginning of a thorough evaluation of the
biotransformation and excretion of HDO by crayfish.

The chemical specificity of the effects observed in the reported experiments could be evaluated by repeating them using 2,4 HDO rather than 2,5 HDO. The 2,4 compound is known to be free of neurotoxic effects under conditions where the 2,5 compound has been demonstrated neurotoxic (Spencer and Bishoff, 1978). The acute effects reported here may indeed be unrelated to the chronic neurotoxicity of HDO. They might be characteristic of polycarbonyl compounds in general. Indeed, the broadening of the action potential observed during acute 1.0 gm % HDO-VH treatment is similar to that reported following superfusion of axons with crosslinking aldehydes such as glutaraldehyde (Shrager et al., 1969).

It is of interest to measure the axonally available ATP in chronically exposed and acutely exposed fibers. The conclusion reached above predicts that ATP would be less available in chronically exposed fibers. The membrane mechanism by which HDO affects the action potential would be most completely described by recording membrane currents during voltage clamp. The broadening effects of MAN-VH and 1.0 gm % HDO-VH on the conducted action potential are particularly appropriate for such investigation because they might arise from prolonged Na current or reduced and/or delayed K current. Clearly much remains to be known
regarding the neurotoxic effects of HDO. The results of the experiments suggested above should further increase the understanding of HDO neurotoxicity.
VII. SUMMARY

1. The anatomy and physiology of normal and diseased nerve fibers are reviewed.

2. Experiments are proposed which are designed to test the hypothesis that myelin is required for the development of conduction velocity decrement during chronic exposure to HDO. In addition these experiments allow the effects of both chronic and acute exposure to HDO on the conducted action potential to be observed.

3. A dose dependent conduction velocity decrement \((-2.95 \text{ m/s/gm}\%\text{HDO})\) occurs following chronic exposure of crayfish to HDO. It may be concluded that myelin is not required for conduction velocity decrease since crayfish axons are devoid of myelin.

4. The amplitude of both the resting and action potentials are decreased following chronic IN VIVO exposure to HDO. This result suggests that HDO may interfere with the maintainance of the ionic concentration gradients which serve as the energy source of the action potential.

5. The action potential is broadened and its conduction velocity is decreased by acute IN VITRO exposure to HDO at 1.0 gm %. No changes in action potential shape
or conduction velocity were detected during acute exposure to 0.1 gm % HDI.

6. Part of the broadening of the action potential observed during acute IN VITRO exposure to 1.0 gm % HDI may be caused by the hyperosmolarity of the test solution, since acute IN VITRO exposure to osmotically equivalent 88 mM Mannitol also tended to prolong the action potential. However, 1.0 gm % HDI treatment produced effects significantly different from those of 88 mM Mannitol treatment.

7. It is hypothesized that chronic IN VIVO exposure to HDI may reduce the axonal ATP pool.

8. The acute IN VITRO effects appear to be qualitatively different from the chronic IN VIVO effects of HDI, and may involve alteration of membrane ionic conductance pathways.

9. The need for and nature of additional study of HDI neurotoxicity is discussed.
VIII. APPENDIX

A. TEMPERATURE CONTROLLER

The device (Tempcon 1), used to control the temperature of the tissue bath was a modification of a design by Uvonics Co., Inc., 767 Adena Dr., Columbus, OH. Tempcon 1 consists of two modules, an interconnecting cable, a heater, and a temperature sense probe. The power module supplies +/- 15 V to the control module, receives a command voltage from the control module and outputs an adjustable voltage to the heater. The control module interconnects and integrates all system components.

CONTROL MODULE

Please refer to figure 25. The temperature is sensed using the temperature coefficient of the forward voltage drop of a silicon diode. The difference between the voltage of the diode (D7) and the ZERO set potentiometer (R12) wiper is amplified by U1B. The difference between the output of U1B and the wiper of R14 is amplified by U2B,A and applied to the command input of the power module. The meter is driven by U1A and displays either the temperature of the
Figure 25

TEMPCON1 CONTROL MODULE
probe or the value of the TEMP SET control (R14). The sensitivity of the meter is controlled by R25.

POWER MODULE

Please refer to figure 26. The power module consists of two sensitive gate phase controlled SCR's (Q1,Q2) which switch the current to capacitor C1. Transistor Q3 is driven by U2A and turns the SCR's on for variable fractions of each AC half cycle. The output power of the power module may be varied between 5 and 113 watts.

CALIBRATION

1) Check for +/- 15 V analog supply voltages by disconnecting the interconnecting cable and measuring the voltage at connector J1.

2) Remove the back of the control module, connect the sense probe, and reconnect the cable between the power and control modules.

3) Prepare two temperature baths at 0 and 40 ºC.

5) Immerse the sense probe in the 0 ºC bath and adjust R12 until the meter reads zero.

6) Immerse the sense probe in the 40 ºC bath and adjust R25 until the meter reads 40 ºC.

7) Repeat steps 5 and 6 until no further adjustment is needed.
TEMPCON1 POWER MODULE

Figure 26
PARTS LIST

Transformers (T)

T1 Stancor 8665 24V, 8A, CT

Switches (S)

S1 SPST toggle
S2 miniature SPDT toggle

Fuses (F)

F1 2A SLO-BLO

Connectors (J)

J1 MS-5S
J2 MS-5S
J3 banana
J4 amphenol series 221 with "reliatac" inserts

Meters (M)

M1 Triplett 321-pl 0.1 ma DC with scale modified to read -10 to 40 °C

Resistors (R) in ohms 10% 1W unless otherwise indicated

R1 151 2W
R2 27
R3 27
R4 2K
R5 2K
R6 0.05 5% 10W
R7 1.5K
R8 1.5 120W
R9 1.5K
R10 511 1% 1/4W
R11 82K
R12 10K trim pot
R13 27K
R14 10K 10 turn pot
R15 1K
R16 56.2K 1% 1/4W
R17 56.2K 1% 1/4W
R18 1M 1% 1/4W
R19 1M 1% 1/4W
R20 100K 1% 1/4W
R21 100K 1% 1/4W
R22 100K 1% 1/4W
R23 100K 1% 1/4W
R24 39K
R25 20K trim pot
R26 10K
R27 9.09K 1% 1/4W
R28 2K 1% 1/4W
R29 82.5K 1% 1/4W

Capacitors (C) mfd
C1 25,000 35 VDC

Diodes (D)
D1 5JF8
D2 5JF8
D3 1N293
D4 5JF8
D5 1N293
D6 1N293
D7 1N293
D8 SV1374 8.2V ZENER
D10 LED
D10 IR10D6

Transistors (Q)
Q1 GE C220 F SCR
Q2 GE C220 F SCR
Q3 GE D4D8 PNP

Integrated Circuits (U)
U1 74747
U2 74747

Power Supply Module (P)
P1 Boston Tech 2.15.100
B. MEMBRANE POTENTIAL AMPLIFIER

An amplifier with a gain of 50 was used to drive the Esterline Angus model AV recording ammeter. This amplifier was originally designed for the differential measurement of transmembrane potential during voltage clamp experiments. It was used here to insure that the equipment of the present study would be similar to that of subsequent voltage clamp studies. This amplifier was used in a single ended mode by connecting the reference input to ground.

Please refer to figure 27. An instrumentation amplifier (U1) was used to convert the differential signal to a single ended ground referenced signal. This signal was amplified further by U2 and routed to U3 which acted as a unity gain line driver.
MEMBRANE POTENTIAL AMPLIFIER

Figure 27
CALIBRATION

1) Check that the power supply voltages are +/- 15 V.
2) Ground both inputs J1 and J2.
3) Set R7 to the middle of its travel.
4) Adjust R6 until the output at J4 equals 0.000 +/- 0.005 V.
5) Apply a 100 mv calibration signal to J2 leaving J3 grounded.
6) Adjust R5 until the output at J4 equals 5.000 +/- 0.005 V.
7) Repeat steps 2 through 6 until no further adjustments are needed.
PARTS LIST

Connectors (J)

J1 vector 644
J2 BNC
J3 BNC
J4 BNC

Resistors (R) ohms 1% 1/4W unless otherwise indicated

R1 9.09K
R2 93.1K
R3 9.09K
R4 39.2K
R5 20K trim pot
R6 100K trim pot
R7 10K trim pot

Integrated Circuits (U)

U1 AD521K
U2 OP 16-GJ
U3 74741
IX. BIBLIOGRAPHY


Couri, D. and Nachtman, J.P.: Biochemical and biophysical
Studies of 2,5-hexanedione neuropathy.


Heslop, J.P.: Axonal flow and fast transport in nerves. 

Hodes, R.: Linear relationship between fiber diameter and conduction velocity in giant axon of squid. 


Lasek, R.J., Gainer, H., Przybylski, R.J.: Transfer of newly


Spencer, P.S. and Schaumberg, H.H.: Pathobiology of neurotoxic axonal degeneration. In: Physiology and


