The effects of flurazepam dihydrochloride on the growth and motility of Tetrahymena pyriformis as seen by the scanning electron microscope

Sonja R. Stovall
Atlanta University

Follow this and additional works at: http://digitalcommons.auctr.edu/dissertations
Part of the Biology Commons

Recommended Citation
The effects of Flurazepam dihydrochloride on the growth and motility of Tetrahymena pyriformis as seen by the Scanning Electron Microscope.

A Thesis
submitted to the faculty of Atlanta University
in partial fulfillment of the requirements
for the degree of Master of Science
by
Sonja R. Stovall
Department of Biology
Atlanta, Georgia
December, 1985
Master of Science Thesis

of

Sonja R. Stovall

Approved:

Major Professor

Thesis Committee Member

Thesis Committee Member

Department Chairman

Dean, School of Arts & Sciences
The effects of Flurazepam dihydrochloride on the growth and motility of *Tetrahymena pyriformis* as seen by the scanning electron microscope.

Advisor: Dr. James H. Penn

The purpose of this study was to analyze the effects of the drug, flurazepam dihydrochloride on the growth and motility of *Tetrahymena pyriformis* maintained under axenic conditions.

Growth cultures containing the drug and the organisms were cultivated at 26°C for five days, with optical readings taken at 24-hr intervals. These readings were determined by turbidity using a Spectronic 20. The organisms were examined by Scanning Electron Microscopy to determine sites of drug activity on the organisms. Results revealed that flurazepam dihydrochloride caused the inhibition of growth and motility of *T. pyriformis*. Scanning Electron Microscope studies revealed that the primary sites of activity of *T. pyriformis* are upon (1) the cilia, preventing motility making the organism unable to reach its food, (2) the buccal cavity, hendering the oral apparatus that also has cilia, and (3) the pellicle, comprising the outer membrane of the organism, which is destroyed.
ACKNOWLEDGEMENTS

I wish to thank my thesis advisor, Dr. James Penn for his guidance and understanding. Sincere thank goes to Dr. Mustapha A. Durojaiye for being there when I needed him most and for pushing me in the right direction.

I would like to give special thanks to God, my mother, other family members, Dr. Barbara Baumstark, and friends who stuck by me through the years of preparing this thesis. I would also like to thank Dr. John Brown for critically reading my thesis.
# TABLE OF CONTENTS

ABSTRACT........................................................................................................ iii.

ACKNOWLEDGEMENTS.................................................................................... iv.

TABLE OF CONTENTS.................................................................................... v.

LIST OF FIGURES........................................................................................... vi.

LIST OF TABLES............................................................................................... ix.

CHAPTER

I Introduction........................................................................................................ 1

II Review of Literature....................................................................................... 4

III Materials and Methods................................................................................. 12

IV Experimental Results................................................................................... 17

V Discussion and Conclusion............................................................................ 44

VI Summary........................................................................................................ 51

Literature Cited................................................................................................... 52
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Structure of the Drug Flurazepam Dihydrochloride</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Graph indicating the effect of .03 mg/ml Flurazepam dihydrochloride on the growth of <em>Tetrahymena pyriformis</em></td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>Graph indicating the effects of .08 mg/ml Flurazepam dihydrochloride on the growth of <em>Tetrahymena pyriformis</em></td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Graph indicating the effect of .13 mg/ml Flurazepam dihydrochloride on the growth of <em>Tetrahymena pyriformis</em></td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>Graph indicating the effect of .18 mg/ml Flurazepam dihydrochloride on the growth of <em>Tetrahymena pyriformis</em></td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>Graph indicating the effect of .23 mg/ml Flurazepam dihydrochloride on the growth of <em>Tetrahymena pyriformis</em></td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>Graph indicating the effect of .28 mg/ml Flurazepam dihydrochloride on the growth of <em>Tetrahymena pyriformis</em></td>
<td>26</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (CONTINUED)

8 Graph indicating the effect of .33 mg/ml Flurazepam dihydrochloride on the growth of *Tetrahymena pyrformis* 27

9 Graph indicating the effect of .38 mg/ml Flurazepam dihydrochloride on the growth of *Tetrahymena pyrformis* 28

10 Graph indicating the effect of .43 mg/ml Flurazepam dihydrochloride on the growth of *Tetrahymena pyrformis* 29

11 Graph indicating the effect of .48 mg/ml Flurazepam dihydrochloride on the growth of *Tetrahymena pyrformis* 31

12 Graph indicating the effect of .53 mg/ml Flurazepam dihydrochloride on the growth of *Tetrahymena pyrformis* 32

13 Graph indicating the effect of .58 mg/ml Flurazepam dihydrochloride on the growth of *Tetrahymena pyrformis* 33

14 Graph indicating the effect of .63 mg/ml Flurazepam dihydrochloride on the growth of *Tetrahymena pyrformis* 34

15 SEM micrograph of a control cell 36
LIST OF FIGURES (CONTINUED)

16 Graph indicating the immobilization of *Tetrahymena pyriformis* 37

17 SEM micrograph of the oral apparatus of a control cell 39

18 SEM micrograph of an experimental cell (.33 mg/ml) of the oral apparatus of *Tetrahymena pyriformis* 40

19 SEM micrograph of .33 mg/ml experimental cell 42

20 SEM micrograph of .63 mg/ml experimental cells 43
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Components of the Proteose peptone medium</td>
</tr>
<tr>
<td>2</td>
<td>Drug Concentrations</td>
</tr>
<tr>
<td>3</td>
<td>Growth study of experimental concentrations</td>
</tr>
<tr>
<td>4</td>
<td>Motility experimental study</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

_Tetrahymena pyriformis_, a pear-shaped, freshwater, ciliated organism, approximately 50 µm in length and 30 µm in width, has been used as a model system in many laboratories to study a variety of biological phenomena such as cell division, cell synchrony, structure of cilia, cilia genetics, cortical patterns, and membrane biosynthesis.

Flurazepam dihydrochloride, 7-chloro-1-L2-(diethylamino)ethyl]-5-(o-flurophenyl)-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one dihydrochloride, (Figure 1) is a member of the group of drugs called benzodiazepines. It is a pale yellow, crystalline compound, freely soluble in U.S.P. alcohol and very soluble in water. Flurazepam dihydrochloride (most commonly known as "dalmane"), one of the benzodiazepines specifically marketed as a hypnotic, is quickly metabolized by the liver to 1-desalkyl flurazepam. However, 1-desalkyl flurazepam is more potent in animals than flurazepam dihydrochloride (Randall, et al. 1969). In man, chronic administration of flurazepam dihydrochloride leads to the accumulation of 1-desalkyl flurazepam for about 7 to 10 days. This kinetic pattern is similar to that found with benzodiazepines used exclusively as anxiolytic agents (Beimer, 1977).

This present study is concerned with the cellular action of flurazepam dihydrochloride on the growth and motility of the protozoan, _Tetrahymena pyriformis_ with the following objectives: 1) to observe the physiological and cellular effects of flurazepam dihydrochloride on
T. pyriformis; 2) to determine whether or not this drug affects the unicellular organism on the ultrastructural level. The growth observations will attempt to determine (1) the degree of structural destruction of the cellular membrane, and (2) the effects the drug will have on food vacuoles with respect to food ingestion and egestion. The motility observations will attempt to determine (1) the rate of movement of the cilia under varied concentrations of the drug, and (2) the time it takes for a complete immobilization to occur using the same concentrations.

Manifestations of dalmane overdosage include somnolence, confusion and coma. Respiration, pulse and blood pressure should be monitored. General supportive measures should be employed, along with immediate gastric lavage. Intravenous fluids should be administered and an adequate airway maintained. Hypotension and Central Nervous System (CNS) depression may be combated by judicious use of appropriate therapeutic agents. The value of dialysis has not been determined. If excitation occurs in patients following dalmane (flurazepam dihydrochloride) overdosage, barbiturates should not be used since multiple agents may have been ingested (Physicians' Desk Reference 38th ed., 1984).

Tetrahymena pyriformis in this type of study is useful because it has been shown that T. pyriformis is an exceptionally useful subject for studying metabolic interrelationships among intracellular membranes (Thompson, 1972). It has also been reported that Tetrahymena, in its nutrition, morphology and reproduction, is similar to a great number of eukaryotes including higher vertebrates (Hill, 1972; Kudo, 1972).
FIG. 1 Structure of the drug Flurazepam dihydrochloride.
CHAPTER II

REVIEW OF LITERATURE

The fluidity of a biological membrane is widely acknowledged to be of great importance in controlling the metabolic functions of that membrane and is primarily a function of (1) lipid composition of the membrane, and (2) the environmental factors, for example, temperature, pH, and ionic strength affecting the membrane at any given time.

The ciliate Tetrahymena pyriformis has been used extensively as a model system to study the response of the membrane to temperature changes (Martin, C.E. et al., 1976, Fukushima et al., 1976, Thompson, 1983).

Cilia of Tetrahymena provide a homogenous system capable of responding to stress via molecular changes in species, yet apparently lacking other key enzymatic mechanisms such as those catalyzing fatty acid desaturation and polar head group alterations, that have been implicated in lipid fluidity alteration (Ramesha, C.S. et al, 1983).

The pellicle of oral ribs, located on the right side of the oral cavity of the organism, is where the food vacuoles form and pinch off into the cell (Sattler and Staehelin, 1976). The oral ribs are delineated on their outer surface by the plasma membrane. Each rib contains six microtubules arranged in two rows; two microtubules in one row and four microtubules in the other. The alveolar sacs are arranged in the form of long tubes which underline the plasma membrane and runs parallel to the ribs between the row of four microtubules of one rib,
and the two of two microtubules in the adjacent rib, (Sattler and Staehelin, 1979).

Nozawa et al., (1979) stated that Tetrahymena pyriformis strain WH-14, grown in medium supplemented with ergosterol (1 mg/100 ml), lead to profound alterations in phospholipid components of the cell membrane.

The behavior and ultrastructure of Tetrahymena pyriformis was assessed after exposure to dosages of 8% and 16% of the lethal concentration of mercury chloride (HgCl₂). The lower dosage caused no abnormal changes in cell motility, in activity of the water expulsion vesicles, or in the cell shape. The higher dosage caused deleterious changes in these parameters. The higher sublethal HgCl₂ concentration (.5 mg/liter), elicited the damage of several cell structures (Tingle, L. et al., 1973).

Grim et al., 1980, removed the outer limiting membranes (the pellicle), through revealing the geometry of the microtubules; which lie at the outer boundary of the ectoplasm, which is useful in the analysis of their organization and functions. It has been shown Grim's membrane removal method provides a better perspective of some sub-pellicular microtubules than other methods previously available. This method will in turn facilitate research on possible cause-and-effect relationships between microtubules and certain membrane components and changes in cell form and shape (Staller and Staehelin, 1975).

Cytotoxic effects of acridine, an organic component of synthetic fossil fuel products, were investigated. Populations of the common freshwater ciliate, Tetrahymena pyriformis, were exposed to various
concentrations from 0 to 40 mg Liter\(^{-1}\) where their behavior, respiration, cytology, and growth were examined. The 24-hr LC\(_{180}\) and 24-hr LC\(_{50}\) were determined to be 35 and 30 mg Liter\(^{-1}\) respectively. At lower concentrations, few if any sublethal alterations were noted in respiration, ultrastructure, cell size, and glycogen and protein contents. However, at 30 mg Liter\(^{-1}\), cell size and glycogen content are significantly reduced. In contrast, population growth is altered at concentrations as low as 2.5 mg Liter\(^{-1}\). This is the most striking toxic response to acridine preceded by shape alteration and contractile vacuole malfunction. Cytolysis may result from a weakened membrane and pellicle by the partitioning of the hydrophylllic acridine into highly lipid membranes (Shultz, et al., 1981).

The oral apparatus of *Tetrahymena pyriformis* is an organelle composed of ciliated and nonciliated basal bodies interconnected by a network of microtubules and filaments. Potassium chloride (KCl) extraction of the isolated oral apparatus resulted in the selective solubilization of oral apparatus basal bodies with remnants of ciliary axonemes and fused body basal plants. Based on their response to KCl extraction, two distinct sets of morphologically similar microtubules can be identified; (a) microtubules which constitute the internal structure of basal bodies and ciliary axonemes, and (b) microtubules which constitute fiber connectives between basal bodies (Gavin, 1977).

Several studies have demonstrated that microtubules differ in their stability and response to various agents. For example, cilia and centrioles were stable to treatments which disrupted cytoplasmic
microtubules (Tilney, L.G. et al., 1973); mitotic microtubules in amoeba differed in respect to conditions which inhibited their reformation following disruption by temperature (Roth, L.E., 1967).

Many compounds of the 1-4-benzodiazepines series display tranquilizing, muscle-relaxant, anti-convulsant and sedative effects. The discovery that the first two members of this family of compounds, chlordiazepoxide and diazepam (valium), have useful pharmacological properties in experimental animals led to their extensive investigation (Sternbach et al., 1964). Today many benzodiazepines are widely used as daytime sedatives, tranquillizers, and sleep inducers (Randall et al., 1974).

Based on the data, close examination of the basic pharmacologic properties of benzodiazepines reveals that numbers 1-, 4-benzodiazepine can be selected exclusively as a hypnotic agent in preference to any other benzodiazepines (Greenblatt et al., 1973). Despite this conclusion, many benzodiazepines are specifically promoted as sleep inducers, whereas others are positioned specifically as anticonvulsants or alternatively as anti-anxiety agents. Even pharmacokinetic properties fail to distinguish between benzodiazepines. It might be argued that benzodiazepines are quickly detoxified and excreted making them suitable as hypnotics (Randall et al., 1973).

Dalmane is a hypnotic agent useful in all types of insomnia characterized by difficulty in falling to sleep, frequent nocturnal awakenings and/or early morning awakening. Dalmane can be used effectively in patients with recurring insomnia or poor sleeping habits,
and in acute or chronic medical situations requiring restful sleep. Since insomnia is often transient and intermittent, the prolonged administration of dalmane is generally not necessary or recommended (Physicians' Desk Reference, 38th ed., 1984).

In animal studies, flurazepam dihydrochloride reduced the pressor response to electrical stimulation of the hypothalamus, and increased the arousal threshold to stimulation of the amygdala and hypothalamus; however, the exact site and mode of action are unknown (Physicians' Desk Reference, 38th ed., 1984).

The pharmacology of flurazepam dihydrochloride has been reviewed extensively by many scientists. Greenblatt, et al., (1975) and Greenblatt, (1978) stated that in animal studies it shares muscle relaxant and anti-convulsant properties with other benzodiazepines although less potentially than diazepam and very roughly with equipotence to chlorodiazepoxide. However, in other measures there are some differences, although a variety of benzodiazepines increase exploratory behavior in mice, flurazepam dihydrochloride does not (Nolan & Parks, 1973). A common test of possible anxiolytes is either their ability to restore behaviors which have been decreased by noxious stimuli, or to reduce behaviors stimulated by noxious stimuli. Although both flurazepam dihydrochloride and chlorodiazepoxide share the latter property, doses necessary for such actions by flurazepam dihydrochloride were close to those which produce motor deficits (Randall et al., 1969). In the immobilized cat, a variety of benzodiazepines differ in
quantitative and qualitative ways in animal studies, although clinical importance of these differences is uncertain.

Flurazepam dihydrochloride is rapidly absorbed after oral administration, and distributed evenly through the body. Although diazepam, chlorodiazepoxide and nitrazepam bind serum albumin thoroughly, flurazepam dihydrochloride may be relatively weakly bound (Miller & Wolbert, 1973). A study in which 28 mg of $^{14}$C-labeled flurazepam dihydrochloride was administered to 2 human subjects indicates that peak concentration of labeled material appeared 1 hr after ingestion (Schwartz & Postma, 1970). It was only at that time that the parent component could be detected, which represented only 1% of the total plasma $^{14}$C, indicating the rapid metabolism of flurazepam dihydrochloride. Approximately half of the labeled material was recovered in the urine in the first 24 hr. After 98 hr, 81% of the total label was found in the urine and 8 to 9% in the feces. The hydroxyethal metabolite conjugated to the glucuronide or sulfate, comprised 22 to 25% of urinary radioactivity. A study of spectrophotofluorometric analyses of blood on urine from 2 volunteers was performed after administration of 90 mg of unlabeled flurazepam dihydrochloride. Peak levels of the parent compound were found after 1 hr, but these quantities were substantially smaller than the two major metabolites (de Silva & Strojny, 1971).

The effect of benzodiazepines on the sleep pattern are similar to those of barbiturates. Both benzodiazepines and barbiturates decrease body movement, the number of awakenings, sleep latency (the time
required to fall asleep), total rapid eye movement (REM) sleep, and the number of shifts in sleep stages. They both increase total sleep, stage 2 sleep, and fast activity (beta activity) in the electroencephalogram (EEG) (Kay et al., 1976; Hauri, et al., 1983). Benzodiazepines may decrease stages 3 and 4 of sleep more than barbiturates, but these reports will have to be confirmed. A possible advantage of some benzodiazepines, especially that of flurazepam dihydrochloride, appears to be that their hypnotic efficacy persists during long-term administration. When compared to barbiturates and other hypnotics in short-term studies, benzodiazepines are equally effective and likely to produce hangover and persistent psychomotor impairment (Hartman, 1975; Cummiskey, et al., 1983) A major advantage of benzodiazepines is their relative safety. Fatalities due to benzodiazepine overdosage alone are rare. When taken together with other drugs, intoxication probably depends largely on the type and quantity of non-benzodiazepines (Greenblatt, et al., 1977). Benzodiazepines do not cause significant enzyme induction in man, and therefore tend to interact with other less frequently than do barbiturates.

The disadvantage of benzodiazepines appears to the slowness of their elimination. Drug-induced abnormalities in the EEG; hangover effects, impairment of psychomotor performance, and ability to fall asleep, persist longer with nitrazepam than with sodium amobarbital (Oswald, 1973). Both benzodiazepines and barbiturates produce rebound abnormalities on withdrawal, with patients spending a disproportionate amount of time in REM sleep with increased intensity. It must be
concluded that a prescription for long term hypnotic use is rarely indicated.
CHAPTER III

MATERIALS AND METHODS

Axenic cultures of *Tetrahymena pyriformis* were obtained from the Carolina Biological Supply Company, Burlington, North Carolina. *Tetrahymena* were maintained in a medium of proteose peptone that was recommended by the Carolina Biological Supply Company. The ingredients of this medium are supplied in Table I.

The growth of *Tetrahymena pyriformis* is affected by light, therefore they were kept at 28°C in the dark. Photo-periodic effects on *Tetrahymena pyriformis* were demonstrated by Willie et al., 1968.

One liter of the medium was dispensed into eight 500 ml Erylenmeyer flasks in quantities of 125 ml per flask. The growth medium was then autoclaved for 10 to 15 min at 141 lb/in² pressure and 121°C. It has been recommended that a reduced time of 10 to 15 min instead of 20 min of autoclaving be used to prevent the breakdown of components such as peptone and tryptone (Prescott, 1972). After the medium was cooled, it was inoculated with *Tetrahymena pyriformis* and placed at 26°C in a covered stationary water bath.

Sterile conditions were used in the experimental procedures. To test for contamination of bacteria, the suspected contaminated flasks were inoculated with 1 g of streptomycin sulfate; before adding the streptomycin to the flask, the *Tetrahymena pyriformis* was streaked on a nutrient agar plate. After inoculating the media, an aliquot was removed and placed in fresh media. The first sample suspected of
Table I

<table>
<thead>
<tr>
<th>Growth Medium</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose Peptone</td>
<td>5 grams</td>
</tr>
<tr>
<td>Bacto Tryptone</td>
<td>5 grams</td>
</tr>
<tr>
<td>Potassium Phosphate ($\text{KH}_2\text{PO}_4$)</td>
<td>0.2 grams</td>
</tr>
<tr>
<td>Distilled Water at pH 7.2</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
contamination with streptomycin in it, is then streaked on a nutrient agar plate. Periodic tests of the same nature were performed to insure that there was no more contamination.

The drug, flurazepam dihydrochloride (lot# 007058) was obtained from the Roche Laborabory, New Jersey. The drug was diluted to several concentrations and stored as stock dilutions at 4°C. Drug dilution concentrations are in Table II.

Growth study procedures were carried out by transferring Tetrahymena pyriformis to fresh medium every 48 hrs, which was during log phase. After cells were transferred, they were placed into 13 x 150 mm test tubes; at a ratio of 1:3:1 (1 ml of Tetrahymena pyriformis: 3 ml of fresh media: 1 ml of the individual drug concentrations). The control test tube contained 4 ml of fresh medium with 1 ml of the organism. There were three experimental test tubes for each drug concentration used. These tubes were placed at 28°C in the covered water bath. Growth study readings were made every 24 hrs for 5 days. These studies were carried out using a Spectronic 20 spectrophotometer (Bausch & Lomb) at 540 nm according to the method of Durojaiye (1979; 1981).

Motility studies were observed using 0.1 ml of Tetrahymena pyriformis and 0.1 ml of the drug concentrations. The experimental tubes were placed in a counting chamber and observed under a light microscope for 60 mins at 5-min intervals for each drug concentration.
Table II

<table>
<thead>
<tr>
<th>Drug Concentrations mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
</tr>
<tr>
<td>----</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>13</td>
</tr>
</tbody>
</table>
The percent of immobilization of *Tetrahymena pyriformis* was determined by using the formula below:

\[
\text{% immobilization} = \frac{\text{total number of immobilized Tetrahymena} \times 100}{\text{total number of Tetrahymena in counting chamber}}
\]

Ultrastructural studies were performed by first pelleting the cells, then they were fixed at room temperature with 2% glutaraldehyde pH 7.2 for 1.5 to 2 hrs. Then the cells were then washed 3 times with 2% sodium cacodylate buffer solution. After washing, they were post fixed with osmium tetraoxide for 1 hr, and were washed 3 times again, with 2% sodium cacodylate. After the last wash, they were run through a graded series of alcohol (ethanol) dehydrations: 25%, 50%, 75%, 90%, 95% and 100%. Further dehydrations were carried out in a graded series of amylacetate dilutions. At this point, they were placed on studs and either left to air dry overnight under a closed hood or were dried with a critical-point dryer, after which the organisms were coated and ready for examination using the OMNISCAN Scanning Electron Microscope (SEM).
CHAPTER IV

EXPERIMENTAL RESULTS

The tests outlined previously were designed to show the ultrastructural effects of flurazepam dihydrochloride on the growth and motility of *Tetrahymena pyriformis*. The information on Table III shows that there was a general stabilizing effect or a slight decrease in the growth of *Tetrahymena pyriformis* after a 5-day reading as the drug concentrations increased. The results on Table III are obtained from an average of three growth readings. Figures 2 through 14 are plotted as optical density against time for 120 hrs (5 days). The drug concentrations are relatively close together, which give the stabilizing effects shown.

The first reading in figures 2 through 14 were taken first at 12 hrs, thereafter 24-hr readings were taken until 120 hrs was reached. Figure 2 shows the growth reading for the lowest drug concentration, .03 mg/ml. The 12-hr reading for this concentration shows a 4 nm difference between the experimental and the control. The 24-hr reading shows an increase of 1 nm more to 5 nm. The largest difference shown is at 48 hrs, where the difference is 8 nm. The last three readings show a constant difference of 6 nm. At this concentration (.03 mg/ml), there is a very short (if any) lag phase (12-24 hrs), a rapid log phase (48 hrs) appearance, and a constant stationary phase in the control. The experimental shows a decrease to a stationary phase.
Table III
Growth Studies

<table>
<thead>
<tr>
<th>Drug Concentrations</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>.03</td>
<td>.04</td>
<td>.03</td>
<td>.02</td>
<td>.02</td>
<td>.02</td>
</tr>
<tr>
<td>2</td>
<td>.08</td>
<td>.04</td>
<td>.02</td>
<td>.01</td>
<td>.01</td>
<td>.01</td>
</tr>
<tr>
<td>3</td>
<td>.13</td>
<td>.03</td>
<td>.01</td>
<td>.01</td>
<td>.01</td>
<td>.01</td>
</tr>
<tr>
<td>4</td>
<td>.18</td>
<td>.04</td>
<td>.01</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>.23</td>
<td>.05</td>
<td>.02</td>
<td>.002</td>
<td>0</td>
<td>.002</td>
</tr>
<tr>
<td>6</td>
<td>.28</td>
<td>.05</td>
<td>.002</td>
<td>.01</td>
<td>.01</td>
<td>.008</td>
</tr>
<tr>
<td>7</td>
<td>.33</td>
<td>.04</td>
<td>.02</td>
<td>.01</td>
<td>.01</td>
<td>.01</td>
</tr>
<tr>
<td>8</td>
<td>.38</td>
<td>.06</td>
<td>.03</td>
<td>.02</td>
<td>.01</td>
<td>.02</td>
</tr>
<tr>
<td>9</td>
<td>.43</td>
<td>.05</td>
<td>.02</td>
<td>.02</td>
<td>.03</td>
<td>.03</td>
</tr>
<tr>
<td>10</td>
<td>.48</td>
<td>.04</td>
<td>.02</td>
<td>.02</td>
<td>.03</td>
<td>.02</td>
</tr>
<tr>
<td>11</td>
<td>.53</td>
<td>.04</td>
<td>.02</td>
<td>.02</td>
<td>.03</td>
<td>.03</td>
</tr>
<tr>
<td>12</td>
<td>.58</td>
<td>.05</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
</tr>
<tr>
<td>13</td>
<td>.63</td>
<td>.06</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
<td>.03</td>
</tr>
<tr>
<td>14</td>
<td>Control</td>
<td>.08</td>
<td>.08</td>
<td>.10</td>
<td>.08</td>
<td>.08</td>
</tr>
</tbody>
</table>
FIG. 2 Growth of *Tetrahymena pyriformis* in 0.03 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

▲ control
● experimental
TIME (HR)
FIG. 3 Growth of *Tetrahymena pyriformis* in 0.08 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

▲ control
● experimental
The results of .08 mg/ml are shown in figure 3. The 12-hr difference is 4 nm, whereas the biggest difference comes at 48 hrs with the control being at .10 nm and the experimental .01 nm. The behavior of the organism at this concentration also shows a stationary phase after 48 hrs (48-120 hrs).

Figure 4 gives the result of .13 mg/ml concentration of the drug. The 12-hr reading gives a difference of 5 nm with the control being .08 nm and the experimental being .03 nm. The largest difference being 9 nm, which is reached at 48 hrs with the control being .10 nm and the experimental being .01 nm. This concentration also exhibits a stationary phase, which last slightly more than the previous concentration from 24 hrs to 120 hrs.

Readings for concentration .18 mg/ml are given in Figure 5. The first reading of 12 hrs shows a difference of 5 nm between the experimental and the control, with the control being .08 nm and the experimental being .04 nm. At 24 hrs, the difference increased to 5 nm. At 48 hrs, the largest difference occurs with the control being .10 nm and the experimental being 0.0, giving a difference of 11 nm. Thereafter there is a death phase of 0.0 for the experimental.

Figure 6 depicts the results of .23 nm concentration of the drug. This concentration also gives the widest range between the control and the experimental, it being 11 nm at 48 hrs. This concentration also undergoes a death phase from 48 to 120 hrs.

The results of .28 mg/ml concentration of the drug are shown in figure 7. The 12-hr reading of this concentration shows that there is a
FIG. 4 Growth of *Tetrahymena pyriformis* in 0.13 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

▲ control
● experimental
FIG. 5 Growth of *Tetrahymena pyriformis* in 0.18 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

▲ control

● experimental
FIG. 6 Growth of *Tetrahymena pyriformis* in 0.23 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

▲ control

● experimental
narrower difference between the control at .08 nm and the experimental at .05 nm, giving a margin of 3 nm. At the 24-hr reading, the gap widens to 8 nm, with the control reading at .08 nm and the experimental at 0.0 nm. The 48-hr reading increased to 9 nm. There was a stationary phase from 48 hrs to 96 hrs. The 120-hr reading also shows no activity with the experimental being at 0.0 nm.

Figure 8 shows the concentration of .33 mg/ml of the drug. The differences between the experimental and the control at 12 hrs is 5 nm, at 48 hrs it is 10 nm, which was the largest margin, at 72 hrs, 96 hrs and 120 hrs the margin was 8 nm.

The .38 mg/ml concentration observations are depicted in Figure 9. There is only a 2 nm difference between the control and the experimental at 12 hrs. This increases to a 8 nm difference at 48 hrs. At 72 hrs this increase drops to 7 nm with the experimental being .01 nm and the control being at .08 nm at 96 hrs and 120 hrs there is a 6 nm difference with the control being at .08 nm and the experimental being at .02 nm.

Figure 10 gives the drug concentration of .43 mg/ml. The 12-hr reading shows the difference between experimental and control being 4 nm and the difference between 24 hrs increased to 7 nm, with the experimental being .08 nm and the experimental being .02 nm. The 48-hr reading gives the widest margin of 9 nm. The 72-hr reading gives a 6 nm difference with the control being .08 nm and the experimental being .03 nm. The 120-hr reading shows an increase in differences to 7 nm by
FIG. 7 Growth of *Tetrahymena pyriformis* in 0.28 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

▲ control

● experimental
FIG. 8 Growth of *Tetrahymena pyriformis* in 0.33 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

▲ control

● experimental
FIG. 9 Growth of *Tetrahymena pyriformis* in 0.38 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

▲ control

● experimental
FIG. 10 Growth of *Tetrahymena pyriformis* in 0.43 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

- control
- experimental
1 nm with the control being .08 nm and the experimental reading dropping to .02 nm.

Results of the .48 mg/ml concentration are shown in Figure 11. The 12-hr reading showed a 4 nm difference between the control and the experimental. At 24 hrs the difference increases to 6 nm. It is still increasing at 48 hrs to 7 nm with the experimental showing .03 nm and the control showing .10 nm. The 72-, 96- and 120-hr readings have a margin of 6 nm, where the control shows .08 nm and the experimental shows .02 nm.

The .53 mg/ml concentration is shown in Figure 12. The 48-hr reading shows a wide margin of 8 nm with the control showing .10 nm and the experimental showing .02 nm. 72-hr and 96-hr readings decrease their margins to show a 5 nm difference with the control being .08 nm and the experimental being .03 nm. The last reading of 120 hrs shows an increase to a 7 nm difference with the experimental showing a .01 nm reading and the control showing a .08 nm reading.

Figure 13 shows the concentration of .58 mg/ml. The readings of the experimental results shows a stationary phase from 24 hrs to 96 hrs. At the 120-hr reading there is an increase in the difference between the experimental and the control of 1 nm to 7 nm, where the experimental reading decreases to .02 nm and the control remains at .08 nm.

The last drug concentration used was .63 mg/ml and it is shown in Figure 14. The margin between the experimental and the control is 2 nm at the first reading of 12 hrs. The margin increases to 5 nm at the
FIG. 11 Growth of *Tetrahymena pyriformis* in 0.48 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

- ▲ control
- ● experimental
FIG. 12 Growth of *Tetrahymena pyriformis* in 0.53 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

▲ control

● experimental
FIG. 13 Growth of *Tetrahymena pyriformis* in 0.58 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

- ▲ control
- ● experimental
FIG. 14 Growth of *Tetrahymena pyriformis* in 0.63 mg/ml Flurazepam dihydrochloride from 0 to 120 hrs at 540 nm.

△ control

● experimental
24-hr reading with the control result being .08 nm and the experimental result being .03 nm. The largest margin is shown at 48 hrs with the experimental being .03 nm and the control being .10 nm. At 72 and 120 hrs a stationary phase is seen in both the experimental and the control.

Light microscopic studies were used to determine the general effects of flurazepam dihydrochloride on tetrahymenas' motility. All organisms were exposed to flurazepam dihydrochloride with the same concentrations shown on Table II. Figure 15 shows an intact control cell of Tetrahymena pyriformis, showing the oral apparatus. Figure 16 and Table IV depict the motility of Tetrahymena pyriformis was not reduced or stopped within the few readings between 0 and 25 mins. The results for the motility studies are shown in Figure 16. The drug concentrations used were 0.18 mg/ml, 23 mg/ml, .33 mg/ml, .43 mg/ml, .53 mg/ml, and .63 mg/ml. No immobility was shown at 0.63 mg/ml until 25 mins was reached, where there was a 27% immobility, with a steady increase to 80% immobility. Signs of immobility start to show at 0.53 mg/ml and 0.43 mg/ml after 30 mins with a steady increase to 67% and 43% respectively. No percent immobility was shown at 0.23 mg/ml until 45 mins where the percent immobility was 6% with an increase to 25%. At 50 mins, 0.18 mg/ml starts with a 5% immobilization with an increase thereafter to 20%.

Figure 17 shows a typical Tetrahymena with the emphasis being on the rows of cilia and oral apparatus. The close up of the oral apparatus of Tetrahymena pyriformis is shown in Figure 18. The oral
FIG. 15 Micrograph of a control cell.
FIG. 16 Graph of specific concentrations of Flurazepam dihydrochloride indicating the % immobilization of *Tetrahymena pyriformis*:

- 0.18 mg/ml
- 0.23 mg/ml
- 0.33 mg/ml
- 0.43 mg/ml
- 0.53 mg/ml
- 0.63 mg/ml
Table 4
Motility Experimental Study

<table>
<thead>
<tr>
<th>mg/ml</th>
<th>TIME (MINUTES)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
</tr>
<tr>
<td>.03</td>
<td>0</td>
</tr>
<tr>
<td>.08</td>
<td>0</td>
</tr>
<tr>
<td>.13</td>
<td>0</td>
</tr>
<tr>
<td>.18</td>
<td>0</td>
</tr>
<tr>
<td>.23</td>
<td>0</td>
</tr>
<tr>
<td>.28</td>
<td>0</td>
</tr>
<tr>
<td>.33</td>
<td>0</td>
</tr>
<tr>
<td>.38</td>
<td>0</td>
</tr>
<tr>
<td>.43</td>
<td>0</td>
</tr>
<tr>
<td>.48</td>
<td>0</td>
</tr>
<tr>
<td>.53</td>
<td>0</td>
</tr>
<tr>
<td>.58</td>
<td>0</td>
</tr>
<tr>
<td>.63</td>
<td>0</td>
</tr>
</tbody>
</table>
FIG. 17 Micrograph of the oral apparatus.
FIG. 18 Experimental micrograph of the oral apparatus of *Tetrahymena pyriformis* exposed to (0.33 mg/ml) Flurazepam dihydrochloride.
apparatus of *Tetrahymena pyriformis* in Figure 19 is an experimental result using the drug concentration of 0.33 mg/ml. Figure 20 depicts the contorted experimental concentration of 0.33 mg/ml of the entire *Tetrahymena*. Figure 21 shows two *Tetrahymenae* at the concentration of 0.63 mg/ml. The drug having taken its effect on the tetrahymenae has perforated the membrane of one *Tetrahymena* and contorted the size of the other.
FIG. 19 Experimental micrograph of *Tetrahymena pyriformis* exposed 0.33 mg/ml Flurazepam dihydrochloride.
FIG. 20 Experimental micrograph of *Tetrahymena pyriformis* exposed 0.63 mg/ml Flurazepam dihydrochloride.
CHAPTER V

DISCUSSION AND CONCLUSION

Several limiting factors that must be considered when observing the effects of any drug on Tetrahymena pyriformis; (1) the time it would take for the drug to enter the cell, whether it is by the cell wall or by the oral apparatus, (2) the concentration of the drug, and (3) the depth of penetration. These three factors center around the fact that the cell membrane of Tetrahymena is very complex. For a number of different drugs the concentration required to reach a good penetration effect will have to be much higher for Tetrahymena than for mammalian cells. The cortex of Tetrahymena consists of a repeating unit of ciliary-kinetosome complex (basal body) with associated microtubules. Tetrahymena is enclosed in a system of membranes: the outer limiting membrane (outer pellicular membrane), the ciliary membrane, and the inner pellicular membrane. The last two membrane layers are connected by a series of cross bridges. (Hill, 1972).

Nutrient entry into Tetrahymena may follow a variety of routes and employ a variety of mechanisms: (1) by diffusion (free, restricted, exchange, facilitated) through the cell membrane uniformly over the surface or a specific loci, and through the food vacuole membrane; and (2) by active transport, pinocytosis of phagocytosis at any of these locations. The paths and modes of entry depend upon the nature of the nutrient under consideration, the environmental circumstances, and the physiological state of the ciliate. Water, ions and small non polar
molecules may traverse the surface by diffusion, active transport and pinocytosis. Macromolecules in solution and particulates can enter by pinocytosis and phagocytosis. Size, charge, lipid solubility and the metabolic activities of the organism influence membrane permeation (Holtz, 1973).

Dopamine is accumulated by cells of *Tetrahymena pyriformis* strain NT-1 and is secreted into their growth medium. These large amounts of dopamine was found to depend primarily upon extracellular non enzymatic conversion of tyrosine to L-Dihydroxyphenyl Alanine (L-Dopa). L-Dopa was then rapidly taken into cells and transformed into dopamine enzymatically. The origin of L-dopa came with the discovery that the enriched proteose peptone growth medium, though free of dopamine, contained high levels of L-dopa after being sterilized by autoclaving (Gunderson and Thompson, 1985; 1983).

When observing the effects of the drug on the organism, the chemistry of the drug is an added parameter. Flurazepam dihydrochloride has a molecular weight of 408.826 (PRR 38th ed., 1984) and is readily soluble in water, with a short duration in the body because it is rapidly metabolized to 1-desalkyl-3-hydroxy flurazepam (de Silva and Strojny, 1971). The harmonic mean apparent half-life of flurazepam dihydrochloride is 2.3 hrs. Clinical observations show that flurazepam dihydrochloride is increasingly effective on the second and third night of consecutive use (Physicians Desk Reference 39th ed., 1985).

The growth rate studies observed in this study were determined by turbidity measurements. Gross (1955) suggests that turbidity determi-
nation is a very good method to ascertain density based on growth. In figures 2 through 14, in the growth studies, the effect of the drug on Tetrahymena shows that there is a peak at the first pay then a decrease until death occurs where there is a stabilizing effect.

In Tetrahymena, cilia are known to possess axonemal ATPase, a 145 and a 305 dynein. The surface membrane (pellicle) also has a fairly high activity of ATPase. In Arrhenius plots of 39°C grown cell preparations of this pellicle bound ATPase shows a break at about 27°C. However, treatment of the membrane with the local anesthetic, dibulaine, or the inhalation anesthetic, methoxyflurane, was observed to inhibit and lower the transition temperature of the ATPase activity to 21°C (Gibbons, 1966). Chua et al., in 1977, found that Ca^{2+}-activated ATPase with a molecular weight of 89,000 was isolated from the cytosol of Tetrahymena pyriformis. The increase in cytosol ATPase activity in stationary growth phase suggests some role in cell division. It is also of great interest to note that this specific enzyme is different from Ca^{2+}-ATPase found in the nuclei, mitochondria, and microsomes.

A body of evidence has accumulated which indicates cyclic AMP and GMP as regulators of many biological functions in the cell. Specifically, cell growth is known to be sensitively controlled by these cyclic nucleotides in a wide variety of cells (Pastan, et al., 1975), even in Tetrahymena (Wolfe, 1973; Dickinson et al., 1976). It was somewhat surprising to find a fairly high activity of adenylate cyclase in a free-living unicellular eukaryote, Tetrahymena pyriformis, which is apparently not exposed to the direct influence of hormones under its
normal growth conditions. However, the enzyme was observed to respond to epinephrine, and its stimulation by this hormone could be abolished by the β-adrenergic blocking agent, propranocol, but not by any α-adrenergic blocker (Rozensweig and Kinder, 1972). The membrane bound cyclase could be partially extracted by washing the membrane fraction with 0.25 M sucrose. The cyclase preparation treated with Triton X-100 was no longer stimulated by epinephrine (Kassis and Kinder, 1975). It has been shown that a predominant enrichment of adenylate cyclase activity in the surface membrane (pellicle) as compared with the other membrane fractions in *Tetrahymena pyriformis* NT-1 (Shimonaka and Nozawa, 1977). Since this enzyme is tightly associated with the pellicle membrane, it seems likely that alterations in membrane structure or composition may drastically change the properties of this enzyme. Indeed the specific activity of adenylate cyclase in ergositerol-replaced pellicle was much lower than that observed in the native pellicle. In Arrhenius plots, a marked change in activity occurred at about 22°C, whereas there was a sharp discontinuity at 28°C to the native pellicle containing *Tetrahymena*. These two temperatures are coincidental with the onset temperatures of membrane particle aggregation (phase separation) in the native and the sterol-replaced pellicle. This may indicate that the activity of adenylate cyclase is dependent upon the physical state of the *Tetrahymena* surface membrane. In addition to adenylate cyclase mentioned above, guanylate cyclase was found in *Tetrahymena* (Gray et al., 1977), and was located in its surface membrane (Nozawa et al., 1979). Whereas guanylate classes from micro-
organisms and mammalian tissue have been reported to require Mn$^{2+}$ as the sole metal cofactor with Mg$^{2+}$ as a very poor substitute, the cyclase of tetrahymena membranes was observed to prefer Mg$^{2+}$ to Mn$^{2+}$ as a cofactor.

Phenethyl alcohol (PEA) is known to affect the phospholipid and fatty acid metabolism in *Escherichia coli* and therefore, alters the phospholipid and its acyl chain composition of the cell membrane (Nunn, 1975, 1977). Evidence shows that PEA inhibits phospholipid synthesis, primarily at the level of Sn-glycerol-3-phosphate acyltransferase and also that fatty acid synthesis would be inhibited as a secondary consequence of the altered phospholipid synthesis (Nunn, 1977). When tetrahymena cells were grown in the presence of 8 mM PEA, it was found that this drug induced a marked alteration in the relative proportions of both phospholipids and fatty acids in pellicles, mitochondria and microsomes (Nozawa et al., 1978).

The experimental results show that flurazepam dihydrochloride causes immobilization and decreased cell growth in *Tetrahymena pyriformis*. This may have been caused by the disruption of the cilia which, through their beating, brings the food to the buccal cavity (oral apparatus), where the food vacuoles form. This drug destroys the pellicle where the vacuoles form, therefore causing the organisms to eventually starve to death (total immobilization).

Factors that influence the motility of cells also affect formation of the pattern of cilia. Under anaerobic conditions, the organism maintains its normal degree of motility at the expense of its glycogen reserves if an external supply of glucose is not present. Much of the
(ATP) adenosinetriphosphate produced by *Tetrahymena pyriformis* is used for motility through the beating of the cilia. Evidence is available to show that a certain intracellular level of ATP is required for the maintenance of motility (Warnock and Van Eys, 1963).

Some inhibitors of motility are physostigmine and diisopropylfluorophosphate, as well as hexamethonium and an antibody prepared from isolated cilia. Hexamethonium leads to a small increase in the intracellular ATP content. Its effect can be reversed by acetylcholine, which was once thought to be involved in impulse transmission along the rows of the cilia (Seaman and Houlihan, 1951).

Dynein has been implicated on the mechanism of ciliary motility, since its presence is required for sensitivity of light-scattering properties of cilia to ATP (Gibbons, 1965).

Colchicine and the inhibitors of protein synthesis block the regeneration of cilia. Colchicine exerts its effect without inhibiting either ribonucleic acid or protein biosynthesis. A suggested action of colchicine is that it interferes with the assembly of ciliary subunit proteins (Rosenbaum and Carlson, 1969). It has also been indicated that colchicine blocks the development of the oral structures (Nelsen, 1970).

Benzimidazoles, benzotriazoles and quinoxalines which contain a nitro group on the benzene ring are inhibitory to *Tetrahymena pyriformis* (Greer, 1958).

The phenothiazine tranquilizer, chloropromazine, immobilizes *Tetrahymena pyriformis* and also increases its permeability. Test of a series of phenothiazines shows that alteration in permeability is
independent of the effectiveness of immobilization. The immobilization by chlorpromazine is reversed by lecithin, Tween 80, and calcium ions (Nathan and Friedman, 1962).

The increased immobilization of *Tetraphymena pyriformis* by flurazepam dihydrochloride can eventually cause starvation for the microorganism and then eventually death. This immobilization causes the ciliary structures not to be formed or destroyed and thus causes the food not to be carried to the oral apparatus. The pellicular structures which form the cilia are destroyed causing the cilia to be destroyed.
CHAPTER VI

SUMMARY

1. Flurazepam dihydrochloride at all concentrations tested reduced the growth rate of *Tetrahymena pyriformis*. Concentrations above 0.33 mg/ml were lethal to the organisms.

2. Flurazepam dihydrochloride at 0.08 mg/ml after 60 min observations caused 20% immobilization. For the same amount of time 0.63 mg/ml were lethal to the organisms. There was 80% immobilization at 0.63 mg/ml.

3. Scanning electron microscope studies revealed that the drug's effect is on the outer surfaces of the pellicle and the oral apparatus.
LITERATURE CITED


Fatty acid synthesis in *Escherichia coli* is directly inhibited by phenethyl alcohol. Biochem. 16:1077-1081.


