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THE EFFECTS OF BENOMYL AND ITS BREAKDOWN PRODUCTS CARBENDAZIM AND BUTYL ISOCYANATE ON THE ULTRASTRUCTURE AND FUNCTION OF TRACHEAL CILIATED CELLS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Stephen P. Kucera

May 1991

DEDICATION

This thesis is dedicated to my parents

Dr. Louis S. Kucera

and

Mrs. JoAnn D. Kucera

who have provided me with invaluable educational opportunities
to pursue and attain my goals.

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ABSTRACT

The effects of benomyl (methyl-1-(butylcarbamoyl) benzimidazole 2yl-carbamate) and its breakdown products carbendazim (methyl-2-benzimidazole carbamate) and butyl isocyanate on the ciliary function and ultrastructure of canine ciliated tracheal epithelial cells were examined. Ciliary beat frequencies from cultured ciliated cells in the tissue outgrowth were measured with an optical spectrum analysis system. A decrease in ciliary frequency described the extent to which these cells were affected by the test compounds over time. Serial dilutions of the test compounds were prepared in 100 percent corn oil and applied to the ciliated cell cultures over 90 minute or 6 hour exposure times with frequencies obtained at 15 minute or 1 hour intervals. Subsequent to exposures, the cell cultures were prepared for examination with a transmission electron microscope.

Benomyl and butyl isocyanate caused dose-dependent decreases in ciliary beat frequencies of tracheal epithelial cells within ninety minutes of exposures. Benomyl at a concentration of 300 μg/ml (3 mM) caused ciliostasis within seventy-five minutes exposure. Butyl isocyanate at a molar concentration three times lower than benomyl (1 mM, 300 μg/ml) caused a similar response although, within thirty minutes. The frequency data obtained from across the ranges of concentrations tested were used to calculate the ED_{50} concentration (effective dose which caused a fifty percent drop in ciliary frequency) for both compounds. The ED_{50} for benomyl was 0.75 mM, while that for butyl isocyanate was 0.52 mM. Carbendazim did not cause statistically significant decreases in frequency over ninety minute exposures. However, with exposures up to 6

hours, moderate decreases were measured with statistical significance seen at 3 hours exposure.

Benomyl exposure caused damage to mitochondria of ciliated epithelial cells which was seen as moderate to severe swelling of mitochondria, disruption of inner mitochondrial membranes, and loss of mitochondrial matrix. Other cellular organelles appeared normal, although a slight proliferation of smooth vesicles was suggested. Butyl isocyanate exposure did not cause the same mitochondrial damage in ciliated epithelial cells as seen with benomyl exposure. Mitochondria, as well as other cellular organelles, appeared normal.

Although ciliostatic responses were seen with both benomyl and butyl isocyanate, the sites and/or modes of action of these two compounds may be different. The toxic effects of benomyl to ciliated tracheal epithelial cells may possibly be attributed primarily to butyl isocyanate and secondarily to carbendazim after benomyl enters the cell and breaksdown to its toxic components.

TABLE OF CONTENTS

CHAPTER		PAGE
1.	INTRODUCTION	1
2.	MATERIALS AND METHODS Preparation of Tissue Quantitation of Ciliary Beat Experimental Compounds Preparation of Tissue for Electron Microscopy	8
3.	RESULTS Effects of Test Compounds on Ciliary Frequency Ultrastructural Changes	16
4.	DISCUSSION	44
LIST OF REFERENCES		53
VITA		59

LIST OF FIGURES

FIGURE		PAGE
1.	Chemical breakdown products of benomyl	2
2.	See List of Plates	
3.	See List of Plates	
4.	Ciliary Beat Frequency Monitoring System	12
5.	See List of Plates	
6.	See List of Plates	
7.	See List of Plates	
8.	See List of Plates	
9.	Ciliary beat frequencies of control cells	22
10.	Inhibition of ciliary beat frequency with exposure to benomyl	23
11.	Comparison of inhibitory effects of benomyl	26
12.	Inhibition of the ciliary beat frequency with carbendazim	28
13.	Inhibition of ciliary beat frequency with exposure to butyl isocyanate	29
14.	Comparison of the inhibitory effects of butyl isocyanate	32
15.	Log-probit analysis dose-response curve for benomyl	33
16.	Log-probit analysis dose-response curve for butyl isocyanate	34
17.	See List of Plates	
18.	See List of Plates	
19.	See List of Plates	

LIST OF FIGURES (Continued)

FIGURE PAGE

20. See List of Plates

21. See List of Plates

22. See List of Plates

23. See List of Plates

24. See List of Plates

LIST OF PLATES

PLATE PAGE

I. Scanning Electron Micrographs of Tracheal Epithelial Cell Cultures 10
Figure 2
Figure 3

II. Transmission Electron Micrographs of Cultured Ciliated Epithelial Cells (Controls) 17
Figure 5
Figure 6

III. High Magnification of Cultured Ciliated Epithelial Cells 19
Figure 7
Figure 8

IV. Ciliated Epithelial Cells Exposed to Benomyl 37
Figure 17
Figure 18

V. Comparison of Mitochondrial Structure of a Control Cell and Benomyl Treated Epithelial Cells 39
Figure 19
Figure 20
Figure 21

VI. Ciliated Epithelial Cells Exposed to Butyl Isocyanate 42
Figure 22
Figure 23
Figure 24

CHAPTER 1

INTRODUCTION

Benomyl (methyl-1-(butylcarbamoyl)benzimidazole 2yl-carbamate) is a systemic fungicide from the carbamate family. It is used on an assortment of fruits, vegetables, and ornamental plants to control a wide variety of plant pathogenic fungi. Although benomyl is best known for its fungicidal properties, it also has inhibitory effects on mite populations (Upham and Delp, 1973; Clemons and Sisler, 1971). Benomyl was developed in the late 1960's as one of the first systemic fungicides (Kavlock et al., 1982) and is manufactured by the E.I. duPont de Nemours Company. It is marketed as Benlate, which contains benomyl (50%) as the active ingredient. Since Benlate is extensively used both in agriculture and in the home, there is a concern in obtaining a toxicological data base for hazard identification (Warheit et al., 1989).

Although benomyl is fairly stable in soil, lasting from six months to two years, it has been shown to degrade in water and organic solvents. The degradation products (Figure 1) carbendazim (methyl-2-benzimidazole carbamate, MBC) and butyl isocyanate (BIC) have been confirmed by gas chromatographic analysis (Hammerschlag and Sisler, 1973). These degradation products have further been confirmed by reversing the pathway and reforming the compound benomyl upon addition of butyl isocyanate to a solution of carbendazim (Chiba, 1977).

The mode of action of benomyl in fungi was determined to be an interference with mitosis (Hammerschlag and Sisler, 1973). This interference was originally thought to be

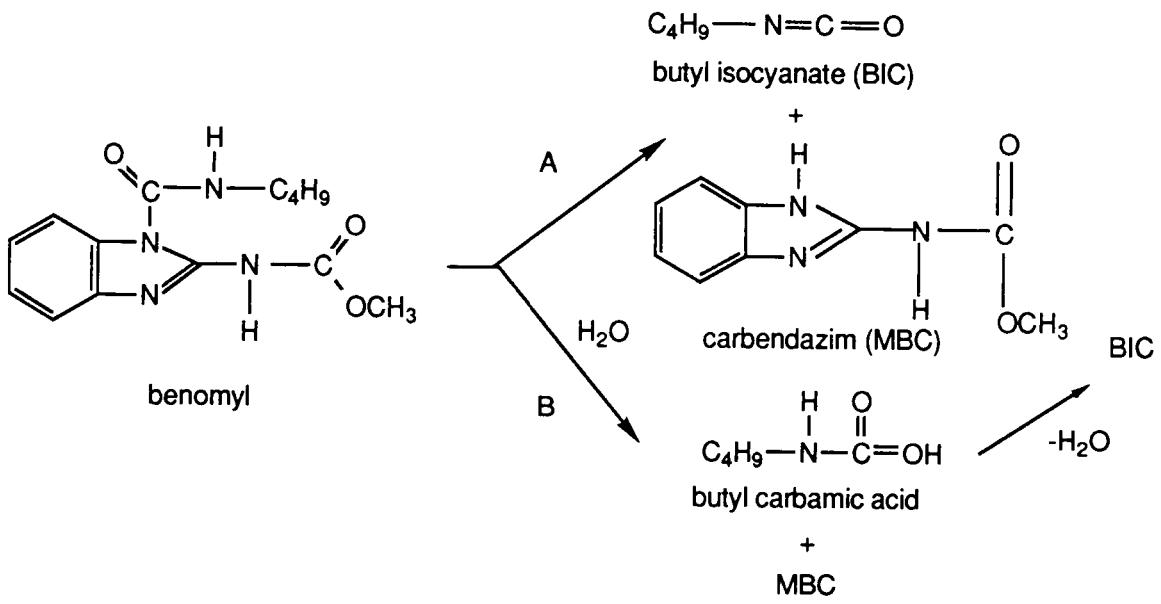


Figure 1. Chemical Breakdown Products of Benomyl. Proposed formation of various breakdown products of benomyl via a non-hydrolytic (A) and a hydrolytic (B) pathway. Both pathways result in the formation of butyl isocyanate (BIC).

due to benomyl binding to fungal tubulin, preventing tubulin polymerization (Davidse and Flach, 1977). However, carbendazim was later suggested to be the actual fungitoxic component of benomyl (Torstensson and Wessen, 1984) because it was equally as toxic to most fungi as benomyl (Clemons and Sisler, 1969). Further research has suggested that toxicity to some fungi comes from butyl isocyanate after benomyl breaks down to carbendazim and butyl isocyanate (Hammerschlag and Sisler, 1973).

Little information concerning the harmful effects of these compounds on non-target organisms is available in the literature. Although benomyl and carbendazim have low affinities for mammalian tubulin (Davidse and Flach, 1977; Ireland et al., 1979), both have been shown to prevent polymerization of bovine tubulin *in vitro* when large concentrations are present (Friedman and Platzer, 1978). The highly reactive nature of butyl isocyanate may also be implicated in toxic responses to other organisms.

Benomyl and carbendazim have been reported to have relatively high oral LD_{50}'s (lethal dose 50) in adult rats of greater than 10,000 mg/kg (Warheit et al., 1989). In contrast, butyl isocyanate has a considerably lower oral LD_{50} (600 mg/kg) in adult rats (Sax and Lewis, 1989). Ninety-day inhalation studies showed that benomyl caused histopathological legions of the olfactory epithelium of rats, specifically degeneration and disorganization of olfactory epithelial cells (Warheit et al., 1989). Based on these pathological observations, the authors determined a no-observable-effect level (NOEL) for male (10 mg/m^3) and female rats (50 mg/m^3). Although scattered lesions of the respiratory epithelium of the nasal cavity were seen in several rats, these same authors did not attribute the lesions to benomyl exposure. Other systemic studies have shown that

benomyl was more toxic (inhibition of growth) to two species of fishes (Lebistes reticulatus and Salmo gairdneri) than carbendazim while the reverse was seen for both an alga (Chlorella pyrenoidosa) and crustacean (Daphnia magna) after a single administration of benomyl (unknown concentration) to the water (Canton, 1976).

In short-term reproductive studies both benomyl and carbendazim have been reported to be a reproductive toxin in adult male rats. Benomyl caused a decrease in testicular and epidydimal weights, decreased sperm count, and testicular lesions (Carter et al., 1985). Carbendazim caused severe seminiferous tubule atrophy (Carter et al., 1984). Long-term reproductive studies by Canton (1976) utilizing Daphnia magna, a sensitive invertebrate often used for such studies, indicated a complete inhibition of reproductive capacity with exposure to carbendazim at a concentration of 50 μg/ℓ in water.

Benomyl, administered via gavage during organogenesis produced teratogenic and fetotoxic effects in offspring of pregnant rats and pregnant mice (Kavlock et al., 1982). Physical anomalies included exencephaly, hydrocephaly, and microphthalmia, which all appeared to be dose related. Carbendazim administered similarly, although at lower doses, increased the incidence of embryonic resorption, decreased fetal weight, and other physical anomalies.

Benomyl and its breakdown products have been shown to inhibit certain intracellular processes. In yeast studies, benomyl inhibited respiration and fermentation more than carbendazim, while butyl isocyanate inhibited respiration more but fermentation less than benomyl (Chiba et al., 1987). Benomyl inhibited glucose and acetate metabolism and DNA, RNA, and protein synthesis in yeast, while carbendazim appeared

only to inhibit cytokinesis or mitosis (Hammerschlag and Sisler, 1972). Benomyl also inhibited [^{3}H]-thymidine incorporation into DNA of thymus, spleen, liver, kidney and testis of the mouse while carbendazim induced a similar effect only in the testis (Hellman and Laryea, 1990). The ED_{50} (effective dose 50) for inhibition of growth in yeast was shown to be 0.4 µg/ml for benomyl and 12 µg/ml for carbendazim (Clemons and Sisler, 1969).

Evidence that benomyl and carbendazim caused genetic damage is conflicting (Lamb and Lilly, 1980). Seiler (1976) concluded that these compounds can produce point mutations by base substitution, and cause abnormalities by acting as spindle poisons. Further, carbendazim produced micro-nuclei in mouse bone marrow cells while benomyl did not (Seiler, 1976).

Variable results in assessing the toxicities of benomyl and its breakdown products may be due in part to the different solubilities of the three compounds in various solvents. Clemons and Sisler (1969) explained that the toxicity differences between benomyl and carbendazim may be due to the failure of carbendazim to penetrate yeast cells as readily as benomyl. Aqueous solutions of these compounds was not possible since both benomyl and carbendazim are insoluble in water and butyl isocyanate is highly water reactive. Several solvent strategies have been utilized to dissolve benomyl including methanol, ethanol, dimethyl sulfoxide, surfactants, wettable powders, acetone, and a mixture of methanol and Tween-20 (Chiba, 1977). To further compound the problem, benomyl was unstable in organic solvents (Koller et al., 1982; Peterson and Edgington, 1969). This resulted in difficulty in controlling benomyl concentrations in test solutions and in interpreting experimental data (Singh and Chiba, 1985).

While human exposure to benomyl has been variable, Everhart and Holt (1982) determined the average potential for exposure (dermal and respiratory) for three different agricultural use-situations. These use-situations were: 1) mixing for aerial application, 2) re-entry into a treated field, and 3) home use. The values obtained were 26, 12, and <1 mg of benomyl per body surface area for dermal exposure and 0.08, 0.03, and 0.003 mg of benomyl (collected on a filter) for respiratory exposure, respectively. Their study indicated that a potential risk exists for exposure to benomyl. Occupational exposure to benomyl is regulated to protect workers. The limit established, the time-weighted average (TWA), is 10 $\mu g/m^3$ for respiratory exposure (NIOSH, 1989-1990). This is an average concentration to which nearly all workers may be exposed, on a day-to-day basis, without adverse health effects. Benomyl is currently unregulated for in-home use.

Ciliary function, an important mechanism in mucociliary clearance, has been shown to be a sensitive indicator of response following both acute and chronic exposures to various air pollutants (Wolff, 1986). Any alteration to this host defense system may jeopardize the health of the host (Adalis et al., 1978). Ciliated epithelium from tracheal rings has previously been utilized to determine the toxicities of potentially respirable metals (Gabridge et at., 1982; Gabridge and Meccoli, 1982; Adalis et at., 1978) and the interactions of metals in tracheal epithelial cells (Porter and Matrone, 1977). Toxicity to this tissue may be manifested in a decrease in ciliary function.

Tracheal epithelial outgrowth cultures have been utilized to study the specificity and early cellular effects of attachment of Bordetella bronchiseptica to canine ciliated epithelium (Bemis and Kennedy, 1981). They have also been used to study the effects

of gossypol acetic acid on the ultrastructure and function of rabbit ciliated epithelium (Duckett et al., 1986). The above studies reported inhibition of ciliary function leading to ciliostasis, which is useful in detecting and assessing certain biologically hazardous agents (Donnelly et al., 1974). Two of the previously mentioned studies (Bemis and Kennedy, 1981, and Duckett et al., 1986) determined toxicity to cultured epithelial cells by quantifying the decrease in ciliary beat frequencies with an optical spectral analysis system (Kennedy and Duckett, 1981). Subsequent electron microscopy was employed to examine morphological changes in cellular organelles.

The present study was conducted utilizing the *in vitro* ciliary bioassay system described above to compare the relative toxicities of benomyl and breakdown products carbendazim and butyl isocyanate. This system, using cultured tracheal epithelial cells from adult mixed-breed dogs, employed a more suitable solvent vehicle. The solvent used was corn oil, which effectively solubilized benomyl and butyl isocyanate. Corn oil was less effective for carbendazim, due to its lower lipid solubility. Corn oil did not adversely affect the functioning of these cells *in vitro*.

CHAPTER 2

MATERIALS AND METHODS

Preparation of Tissue

Tracheas from adult mixed-breed dogs were excised from just below the cricoid cartilage to the bifurcation of the bronchi. The excised tracheas were placed in a flask of sterile Dulbecos' phosphate buffered saline and transported to the laboratory within 30 minutes of excision. The tracheas were cut longitudinally into four strips and placed into a dish containing Waymouths' MB 752/1 tissue culture medium (Sigma Chemical Co.) (Kennedy and Ranyard, 1983), supplemented with 10% (v/v) Nu serum (Collaborative Research, Inc.) and antibiotics (penicillin 50 units/ml, streptomycin 50 μg/ml, and gentamicin 20 μg/ml). The psuedostratified ciliated epithelium along with some of the underlying connective tissue was dissected away from the cartilaginous tissue with microdissecting scissors and forceps under a dissecting microscope. The strips of epithelium were placed in a 100 x 15 mm petri dish containing fresh medium and antibiotics for holding.

The large strips of tissue were cut into smaller strips approximately 2 mm x 6 mm, rinsed in fresh medium and placed into 50 ml tissue culture flasks (3 strips per flask) containing 10 ml of culture medium with serum and antibiotics. These flasks were placed in a 37°C humidified incubator (95% air/5% CO_2) overnight. This allowed for mucus to separate from the surface of the epithelium, providing easier handling of the tissue.

After 24 hours incubation, the epithelial strips were cut into explants approximately 1 mm square and placed (3 per dish) into 35 x 10 mm Falcon or Lux tissue culture dishes. Culture medium (0.7 ml), containing serum and antibiotics, was added to the dishes and incubated at 37°C. The medium was decanted from the dishes and replaced with fresh medium at three day intervals. Within 8-10 days, an outgrowth containing both ciliated and non-ciliated cells (Kennedy and Ranyard, 1983) was established. It was from these isolated ciliated tracheal epithelial cells (Plate 1 - Figures 2 and 3) that ciliary beat frequencies (CBF) were measured after exposure to the test chemicals. These cultures were maintained for up to 2-4 weeks with medium changes at three day intervals. Additional tissue was maintained in 50 ml tissue culture flasks containing serum and antibiotics for subsequent culturing.

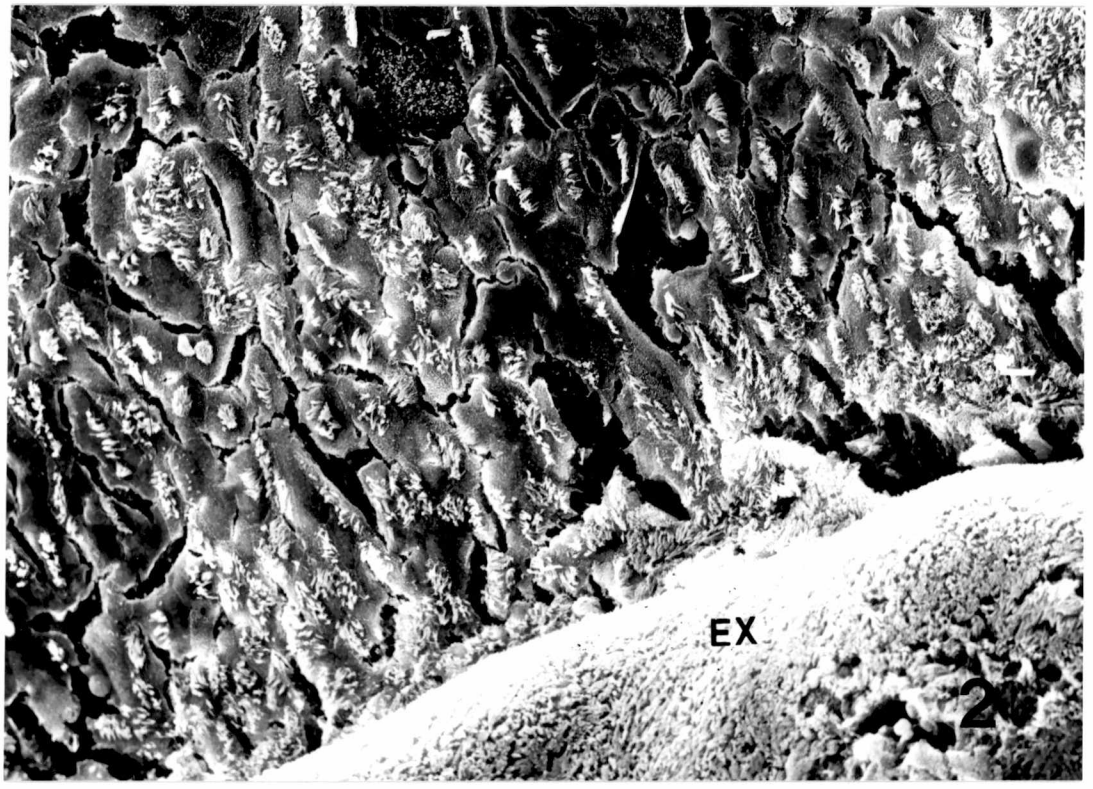
Quantitation of Ciliary Beat

For measuring ciliary frequencies, a ciliated outgrowth culture was placed on a Nikon inverted phase contrast microscope and heated to 37°C with a Nicholson airstream stage incubator. These ciliated cells, attached to the bottom of the culture dish, could be placed in the optical path of the microscope. An RCA TV camera, mounted to the side of the microscope, projected the enlarged outgrowth image onto a RCA TV monitor. A photomultiplier detection head, with a 40X objective lense, was placed against the monitor near the edge of beating cilia of a cell. The variation in transmitted light resulting from ciliary motion on the TV monitor was converted to an analog signal. This signal was then converted by Fast Fourier transform into a frequency function (Kennedy and Duckett, 1981) with a Hewlett-Packard spectrum analyzer model 358A (Figure 4).

Plate 1: Scanning Electron Micrographs of Tracheal Epithelial Cell Cultures

Figure 2. Scanning electron micrograph of tracheal explant (EX) and ciliated outgrowth cells. X120.

Figure 3. Higher magnification of ciliated outgrowth cells (C) and non-ciliated cells (NC). Both types of cells contain microvilli (MV) on their apical surfaces. Ciliary frequency was obtained from uniformly beating tuffs of cilia on individual outgrowth cells. X620.


EX
2


NC
mv
C
3

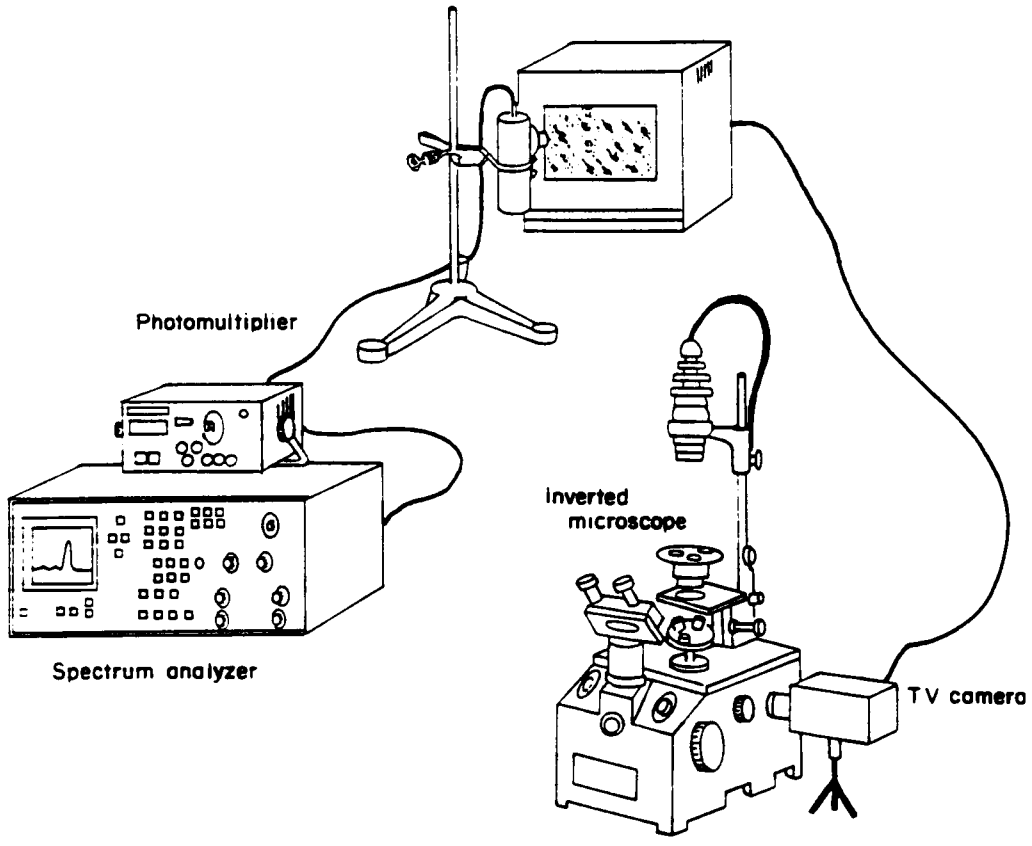


Figure 4. Ciliary Beat Frequency Monitoring System. The system for quantitating ciliary beat frequency of ciliated outgrowth cells consists of an inverted phase-contrast microscope. The field image is projected onto a TV monitor and a photomultiplier unit converts the variation in transmitted light resulting from ciliary motion into an electrical analog signal. This signal is converted by Fast Fourier transform into a frequency function using a spectrum analyzer.

Ciliary beat frequencies (CBF) were acquired at time zero and at fifteen minute intervals over ninety minute exposure times or 1 hour intervals over 6 hour exposure times. A decrease in CBF over time describes the extent to which the test compounds affect ciliary function (Duckett et al., 1986). Prior to chemical exposures, 100% corn oil (Mazola Brand) was exchanged for the culture medium and the culture was incubated for fifteen minutes. Corn oil, which had no adverse effect on ciliary frequency, tended to normalize ciliary frequencies within a relatively narrow range. Data obtained at the specified time, T=0, denotes normal baseline beat frequencies. This provided beat frequency data to which a reference comparison could be made during exposure to the test compounds. Exposure times where measured after the exchange of 100% corn oil with the corn oil/test compound solution. Frequencies from 10 randomly selected cells were obtained at each measurement time and a mean and standard deviation was calculated.

Experimental Compounds

Benomyl, 95% technical grade, and carbendazim, 98% technical grade, were supplied by Dr. Rex Hess (University of Illinois, Urbana) and butyl isocyanate was obtained from Eastman Kodak Company. These compounds were dissolved in 100% corn oil, Mazola brand, due to the lipophilic nature of the test compounds. Control experiments indicated that corn oil did not adversely affect ciliary function. Ten-fold serial dilutions were prepared in corn oil ranging from 1 μg/ml ($3.3X10^{-6}M$) to 300 μg/ml ($1X10^{-3}M$) for benomyl and 1 μg/ml ($1X10^{-5}M$) to 300 μg/ml ($3X10^{-3}M$) for butyl

isocyanate. Carbendazim was tested at a concentration of 300 μg/ml ($1.5X10^{-3}M$). Benomyl and butyl isocyanate completely dissolved in the corn oil while carbendazim formed a suspension due to this compounds lower lipid solubility. Exposures of tracheal epithelial cultures to benomyl, butyl isocyanate, and carbendazim were initiated with fresh preparations to minimize errors as a result of the degradation of these compounds.

Preparation of Tissue for Electron Microscopy

For transmission electron microscopy, ciliated tracheal epithelial cell cultures were fixed in a mixture of osmium tetroxide and glutaraldehyde, (Kennedy and Allen, 1980). This fixative was prepared by mixing 2 ml of 0.2M phosphate buffer (pH 7.3) with 1 ml of 5% (w/v) osmium tetroxide for a final 1.66% (v/v) solution of OsO_4 in 0.133M phosphate buffer. The phosphate buffered osmium tetroxide was mixed 1:1 with 2.5% (v/v) glutaraldehyde in distilled water prior to use and maintained in an ice bath. After several rinses with phosphate buffer to remove the test compound/oil mixture, 1.5 ml of the mixed fixative was added to the culture dishes and the dishes were placed in an ice bath for 1 hour. After fixation, the tissue was rinsed with phosphate buffer and dehydrated in a graded series of ethyl alcohol. With the use of the Lux permanox culture dishes, two additional changes of propylene oxide were made after dehydration.

After dehydration, the explants were removed and placed overnight in a vial with a 50:50 mixture of propylene oxide and resin (Ladd Chemical LX-112). The explants were embedded in resin and polymerized in a 60°C oven for 2 days. The tissue blocks were trimmed and sections were cut using glass knives on a Sorvall MT-1

ultramicrotome. Sections were picked up on 400 mesh copper grids and stained for 20 minutes in 4% (w/v) saturated uranyl acetate in distilled water mixed 50:50 with methanol, followed by two minutes in lead citrate (0.03g lead citrate per 10 ml distilled water and 0.1 ml 10N NaOH). The grids were rinsed in distilled water, dried on filter paper and viewed with a Hitachi H-600 transmission electron microscope.

For scanning electron microscopy, the ciliated epithelial cell cultures were fixed in 3% glutaraldehyde in 0.025M cacodylate buffer followed by a post fixation in a 1:1 solution of 2% osmium tetroxide and cacodylate buffer (pH 7.2) for forty-five minutes. The tissue was dehydrated in a graded series of acetone and dried in a critical point drying apparatus (Ladd Research Industries, Birlington, VT). Specimens were mounted on studs, coated with gold palladium wire (60:40), and examined in an ETEC Autoscan scanning electron microscope (ETEC, Haywood, CA).

CHAPTER 3

RESULTS

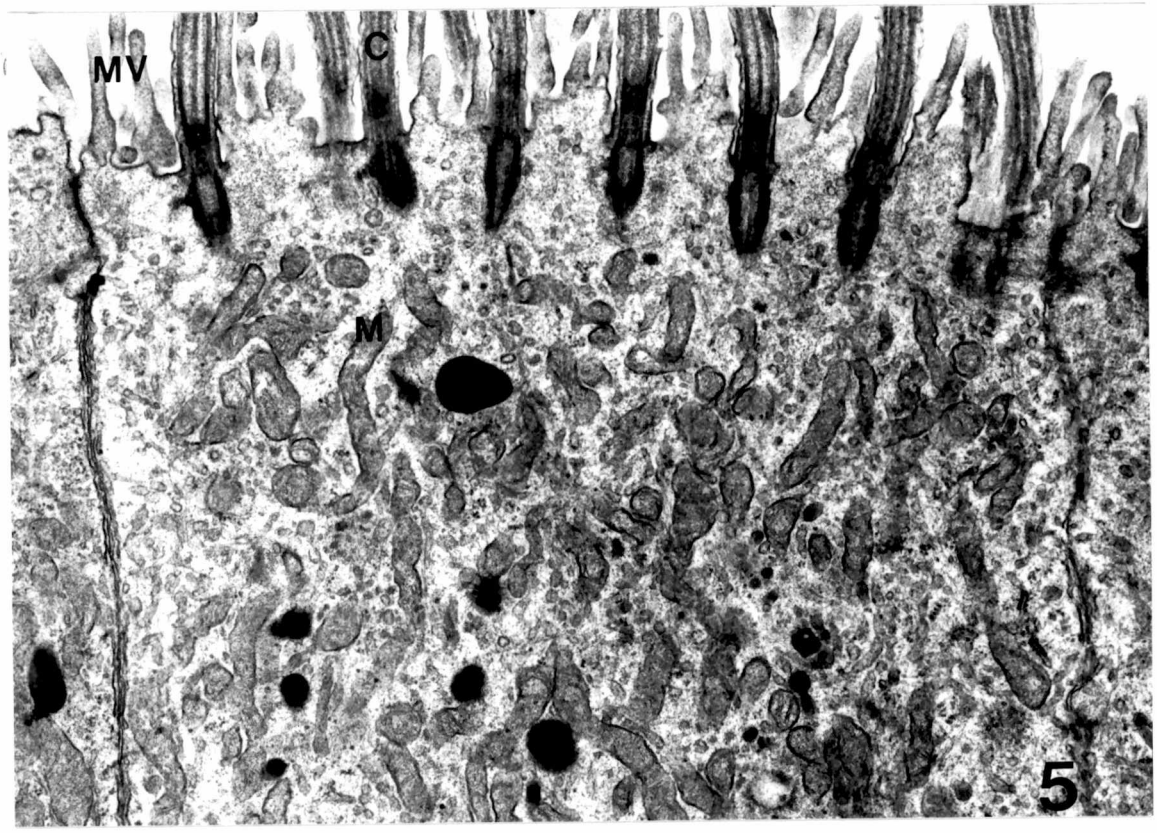
Canine ciliated tracheal epithelial cell cultures were used in an *in vitro* comparative study of the ciliotoxic effects of benomyl and its breakdown products carbendazim and butyl isocyanate. Ciliary beat frequency (CBF) data was obtained from the individual ciliated cells within the tissue outgrowth (Figure 2). Decrease in CBF over time was used as a measure of the extent to which these test compounds affect ciliary function.

Cultured cells, although squamous rather than columnar shaped, maintained normal ciliary morphology and function. Most tracheal epithelial cells contained both cilia and microvilli (Figure 3) on their apical surfaces. Explant cells appeared characteristically columnar in shape with long slender microvilli interspersed between the cilia (Figure 5). Outgrowth cells, while generally not as ciliated as explant cells, contained numerous microvilli (Figure 6). Cells in Figures 5 and 6 are from control cultures which were exposed to 100% corn oil for 6 hours. These cells contained long slender mitochondria with a high concentration of these organelles in the apical portion of the explant cells. Within the mitochondria were thin cristae usually at right angles to the long axis of the mitochondria (Figure 7). Cells contained multiple strands of granular endoplasmic reticulum surrounding the nucleus (Figure 8). Some strands of endoplasmic reticulum lay parallel to the nucleus and others terminated in a series of small vesicles of smooth endoplasmic reticulum. Scattered Golgi bodies and numerous polysomes were also evident.

Plate II: Transmission Electron Micrographs of Cultured Ciliated Epithelial Cells (Controls)

Figure 5. Portion of a ciliated explant cell from a culture exposed to 100% corn oil for 6 hours. Normal apical distribution and filamentous shape of mitochondria (M). Cilia (C) and microvilli (MV) appear normal. X 10,000.

Figure 6. Portion of a ciliated outgrowth cell from a culture exposed to 100% corn oil for 6 hours. Normal mean ciliary beat frequencies (between 9.8 and 14 beat/sec) were recorded. Normal filamentous shape of mitochondria (M) and normal ciliary structure (C) are evident. X 10,000.


MV
C
M
5

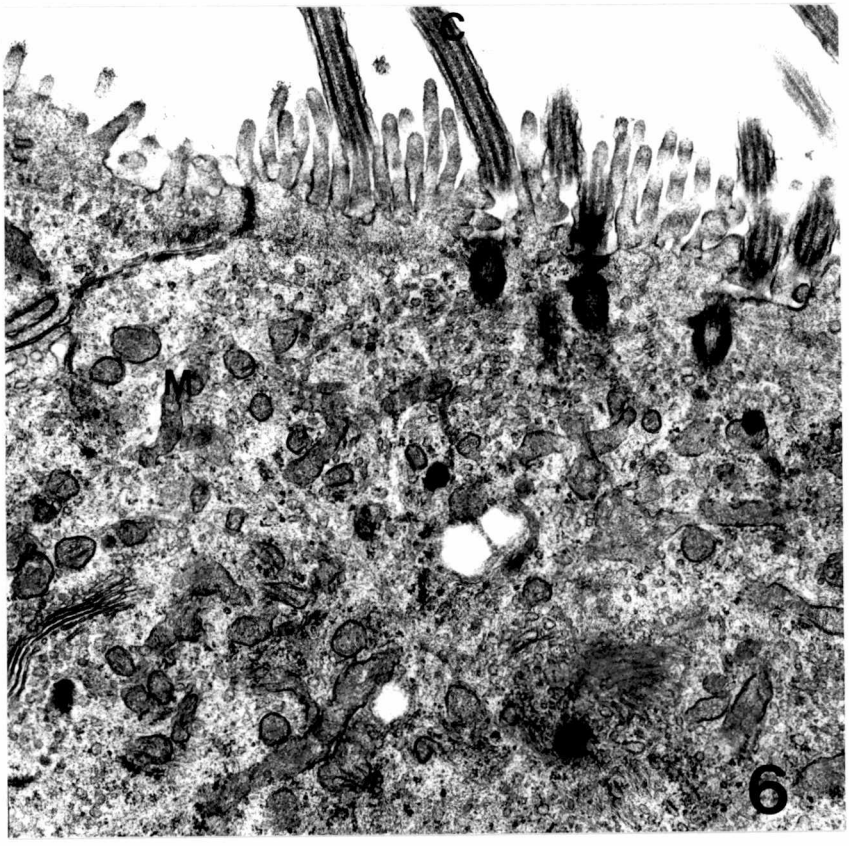
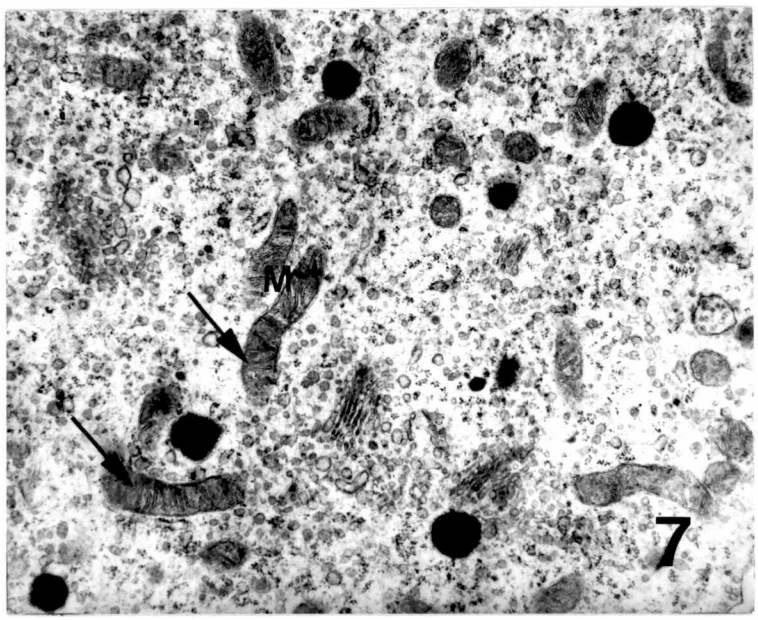
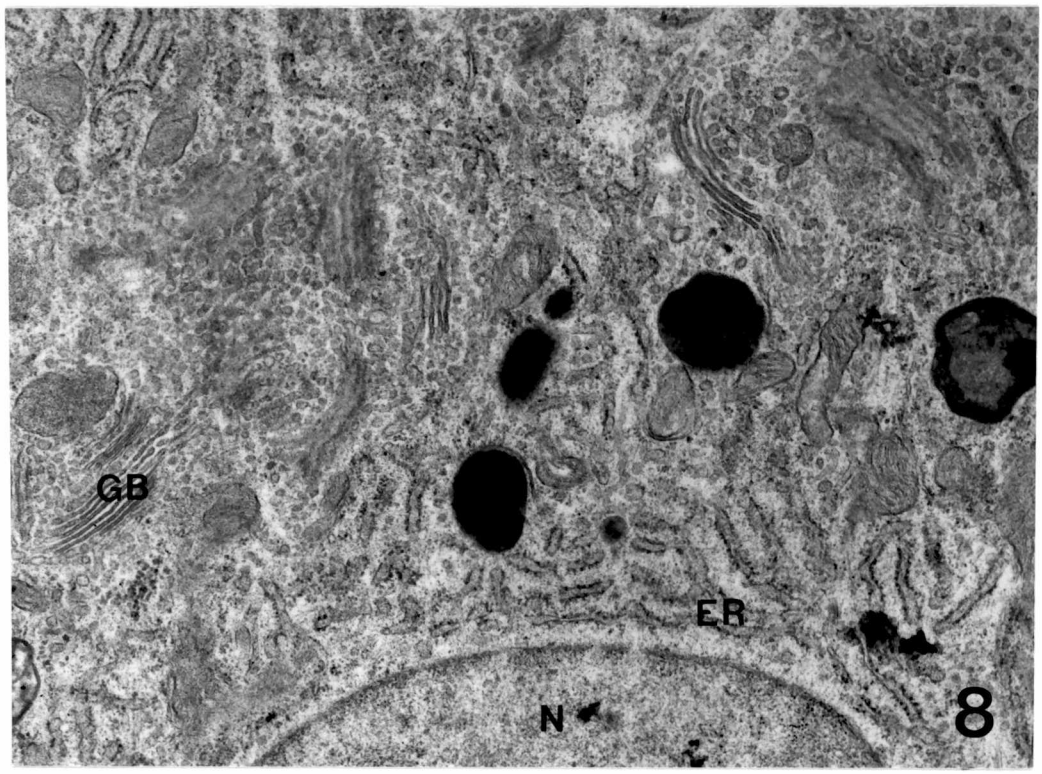

C
M
6

Plate III: High Magnification of Cultured Ciliated Epithelial Cells

Figure 7. High magnification of an outgrowth cell exposed to 100% corn oil for 6 hours. Mitochondria (M) appear normal with cristae (arrows) at characteristic right angles to the long axis of the mitochondria. X 15,000.

Figure 8. High magnification of an outgrowth cell exposed to 100% corn oil for 6 hours. Organelles such as endoplasmic reticulum (ER), Golgi bodies (GB), and nucleus (N) appear normal. X 15,000.


M
7


GB
ER
N
8

The average ciliary beat frequencies of healthy canine tracheal outgrowth cells in media varied from 6-16 beats/sec. However, baseline frequencies in corn oil varied from 8.5 to 12 beats/sec. With baseline frequencies all beginning within this narrow, normalized range, comparisons could be made between different cultures exposed to varying concentrations of test chemicals. Figure 9 shows the means (+/- 1 SD) from 4 different tracheal epithelial cell cultures exposed to 100% corn oil for ninety minutes. As indicated by the relatively flat curves, ciliary frequencies were maintained within a very narrow range over the ninety minute exposure time, with no statistically significant decreases in ciliary frequencies seen.

Effects of Test Compounds on Ciliary Frequency

Ciliated epithelial cells showed a dose-dependent decrease in ciliary frequency at the 3 concentrations of benomyl tested. At a concentration of 300 μg/ml (1 mM) (Figure 10), cells exhibited a precipitous decline in ciliary beat frequencies with ciliostasis occurring in some cells within sixty minutes. Four representative cultures are shown in each figure indicating that the measured effects are reproducible. All 4 cultures became ciliostatic within ninety minutes of exposure at this highest concentration. With an intermediate concentration of benomyl, 100 μg/ml (0.33 mM), there also was a decrease in ciliary function, although the rate of decrease was more moderate than the higher concentration. Benomyl at this concentration caused a reduction in ciliary beat frequencies from between 8.5-11.2 beats/sec to between 4.3-6.4 beats/sec. (Figure 10) over ninety minutes exposure time.

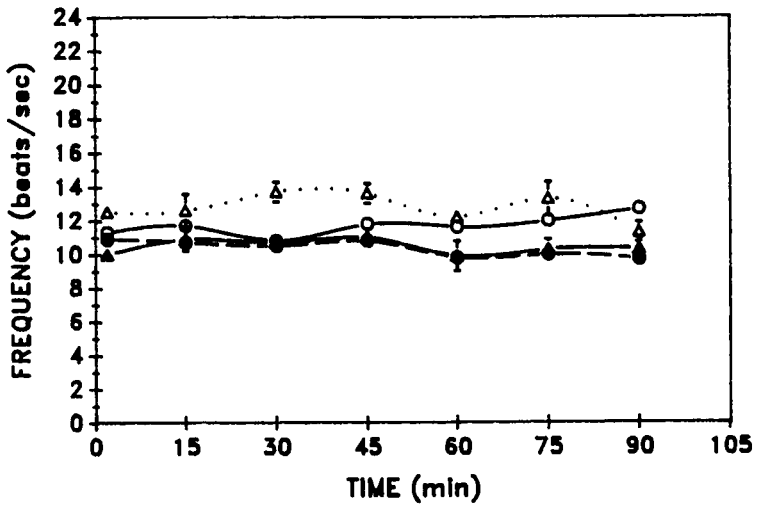


Figure 9. Ciliary beat frequencies of control cells. Ciliary beat frequencies of 4 different tracheal epithelial cell cultures exposed to 100% corn oil for ninety minutes. Each data point of each curve represents the mean +/- 1 standard deviation (SD) of 10 randomly selected cells. Data points not showing SD bars indicate that the small SD is overdrawn by the data symbol.

Figure 10. Inhibition of ciliary beat frequency with exposure to benomyl. Inhibition of ciliary beat frequency by 3 concentrations of benomyl (300 μg/ml (1 mM) (A), 100 μg/ml (0.33 mM) (B), and 10 μg/ml (0.033 mM) (C)) with time. Each concentration is represented by 4 different cultures. Each data point represents the mean +/- 1 SD from 10 randomly selected cells.

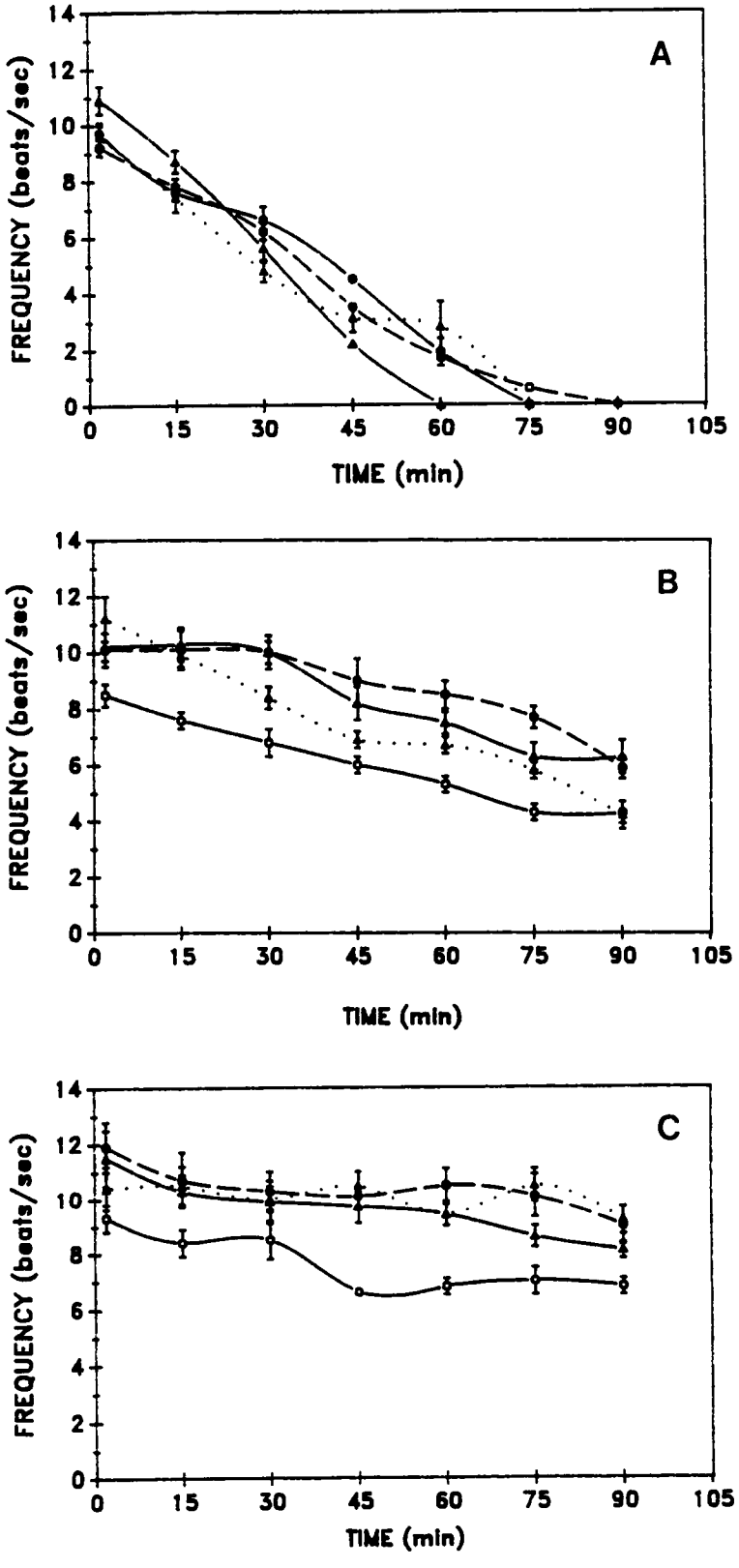

A
B
C
FREQUENCY (beats/sec)
TIME (min)
0
2
4
6
8
10
12
14
0
15
30
45
60
75
90
105

Figure 10.

A ten-fold dilution (or 300 fold depending on reference point) in concentration of benomyl to 10 μg/ml (0.033 mM) resulted in very gradual decreases in ciliary function over the same exposure time. Ciliary beat frequencies dropped from between 8.4-11.8 beats/sec to between 7.2-9.1 beats/sec (Figure 10).

Figure 11 shows a comparison of the decreases in ciliary beat frequencies for the 3 concentrations of benomyl tested. Data points on each of curve represent the mean frequency (+/- 1 SD) of the 40 cells from each of 4 cultures at the three concentrations of benomyl tested. A T-test was performed to identify statistically significant decreases in mean CBF when compared to normal baseline ciliary beat frequencies (T=0). Statistically significant differences in mean ciliary frequencies ($p<0.01$) occurred for all 3 concentrations of benomyl tested, although the time period in which significant differences were seen varied with concentration. Benomyl at the highest concentration, 300 μg/ml (1 mM), resulted in an average time of ciliostasis between seventy-five and ninety minutes exposure. This concentration resulted in a statistically significant decrease in ciliary frequency within the first fifteen minute measurement interval. A significant decrease was seen both at thirty and forty-five minutes exposure to benomyl at concentrations of 100 μg/ml (0.33 mM) and 10 μg/ml (0.033 mM) respectively.

Carbendazim, the supposed fungitoxic component of benomyl, did not result in any significant decrease in ciliary beat over ninety minutes exposure to a 300 μg/ml (1.5 mM). Exposures to carbendazim at this same concentration were continued for up to 6 hours to determine if decreases in ciliary beat frequencies occurred. Of 6 experiments performed, 3 indicated very little decrease in CBF (from between 9.7-10.6 to 8.4-9.0

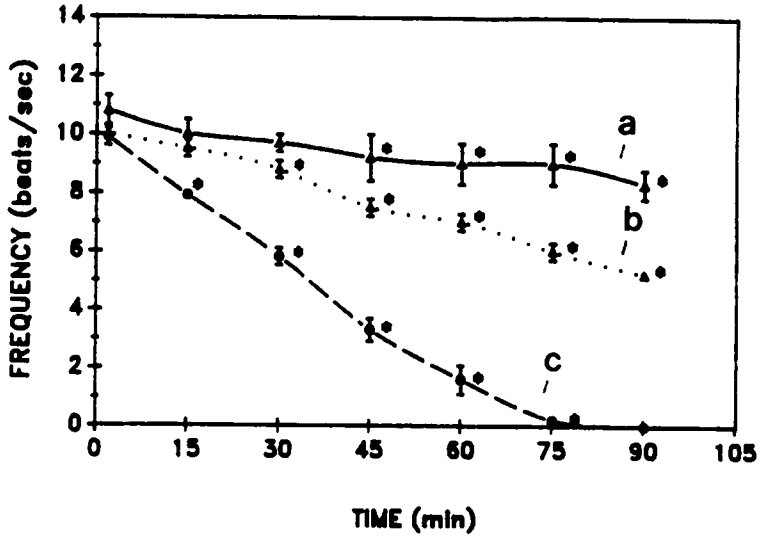


Figure 11. Comparison of inhibitory effects of benomyl. Comparison of the inhibition of ciliary beat frequency by 3 concentrations of benomyl (a, 10 μg/ml (0.033 mM), b, 100 μg/ml (0.33 mM), and c, 300 μg/ml (1 mM)) with time. Each data point represents the mean +/- 1 SD from forty cells. (*) Indicates a significant difference in CBF compared to baseline (T=0) using a Student T-test, $p<0.01$.

beats/sec). The other 3 showed a slightly greater decrease (from between 9.3-9.9 to 5.5-6.2 beats/sec). The variable ciliary frequency results obtained with carbendazim may be consistent with the fact that carbendazim did not dissolve in the corn oil, but rather formed a cloudy suspension. Figure 12 shows the curves representing the pooled means and standard deviations of these 6 cultures. A statistically significant decrease (T-test, $p<0.01$) occurred at 3 hours exposure. The top curve in Figure 12 represents the means and standard deviations from control cultures (100% corn oil) over 6 hours. Ciliary frequencies of control cultures were maintained between 10 beats/sec and 11.4 beats/sec throughout the 6 hour exposure time.

Butyl isocyanate, a breakdown product of benomyl, also caused a similar dose-dependent decrease in ciliary beat over the 3 concentrations tested. Although the concentrations of butyl isocyanate were close to an order of magnitude lower in molarity, the rate of decrease was greater in comparison to benomyl. Butyl isocyanate at the highest concentration tested, 300 µg/ml (3 mM) resulted in a very rapid decrease in ciliary beat. Ciliostasis occurred in all 4 cultures within thirty minutes exposure (Figure 13). With a decrease in concentration to 100 µg/ml (1 mM), there was a corresponding decrease in the time of ciliostasis (Figure 13) to between sixty and seventy-five minutes. The rate of decrease in ciliary beat at this concentration closely resembles that of benomyl at the highest concentration tested, 300 µg/ml (1 mM). Butyl isocyanate at 10 µg/ml (0.03 mM) resulted in a very minor drop in ciliary beat (Figure 13). A T-test was also performed to identify statistically significant decreases in mean CBF when compared to normal baseline ciliary beat frequencies (T=0). A comparison of the decreases in mean

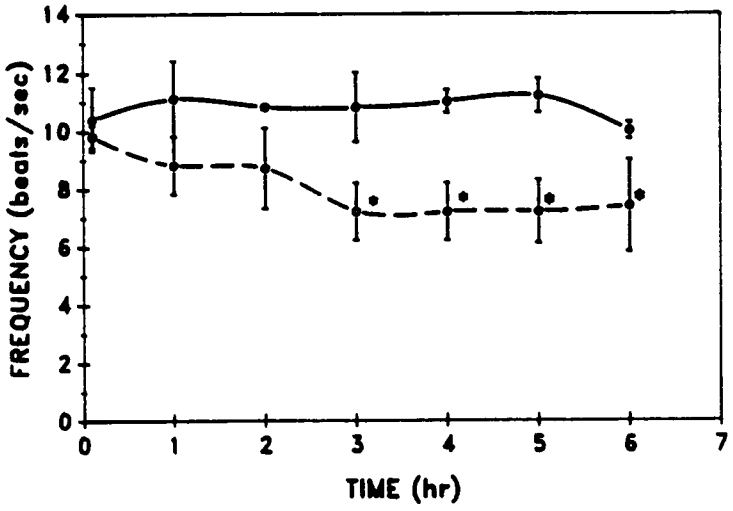


Figure 12. Inhibition of the ciliary beat frequency with carbendazim. Inhibition of ciliary beat frequency by carbendazim (1.5 mM, 300 µg/ml) with time compared to a control. The data points on the control curve, ●———●, represent the mean +/- 1 SD from twenty cells (10 cells from 2 different cultures). The data points on the exposure curve, o------o, represent the mean +/- 1 SD from sixty cells (10 cells from 6 different cultures). (*) Indicates a significant difference in CBF compared to baseline (T=0) using a Student T-test, $p<0.01$.

Figure 13. Inhibition of ciliary beat frequency with exposure to butyl isocyanate. Inhibition of ciliary beat frequency by 3 concentrations of butyl isocyanate (300 µg/ml (3 mM) (A), 100 µg/ml (1 mM) (B), and 10 µg/ml (.1 mM) (C)) with time. Each concentration is represented by 4 different cultures. Each data point represents the mean +/- 1 SD from 10 randomly selected cells.

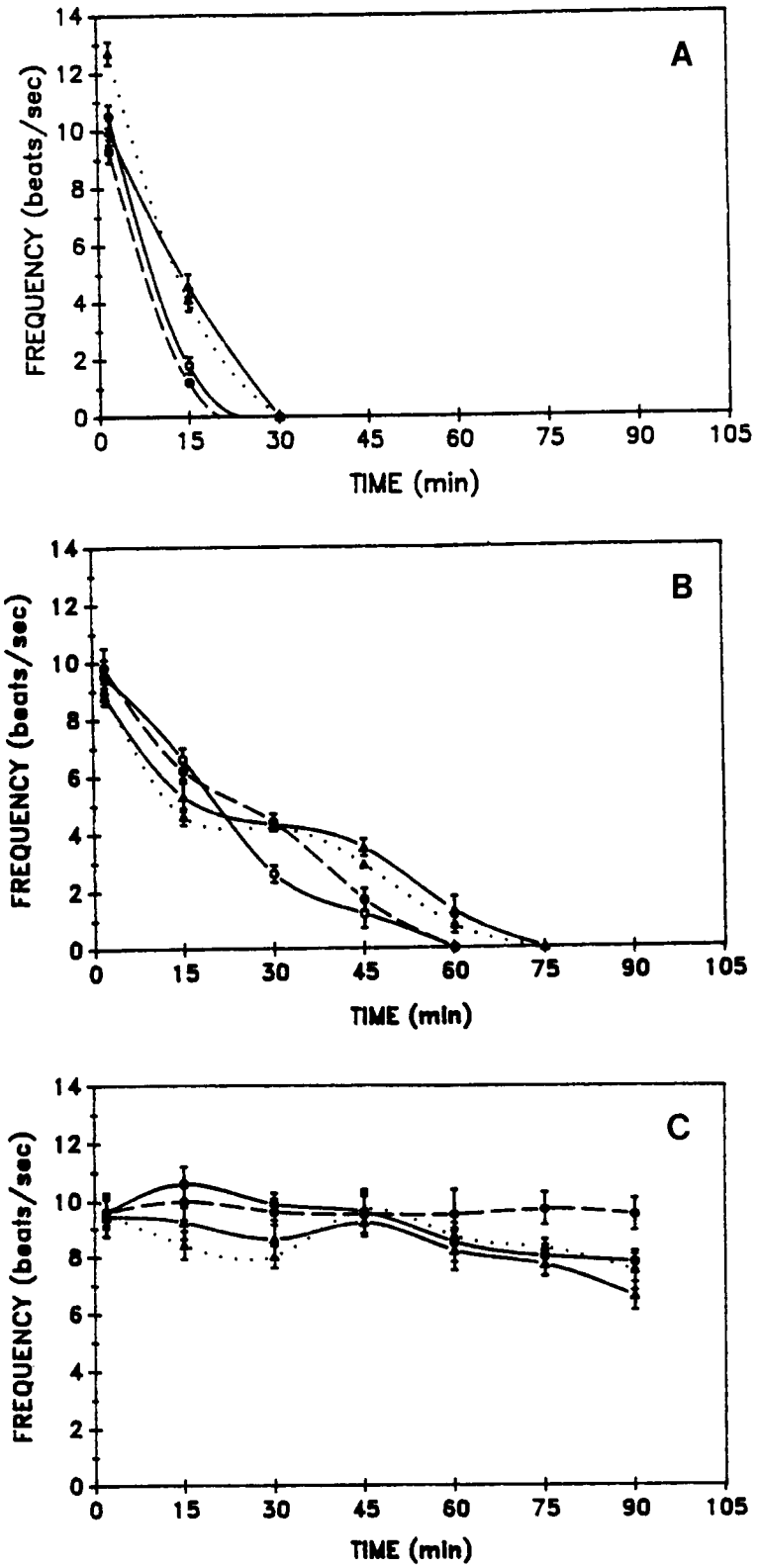

A
B
C
FREQUENCY (beats/sec)
TIME (min)
0
2
4
6
8
10
12
14
0
15
30
45
60
75
90
105

Figure 13.

ciliary frequencies for the 3 concentrations of butyl isocyanate tested is shown in Figure 14. All 3 concentrations resulted in significantly different mean ciliary beat frequencies ($p<0.01$) at various time intervals. Butyl isocyanate at a concentration of 300 μg/ml (3 mM) resulted in a drop in frequency from 10.4 beats/sec to zero within an average time of thirty minutes exposure. A concentration of 100 μg/ml (1 mM) resulted in ciliostasis also, but at a slower rate (75 min). Both concentrations, 300 μg/ml (3 mM) and 100 μg/ml (1 mM), resulted in statistically significant decreases in mean ciliary frequencies ($p<0.01$) within fifteen minutes exposure. The lowest concentration of butyl isocyanate, 10 μg/ml (0.1 mM) showed a slight decrease in ciliary beat, with a statistically significant decrease occurring at sixty minutes exposure.

Cultures were exposed to a range of concentrations of benomyl and butyl isocyanate beyond those described above. These concentrations ranged from 200 μg/ml (0.68 mM) to 1 μg/ml (0.0033 mM) for benomyl and 200 μg/ml (2 mM) to 1 μg/ml (0.01 mM) for butyl isocyanate. Ciliary beat frequencies were also measured at each concentration. Dose-dependent decreases in CBF were seen over the full ranges tested for both compounds. The data from all of these concentrations were used, in conjunction with the data presented previously to perform a probit analysis on dose. A probit transformation adjusts the ciliary beat frequencies to an assumed normal distribution. With the use of Statistical Analysis System (SAS), maximum-likelihood estimates of the slope, intercept, and threshold response rates were calculated. This data is presented graphically as dose vs. probability value (decrease in CBF). Graphs presented in Figures 15 and 16 represent CBF data collected at the forty-five minute measurement interval.

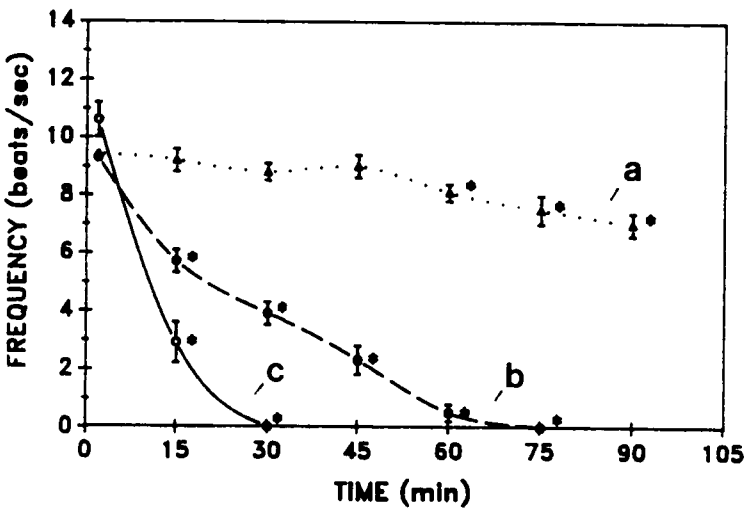


Figure 14. Comparison of the inhibitory effects of butyl isocyanate. Comparison of the inhibition of ciliary beat frequency by 3 concentrations of butyl isocyanate (a, 10 μg/ml (.1 Mm), b, 100 μg/ml (1 Mm), and c, 300 μg/ml (3 mM)) with time. Each data point represents the mean +/- 1 SD from forty cells. (*)Indicates a significant difference in CBF compared to baseline (T=0) using a Student T-test, $p<0.01$.

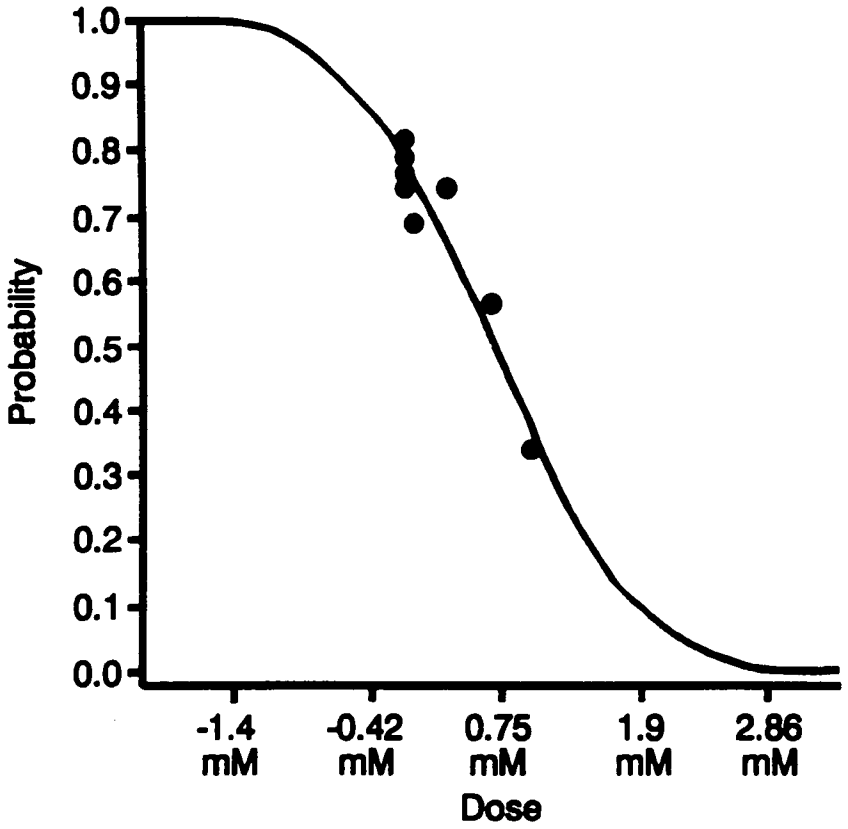


Figure 15. Log-probit analysis dose-response curve for benomyl. Data points used to generate the dose-response curve are the CBF means from 8 different concentrations of benomyl at T=45 min. (χ^2=0.9466). The ED_{50} for inhibition of ciliary beat frequency is 0.75 mM (224 μg/ml).

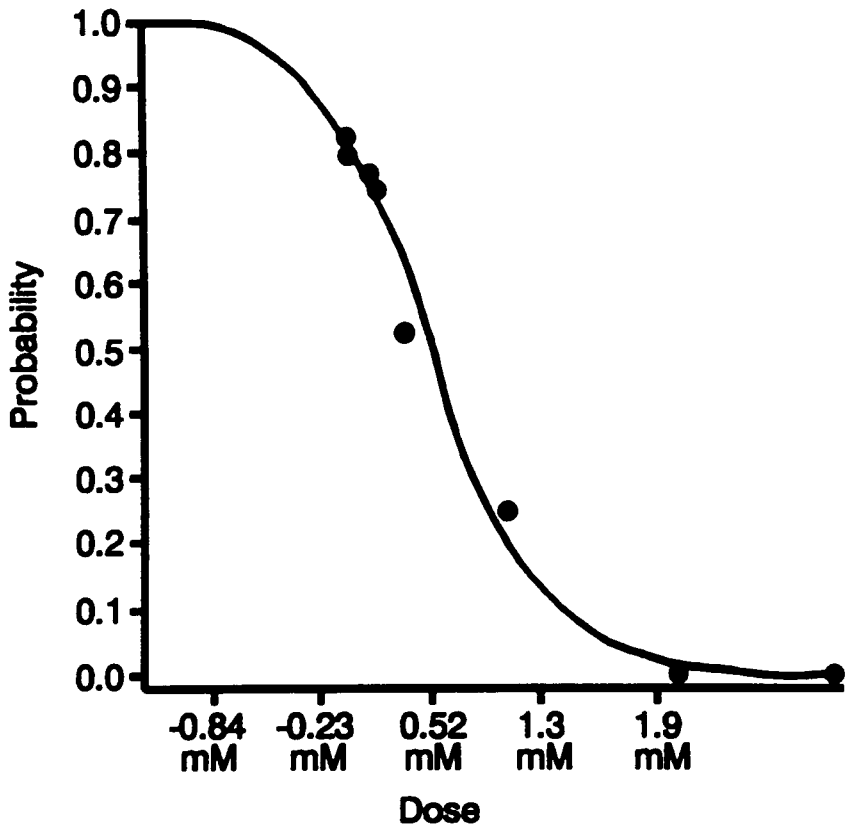


Figure 16. Log-probit analysis dose-response curve for butyl isocyanate. Data points used to generate the dose-response curve are the CBF means from 8 different concentrations of butyl isocyanate at T=45 min. (χ^2=0.8530). The ED_{50} for inhibition of ciliary beat frequency is 0.52 mM (52 μg/ml).

This measurement interval was chosen from the probit analysis due to high chi-squares (goodness of fit) for both benomyl (χ^2=0.9466) and butyl isocyanate (χ^2=0.8530). Figure 15 shows the curve resulting from the probit analysis over several concentrations of benomyl. From this analysis, the ED_{50}'s, (effective dose 50) for the inhibition of ciliary beat frequency was determined. This is the dose which caused a 50% reduction in ciliary beat frequency during a ninety minute exposure to benomyl. The ED_{50} was obtained by drawing a horizontal line from the probability value 0.5, which is the fifty percent CBF point, to the dose-response curve. At the point of intersection a vertical line was drawn intersecting the abscissa at the ED_{50} point. For benomyl, the ED_{50} obtained was 0.75 mM (224 μg/ml). Figure 16 shows the curve resulting from the probit analysis on several concentrations of butyl isocyanate. For butyl isocyanate, the ED_{50} obtained was 0.52 mM (52.5 μg/ml).

The estimations of the ED_{50}'s indicate the concentrations of benomyl or butyl isocyanate that would be expected to cause a fifty percent reduction in ciliary beat frequencies in cells exposed for ninety minutes. The ED_{50} for benomyl obtained was slightly higher than that of butyl isocyanate. In comparing the ED_{50} for benomyl and butyl isocyanate, one-third more benomyl is required to produce the same response caused by butyl isocyanate. In essence, butyl isocyanate was more potent that benomyl in reducing ciliary beat frequencies of tracheal epithelial cells. This assumes, however, that both compounds were absorbed into the cells at an equal rate.

The frequency data also indicated that the rate of ciliary frequency decrease was similar for equal molar concentrations of both benomyl, 1 mM (300 μg/ml), and butyl

isocyanate, 1 mM (100 μg/ml), over ninety minutes exposure (Figures 11 and 14). A similar rate of ciliary frequency decrease was observed for 0.033 mM (10 μg/ml) benomyl and 0.1 mM (10 μg/ml) butyl isocyanate (Figures 10 and 11), although the molar concentrations differed by nearly an order of magnitude.

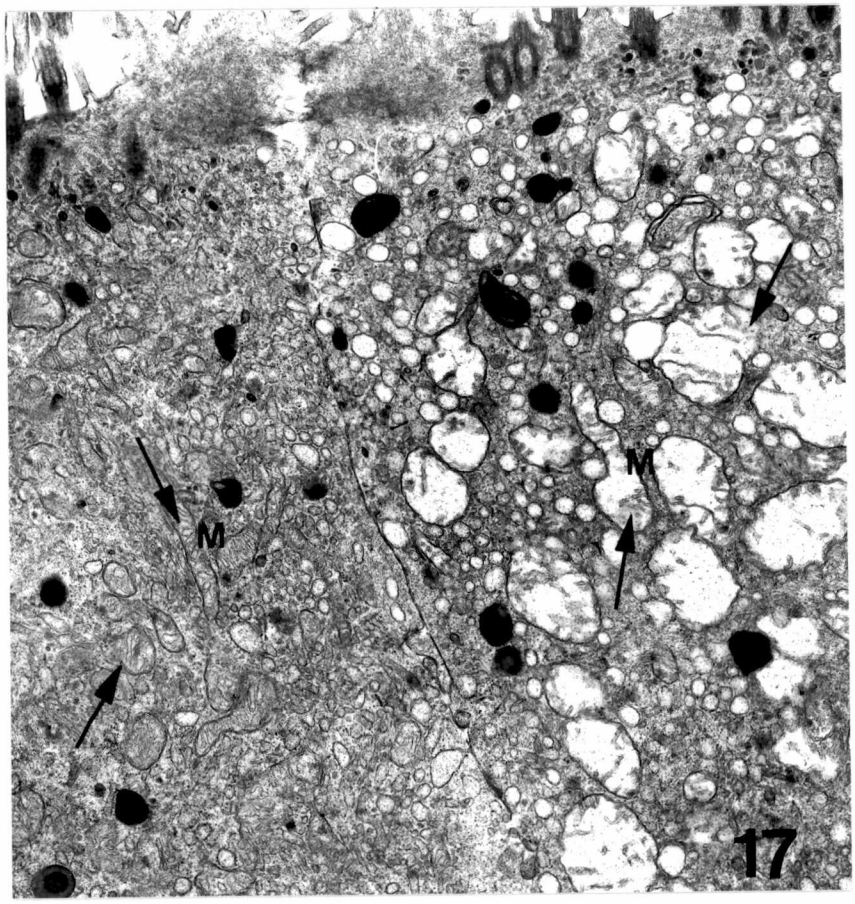
Ultrastructural Changes

Ciliated outgrowth cells exposed to 300 μg/ml (1 mM) benomyl showed a complete inhibition of ciliary beat within ninety minutes. A temporal difference was seen between outgrowth and explant cells, as explant cells generally did not show a decrease in frequency until after several hours. Therefore, outgrowth cells were examined after 2 hours exposure while explant cells were examined after 4 hours exposure. Ultrastructural examination of explant cells showed a marked swelling of mitochondrial, disruption of cristae, and loss of mitochondria matrix after 4 hours exposure to 300 μg/ml (1 mM) benomyl (Figure 17). Differences in magnitude of damage to mitochondria could be seen between adjacent explant cells. Some appeared only slightly swollen, while others showed extreme swelling with complete loss of matrix material. Similar swelling of mitochondria was also seen in outgrowth cells (Figure 18). A comparison of mitochondria from control cells (Figure 19) with benomyl treated cells (Figures 20 and 21) illustrates the magnitude of damage to mitochondria in cells in which ciliostasis had occurred. There also appeared to be an increase in smooth endoplasmic reticulum and other vesicles within the cytoplasm of the benomyl treated cells (Figures 19, 20 and 21), although this apparent increase is difficult to quantitate.

Plate IV. Ciliated Epithelial Cells Exposed to Benomyl

Figure 17. Portions of two adjacent explant cells exposed to benomyl (1 mM) for 4 hours. These cells show varying degrees of damage to mitochondria (M). The cell on the left shows distention of inner mitochondrial membranes (arrows) and slight swelling of mitochondria. The cell on the right shows more extensive swelling of mitochondria (M), complete separation of mitochondrial membranes (arrows), and loss of mitochondrial matrix. X 10,000.

Figure 18. Portion of an outgrowth cell exposed to benomyl (1 mM) for 2 hours. Similar swelling of mitochondria (M) was seen. Endoplasmic reticulum (ER) and nucleus (N) appear normal. X 10,000.


M
M
17

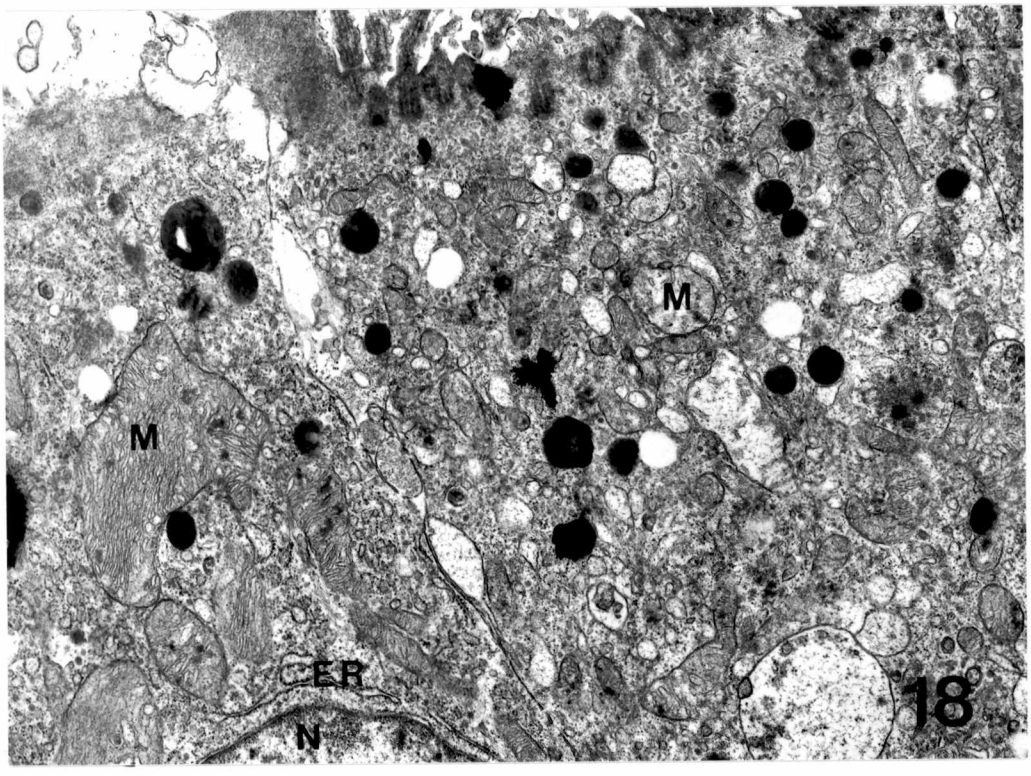
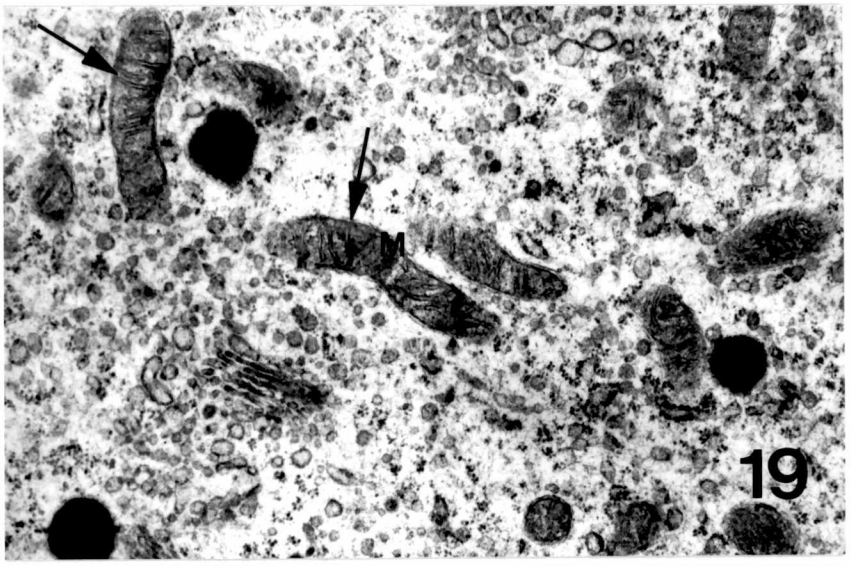

M
M
ER
N
18

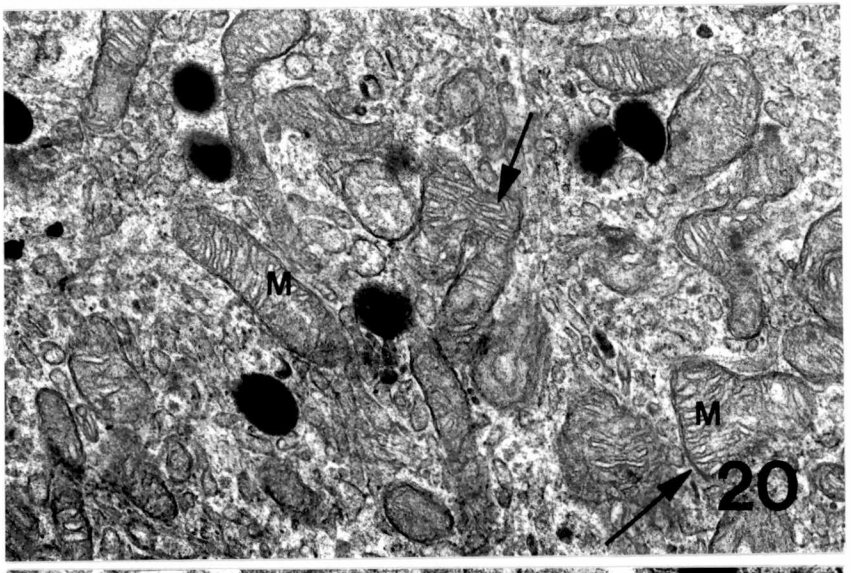
Plate V. Comparison of Mitochondrial Structure of a Control Epithelial Cell and Benomyl Treated Epithelial Cells

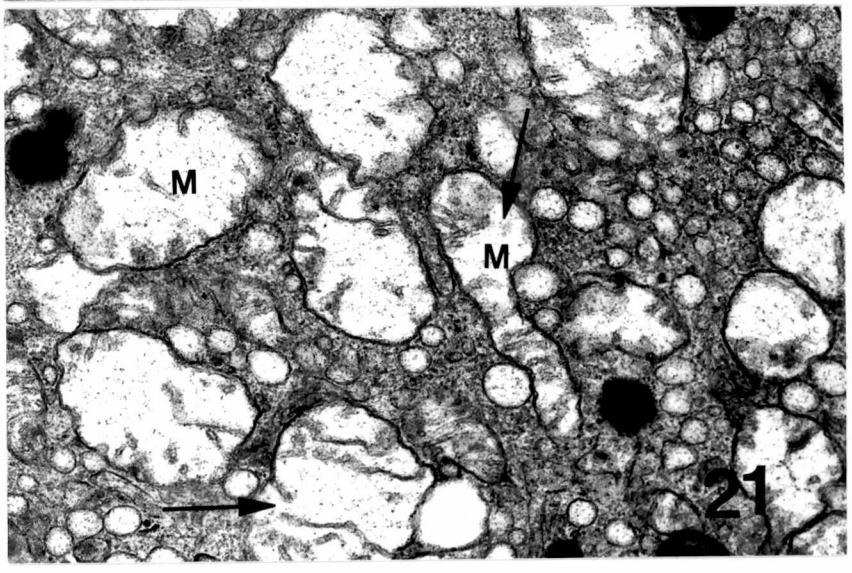
Figure 19. Mitochondria (M) of a control explant ciliated epithelial cell. Structure is normal with inner mitochondrial membranes generally at right angles to the long axis of the mitochondria (arrows). X 20,000.

Figure 20. Intermediate damage of mitochondria (M) of an explant ciliated epithelial cell exposed to benomyl (1 mM) for 4 hours. Distention and separation of inner mitochondrial membranes is evident (arrows). Mitochondrial matrix appears slightly washed out. X 20,000.

Figure 21. Extensive damage of mitochondria of an explant ciliated epithelial cell (1 mM) exposed to benomyl for 4 hours. Mitochondria (M) appear grossly enlarged with separation of inner mitochondrial membranes (arrows). Mitochondrial matrix is nearly completely washed out. X 20,000.


M
19


M
M
20


M
M
21

Similar ultrastructural damage observed in benomyl treated cells was not seen in either outgrowth (Figure 22) or explant cells (Figure 23) exposed to butyl isocyanate. Exposure to 100 μg/ml (1 mM) butyl isocyanate (Figure 22) resulted in ciliostasis within an average time of thirty minutes in outgrowth cells. Again temporal differences in reduction in CBF were seen between outgrowth and explant cells. Both cell types were examined after 1 hour exposure. This time was chosen because exposure greater than 1 hour caused cellular membrane damage and generalized cell swelling possible precluding any determination of subcellular organelle damage. Conclusive evidence suggesting damage to mitochondria was not seen with exposure to butyl isocyanate. Neither severe swelling of mitochondria nor loss of mitochondrial matrix material seemed to occur. Figure 24 shows a photomicrograph of an explant cell treated with butyl isocyanate at high magnification, identifying normal mitochondrial appearance. Ciliary structure also appeared normal as did other cellular organelles. These differences in ultrastructural appearances suggest that butyl isocyanate affects ciliated epithelial cells differently than benomyl.

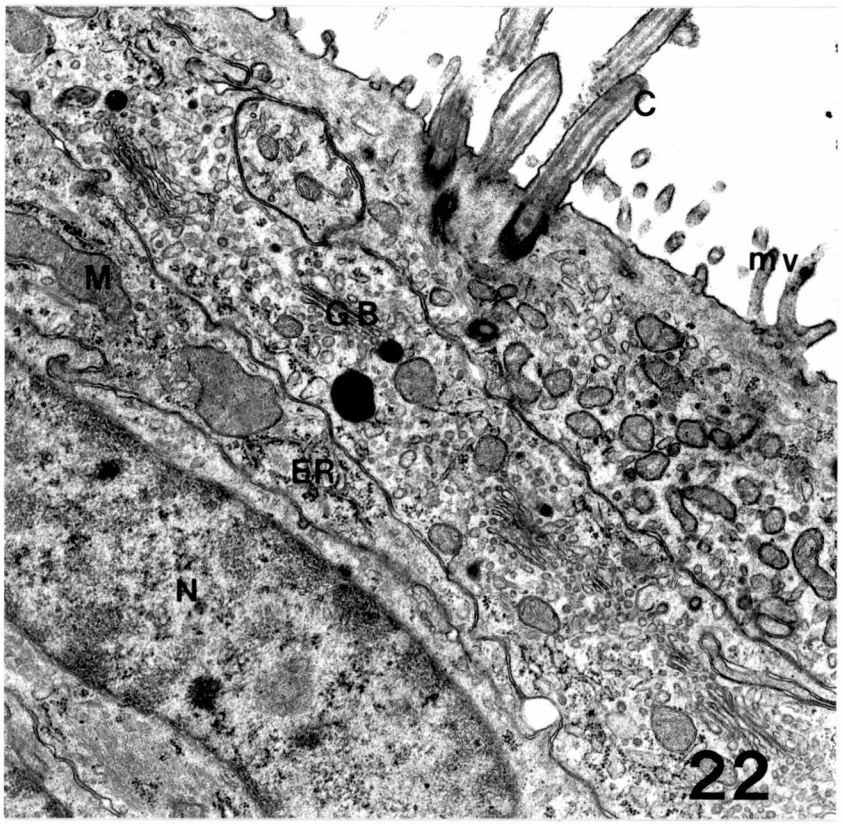
These electron microscopy studies supported the ciliary frequency data which indicated a toxic response of ciliated tracheal epithelial cells to benomyl. The site of action appears to be on the mitochondria, perhaps disrupting the normal functioning of these organelles resulting in a drop in intracellular ATP levels and manifested as a decrease in ciliary beat frequency. These same conclusions could not be made in the case of butyl isocyanate exposure. Although toxicity had occurred as measured by the drop in ciliary beat frequency, ultrastructural evidences did not indicate site of activity.

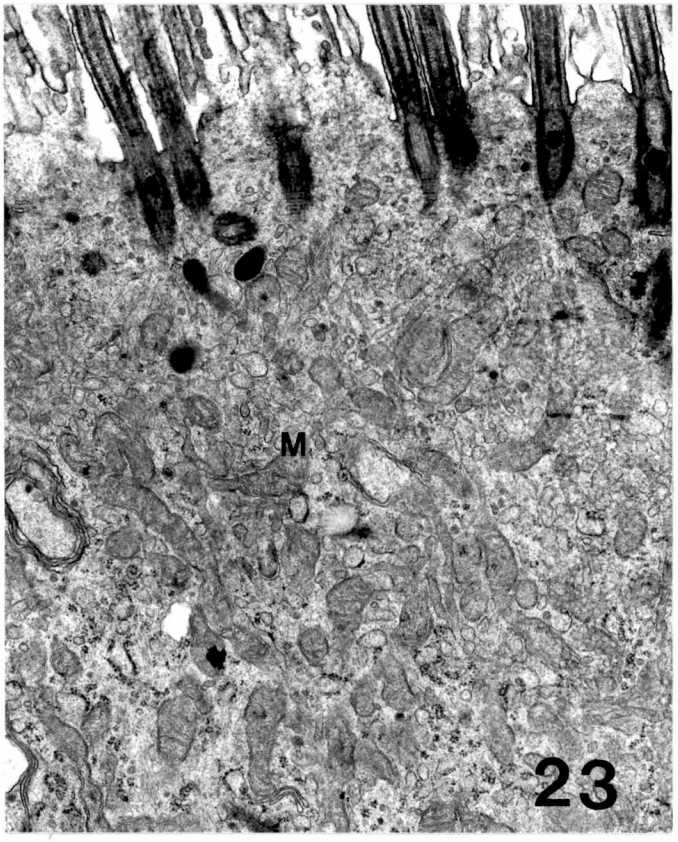
Plate VI. Ciliated Epithelial Cells Exposed to Butyl Isocyanate.

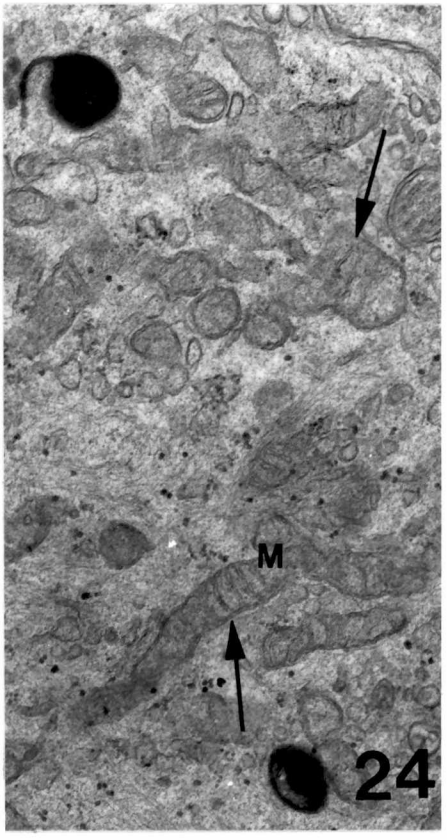
Figure 22. Ciliated outgrowth cell exposed to butyl isocyanate (1 mM) for 1 hour. Mitochondria (M) appear normal as do golgi bodies (GB), granular endoplasmic reticulum (ER), nucleus (N), and cilia (C). Also, microvilli (mv), and endoplasmic reticulum (ER) appear normal. X 10,000.

Figure 23. Ciliated explant cell exposed to butyl isocyanate (1 mM) for 1 hour. Again all organelles appear normal, with mitochondria (M) located in the apical portion of the cell. X 10,000.

Figure 24. High magnification of mitochondria from a ciliated explant cell exposed to butyl isocyanate (1 mM) for 1 hour. Mitochondria (M) appear normal as do the inner mitochondrial membranes (arrows). X 20,000.


C
mv
M
GB
ER
N
22


M
23


M
24

CHAPTER 4

DISCUSSION

Benomyl toxicity has been studied for many years due to its widespread agricultural and domestic use. Benomyl is commercially applied primarily through aerial field spraying, thereby presenting a possibility for inhalation and/or dermal exposure to field workers (Warheit et al., 1990). In-home use of benomyl requires it to be aerosolized for application. Because benomyl in suspension remains largely intact at recommended dilutions for spray applications (Upham and Delp, 1973), this method of application also presents a probable inhalation exposure route.

The ciliated and non-ciliated cells of the upper respiratory tract make up the mucociliary escalator, an important host defense system. This system is responsible for trapping inhaled particulate matter and chemicals suspended in air in the mucus on the epithelial surface. Ciliary action propels the mucus layer towards the pharynx, where it is swallowed or expectorated, removing any trapped materials from the epithelial surface (Sleigh et al., 1988). Thus, the ciliated tracheal epithelium is a potential target for the deposition of environmental pollutants (Schumann et al., 1988). The failure of this specialized function may enhance respiratory disease by permitting inhaled noxious agents to reach deeper surfaces and affect cells within the lower respiratory tract (Ballanger, 1988).

Tissue culture techniques have been useful in toxicity studies in that they allow a comparison to be made between the responses of animal and human cells under more

controllable experimental conditions (Mohr and Emura, 1985). Tracheal explant cultures have been considered to be valuable in studying the effects of inhaled substances because they contain the target tissue of interest, tracheal epithelium (Gabridge et al., 1982) and they show the sensitivity of the mucociliary system to insult and provide a means for ranking such insults (Wolff, 1986). Ciliated epithelial cell cultures have been previously used to study a variety of potentially respirable toxicants. Gabridge et al. (1982) and Gabridge and Meccoli (1982) used tracheal rings to study the relative cytotoxic and ciliotoxic effects of heavy metals, while Porter and Matrone (1977) used similar techniques to study interactions of combinations of metals. Bemis and Kennedy (1981) used rabbit tracheal epithelial cell cultures to study the effects of Bordetella bronchiseptica on ciliary activity. A more recent study by Duckett et al. (1986) reported the dose-dependent ciliotoxic effects of gossypol on ciliated tracheal epithelial cells.

Because of the proven effectiveness of the tracheal explant and outgrowth system for studying respirable toxicants, this study employed canine tracheal epithelial cultures. The system provided a highly sensitive and reliable method for measuring the toxic effects of benomyl and its breakdown products, carbendazim and butyl isocyanate, on the structure and function of ciliated epithelial cells. Typical commercial applications of benomyl for plant protection from fungi contain between 100 to 200 μg/ml of active benomyl (Upham and Delp, 1973). These same concentrations were included in the range of concentrations examined in this study to evaluate the ciliotoxic potential of benomyl and its breakdown products.

Most fungicides act by inhibition of non-specific enzymatic processes (Klaassan et al., 1986). Since the fungitoxic component of benomyl, carbendazim, is only toxic to growing fungal cells this suggests that it is affecting anabolic rather than catabolic phases of metabolism (Clemons and Sisler, 1791). The mode of action of benomyl in fungi is the inhibition of mitosis and cytokinesis by the breakdown product carbendazim via binding to and preventing tubulin polymerization (Hammerschlag and Sisler, 1972). Although the affinity of benomyl and carbendazim for mammalian tubulin is considered to be low (Davidse and Flach, 1977; Ireland et al., 1979), both have been shown to prevent polymerization of bovine tubulin *in vitro* when large concentrations are present (Friedman and Platzer, 1978). However, due to the short duration in which decrease in ciliary function by benomyl or carbendazim is seen, microtubule inhibition should not be considered a mode of toxicity to tracheal epithelial cells in culture. Furthermore, ultrastructural analysis indicated no alteration of axonemal microtubules at the time of ciliostasis. Thus, it is doubtful that benomyl affects non-target tissues such as tracheal epithelial cells by blocking microtubule function as it does in fungi.

The molecular structures of benomyl and carbendazim contain the benzimidazole moiety. Benzimidazoles are known uncoupling agents of oxidative phophorylation (Terada, 1990). Jones and Watson (1967) showed that a variety of 2-triflouromethyl-benzimidazole compounds uncoupled oxidative phophorylation in rat liver mitochondrial preparations. They correlated the uncoupling activity with the acidity of the imidazole -NH group at the 1- position. Common features of uncouplers of oxidative phosphorylation are: 1) an acidic dissociable group; 2) a strong electron withdrawing

moiety; and 3) bulky hydrophobic group(s) (Terada, 1990). Benomyl would not be expected to be an uncoupling agent due to the butylcarbamoyl substitution at the 1-position (see Figure 1). Also, Clemons and Sisler (1971) reported that carbendazim would not be expected to be an uncoupling agent due to the fact that it is not substituted at the 2-position with a strong electrophilic group. However, slight but significant decreases in CBF were observed with exposure to carbendazim for up to six hours. Perhaps carbendazim is in fact a weak uncoupling agent of oxidative phosphorylation resulting in a drop in intracellular ATP levels essential for ciliary motility. The slow rate of decrease in CBF may be due to the low rate of penetration of carbendazim into epithelial cells. This interpretation would be consistent with the hypothesis of Clemons and Sisler (1969) that carbendazim failed to penetrate cells as readily as benomyl. Studies of Upham and Delp (1973) support this hypothesis when they showed less plant penetration with carbendazim than benomyl, indicating improved performance of benomyl over carbendazim as a fungicide.

The differential effects on ultrastructure seen with exposure to benomyl in part support a hypothesis of uncoupling of oxidative phophorylation. The primary organelle affected in benomyl treated cells appeared to be mitochondria. Benomyl exposure resulted in varying degrees of swelling of mitochondria, distention of inner mitochondrial membranes, and loss of mitochondrial matrix. These results are in contrast to those reported by Richmond and Pring (1971) for the fungus Botrytis fabae. While mitochondria in that organism exhibited looped cristate membranes after exposure to sublethal concentrations of benomyl (0.1 to 0.3 μg/ml), the authors did not attribute this effect to benomyl.

Because of the extensive damage to mitochondria and the precipitous decline in CBF in ciliated tracheal epithelial cells exposed to benomyl, it appears that benomyl or one of its breakdown products does indeed affect metabolism. This may be due to a physical disruption of inner mitochondrial membrane integrity leading to an alteration of functionally associated membrane bound biochemical processes (Woods and Fowler, 1986). This disturbance of mitochondrial structure may have resulted in an inability of ciliated cells to manufacture ATP. The energy supplied by ATP is essential for ciliary motility. A decrease in intracellular ATP levels may be correlated with a drop in ciliary beat frequency. Gabridge et al. (1982) reported that a decrease in total ATP content directly resulted in a loss of ciliary motility in tracheal epithelial rings exposed to metal salts. It has also been suggested that a prolonged lack of intracellular ATP could reduce ion pump efficiency necessary to maintain normal intracellular osmotic and ionic pressures (Gabridge and Meccoli, 1982). Mitochondrial damage and ciliary inhibition similar to that seen for benomyl exposed cells has also been observed in ciliated tracheal epithelial cells exposed to gossypol (Duckett et al., 1986). Schultz and Kennedy (1976) suggested that the mitochondrial swelling seen in Daphnia pulex with exposure to 3-amino-1,2,4-triazole, resulted in inhibition of enzyme reactions which lead to the breakdown of osmoregulatory mechanisms due to lack of ATP.

Bourgois et al. (1977) reported that benomyl caused the fragmentation of granular endoplasmic reticulum in Fusarium oxysporum. Richmond and Pring (1971) also noted this same effect in Botrytis fabae and they suggested that this ultrastructural alteration may result in the disruption of essential metabolic activity controlling the direction of

overall cellular activities. This type of ultrastructural alteration was not seen in ciliated epithelial cells exposed to benomyl.

It has recently been reported that a second breakdown product of benomyl may be the toxic component rather than carbendazim. Chiba et al. (1987) suggested that the activity of benomyl in yeast came from butyl isocyanate after benomyl degraded to butyl isocyanate and carbendazim. Isocyanates are very water reactive and degrade to form CO_2 and the appropriate amine, n-butylamine in the case of butyl isocyanate (Hammerschlag and Sisler, 1973). Axness and Fleeker (1979) studies the metabolism of benomyl in rats after oral feeding, with radioisotope labeling on the butylcarbamoyl moiety. They found that a large portion of radioisotopes were excreted in the urine, suggesting the formation of metabolites reacting with the intact butylcarbamoyl moiety. This demonstrated that the butylcarbamoyl moiety of benomyl, from which butyl isocyanate originates, is reactive and complexes with metabolites *in vivo*. This type of reaction was earlier proposed by Hammershlag and Sisler (1973) in which they suggested that butyl isocyanate may react with cells or certain metabolites instead of water to form various other deleterious products. The fact that the highly reactive compound, butyl isocyanate resulted from the breakdown of benomyl suggests that benomyl may be a broad spectrum toxicant in non-target organisms (Torstensson and Wesson, 1984).

Butyl isocyanate is an alkyl isocyanate. Alkyl isocyanates as a group are highly reactive to a variety of functional groups such as amines, thiols, alcohols, carboxyl groups (Axness and Fleeker, 1979) as well as, sulfhydryl and imidazole groups (Brown and Wold, 1979b). Hermans (1990) suggested that butyl isocyanate, being an electrophile,

could react with nucleophilic sites in biological macromolecules such as amino ($-NH^2$), hydroxyl (-OH), and sulfhydryl (-SH) groups of proteins. For example, Krupka (1974) suggested that butyl isocyanate, resulting from the breakdown of benomyl, inhibited acetylcholinesterase by binding to the enzymes active site. Koller et al. (1982) reported that butyl isocyanate inhibited fungal cutinase, an important enzyme for fungal penetration into herbaceous plants. Brown and Wold (1973a) reported that alkyl isocyanates were highly specific active-site reagents for serine proteases. Chymotrypsin, trypsin, and elastase all contain an active-site serine. They reported that butyl isocyanate while having no effect on trypsin, inactivated both chymotrypsin and elastase in a site specific manner. Furthermore, structurally similar molecules to butyl isocyanate, isothiocyanates such as 4-bromophenylisothiocyanate, have been found to be uncouplers of oxidative phosphorylation (Terada and Kubota, 1979). Terada and Kubota (1979) reported that many hydrophobic isothiocyanates were reactive with non-proteinous sulfhydryl and amino groups forming weakly acidic dithiocarbamates, which were uncoupling agents probably by disruption of the proton gradient. Mailmen et al. (1972) reported that growth of Saccharomyces cerevisiae was inhibited by benomyl, an effect which may be attributed to butyl isocyanate, but this inhibition was reversed upon the addition of cysteine and other thiols of the structure $R\text{-}CH_2\text{-}SH$. These compounds probably formed complexes with butyl isocyanate rendering it ineffective. Perhaps the ciliostatic effects seen with exposure to butyl isocyanate were a result of the inhibition of important metabolic intermediates necessary for ciliary motility. Butyl isocyanate may react with the nucleophilic portions of enzymes such as ciliary ATPase, thus inhibiting their action. Or

further, butyl isocyanate upon reacting with certain metabolites may form complexes that are uncouplers of mitochondrial oxidative phosphorylation resulting in a drop in intracellular ATP.

Butyl isocyanate while causing an inhibition of CBF, did not cause any observable alterations in ultrastructure. This suggested that butyl isocyanate may be interacting with and inhibiting certain cellular functions important in ciliary motility at sites other than the mitochondrion or prior to any effect on mitochondria. Duckett et at. (1986) demonstrated that exposure of isolated cilia to gossypol resulted in inhibition of ciliary beat at the level of the axoneme possibly acting on axonemal ATPase. They suggested that the action of gossypol may be two fold, inhibiting ciliary ATPase as well as mitochondrial ATPase. Perhaps butyl isocyanate reacts similarly, resulting in a loss of ciliary motility. Further, the electrophilic nature of butyl isocyanate may cause it to react with nucleophilic groups on cellular membrane pores leading to a disruptions in total cell osmoregulation or perhaps blocking important ion channels such as for calcium. Ca^{+} is an important ion in the control of ciliary activity (Girard and Kennedy, 1986). This interpretation would be consistent with that of Brown and Wold (1973b) that alkyl isocyanates form reasonably stable derivatives with amino and hydroxyl groups on proteins. Exposure to butyl isocyanate for two hours resulted in a generalized disruption of cell membranes and generalized swelling of the cell suggesting a total loss of the cells' ability to osmoregulate. If in fact butyl isocyanate affects either ciliary ATPase or other axonemal components and/or important plasma membrane proteins such as ion channels it may directly shut down ciliary function without causing ultrastructural damage until

osmoregulation is also lost. The observed effect of benomyl on mitochondrial ultrastructure may result from a delayed release of butyl isocyanate after it has been carried into the mitochondrion as a part of the benomyl molecule. certainly this interpretation is plausible since butyl isocyanate inhibited ciliary function more rapidly and at slightly lower molar concentrations than benomyl.

Thus, the broad spectrum toxicity of benomyl to ciliated tracheal epithelial cells may be due primarily to butyl isocyanate with benomyl acting as a carrier for the toxic components to gain entry into cells. The role of carbendazim is less clear at this time.

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