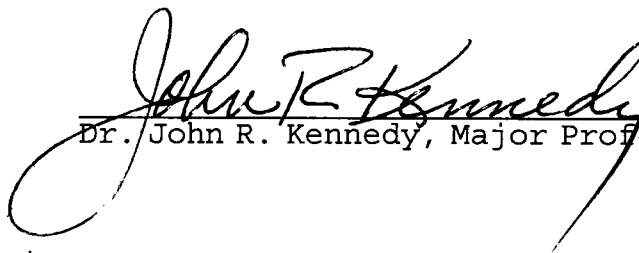
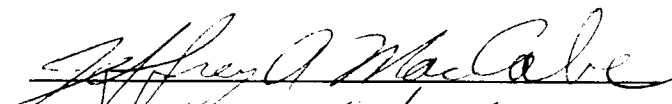
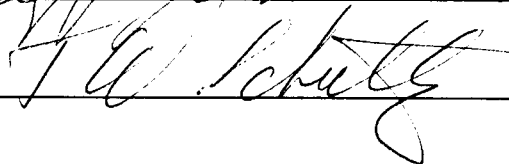


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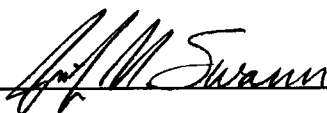

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THE FUNCTIONAL AND ULTRASTRUCTURAL EFFECTS
OF THE ORGANOPHOSPHOROUS INSECTICIDES DURSABAN AND LORSBAN
ON THE CILIATED EPITHELIUM OF THE FROG PALATE IN VITRO

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Jerilyn M. Swann

August 1994

DEDICATION

This thesis is dedicated to my parents,
Jerry and Sharon Mitchell.

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. John Kennedy, for his guidance and support throughout this study. I am fortunate to have Dr. Kennedy as a mentor in both research and classroom instruction. I would also like to thank my committee members Drs. Terry Schultz and Jeffrey MacCabe for their advice and review of the thesis. I am grateful to Mr. Rodney Bunn for his assistance with the computer graphics program. I would like to thank the Department of Zoology for providing me with a teaching assistantship and financially supporting this study. I would like to express my thanks to my parents, Jerry and Sharon Mitchell, for always believing in me and encouraging me in all my pursuits. Finally, I would like to thank my husband, Bill, for his love, support, and patience through the course of this study.

ABSTRACT

This study was conducted to assess the ciliotoxic potential of the organophosphorous insecticides Dursban and Lorsban, their active ingredient, chlorpyrifos and their carrier ingredients (blanks). The ciliated epithelium of the frog palate was used as the model for an innervated ciliated system, since chlorpyrifos inhibits nervous system acetylcholinesterase. Frog palates were excised and cultured to form ciliated outgrowths. Outgrowths were monitored with an inverted phase contrast microscope, and ciliary beat frequencies were determined using an optical spectrum analysis system. All compounds caused a decrease in frequency of ciliary beat over time. Concentration-response curves were constructed from data of time to inhibition of ciliary beating. The order of toxicity was Lorsban, Dursban, Lorsban Blank, Dursban Blank, and chlorpyrifos, with Lorsban being the most toxic and chlorpyrifos being the least toxic. EC_{50} estimations were also calculated from these data; and they followed the same order of toxicity as the time to inhibition studies. Samples were examined by electron microscopy to evaluate any ultrastructural effects. All compounds were found to cause mitochondrial damage, including swelling, disruption of cristae, and loss of matrix.

The response of individual cells was monitored

immediately after addition of test compounds. Stimulation of ciliary beating was evident immediately after exposure to all compounds. Stimulation was ultimately followed by inhibition, within a time frame (a few minutes to hours) depending on the concentration of the compound in the test medium. Dursban, Lorsban, and both blanks elicited stimulatory effects in the presence of atropine. Atropine blocked the initial stimulatory response only with chlorpyrifos. It appears that in addition to chlorpyrifos some component(s) of the inert ingredients were initially stimulatory but ultimately inhibitory to ciliary beating in the frog palate model.

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1. INTRODUCTION

Chlorpyrifos [o,o-diethyl-o-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] is an organophosphorous insecticide with widespread commercial use on field crops and household pests (Matsumura, 1985). It has a low vapor pressure (20 mm Hg) and low solubility in water (2 ppm at 25 C) (Racke, 1993), but is readily solubilized in several organic solvents including acetone and ethanol. It is combined with xylene-like aromatic solvents and emulsifiers (carrier ingredients) into two basic formulations, Dursban and Lorsban. The types and concentrations of individual components in the inactive ingredients of Dursban and Lorsban are proprietary information. Dursban 2E (an emulsifiable concentrate) is diluted either in water or in oil, while Lorsban 4E forms an emulsion when diluted in water (DowElanco Materials Safety Data Sheet). Both are used to control ants, aphids, cockroaches, grasshoppers, spiders, mosquitoes, fleas, ticks, weevils, mites, moths, and various worms. Dursban is reported to have a relatively high ratio of accumulation of residues in soil, with short residual time on foliage, and animals reportedly do not detoxify the compound (Matsumura, 1985). Racke (1993) reported that chlorpyrifos is readily taken up by the soil, with little vertical mobility. Chlorpyrifos does not erode into water systems, even when

heavy precipitation is simulated. Biotic and abiotic systems do degrade chlorpyrifos through transformation processes (Racke, 1993).

Several studies report age-related differences in susceptibility to and recovery from chlorpyrifos exposure. Long et al. (1986) applied a concentration of 2.5% chlorpyrifos dermally to the tail and umbilical region of newborn piglets. Applications made before the 30-hour mark resulted in toxicosis and death in 10 of 12 piglets; applications made after 30 hours post-birth did not cause these affects. Meyers et al. (1992), found that nestlings of red-winged blackbirds (Agelaius phoeniceus; blackbird) and European starlings (Sturnus vulgaris; starling) were both more vulnerable to chlorpyrifos than adults of the same species. This increased sensitivity of nestlings resulted in temporary (24 hr) growth depression at a concentration of 2 mg/kg body mass. Pope et al. (1991), reported that for subcutaneous injections the maximum tolerated doses of chlorpyrifos in neonatal and adult rats was 45 and 279 mg/kg, respectively. The degree of inhibition of brain cholinesterase in neonates and adults was similar, but recovery time was shorter in neonates.

Studies assessing different facets of reproduction report various vulnerabilities to chlorpyrifos exposure. Dermal exposure of Holstein bulls to Dursban 44

(43.2% chlorpyrifos), used to control lice, caused an increase in the percentage of post-thaw discards of ejaculate material from all 185 bulls in the study (Everett, 1982). Sublethal concentrations of chlorpyrifos (0.05-0.25 ppm) applied to the snail Biomphalaria alexandrina resulted in reduced egg production and egg hatchability (Ibrahim, 1992). In California grunion (Leuresthes tenuis) exposed to low concentrations of chlorpyrifos (Goodman et al., 1985), no effect was observed on percentage hatching of embryos, however fry survival was decreased in groups receiving concentrations as low as 1 ug/L. Average weights of survivors were significantly less than controls at exposures of 0.5 ug/L or more. Jarvinen et al. (1983), reported reduced growth of fathead minnows (Pimephales promelas) from chlorpyrifos concentrations as low as 0.12 ug/L for second-generation fish. Growth reduction and increased deformities in first-generation fish resulted from exposure to 2.68 ug/L. All concentrations (0.12 to 2.68 ug/L) resulted in reduced maturation of first-generation fish, and concentrations greater than or equal to 0.63 ug/L reduced reproduction significantly. They also observed 10% to 89% acetylcholinesterase inhibition at the concentrations employed. A later study by Jarvinen et al. (1988) on the same species reported that deformities and growth reduction occurred within 5 hours of exposure to a chlorpyrifos

concentration similar to a 96-hour LC_{50} value of 122.2 ug/L.

Embryotoxicity and fetotoxicity were evaluated among pregnant mice dosed with 1, 10 or 25 mg chlorpyrifos/kg/day by gavage (Deacon et al., 1980). The study reported increased maternal deaths in the 25 mg group. In the 1 mg group, a significant increase in exencephaly was observed, but not in the 10 or 25 mg groups. In a subsequent test of 0.1, 1.0 or 10 mg chlorpyrifos/kg/day, no instance of exencephaly was reported, suggesting that chlorpyrifos was not teratogenic to mice at these doses (Deacon et al., 1980). However, these concentrations did result in decreased plasma and erythrocyte cholinesterase activity.

Reports vary as to the genotoxic potential of chlorpyrifos, due most likely to species variation and concentrations tested. Patnaik et al. (1992), determined that Durmet (20% chlorpyrifos) at $5 \times 10^{-6}\%$ was genotoxic to Drosophila germ cells and somatic cells. Benova et al. (1989) found that 30.5 mg/kg of Dursban (chlorpyrifos), half of the oral LD_{50} , caused a significant increase in micronucleated polychromatic erythrocytes in mice bone marrow. A 25% LD_{50} dose caused no mutagenesis in this assay. No alteration of sister chromatid exchange was observed when chick embryos were exposed to an LD_{50} of 1500 ug/embryo or when Chinese hamster ovary cells were exposed to 1, 10 or 100 ug chlorpyrifos/ml (Muscarella et al., 1984). However,

increased frequencies of sister chromatid exchanges were observed in cultures of mouse spleen cells after exposure to 0.5-4.0 ug Dursban/ml (Amer and Aly, 1992). Mostafa et al. (1983), reported increased alkylation of DNA and RNA in the liver of mice intraperitoneally injected with 5-15 mg radiolabeled chlorpyrifos/kg body mass.

The most generally accepted mode of action of chlorpyrifos is acetylcholinesterase inhibition (Abou-Donia and Lapadula, 1989). Intraperitoneal injection of 0.2 mmol chlorpyrifos/kg body weight into male Wistar rats caused significant decreases in brain, plasma, red cell, and retinochoroid cholinesterases (Yoshikawa, 1990). Cortical and striatal cholinesterase inhibition in rats was observed up to 6 weeks after treatment of a single dose of 279 mg/kg subcutaneously (Pope et al., 1992). Similar cholinesterase inhibition in rat brains was reported (McCollister et al., 1974) at oral doses of 3 mg/kg/day. The same study reported decreased plasma cholinesterase in dogs at doses of 0.1-3 mg/kg/day, for erythrocyte cholinesterase at 3 mg/kg/day, and for erythrocyte and brain cholinesterase at 3 mg/kg/day. [Cholinesterase inhibition was found to be reversible when chlorpyrifos diets were replaced with diets containing no chlorpyrifos.] Yeary (1984) also observed plasma cholinesterase inhibition (50%) in dogs fed chlorpyrifos in a mixture of fertilizers and herbicides. A

study of hens exposed to single oral doses of 60-90 mg chlorpyrifos per kg showed significant acetylcholinesterase inhibition (Capodicasa et al., 1991).

Organophosphorous insecticide inhibition of acetylcholinesterase prolongs the action of acetylcholine at cholinergic synapses (Sattelle and Lummis, 1986). In higher animals, organophosphorous insecticides cause delayed neurotoxicity, where ataxia and paralysis occur 1-2 weeks after exposure. Histologically, demyelination occurs in the central and peripheral nervous systems (Abou-Donia and Lapadula, 1989). Rotorod tests on rats exposed prenatally to 0.3 mg chlorpyrifos/kg confirmed behavioral neurotoxicity on postnatal days 3, 10, 12 and 16, which the authors conclude resulted from the repeated prenatal exposures (Muto et al., 1992). Acute exposure to chlorpyrifos (279 mg/kg, subcutaneous injection) resulted in reduced locomotor activity in rats, with normal function returning within 2 days after treatment (Pope et al., 1992). Capodicasa et al. (1991) used hens to model the human neurological response to chlorpyrifos. Single, high oral doses of 60-90 mg/kg (4-6 times LD₅₀) caused delayed polyneuropathy. A recent report (Kaplan et al., 1993) documents sensory neuropathy in 8 humans resulting from environmental exposure to chlorpyrifos (Dursban). All exposures were either dermal or inhaled and followed commercial fumigation of the home or

workplace. Symptoms ranged from nausea, muscle cramps, numbness, paresthesias, urinary frequency, and lacrimation to relatively long-term memory impairment, slowed cognition, and reduced intellectual functioning. Most patients experienced full recovery within 6 months to 1 year. Kaplan et al. (1993) conclude that chlorpyrifos (Dursban) should be handled carefully and its safety should be reevaluated.

The ciliated epithelium which lines most of the respiratory tract functions to trap inhaled particles and remove them from the airway (Nadel et al., 1979). This is a major element of the respiratory tract's defense system (Puchelle et al., 1984). Risk of infection may be elevated if there is decreased action of the cilia, since potentially harmful material may reside longer inside the airway (Adalis et al., 1978). Several studies have reported decreased mucociliary clearance after exposure to a variety of airborne pollutants (Wolff, 1986). Cultures of ciliated tracheal epithelium have been used to study regulatory mechanisms and the effects of toxic agents on ciliary action. It has been argued that, while in vitro studies can not model the entire in vivo response, they are extremely useful as a screen to assess the relative toxicities of chemicals from which compounds may be selected for the more involved, more expensive in vivo studies (Adalis et al., 1978; Romet et al., 1990; Blanquart et al., 1991). Palatine tissue from the frog

is ciliated and has also been used to study the regulatory mechanisms of ciliary motility (Maruyama et al., 1983; Aiello et al., 1991; Hernandez and Aiello, 1992). Unlike tracheal ciliated epithelium, frog palate ciliary beating is regulated by the central nervous system via cholinergic nerves branching and extending directly to the palate (Seo, 1931; Lucas, 1935; Slaughter and Aiello, 1982; Chu and Kennedy, 1994). Ciliated cells from the frog palate were found to be directly stimulated by acetylcholine nerve endings in vitro (Chu and Kennedy, 1994) just as they are in vivo (Hernandez and Aiello, 1992). It is for this reason that the frog palate is a good model for studying the effects of chlorpyrifos, a known acetylcholinesterase inhibitor.

This study was undertaken to evaluate the functional and ultrastructural effects of Dursban and Lorsban on the ciliated epithelial cells of the frog palate in vitro. The two main components of both insecticides, chlorpyrifos and individual inert ingredients, as well as the complete formulations of Dursban and Lorsban, were evaluated in concentration-response studies, with 90% inhibition of ciliary beating as the endpoint. EC_{50} values were estimated from the concentration-response studies. Transmission electron microscopy was used to evaluate the ultrastructural effects of these compounds on the ciliated epithelium.

2. MATERIALS AND METHODS

Culture Preparation

Rana pipiens was obtained from Carolina Biological Supply. The brain and spinal cord were pithed and the palate excised and placed in Amphibian culture medium (Wolf and Quimby, Gibco) supplemented with 10% NuSerum (Collaborative Research) and 0.15cc Gentamicin (Sigma) per 100 ml of media. The tissue was cut into 1-2mm square explants and placed connective tissue-side down in 35mm diameter plastic culture dishes with 0.2ml of medium and allowed to attach. Ciliated epithelial cells migrated outward within 24 hours and outgrowth continued for 2-3 weeks. Cultures were incubated in an humidified room-temperature incubator. Culture medium was changed every 2 days once attachment had occurred. Culture age ranged from 3-8 days at the beginning of all experiments.

Quantitation of Ciliary Beating

Ciliary beat frequency was measured using the method described by Kennedy and Duckett (1981). A culture dish with the epithelial outgrowth to be studied was placed on the stage of an inverted phase contrast microscope (Nikon Diaphot). A television camera (RCA Model #TC1005) was positioned in parallel with the microscope eyepieces and the

image of the beating cilia was displayed on a television monitor (RCA Model #TC1112) and when necessary, recorded with a video cassette recorder (Panasonic Model #AG1950).

A photomultiplier (Oriel Model #EN6225) with a limiting aperture in front of the photo cell was placed in front of the TV monitor over the image of the beating cilia and the analog signal was processed with a frequency spectrum analyzer (Hewlett Packard Model #3582A). The analog signal was converted by a Fast Fourier transform into a frequency spectrum and displayed on an oscilloscope screen. The frequency analyzer updated the spectrum every half second, with the major peak corresponding to the dominant frequency of the cilia.

Concentration-Response Curves

Ciliary beat frequency was monitored at varying time intervals after exposure for each of the five compounds tested. Once percent motility fell to 10% or less, the experiment was terminated. Ninety percent inhibition of ciliary beat was therefore the predetermined endpoint. Times to inhibition of ciliary beating were recorded and from these data concentration-response curves were constructed for each compound. Cultures for these experiments were rinsed (3X) and replaced with 0.9ml of frog Ringer's solution and allowed to equilibrate for 15-20 minutes. Acute experiments

(0-24 hours exposure) were conducted in frog Ringer's solution. Chronic exposure experiments (longer than 24 hours exposure) were performed in Amphibian culture medium. Dursban contains 6.7% chlorpyrifos, and Lorsban contains 40.7% chlorpyrifos. Test agent concentrations were formulated so that addition of 0.1ml of the test agent to the culture dish gave the desired final concentration. Concentrations tested ranged from 10^{-5} to 10^{-9} M chlorpyrifos in Dursban and Lorsban. [Dilution to 10^{-5} M chlorpyrifos in either the Dursban or Lorsban compound is the highest concentration recommended for use in the home or in the field by the manufacturer (DowElanco)]. The molar concentrations of chlorpyrifos tested (10^{-5} to 10^{-9}) corresponded to $5 \times 10^{-1}\%$ (0.5%) to $5 \times 10^{-5}\%$ (0.00005%) solutions of Dursban, and $1 \times 10^{-1}\%$ (0.1%) to $1 \times 10^{-5}\%$ (0.00001%) solutions of Lorsban. Pure chlorpyrifos was tested at concentrations ranging from 10^{-6} M to 10^{-9} M. Chlorpyrifos could not be tested at 10^{-5} M because its solubility in water (or Ringers) is only at 2×10^{-6} M.

Dursban Blank and Lorsban Blank are the carriers for chlorpyrifos in Dursban and Lorsban, respectively. The blanks are made of xylene-range aromatic solvents and emulsifiers, and are referred to as "inert" by the manufacturer. Specific composition of each blank is proprietary information of DowElanco. Formulations of

Dursban Blank and Lorsban Blank were adjusted to correspond to the same concentration employed for Dursban (5×10^{-1} to $5 \times 10^{-5}\%$) and Lorsban (1×10^{-1} to $1 \times 10^{-5}\%$), respectively. Experiments were repeated five times for each concentration tested. Concentrations were monitored for intervals as short as 5 minutes to as long as 24 hours, depending on the concentration employed. Dursban 2E was obtained locally, while samples of Lorsban 4E, Lorsban 4E Blank (active ingredient absent), Dursban Blank, and chlorpyrifos were kindly donated by DowElanco.

EC₅₀ Estimations

EC₅₀ values were estimated from concentration-inhibition curves using the probit procedure in the Statistical Analysis System (SAS). The probit procedure transformed inhibition data into a normal distribution, from which a line was calculated based on concentration. Concentrations were estimated for several inhibition percentages. Estimations for 50% inhibition are presented.

Transmission Electron Microscopy

For electron microscopy, cultures were treated with Dursban for varying lengths of time at various concentrations, typically between 10^{-6} M and 10^{-7} M chlorpyrifos. Samples were exposed to Lorsban and

chlorpyrifos at concentrations of 10^{-6} M (for chlorpyrifos) for 60 minutes. Samples were also exposed to Lorsban Blank (0.002%) and Dursban Blank (0.005%) for 60 minutes. Control samples were incubated in Ringer's solution. All samples were fixed on ice using a 1:1 mixture of 2.5% glutaraldehyde: 1.66% osmium tetroxide in 0.12M phosphate buffer. Samples were dehydrated in ethyl alcohol and embedded in LX-112 Resin (Ladd). Thin sections were cut with glass knives on a Porter-Blum Ultramicrotome MT-1 (Sorvall) and stained with uranyl acetate and lead citrate. Sections were viewed with a Hitachi H-600 transmission electron microscope.

Early Individual Cell Responses

In order to monitor the response of individual cells over time, the ciliary beat frequency of an exposed cell was monitored second by second with the spectrum analyzer. Frequencies were transferred to an IBM/AT computer (Model 50Z) for frequency histogram construction. Measurements were made either in real time or from video taped recordings. Control experiments with Ringer's solution were performed to establish baseline frequencies. Once a test compound was added to the medium, an increase or decrease in ciliary beat frequency denoted an effect due to the test compound. Experiments were repeated at least three times for each response shown. Acetylcholine and atropine

were obtained from Sigma.

3. RESULTS

Concentration-Response

In order to examine relative toxicities of Dursban and Lorsban on frog palate epithelial cells, cultures were exposed to varying concentrations (measured as molarity of chlorpyrifos) of these compounds and monitored for changes in frequency of ciliary beat. A representative response for Dursban at three concentrations is presented in Figure 1. Exposure to Dursban resulted in a drop in ciliary beat frequency with a rate of decrease that depended on the concentration used. As the concentration decreased, the time to inhibition of ciliary beating increased. At the highest concentration shown, 5×10^{-6} M chlorpyrifos, cilia were inhibited in less than 30 minutes. At 10^{-6} M, cilia stopped after about 45 minutes of exposure. At 2×10^{-7} M, the time to inhibition was 4 hours. [Dye exclusion tests (0.5% trypan blue) performed after 90% inhibition of ciliary beating confirmed that the majority of the cells were dead, as dye was taken up by most cells.] The result from exposure to all concentrations studied was the same, inhibition of ciliary beat frequency. It was the time to inhibition which varied. This typified the kind of response obtained for all compounds examined. It was from curves such as these that data to time of 90% inhibition was obtained to construct concentration-

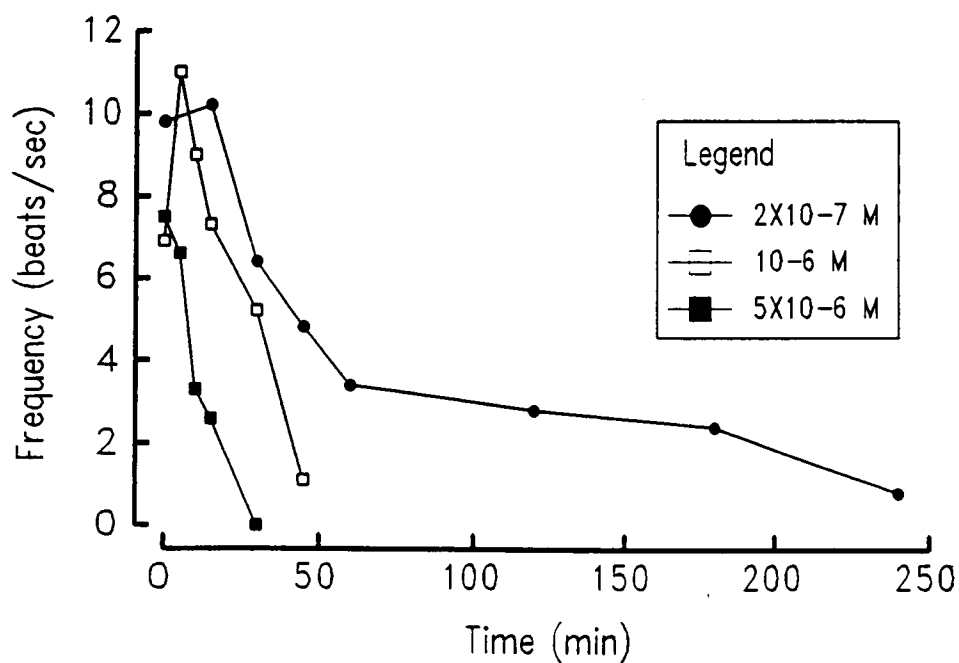


Figure 1. Representative frequency curves for Dursban. Each line corresponds to a different molarity of chlorpyrifos in Dursban and represents the typical response for that concentration. Each point represents the average frequency of 10 cells.

response curves.

Because concentration of each compound could be measured in two ways, as a function of concentration of chlorpyrifos or of inert ingredients, two types of analyses were made. One analysis compared activity as a function of the molar concentration of chlorpyrifos. The other compared activity as a function of the percentage of whole product. A concentration-response curve was constructed (Fig. 2) for Dursban and Lorsban at five molar dilutions (10^{-5} to 10^{-9} M) of the active ingredient, chlorpyrifos. The responses were compared with pure chlorpyrifos at the same concentrations. Pure chlorpyrifos could not be evaluated at 10^{-5} M because its solubility in water is 2×10^{-6} M. At concentrations of 10^{-5} and 10^{-6} M, Dursban and Lorsban were highly inhibitory to ciliary beating, 90% inhibition occurring within 1 hour. With chlorpyrifos (10^{-6} M) inhibition occurred within 20 hours. At 10^{-7} M, a larger variability in response between compounds was observed. Chlorpyrifos at 10^{-7} M showed a significant increase in time to inhibition (6 days) over that at 10^{-6} M, while Lorsban (10^{-7} M chlorpyrifos) was about the same as at 10^{-6} M (<1 hour). The increased time to inhibition with chlorpyrifos at 10^{-7} M over 10^{-6} M may be due to reduced availability of chlorpyrifos as a result of nutrient binding in the medium. The 10^{-6} M pure chlorpyrifos experiment was performed in Ringer's solution, while the 10^{-7} M experiment

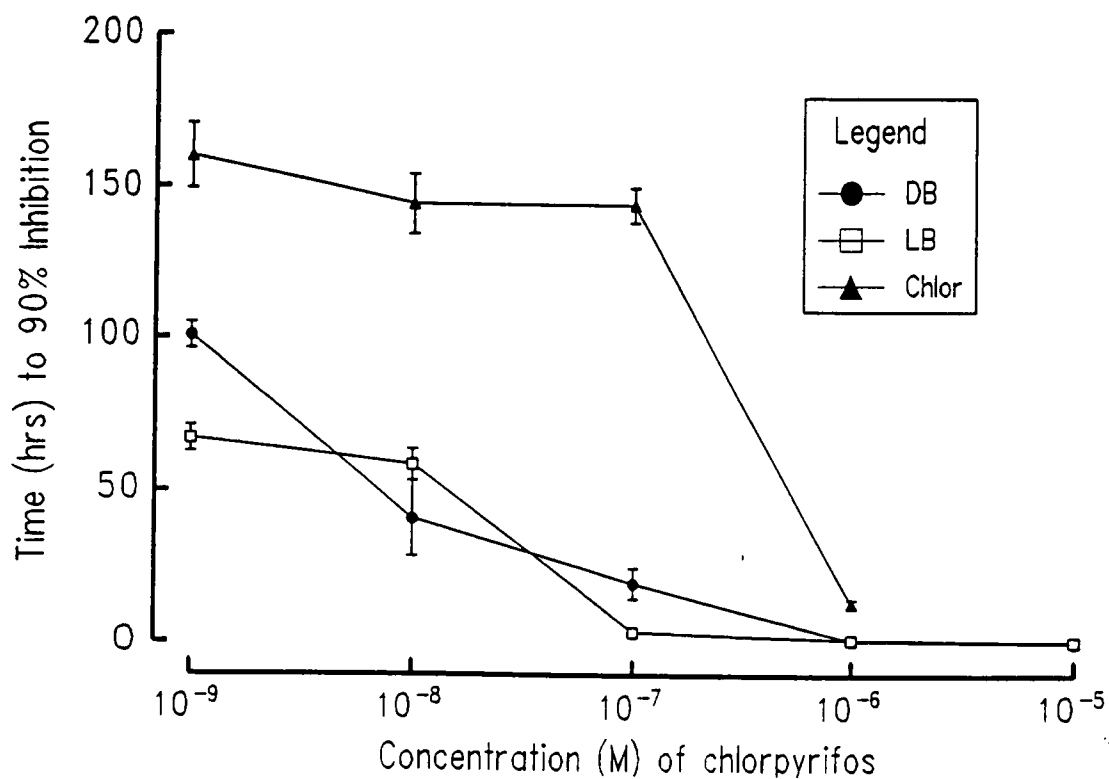


Figure 2. Concentration-response curves for Dursban, Lorsban, and chlorpyrifos based on molarity of chlorpyrifos. Data points correspond to the average time (hrs) to 90% inhibition of ciliary beating for each concentration. Each curve represents the average of five runs $\pm$ standard error. Data points not showing standard error bars indicate that the small standard error is overdrawn by the data symbol.

was performed in Amphibian medium. Dursban at 10^{-7} M showed a slight but significant increase in time to inhibition (1 day) over that at 10^{-6} M (< 1 hour). The most inhibitory compounds at 10^{-8} M were Dursban (<2 days) and Lorsban (2 1/2 days), with chlorpyrifos being much less toxic (6 days). At 10^{-9} M, Lorsban (3 days) was more inhibitory than Dursban (4 days), but both were considerably more inhibitory than chlorpyrifos (7 days). Thus it is evident that since Lorsban and Dursban were much more active than chlorpyrifos, the activity observed in Lorsban and Dursban must be due not only to chlorpyrifos, but to a component(s) in the carrier ingredients as well.

A comparison was also made between Dursban and Lorsban by adjusting the molar chlorpyrifos data to correspond to percentage dilutions ranging from $5 \times 10^{-1}\%$ (0.5%) to $5 \times 10^{-5}\%$ (0.00005%) of whole product for Dursban and from $6 \times 10^{-2}\%$ (0.06%) to $6 \times 10^{-6}\%$ (0.000006%) of whole product for Lorsban. When this adjustment is made, the chlorpyrifos concentration in Lorsban is 6.1 times higher than in Dursban. However, the two compounds can then be compared on a percent basis with their respective blanks (carriers without chlorpyrifos). Thus while in Figure 2 the chlorpyrifos concentration is constant, in Figure 3 the amount of carrier ingredients is constant. At concentrations of $10^{-2}\%$ (0.01%) and higher, all compounds were toxic. From $5 \times 10^{-3}\%$ (0.005%)

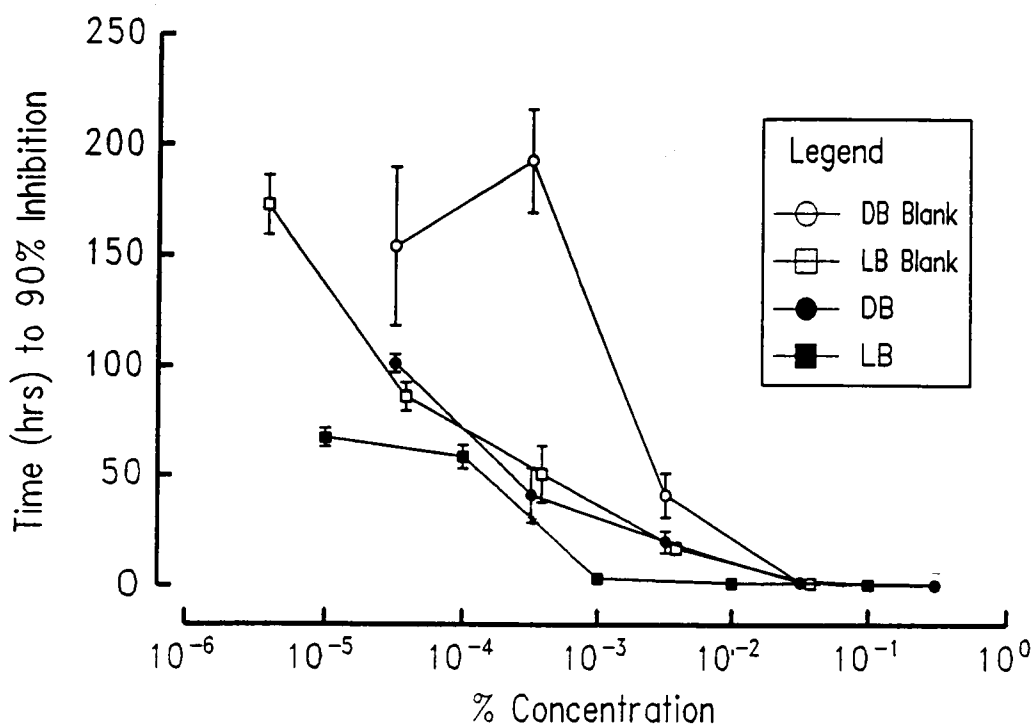


Figure 3. Concentration-response curves for Lorsban, Dursban, Lorsban Blank, and Dursban Blank based on percent solution of whole product. Data points correspond to the average time (hrs) to 90% inhibition of ciliary beating for each concentration. Each curve represents the average of five runs $\pm$ standard error. Data points not showing standard error bars indicate that the small standard error is overdrawn by the data symbol.

to $10^{-4}\%$ (0.0001%), Dursban and Lorsban appear to have the same level of toxicity, even though there is a six-fold difference in chlorpyrifos concentration between the two. At $5 \times 10^{-5}\%$ (0.00005%), Lorsban is more toxic than Dursban. However, at this concentration the difference may be due to the increase in chlorpyrifos concentration in Lorsban.

Dursban and Dursban Blank were also compared (Fig. 3) based on percent of total product (holding the carrier ingredient constant). Both Dursban and Dursban Blank were similar in their effects from $5 \times 10^{-1}\%$ to $5 \times 10^{-2}\%$, inhibition occurring within 1 hour. Dursban was significantly more toxic than Dursban Blank at the concentrations of $5 \times 10^{-3}\%$ and below. Dursban Blank showed a major drop in toxicity between $5 \times 10^{-3}\%$ and $5 \times 10^{-4}\%$, with time to inhibition increasing from less than 2 days to 8 days. The time to inhibition for Dursban increased steadily over decreasing concentrations, requiring 4 days to inhibit at the lowest ($5 \times 10^{-5}\%$) concentration studied. The combined toxicity of chlorpyrifos and carrier ingredients in the whole product of Dursban is probably less than additive, according to the classifications given by Dawson (1994).

The same relationship was seen between Lorsban and Lorsban Blank (Figure 3). At $1 \times 10^{-1}\%$, both compounds were similarly inhibitory (<1 hour to 90% cessation of ciliary beat). Lorsban remained at this level of inhibition down to

the $1 \times 10^{-3}\%$ dilution. Lorsban showed a considerable increase in time to inhibition at $1 \times 10^{-4}\%$ (59 hours) over the first three dilutions. There was no significant difference in time to inhibition for Lorsban at $1 \times 10^{-4}\%$ and $1 \times 10^{-5}\%$. Lorsban Blank increased in time to inhibition steadily over all dilutions, and required significantly longer times than Lorsban over the last four concentrations studied.

A comparison of Dursban Blank and Lorsban Blank at the same percentage dilutions was also made (Fig. 3). At the $5 \times 10^{-2}\%$ and $6 \times 10^{-2}\%$ concentrations, both were inhibitory within 1 hour of exposure. Both blanks required longer times to 90% inhibition at $5 \times 10^{-3}\%$ and $6 \times 10^{-3}\%$, but Lorsban Blank reached 90% inhibition within 17 hours while Dursban Blank took 41 hours. At $5 \times 10^{-4}\%$, Dursban Blank required substantially more time to cause 90% inhibition (8 days) than Lorsban Blank at $6 \times 10^{-4}\%$ (>2 days). At $5 \times 10^{-5}\%$ and $6 \times 10^{-5}\%$, the difference between Dursban Blank and Lorsban Blank, respectively, was smaller, but Lorsban Blank (<4 days) was still more inhibitory than Dursban Blank (6 1/2 days). The carrier ingredients of Lorsban and Dursban are obviously of different toxicity, and some element of Lorsban Blank makes it more active than Dursban Blank. Thus the general order of toxicity between the five compounds was as follows: Lorsban, Dursban, Lorsban Blank, Dursban Blank, and

chlorpyrifos, with Lorsban being the most toxic and chlorpyrifos being the least toxic.

EC₅₀ Estimations

A 24-hour EC₅₀ (effective concentration for 50% inhibition of ciliary beating) was calculated for all five compounds. The predicted 24-hr EC₅₀ values for Lorsban, Dursban, and pure chlorpyrifos were 2.6×10^{-8} M, 2.1×10^{-7} M, and 4.8×10^{-7} M, respectively. These EC₅₀ values are based on the molar concentration of chlorpyrifos present in each product. The ranges given by 95% confidence limits overlapped for the compounds tested at 24 hours. The 24-hr values show that it required more pure chlorpyrifos to elicit the same effect as Dursban and Lorsban at 24 hours. It also showed that of the three, Lorsban required the least amount of chlorpyrifos to elicit the same response. This correlates with the data in Figure 2 which shows that Lorsban was the most inhibitory and pure chlorpyrifos was the least inhibitory. Six-hour EC₅₀ values were estimated for Dursban and Lorsban. The 6-hour EC₅₀ value for Dursban was 3.4×10^{-7} M and for Lorsban 2.8×10^{-8} M. Thus, Lorsban at 6 hours again required a lower concentration to cause the same effect as Dursban. The values for Lorsban at 6 and 24 hours were almost the same, indicating that the actual time to effect (50% inhibition) for Lorsban was closer to 6 hours.

EC₅₀ estimations were also made for Dursban Blank and Lorsban Blank based on percent solution. The 6-hr estimations are relatively similar, 2.8×10^{-2} and $6.6 \times 10^{-2}\%$, respectively. The EC₅₀ (% solution) estimations for Dursban and Lorsban are 2.8×10^{-2} and $6.1 \times 10^{-4}\%$, respectively. Lorsban is again shown to be the most toxic, as a lesser amount of it is required to elicit the same effect as the other compounds. The estimations for Dursban and Dursban Blank are the same, indicating the possibility that most of Dursban's effects are attributable to its carrier.

Ultrastructural Effects

Electron microscopy was employed to examine the ultrastructural effects of each compound on the frog palate epithelium. Under normal circumstances, the ciliated epithelium of the frog palate (Fig. 4) is composed of ciliated and non-ciliated cells, including mucin-secreting goblet cells. The primary cell type is the ciliated cell (Fig. 4a). Along the apical border are basal bodies from which cilia project into the oral cavity. Cilia function to move trapped food and mucus. The ciliated cells contain numerous apical mitochondria. The mitochondria of all cell types in the epithelium are small and contain fine shelf-like cristae. Also present are profiles of granular endoplasmic

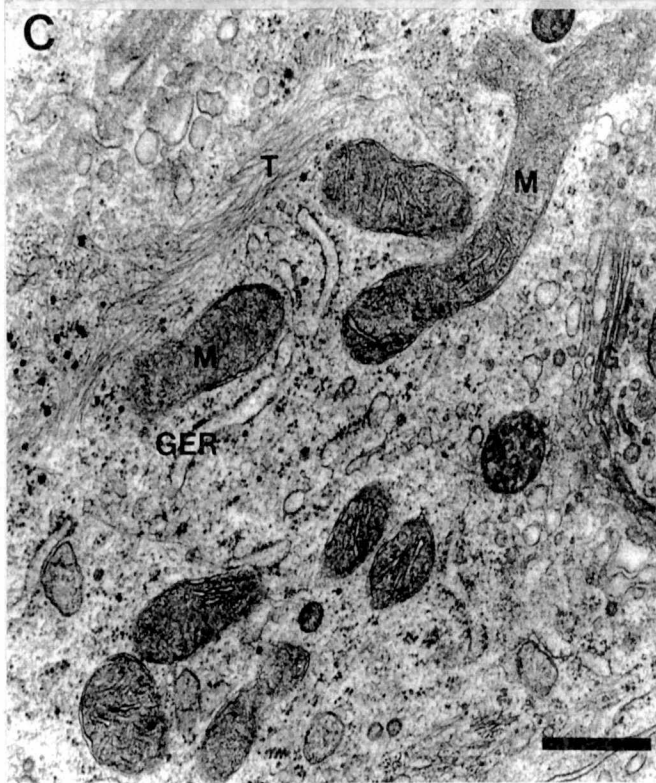
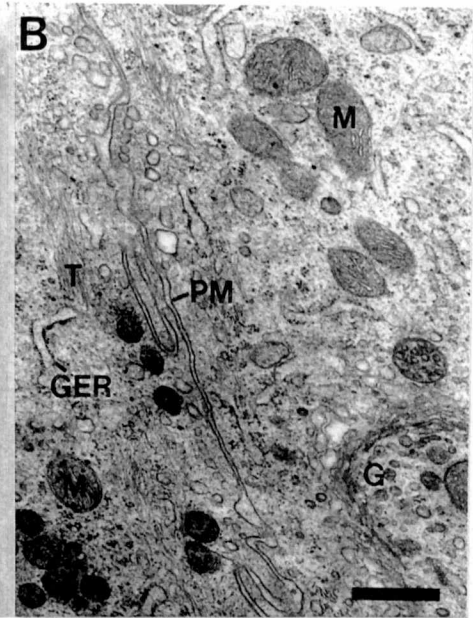
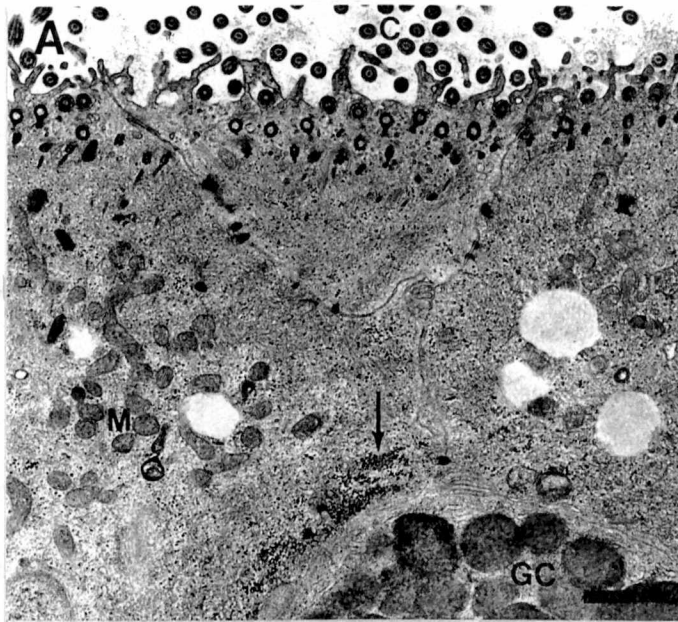
Figure 4. Electron photomicrographs of normal frog palate.

(A). Several ciliated cells at the apical surface of normal palate epithelium. A goblet cell (GC) is seen in the lower portion of the micrograph. Some mitochondria (M) are rounded, others are more elongate. Also visible are glycogen granules (arrow) and cilia (C). Bar 1 μ m.

(B). Portions of two epithelial cells from frog palate in vitro, showing the plasma membranes (PM) of two adjacent cells, normal mitochondria (M), Golgi apparatus (G), tonofilaments (T), and granular endoplasmic reticulum (GER). Bar 0.5 μ m.

(C). A region of mitochondria (M), granular endoplasmic reticulum (GER), tonofilaments (T), and Golgi apparatus (G) from a control culture of frog palatine epithelial tissue. Bar 0.5 μ m.

(D). Apical surface of a non-ciliated cell from a control culture showing the fine glycocalyx (Gx) surrounding the microvilli (Mv). Bar 0.25 μ m.



reticulum and many fine tonofilaments (Figs. 4b,c). Other structures present include desmosomes, lipid droplets, Golgi bodies, and glycogen. Projecting from the apical surface between cilia are microvilli. Covering the apical border of both ciliated and non-ciliated surface cells is a fine glycocalyx (Fig. 4d). Scattered between ciliated cells are goblet cells (Fig. 4a) filled with mucin granules. The goblet cell contains an extensive network of granular endoplasmic reticulum, large mucin granules, and small scattered glycogen granules.

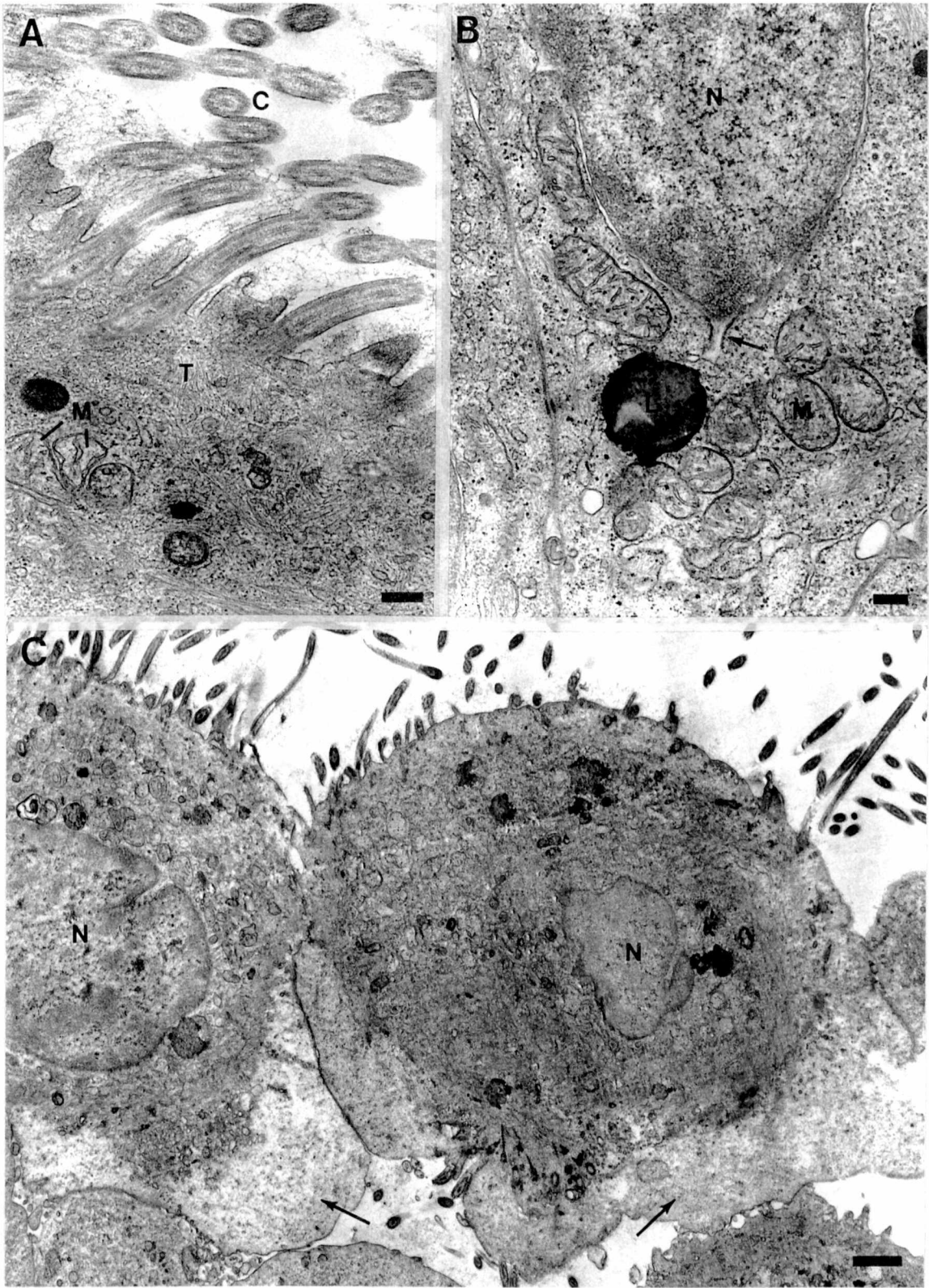
After thirty minutes of exposure to Dursban ($5 \times 10^{-2}\%$; 10^{-6} M chlorpyrifos), general structural integrity of the cell is still maintained (Figs. 5a,b). Nuclei, plasma membranes, desmosomes, glycogen, granular endoplasmic reticulum, and tonofilaments seem to be basically unaffected. Modest swelling of the mitochondria is apparent, however. Figure 5a shows mitochondria at the apical end of a ciliated cell. The mitochondria are swollen, only a few cristae are present, and there is loss of matrix. Mitochondria near the nuclei (Fig. 5b) show the same swelling and loss of matrix. Cultures treated for one hour with Dursban ($5 \times 10^{-2}\%$; 10^{-6} M chlorpyrifos) begin to show cytoplasmic swelling (Fig. 5c). The cortical regions (arrows) of the cells have begun to swell, as seen by the lighter staining of these areas. Cells round up and when left in culture, ultimately detach from the

Figure 5. Electron photomicrographs of frog palate epithelium exposed to Dursban (10^{-6} M chlorpyrifos).

(A). Apical surface of a ciliated cell from cultures treated for 30 minutes with Dursban. Mitochondria (M) show damage. Tonofilaments (T) and cilia (C) appear normal. Bar 0.25 μ m.

(B). Portions of two cells from a culture exposed to Dursban for 30 minutes. Mitochondria (M) appear somewhat swollen. Regions of the membrane of the nucleus (N) are continuous with granular endoplasmic reticulum (arrow). L = lipid droplet. Bar 0.25 μ m.

(C). Rounding up of ciliated cells in the outgrowth after 60 minutes exposure to Dursban. Cortical region along the basal surface of cells is swollen (arrows). N = nucleus. Bar 1 μ m.



culture dish.

Mitochondria from the 30-minute experimental group (Fig. 6b) and the 60-minute experimental group (Fig. 6c) show the progressive deterioration from exposure to Dursban when compared to the control (Fig. 6a). All three electron photomicrographs are enlarged to the same magnification (52,300X). Average mitochondrial diameters from each group show that mitochondria from the 30-minute group are twice as large as the control, and those from the 60-minute group are three times as large as the control. It also appears that swelling of the cell and washing out of the cytoplasm in the 60-minute experimental group (Fig. 6c) is much greater than in the 30-minute experimental group (Fig. 6b). Thus, it appears that the effects of Dursban exposure increase with time, and that the initial insult is to the mitochondria.

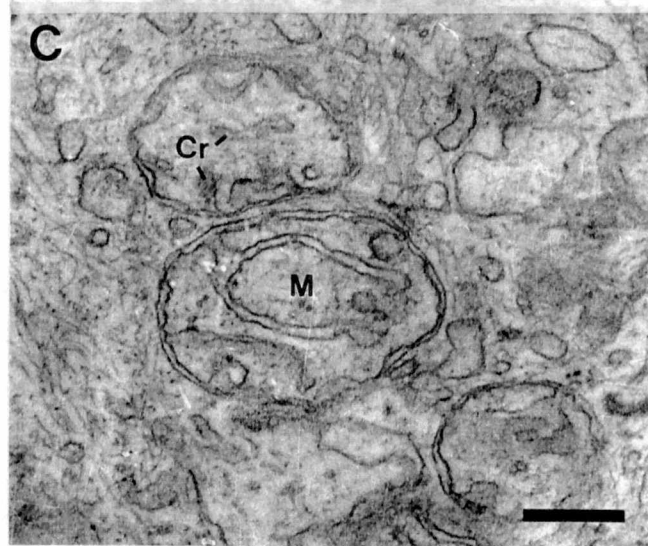
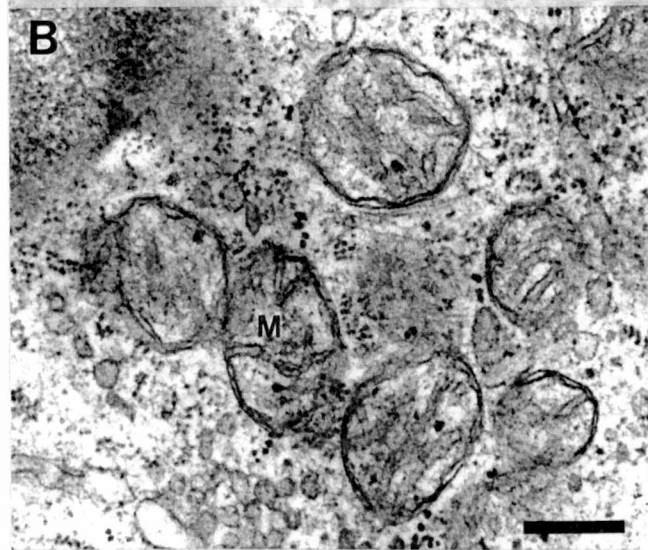
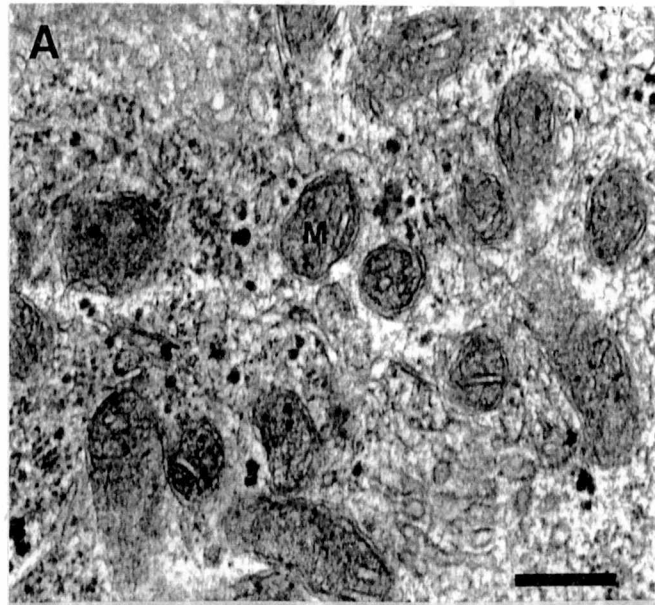
Results of 60 minutes exposure to Lorsban ($10^{-2}\%$; 10^{-6} M chlorpyrifos) and Dursban Blank ($5 \times 10^{-3}\%$) are comparable to the results seen after 60 minutes exposure to Dursban ($5 \times 10^{-2}\%$; 10^{-6} M chlorpyrifos). Mitochondrial matrices are disrupted, cristae are not present, and the mitochondria as a whole are swollen. Lorsban Blank ($2 \times 10^{-3}\%$) causes the most damage to the epithelial cells. All organelles are swollen, with major damage to the nucleus and mitochondria, evidenced both by swelling and matrix disruption. Chlorpyrifos (10^{-6} M) causes mitochondrial swelling as well,

Figure 6. Electron photomicrographs of the frog palate mitochondria from control and experimental groups.

(A). Mitochondria (M) from control cultures have normal structure. Bar 0.25 μ m.

(B). Mitochondria (M) from a culture exposed to Dursban (10^{-6} M chlorpyrifos) for 30 minutes. Mitochondria are swollen to twice the size of mitochondria from control (Fig. 6a). Bar 0.25 μ m.

(C). Mitochondria (M) from a culture exposed to Dursban (10^{-6} M chlorpyrifos) for 60 minutes. Mitochondria are swollen to 3 times their normal size and cristae (Cr) are damaged. Bar 0.25 μ m.



but to a lesser extent than the other compounds. The mitochondrial damage common to all compounds tested shows that toxic insult from Lorsban or Dursban results from a combination of the active ingredient chlorpyrifos and the carrier ingredients. This confirms the results of the concentration-response studies which indicated an additive effect of active and "inactive" ingredients.

Comparison of cell populations with and without non-beating cells

In evaluating the effects of Dursban and Lorsban on ciliary beat we noted that all cells did not slow in unison but that some cells continued to beat rapidly while others slowed. A comparison was made between the average frequencies of beating cells and the average frequencies of all cells, including those cells whose frequencies were zero. Figure 7 shows average frequencies of beat with and without inhibited cells from 3 runs (10 cells each) after exposure to Dursban at 10^{-7} M chlorpyrifos and Lorsban at 10^{-7} M chlorpyrifos. One obvious difference is that Lorsban was much more inhibitory to ciliary beating (4 hours) than Dursban (36 hours) at the same molar concentration of chlorpyrifos. Pure chlorpyrifos (not shown) showed a response comparable to that of Dursban and Lorsban when frequencies of beating cells and frequencies of all cells,

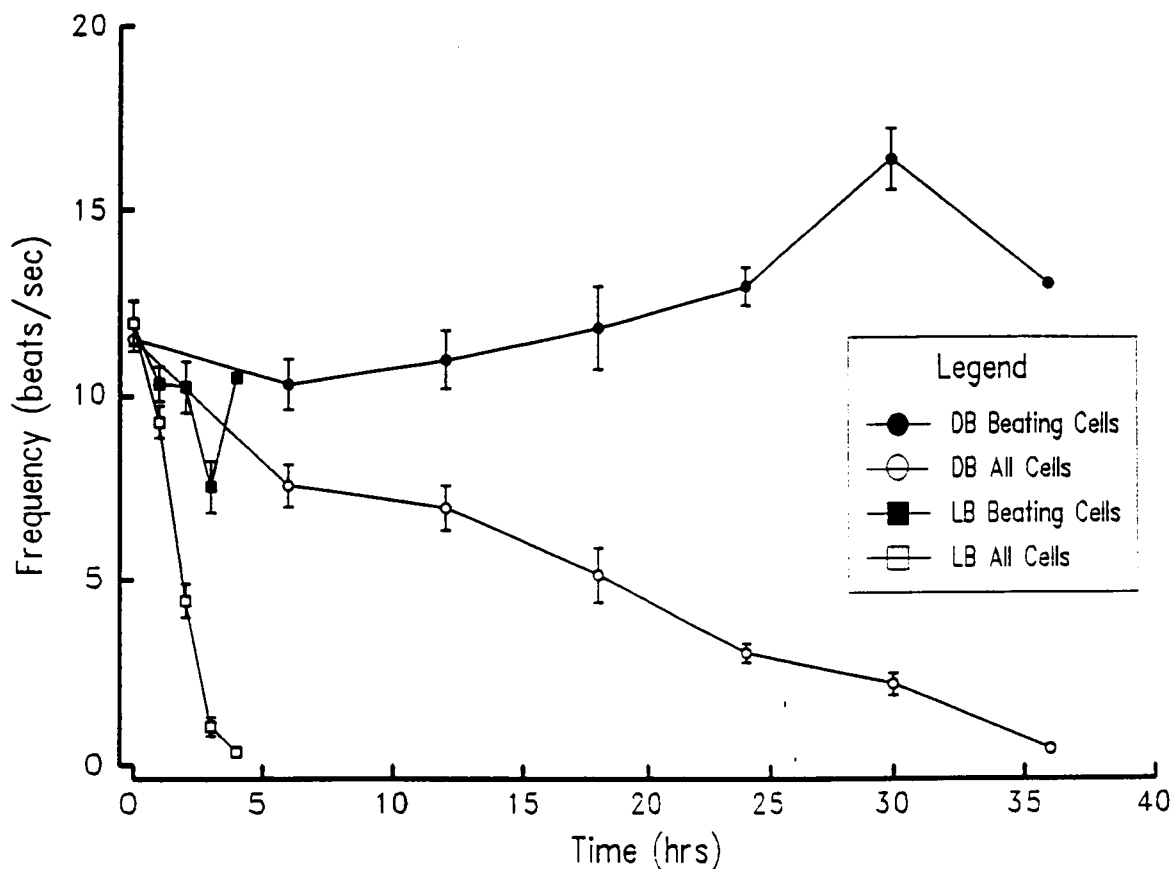


Figure 7. Comparisons of the ciliary beat frequencies of Dursban and Lorsban (both at 10^{-7} M chlorpyrifos) computed by two different methods. In the legend, "beating cells" indicates that means for that curve were determined by averaging the frequencies of beating cells only. "All cells" indicates that means were determined by averaging frequencies of all cells, including zeros for those cells that were not beating. Each data point shows the mean $\pm$ SE for 10 randomly selected cells from each of three runs. Data points not showing standard error bars indicate that the small standard error is overdrawn by the data symbol.

including non-beating cells were compared. The time to inhibition for 10^{-7} M chlorpyrifos (6 days) was much greater than either Lorsban (4 hours) or Dursban (36 hours).

The two curves shown for Dursban and for Lorsban were each taken from the same runs, and it is evident that cells beat at a relatively constant frequency until they were inhibited. Statistical (t) tests comparing the slopes of the two Dursban lines show that the lines are significantly different ($p=0.0001$). Similarly, t-tests for the two Lorsban lines show that the lines are significantly different ($p=0.0045$). The fact that cilia beat more or less at constant frequency until they are inhibited may be due in part to the mitochondrial damage seen with electron microscopy. Mitochondria seem to be able to produce enough ATP to maintain ciliary function even during swelling, but at some point mitochondrial function and cellular osmoregulation are disrupted and the cilia abruptly cease beating. Comparable effects on ciliary function were also observed with Dursban and Lorsban Blanks and with chlorpyrifos.

Effects on Individual Cells Over Time

The initial effects of all five compounds on ciliary beating were examined on individual cells over time. The most obvious response for all concentrations studied in this manner was the initial stimulation of ciliary beat frequency.

Figure 8 is a representative series of concentrations of Dursban. Most stimulations occurred within 2-3 minutes of application of the compounds. Usually, the initial increase in frequency was the maximal frequency obtained during the stimulatory event. The duration of stimulation depended on the concentration of the insecticide applied. Higher concentrations (Fig. 8a; Dursban, 5×10^{-5} M) resulted in shorter stimulation times. Lower concentrations (Fig. 8c; 5×10^{-7} M) resulted in longer stimulatory events; in this case, the stimulation lasted almost half an hour. The initial stimulation was followed ultimately by inhibition of ciliary beating. At concentrations of Dursban (Fig. 8) at 5×10^{-5} M (a), 5×10^{-6} M (b), and 5×10^{-7} M chlorpyrifos (c), a graded level of time to inhibition was evident relative to the concentration applied. At 5×10^{-5} M, inhibition occurred within 5 minutes after application, as compared to almost 1 hour (endpoint not shown) for 5×10^{-7} M. Similar results were observed for Lorsban, both blanks, and chlorpyrifos. At very low concentrations (10^{-9} M chlorpyrifos or $5 \times 10^{-5}\%$ or lower solutions of carriers), no initial stimulation of ciliary beat frequency was evident, however inhibition still ultimately occurred.

Atropine Studies

Chlorpyrifos is reportedly an acetylcholinesterase

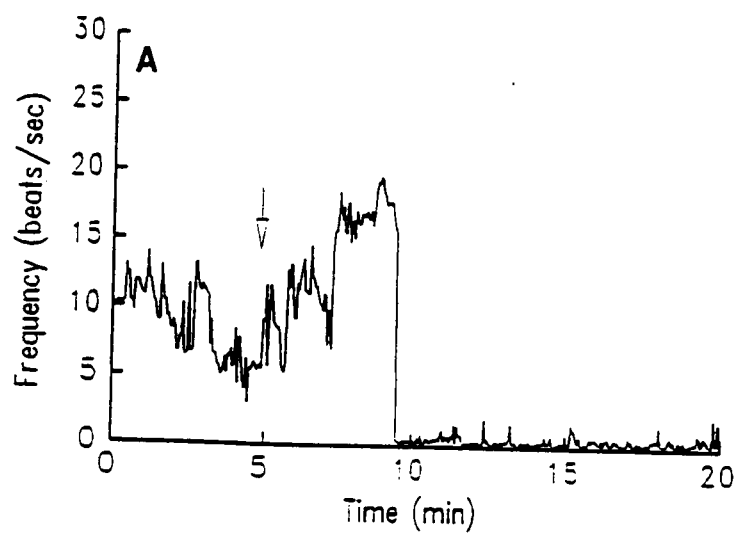
Figure 8. Individual cell responses to 3 concentrations of Dursban.

(A). Typical frequency response of cell to Dursban (5×10^{-5} M chlorpyrifos). Arrow indicates time of Dursban addition (5 minutes). Stimulation occurs within 2 minutes of addition, followed by inhibition (>90%) within 5 minutes.

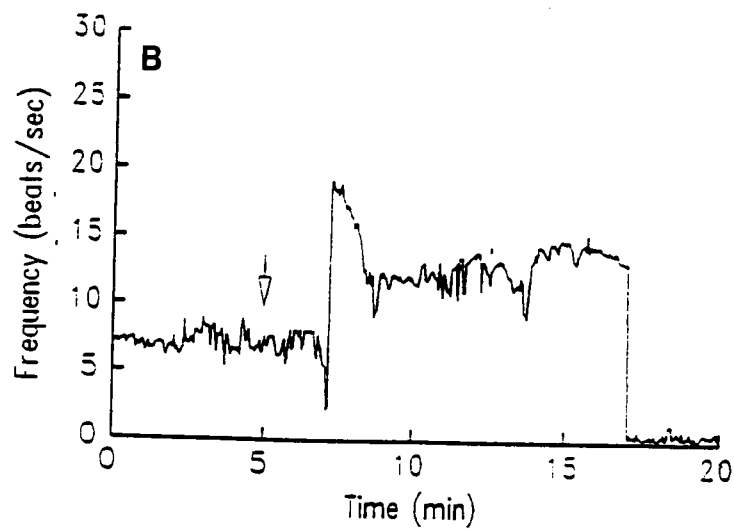
(B). Typical frequency response of cell to Dursban (5×10^{-6} M chlorpyrifos). Stimulation occurs within 2 minutes of Dursban addition (arrow). Stimulatory event lasts longer (10 minutes) at this concentration than at higher concentrations (<5 minutes, Fig. 8a). Inhibition occurs within 12 minutes of addition.

(C). Typical frequency response of cell to Dursban (5×10^{-7} M chlorpyrifos). Stimulation occurs within 3 minutes of Dursban addition (arrow). Stimulation lasts for remainder of test period (>13 minutes).

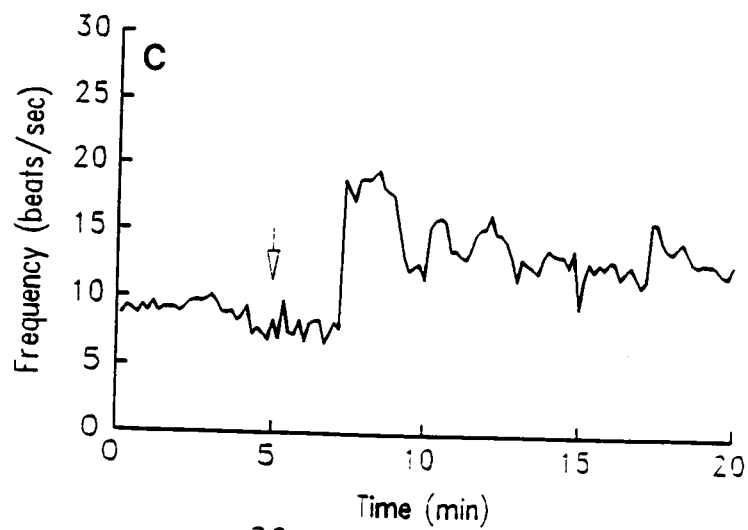
5×10^{-5} M Dursban



5×10^{-6} M Dursban



5×10^{-7} M Dursban



inhibitor. Inhibition of acetylcholinesterase should result in a buildup of acetylcholine at cholinergic synapses. The stimulation observed in ciliary beat frequency may be due to excess acetylcholine at the synapses because of the breakdown of acetylcholinesterase by chlorpyrifos. Atropine, which blocks acetylcholine receptors, was used to in an attempt to block the initial stimulatory response to pure chlorpyrifos. The stimulatory effect of pure chlorpyrifos on ciliary beat frequency (Fig. 9a) was similar to the stimulatory effect seen with Dursban (Fig. 8). Atropine (Fig. 9b) effectively blocked the stimulation of pure chlorpyrifos. Acetylcholine (Fig. 9b) was added after the atropine to confirm blockage of acetylcholine receptors. Pure chlorpyrifos was then added and no stimulation occurred. Since blockage of the acetylcholine receptors apparently prevented an increase in ciliary beat frequency by chlorpyrifos, stimulation by chlorpyrifos may be due to a buildup of acetylcholine at synapses. To confirm atropine blockage of stimulation by the whole product, the test was performed with Dursban (Fig. 9c). Dursban was able to elicit an increase in frequency even after acetylcholine receptors were blocked with atropine. It thus appears that some component of the inert ingredient is able to stimulate an increase in beat frequency through direct action on ciliated cells. When the test was performed on both Dursban and Lorsban carrier compounds, the results

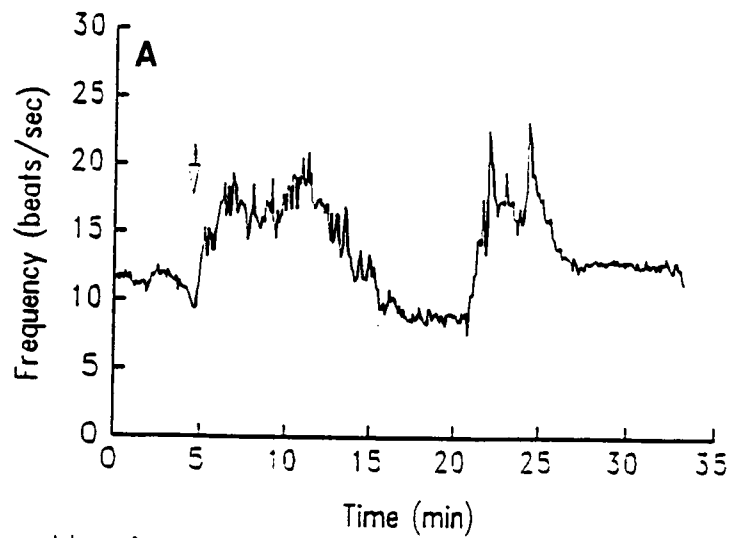
Figure 9. Ciliary responses to chlorpyrifos, atropine, and Dursban.

(A). Typical stimulatory effect of pure chlorpyrifos (10^{-6} M) on ciliary beat frequency. Arrow indicates time of addition.

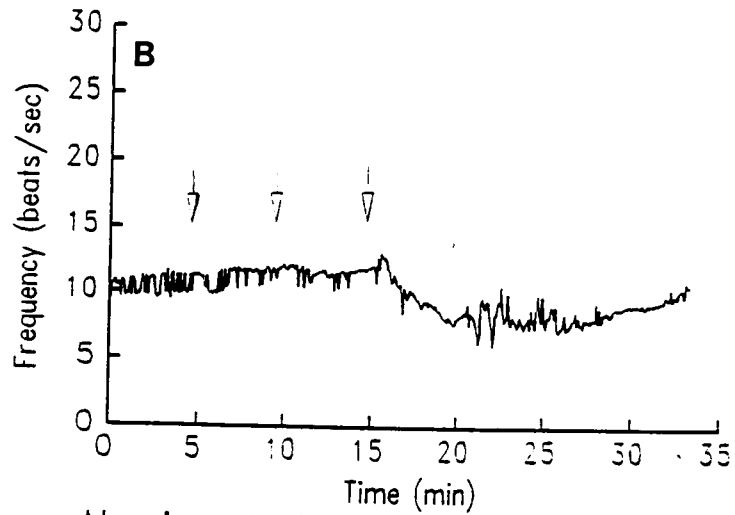
(B). First arrow indicates time of addition of atropine (10^{-3} M). Second arrow corresponds to addition of acetylcholine (10^{-4} M). Third arrow shows time of addition of 10^{-6} M chlorpyrifos. Atropine effectively blocked stimulation of frequency by chlorpyrifos.

(C). Arrows show times of addition of atropine (10^{-4} M), acetylcholine (10^{-4} M), and Dursban (10^{-6} M chlorpyrifos). Atropine was unable to block stimulatory effect of Dursban.

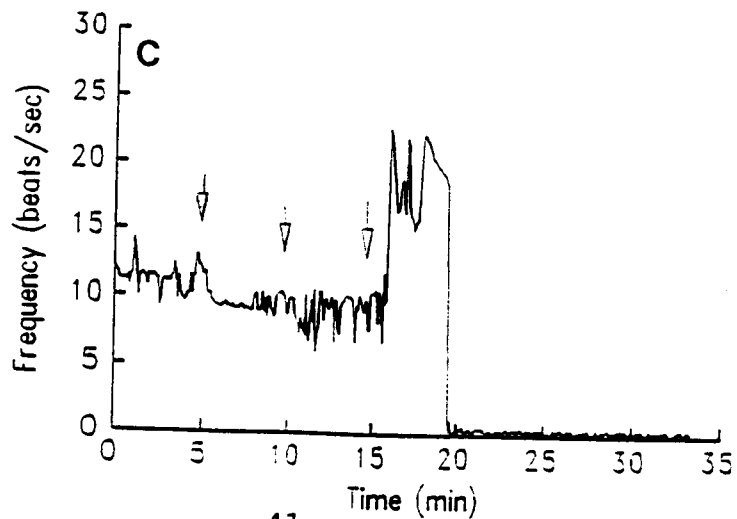
10⁻⁶ M Chlorpyrifos



Atropine, Acetylcholine, 10⁻⁶ M Chlorpyrifos



Atropine, Acetylcholine, 10⁻⁶ M Dursban



were the same as those for Dursban. Both carriers were able to stimulate an ciliary beating frequency after atropine had blocked acetylcholine receptors. Since atropine could not block the stimulation of either carrier, stimulation by the carriers must be by some mechanism other than acetylcholinesterase hydrolysis. When Figures 9a and 9c are compared, the greater toxicity of Dursban over pure chlorpyrifos is seen. Dursban (Fig. 9c) caused complete inhibition within 5 minutes of application. Cells exposed to pure chlorpyrifos (Fig. 9a) were still beating after almost 30 minutes of exposure. However, while the mechanisms of action of chlorpyrifos and its carriers are possibly different, the results are the same. Ciliary beat frequency is stimulated upon application of either compound, then ciliary beating decreases, and ultimately the cells die.

4. DISCUSSION

Many studies report decreased ciliary activity after exposures to high concentrations of respiratory irritants. Ciliary beat frequency of hamster tracheal epithelium decreased 29.5% after exposure to sulfuric acid (Grose et al. 1980). Similarly, decreases in tracheobronchial mucociliary clearance were reported in rabbits (Chen and Schlesinger, 1983), dogs (Wolff et al. 1981), donkeys (Schlesinger et al. 1978), and humans (Liekauf et al. 1981) all exposed to sulfuric acid aerosol. The inhibitory effects of Dursban and Lorsban on ciliary beat followed the typical concentration-response relationship for toxic compounds. Inhibition (>90%) occurred at all concentrations tested, the time to inhibition varying as a function of the concentration of the test agent applied; the higher the concentration used, the shorter the time to inhibition of ciliary beat.

Concentration-response curves were constructed for all five compounds based on molar concentrations of chlorpyrifos or percent solution of whole product. Overall, Dursban and Lorsban were similar in their inhibitory effects at each concentration tested. Pure chlorpyrifos was not as inhibitory as Dursban and Lorsban or either of their carriers. However, both carriers were consistently less toxic than Dursban or Lorsban at more dilute concentrations

of products. EC_{50} estimations supported the conclusion that chlorpyrifos was the least toxic, followed by the carriers, and the most toxic compounds were Dursban and Lorsban. The relative toxicities of these compounds indicate the possibility that the combination of chlorpyrifos with a carrier results in an additive effect on toxicity. The result common to all compounds examined in this study was mitochondrial swelling.

Other organophosphorous compounds have produced mitochondrial damage in test systems. Methylbromofenvinphos, an organophosphorous insecticide, has been shown to cause mitochondrial swelling in avian hepatocytes (Chishti and Rotkiewicz, 1993). Diazinon caused mitochondrial swelling in muscle cells of fish (Sakr and Gabr, 1992). We found that pure chlorpyrifos caused mitochondrial swelling as well, but swelling was modest in comparison to Dursban, Lorsban, and both carrier compounds. The actual mechanism by which these compounds affect mitochondrial swelling is unclear. The loss of ATP production due to the swelling of the mitochondria would cause the cell to lose its ability to osmoregulate, and this could account for the cytoplasmic swelling noticed after one hour exposure. Prolonged deficiencies of ATP were shown to reduce ion pumping efficiency and resulted in no maintenance of osmotic pressure in the cells of hamster tracheal explants (Gabridge and Meccoli, 1982). Schultz and

Kennedy (1976) reported mitochondrial swelling in Daphnia after exposure to the herbicide 3-amino-1,2,4-triazole, and suggested that this resulted in failure of osmoregulation. However, permanent damage to the mitochondria is likely responsible for the ultimate deaths of the outgrowth cells. These findings are consistent with those of Duckett et al. (1986) who found that mitochondrial damage and inhibition of ciliary beat were the principal effects of gossypol on cultured epithelial cells of rabbit trachea.

Schmuck et al. (1994) reported that carrier ingredients usually have minimal, if any, toxic effects in test systems. They found that typically, the active ingredient is more toxic than its carrier, except in cases where the carrier is an emulsifier, which is the case with both the Dursban and Lorsban carriers (Blanks). The mechanism by which emulsifiers in the carriers adversely affect cells is unclear. The first goal of any toxicant is to reach the site of action. If a toxicant cannot reach the site of action, it cannot cause a biological response (Dearden, 1990). Emulsifiers weaken lipid membranes by fractioning lipids into small droplets which become suspended in the water portion of the membrane (Mathews and van Holde, 1990). This detergent action alters membrane structure and makes cells more permeable (Mathews and van Holde, 1990). Methylbromfenvinphos, an organophosphorous insecticide,

causes phase separation in model lipid membranes (Blasiak, 1993). Gossypol has been shown to alter membrane lipids and consequently, protein function (Cuellar and Ramirez, 1993). Malathion and Parathion, two organophosphorous insecticides, are also found to decrease membrane fluidity at concentrations below that which cause acetylcholinesterase inhibition (Domenech et al, 1977). However no distinction has been made between the action of the active ingredient and that of the carrier compound.

It is possible that membrane disruption is a factor in the toxicity of the Dursban and Lorsban carriers. The toxicological effect of both carriers on mitochondria support the idea of membrane disruption by the carriers. Mitochondrial swelling as a result of calcium saturation has been cited as evidence of inner membrane permeability changes in isolated rat liver (Lapidus and Sokolove, 1993). In this study, Lorsban Blank was more damaging to mitochondria than Dursban Blank. Lorsban Blank was also more damaging to the epithelial cells than all other compounds tested. Therefore it may have a more lipophilic nature than Dursban Blank.

We observed that frequency of beating cilia on some cells seemed relatively high even as other cells were being inhibited. Thus comparisons were made between populations of beating cells and populations of cells containing representative numbers of inhibited cells over time. It was

determined for both Dursban and Lorsban that there was a significant difference between average frequencies of beating cells only and for all cells, including cells which had stopped beating (Dursban $p=0.0001$, Lorsban $p=0.0045$). The percentage of active cells over time seems to decrease in a concentration-response manner. However, toward the end of an experiment, if the frequencies of cells still beating are compared to the initial beat frequencies, it is found that frequencies have remained fairly constant (or have increased) throughout the course of the experiment. It appears that once the degree of cell damage is high enough, the cessation of beat is immediate. This response can be seen in the individual cell response curves. The end of the stimulatory event is abrupt and precipitous. This response pattern may vary between species. In studies which monitored ciliary beat frequency of canine tracheal epithelium after exposure to the fungicide benomyl, ciliary beat frequency of individual cells was found to decrease steadily over the course of the experiment (Kennedy, personal communication). Similarly, gossypol caused a steady decline in ciliary beat frequency in rabbit tracheal cells (Kennedy, personal communication). To our knowledge, this is the first report of this type of frequency inhibition response in ciliary function.

Chlorpyrifos inhibition of acetylcholinesterase is well

documented. Brain, plasma, and erythrocyte acetylcholinesterase levels decrease after exposure to chlorpyrifos (Pope et al, 1991; Yoshikawa et al., 1990; McCollister et al., 1974). Similar reports of reduced cholinesterase levels in the presence of chlorpyrifos have been shown in dogs (Yeary, 1984), hens (Capodicasa et al., 1991), mice (Deacon et al., 1980), piglets (Long et al., 1986), and minnows (Jarvinen et al., 1983). If acetylcholinesterase is inhibited by chlorpyrifos in palate ciliated cell cultures, acetylcholine concentrations should increase. Increasing levels of acetylcholine are known to cause an increase in ciliary beat frequency of frog palate in vitro (Aiello et al., 1991), in vivo (Slaughter and Aiello, 1982), and in situ (Maruyama et al. 1983; and Morley and Sanjar 1984).

Atropine was used in an attempt to block the initial stimulation caused by chlorpyrifos. Atropine blocks acetylcholine receptors (Aiello et al., 1991), so that even if excess acetylcholine is present, it should not be able to elicit a stimulatory event because of reduced receptor availability. We found that chlorpyrifos stimulation could be blocked by atropine. However, Dursban was still able to elicit a stimulatory response from the ciliated outgrowths after atropine had been applied. Dursban Blank and Lorsban Blank also caused the same type of initial stimulation after

atropine addition as was seen with Dursban. Since atropine was able to block stimulation by chlorpyrifos, but not any of the other chemicals tested, some component(s) of the Blanks must be at least partly responsible for the initial stimulatory event. Furthermore, these Blanks must have elicited their response via some mechanism other than acetylcholinesterase inhibition. Girard and Kennedy (1986) showed that an increase in cytoplasmic calcium levels stimulated increased frequency of ciliary beat in rabbit trachea. Chu (1993) showed that a calcium release from mitochondrial stores produced a brief stimulation of ciliary beat frequency in frog palate. He also showed that an influx of extracellular calcium resulted in prolonged stimulation of ciliary beating. Weiss et al. (1992) found that an increase in the concentration of calcium in the cytosol opened potassium channels in the cell membrane and caused a depolarization of the cell which resulted in increased ciliary beat frequency. Some component(s) of the carriers may be eliciting a nonspecific release of calcium, possibly by membrane damage, initially causing an increase in ciliary beat frequency. Constant influx and efflux of calcium has been shown to cause mitochondrial fatigue, such that mitochondria could no longer produce ATP (Richter et al., 1992). The resultant loss of the cells ability to osmoregulate would conceivably result in inhibition of

ciliary beating and ultimately cell death.

In summary, the toxicity of Dursban and Lorsban appears to be due to a combination of the effects of chlorpyrifos and the effects of the carriers. The mechanism of chlorpyrifos action is most likely inhibition of acetylcholinesterase, which at chronic exposure ultimately results in cell death. The mechanism of carrier toxicity may be disruption of membrane integrity, which would result in generalized damage to cells. Indeed, the combination of chlorpyrifos with its carrier ingredients at manufacturer-recommended concentrations may result in a primary toxicity to frog palate epithelium unrelated to acetylcholinesterase inhibition.

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