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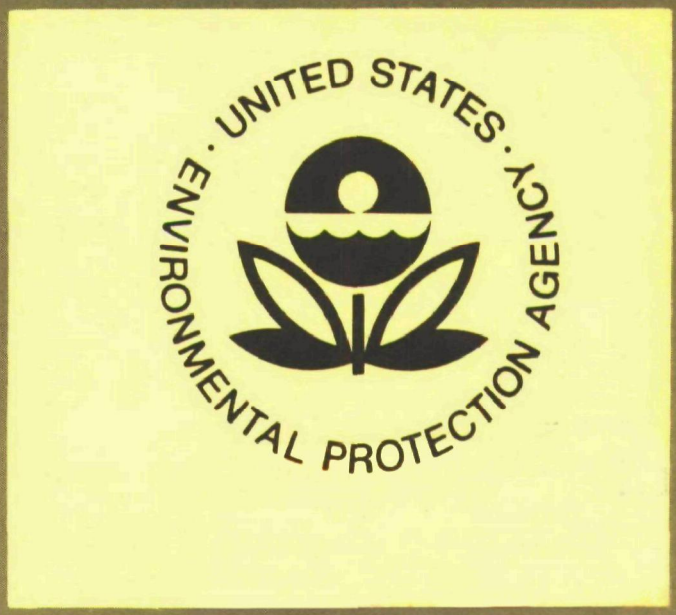
Environmental Health Effects Research Series

MODE OF ACTION OF CYCLODIENE INSECTICIDES



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MODE OF ACTION OF CYCLODIENE INSECTICIDES

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ABSTRACT

This report contains information concerning the mode of action, excretion, and metabolism of the cyclodiene insecticides. Toxaphene was the primary candidate for investigation with major emphasis on the mammalian system.

Excretion of ^{36}Cl-toxaphene was studied in the laboratory rat. Upon extraction, most of the radioactivity occurred in the water fractions of urine and feces as ionic chloride, indicating considerable metabolism of toxaphene. Only minimal storage appeared to occur.

Uptake of radioactivity in several tissues of Leucophaea maderae was determined after injections of ^{36}Cl-toxaphene. In subcellular particles of ventral nerve cord and brain, significant levels of ^{36}Cl occurred in the larger cell fragments; microsomes were also labelled. Ventral nerve cords of L. maderae and Periplaneta americana showed increased activity when exposed to toxaphene.

The toxicity of toxaphene to Gambusia affinis was divided into 5 stages, and the residue level at each stage was determined. Metabolic alteration of toxaphene appeared to be minimal. Excretion was not observed.

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SECTION I

CONCLUSIONS

In the rat, approximately one-half of an oral dose of toxaphene (20 mg/kg) is excreted within 9 days. Most of that excreted occurs as ionic chloride, indicating considerable metabolism of toxaphene. It is suggested that other organelles besides microsomes may be involved in this metabolism. Only minimal storage occurs 1 day following treatment. A second dose given on the 9th day is excreted in a similar manner.

Toxaphene accumulates in ventral nerve cords of Leucophaea maderae. The large amount retained after rinsing indicates penetration or binding of toxaphene by the nerve cord. Toxaphene localizes in the larger cell fragments, e.g., fragments of nerve sheath, nuclei, and unbroken cells. Microsomes are also labelled.

Overt symptoms of toxaphene poisoning for L. maderae follow a pattern similar to that reported for other cyclodienes. At the moribund stage, the ventral cord gives evidence of endogenous activity. Ventral nerve cords of Periplaneta americana show increased nerve activity when exposed to toxaphene. Nerves affected by toxaphene show prolonged volleys of spikes (13-24 spikes per burst and 8-12 bursts per minute) until apparent death of the nerve.

Sorption of toxaphene into Gambusia affinis is a linear function with respect to time. Excretion is not observed. Metabolic alteration of toxaphene appears to be minimal. Differences in individual mortality appear to be due to differences in uptake rather than in ability to tolerate particular body loads of toxaphene.

SECTION II

RECOMMENDATIONS

1. Currently, toxaphene is commonly used in combination with methyl-parathion, and more recently with methyl-parathion and chlordimeform. It is recommended that it be determined whether the uptake, metabolism, storage, and excretion of toxaphene in the mammal is influenced by these other insecticides. Furthermore, experiments should be conducted to quantitate the potentiation, if any, of one insecticide upon another in these combinations. This would be important in determining efficacy in insects and potential hazards in various non-target organisms.

2. Specific localization of cyclodienes and formation of nerve-cyclodiene complexes was studied principally in insects during this project. This work should be continued to include the mammalian systems. By measuring the rate of nerve-cyclodiene complex formation, the time course and amounts complexed could then be correlated with electrophysiological and ionic events.

3. It is recommended that studies of the effect of cyclodienes on ion fluxes in nerve tissue continue and be expanded to include mammalian nerve. During this project, toxaphene's effect on spontaneous nerve activity was studied; however, single-cell electrophysiological recordings of its effect on individual neurons is necessary to help elucidate the mode of action. Furthermore, it is suggested that ion fluxes (influx and efflux) in the nervous system be measured with radioactive potassium, sodium, calcium, and chloride. Only chloride influx was examined in this project. Related to toxaphene's current usage in combination with other insecticides previously described, the effect of toxaphene on ion fluxes in nerve tissue should also be explored.

SECTION III

INTRODUCTION

Toxaphene (chlorinated camphene with a chlorine content of 67-69%) is the major chlorinated hydrocarbon insecticide used on cotton. Studies on the mode of action of toxaphene are of primary importance to the EPA and other regulatory agencies, and as such this insecticide was selected for investigation.

As is the case with other chlorinated hydrocarbon insecticides, the mode of action of toxaphene and other cyclodienes has not been elucidated. What little is known concerning their mode of action has been presented in reviews by Dahm[1], Winteringham and Lewis[2], Roan and Hopkins[3], O'Brien[4,5], and more recently by Brooks[6]. Most of these authors concurred that cyclodienes probably act by interfering with nerve transmission rather than as enzyme inhibitors.

Cyclodiene insecticides are believed to have a mode of action similar to that of other chlorinated hydrocarbon insecticides, e.g., DDT and lindane; neurophysiological evidence indicates action on the central nervous system (Lalonde and Brown[7], Wang and Matsumura[8], Shankland and Schroeder[9]). It has been suggested that some cyclodienes must be metabolized into a toxic form before they have pronounced neurotoxic effects (Wang and Matsumura[8]). No theory has been put forth for the mode of action of cyclodiene insecticides, but three attractive hypotheses have been generated for DDT and lindane (Mullins[10], Matsumura and O'Brien[11,12], and Holan[13]). Experimentation centered around these theories might aid in elucidating the mode of action of cyclodienes.

It was hypothesized that cyclodienes may interfere with transport mechanisms in membranes. Interactions with nerve membranes could lead to alterations in ionic transport across the membrane and, therefore, result in electrical potential modifications. By employing techniques of electrophysiology, potentials and ionic fluxes could be determined in relationship to cyclodiene poisoning. Additionally, a knowledge of uptake, metabolism, and excretion of cyclodienes could be considered along with electrophysiological and symptomological studies.

The objectives of the project were to study:

(1) Metabolism and excretion of cyclodienes in various animals.

(2) Specific localization of cyclodienes in various tissues with special emphasis on the nervous system.

(3) Nerve-cyclodiene complexes as related to nerve disruption following acute poisoning.

(4) Ionic fluxes across nerve membranes from cyclodiene-treated animals.

SECTION IV

METHODS AND MATERIALS

A. FATE OF TOXAPHENE IN THE RAT

1. Uptake and Excretion Experiments

a. Experimental Design -

Thirty-day-old albino rats (Holtzmann Co.), weighing an average of 114 g, were deprived of food 24 hrs prior to dosage. Twenty mg/kg of technical grade ^{36}Cl-toxaphene (42 μCi/g; Hercules, Inc.) in 0.5 ml of a peanut oil-gum acacia solution was orally administered via a stomach tube. Controls were dosed with 0.5 ml of the peanut oil-gum acacia solution. In each of 2 experiments, 3 treated and 3 control rats were placed into glass metabolism chambers, which provided for separate collection of urine and feces (Halladay[14]). Another group of treated and control animals were held for organ and tissue sampling at 9 time intervals; 3 treated and 1 control were used at each interval. On the ninth day, 3 treated and 3 control rats were given an additional dose of 20 mg/kg; these animals were referred to as "redosed". All holding and metabolism cages were maintained in an air conditioned environment (22-25°C; 50% RH; L:D - 11:13). The animals were provided Purina Laboratory Chow and water ad libitum.

Urine and feces were collected daily, weighed, and stored at 0°C to await further analysis. At scheduled time intervals, rats were sacrificed and their organs and tissues excised, weighed, and stored at 0°C. Additionally, the animals employed in the excretion experiment were sacrificed for organs and tissue samples at the end of 9 and 20 days. Blood obtained from heart punctures was immediately centrifuged at 3,000 rpm for 5 minutes in a refrigerated superspeed Sorvall centrifuge, model RC2-B, to precipitate cellular matter.

b. Analytical Procedures -

Feces were thawed, air-dried, ground to a powder, and 1 g samples extracted with 25 ml each of hexane and water. Urine samples were also extracted with hexane and water. Aliquots of all extracts were then digested and solubilized in NCS® (Amersham/Searle Corp.) and Triton X-100, using heat to aid digestion. Tissue samples were thawed, minced with scissors, and homogenized with NCS® over heat.

Determination of ^{36}Cl in urine and feces water extracts was accomplished with acidification (3-4 drops of 1 M HNO_3) followed by precipitation (several drops of 0.5M $AgNO_3$). After centrifugation, the precipitant was discarded and the procedure repeated until $AgNO_3$ saturation was attained. The supernatants were radioassayed to determine non-ionic ^{36}Cl; the ionic ^{36}Cl was then calculated by subtracting non-ionic ^{36}Cl from total ^{36}Cl.

Toluene based fluor (5 g PPO and 0.06 g POPOP/1 toluene) was added to all samples. Radioassay was performed on a dual channel (Nuclear-Chicago Model 6822) liquid scintillation spectrophotometer. Quench was corrected using the external standard method.

2. Metabolism by the Liver

Adult male rats were sacrificed and their livers removed. A 3 g portion of each liver was placed in 30 ml of ice-cold 0.25 M sucrose and homogenized. The homogenate was centrifuged at ca. 12,000 x G for 10 min to obtain the microsomal supernatant.

One ml samples of homogenate and microsomal supernatant (90 mg tissue/ml) were placed in reaction vessels for incubation. The following was added to each vessel: 0.05 ml glucose-6-phosphate dehydrogenase (0.1 mg/ml in water), 1 ml glucose-6-phosphate (4.67 mg/ml in 50 mM phosphate buffer), 0.085 ml of 2.7 mM KCl, 0.125 ml NADP (8 mg/ml in 50 mM phosphate buffer), and 8 ml of phosphate buffer. One µl of ^{36}Cl-toxaphene (50 µg) in acetone was added to the vessels of both samples; 1 µl acetone was used as the control. The vessels were incubated for 15 hours in a shaking water bath at 37°C. Following incubation, samples were extracted twice with hexane and water. Precipitated tissue was removed and digested with 2.5 ml of NCS®. All samples were then radioassayed.

B. FATE OF TOXAPHENE IN COCKROACHES

1. Uptake in the American Cockroach, Periplaneta americana

P. americana cockroaches were topically dosed with 75 µg of ^{36}Cl-toxaphene in 1 µl acetone. At each of several time intervals, cockroaches were weighed and rinsed with acetone. Hemolymph was extracted according to the method of Sternberg and Corrigan[15]. Remaining cockroach carcasses were homogenized in insect saline (Yamasaki and Narahashi[16]) and filtered through 2 layers of cheesecloth. Samples of the homogenates as well as the hemolymph were prepared for scintillation counting and radioactivity

determined. Quench was corrected via the external standard method.

2. Uptake in Leucophaea maderae

a. Distribution Following an Injected Dose -

Adult male L. maderae, 2-6 weeks post final molt, were injected with 0.05 cc toxaphene in mineral oil (175.4 μg/dose) in the third abdominal segment between tergites. All punctures were sealed with paraffin to avoid the loss of the injected dose.

Controls consisting of both non-injected and insects injected with 0.05 cc mineral oil were prepared. Test or control insects to be dissected within 24 hours were not provided with nourishment. Water was given to insects sacrificed at 48, 72, 96, and 120 hours after injection.

Following incubation periods of 2, 4, 6, 8, 12, 24, 48, 72, 96, and 120 hours, insect hemolymph was collected by the method of Sternberg and Corrigan[15], weighed, and then prepared for scintillation counting. Each insect was then dissected for removal of fat body, entire alimentary canal, and abdominal nerve cord. Weights of all tissues were recorded and each was prepared for radioassay.

To determine the nature of the radio-labelled material found, tissues of asymptomatic and symptomatic L. maderae were gas-chromatographed following extraction and clean-up. This included tissues which were prepared for radioassay and freshly prepared samples. Fresh samples were handled in the same manner as above, but time of incubation and tissue preparation differed (Fig. 1). The ethyl ether layer was also prepared for scintillation counting to determine ^{36}Cl content.

b. In Vitro Studies of Distribution in Nerve -

Six trials were performed using 3 ventral nerve cords of L. maderae, 2nd to 6th ganglia--a total of 104 in all for each concentration of toxaphene, 6 concentrations in all, 10^{-2} - 10^{-7}M. Following dissection, 3 nerve cords were weighed and placed in each of the center wells of Warburg flasks containing a 0.5 ml concentration of toxaphene dissolved in mineral oil. The Warburg flasks were incubated on a shaker for 24 hours at 37°C. Flasks were also prepared for controls as above with 3 cords incubated in 0.5 ml mineral oil.

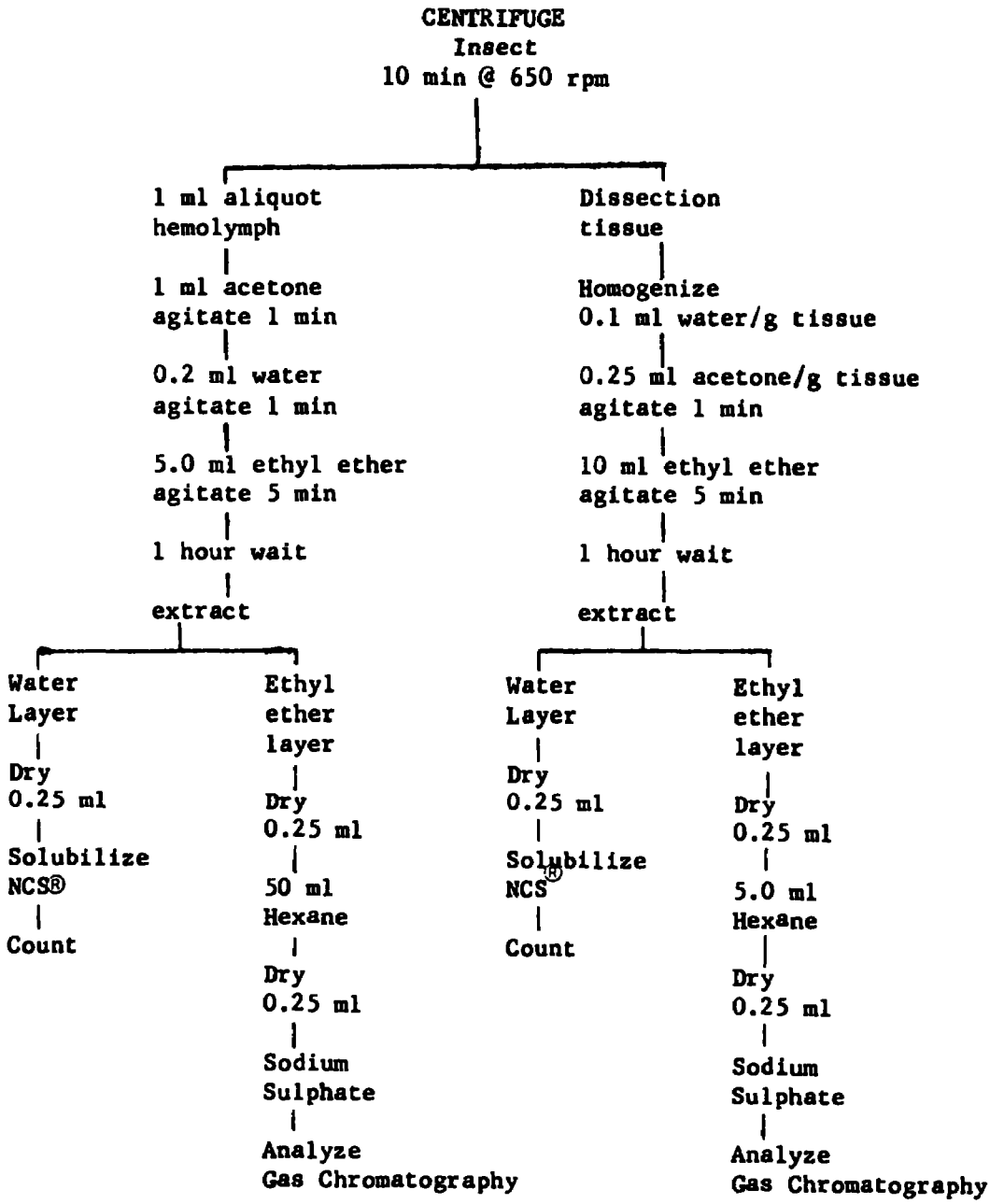

Figure 1. Flow sheet representing preparation of residue of insect tissue for gas-chromatographic analysis.

After incubation, the cords were removed from the flasks and sent through a series of 4 rinses at different time intervals for each rinse: rapid 0-5 sec, 100 sec, 10 min, and 20 min; one ml insect saline (Yamasaki and Narahashi[16]) was used for each rinse. The cords were again weighed at the 20 min rinse.

Rinse vials, ambient solutions left in the Warburg flasks, and nerve cords were then prepared for scintillation counting. Contents of rinse vials were concentrated, then solubilized in NCS®. Nerve cords were homogenized in ground glass tissue grinders with 1.0 ml NCS® until a clear liquid was formed. Ambient solutions were quickly transferred to scintillation vials for radioassay.

Homogenized nerves from male L. maderae were assayed for toxaphene binding in a way similar to that for whole nerves. Nerve cords were homogenized in 3 ml phosphate buffer pH 7.4 and then incubated for 24 hours with ^{36}Cl-toxaphene. Following incubation, the homogenate was centrifuged for 10 min at 1,000 x G. The precipitate was washed 3 times and radioassayed; the rinses and supernatant were also radioassayed.

In a related experiment, male L. maderae were removed from the colony and their head, legs, and wings were removed, with the head being retained for brain removal. The body was opened dorsally, and viscera removed. Two or 3 drops of insect saline were then added to the body cavity to keep the nerve cord both alive and moist. The nerve was further exposed by cleaning from it all fat body, trachea, and muscle. The nerve cord, from the 3rd thoracic to the 6th abdominal ganglia, and brain were removed. The isolated tissues were placed in 5 ml of ice-cold 0.25M sucrose solution and homogenized, with the homogenate kept at or below 4°C. The homogenates were then transferred to centrifuge tubes. ^{36}Cl-toxaphene in acetone, giving a final concentration of 4.14 μg/ml or 10^{-5}M, was added to each homogenate. Controls were treated with equal volumes of acetone. Incubation occurred at room temperature (24°C) for one hour, after which the reaction was stopped by immersion in an ice bath. Homogenates were then centrifuged at 20,000 x G for 45 min to settle all but the microsomes. The supernatant was decanted, the sediment resuspended, recentrifuged, and the supernatant added to the first. The combined supernatants were then centrifuged at 100,000 x G for 45 min. The sediment containing microsomes from the 20,000 x G centrifugation was resuspended in 0.25M sucrose and placed on a discontinuous density gradient consisting of 0.8, 1.0,

1.2, 1.5, and 1.8 M sucrose layers, and centrifuged at 90,000 x G for 2 hours. The various sediments were removed, dried, and radioassayed.

C. UPTAKE OF ^{36}Cl-TOXAPHENE IN MOSQUITOFISH

1. Selection of Gambusia Population

G. affinis were collected from a total of 5 locations in Pima and Pinal County, Arizona. Four were eliminated due to their high susceptibility to toxaphene or the seasonable instability of their environment. Fish for this research were obtained from a sewage oxidation pond located in Tucson, Arizona. Fish were maintained in the laboratory in aged tap water for 24 hours prior to testing. Fish used in experiments were selected by randomly dip-netting fish from the stock containers. The sex of individual fish was not recorded. Mean weight was 1.082 $\pm$ S.D. of 0.627 g.

2. Preparation of Solutions

Solutions used for mortality experiments to screen populations of G. affinis were prepared by the appropriate aqueous dilution of a 1% acetone-based stock solution of ^{36}Cl-toxaphene (0.042 μCi/mg) in a method described by Boyde and Ferguson[17]. All other experimental solutions were prepared by appropriate aqueous dilution of a 10^{-2}M solution of ^{36}Cl-toxaphene in redistilled acetone. Experimental solutions employed tap water which was aged for at least 24 hours at room temperature prior to use. A solvent control test was conducted at the rate of 2 ml redistilled acetone per 1 of water and resulted in 0% mortality after 48 hours. This concentration of solvent was roughly 5 times as high as in the highest experimental solution. All experiments were conducted at room temperature.

3. Extraction Techniques

Fish were homogenized in 40 ml of redistilled acetone in a ground glass tissue grinder. After grinding, the extract was filtered through filter paper, evaporated to dryness, and redissolved in 0.25 ml of distilled water and 0.25 ml of hexane. Extraction of 10 fish topically dosed with 0.1 ml of ^{36}Cl-toxaphene solution prior to extraction resulted in an average recovery rate of 96.01 $\pm$ S.D. of 5.28%. A recovery of 96% was used in the calculation of all experimental data.

4. Radioassay

Fluor used in preliminary experiments was toluene based containing 0.06 g POPOP and 5.0 g PPO in one l of reagent grade toluene. Bray's solution, a

dioxane based fluor, was employed in all other experiments. Bray's solution contains 60 g naphthalene, 10 ml methanol, 20 ml ethylene glycol, 4 g PPO, 0.2 g POPOP and one l reagent grade dioxane. This fluor was stored in darkness until use and due to the instability of dioxane, added to samples immediately before counting.

5. Toxicity Experiments

On the basis of preliminary tests, it was found that fish exposed to lethal concentrations of toxaphene exhibited a particular chain of toxic symptoms. This chain was divided into 5 characteristic stages as follows:

STAGE	DESCRIPTION
1	Fish apparently healthy
2	Fish swimming at surface, often swimming perpendicularly into the side of the aquarium.
3	Fish losing equilibrium; no longer swimming in horizontal attitude. Tail-end down, swimming against sides of container; sometimes rolling-over as they swim.
4	Fish prostrate on bottom of aquarium; gills ventilating rapidly; occasional darting behavior.
5	Death as indicated by the cessation of gill movements.

To correlate the amount of toxaphene residues with these symptoms, G. affinis were placed in 5 l aquaria containing 2 ppm of ^{36}Cl-toxaphene. Samples were taken during the next 10 hours so that there were 10 fish sampled at each of the 5 toxic stages. Samples were rinsed in tap water and analyzed as previously described.

6. Uptake Experiments

Two 5 l aquaria were set up with aged tap water containing 2 ppm of ^{36}Cl-toxaphene. G. affinis were introduced to each aquarium at 0800 and sampled hourly for 8 hours. Each sample consisted of 10 fish which were rinsed with fresh tap water and frozen individually for subsequent extraction. At the time of sampling, the toxic stage of each fish was recorded. This experiment was duplicated.

7. Excretion Experiments

Two 5 l aquaria were set up as in the uptake experiments and fish introduced at 0800. After 8 hours exposure to 2 ppm of ^{36}Cl-toxaphene, fish were transferred to 5 l aquaria containing untreated, aged tap water. Dead

fish were removed, rinsed in tap water, and frozen for subsequent analysis. Also at the time of transfer, a sample of 10 fish was collected, rinsed, and frozen. Samples were taken every hour until all fish were removed. At the time of sampling, the toxic symptoms of each fish were recorded. This experiment was duplicated.

8. Partitioning of Metabolites

A total of 34 fish killed by an 8 hour exposure to 2 ppm ^{36}Cl-toxaphene was used to characterize the composition of radioactive fish. Fish were homogenized in 400 ml acetone. Samples of this homogenate were preapred for radioassay. Two hundred ml of homogenate were evaporated to dryness and partitioned in 100 ml distilled water, and 100 ml of hexane added to the nonpolar fraction. Aliquots of both fractions were evaporated to dryness, redissolved in 0.25 ml distilled water and 0.25 ml hexane, and prepared for radioassay.

D. EFFECT OF TOXAPHENE ON THE NERVOUS SYSTEM

1. Toxicity Experiments

LD_{50}, LC_{50}, and LT_{50} studies were made using male adult L. maderae. The animals were treated by injection. After treatment, they were kept in Mason jars with adequate food and water. Depending on the test, 2 variables were kept constant. For the LD_{50} and LC_{50}, times varied from 24-120 hours. The LT_{50} was made from the most lethal dose and concentration found from the LD_{50} and LC_{50}. Control insects were injected similarly to the test animals and maintained as above.

2. Electrophysiological Studies of Cockroach Nerves

Electrophysiological studies were made using an extracellular suction electrode system (Florey and Kriebal[18]). Glass capillary tubes, fashioned to approximate the outer diameter of a length of ventral nerve cord, were used in drawing the nerve in contact with a Ag-AgCl cathode through a saline bridge. Nervous activity was observed on a Tektronix Type 5103 Dual Beam Storage Oscilloscope amplified by a Grass P-5 Type Preamplifier. A Ag-AgCl rod, 0.5 mm diameter and 3.0 cm long, was immersed in a saline bathing solution and used as the ground. The nerves were bathed in insect saline, pH 7.1, (Yamasaki and Narahashi[16]) contained in a polyurethane well, 2.0 x 2.0 x 6.0 cm. Temperature of the saline varied between 22-26^{o}C. Frequency of spikes and bursts of spikes were measured using a Tektronix DC502 Frequency Counter. Spike and burst intervals were observed on a Mentor N-750 spike analyzer.

Nerve cords were obtained from male P. americana. A length of ventral nerve cord including the 3rd through 6th abdominal ganglia was used in the preparations.

Perfusion of insect saline at 1.0 ml/min into the cell was continuous. Temperature and pH of the saline were observed during each preparation.

Toxaphene dissolved in mineral oil was used throughout the studies. Five concentrations were used: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}M. Toxaphene was introduced in close approximation to the nerve by injection from a 22 gauge Yale needle (0.05 ml at all concentrations).

Specimens were observed for spontaneous nerve activity before pharmacological tests were made. Mineral oil was introduced as a control before testing the effects of toxaphene. Activity of a nerve was observed from the time of dissection until pharmacological tests were concluded.

3. Effect on Ion Fluxes in Cockroach Nerves

Uptake of chloride ion by abdominal and thoracic segments of ventral nerve cord of P. americana was studied. Adult male cockroaches were removed from the colony, the body opened dorsally, and the viscera removed to expose the ventral nerve cord. The nerve cord was then cut between the 3rd thoracic and 1st abdominal ganglia. Both segments were removed and placed in ice-cold saline (Yamasaki and Narahashi[16]). Once all the nerve segments were removed, they were transferred to ^{36}Cl-labelled saline, with or without toxaphene, and allowed to incubate for various times at room temperature (24°C). Following incubation, the segments were weighed, solubilized with NCS®, and radioassayed.

SECTION V

DISCUSSION

A. FATE OF TOXAPHENE IN THE RAT

1. Uptake and Excretion

Due to the paucity of information concerning the fate of toxaphene in the mammalian system, the following study was undertaken. Reported herein are routes and rates of excretion as well as amount and loci of accumulation in various tissues of male rats administered an oral dose of ^{36}Cl-toxaphene.

Average excretion of radioactivity derived from ^{36}Cl-toxaphene, represented as percent of administered dose is reported in Table 1. During 9 days, 52.6% of a single dose was excreted. About one-half of this occurred the first day. Approximately 30% was excreted in urine while 70% appeared in feces. Excretion on an accumulative basis is presented in Fig. 5; feces equilibrated between 2-3 days while urine excretion continued an upward trend. Feces have also been reported as the major excretion route for dieldrin (Matthews et al.[19]) and mirex (Mehendale et al.[20] and Gibson et al.[21]) in rats. However, the amount of toxaphene excretion in urine demonstrated herein was greater than observed with either dieldrin or mirex.

Following a second dose, urine played a greater role in excreting ^{36}Cl than with the single dose (Table 1). The peak of excretion in feces appeared delayed about 1 day longer than in the single dose; here it took 2 days for 50% of the excretion to occur. Again similar trends were noted--excretion in feces equilibrated early while urine continued an upward trend (Fig. 2). Based upon total recovery expressed as 100%, urine increased while feces decreased (Fig. 3). On the third day, relative percents of urine and feces were equal. This importance of urine excretion during the later days was evident in both single-dosed and redosed animals. It was observed that less of the toxaphene dose was excreted in redosed than single-dosed. This contrasts to the report by Gibson et al.[21] that redosed rats eliminated 25% of an administered dose of mirex as opposed to 18% for single-dosed.

Table 1. EXCRETION OF ^{36}Cl IN URINE AND FECES OF RATS FOLLOWING ^{36}Cl - TOXAPHENE, 20mg/kg
(percent administered dose)

Single Dose				Redose[a]			
Day	Urine	Feces	Total	Day	Urine	Feces	Total
1	1.46	23.95	25.4	10	1.81	6.00	7.8
2	3.20	7.45	10.6	11	3.55	11.60	15.2
3	2.89	1.25	4.1	12	2.26	1.40	3.7
4	2.35	1.10	3.5	13	3.08	1.10	4.2
5	1.82	1.06	2.9	14	3.09	1.20	4.3
6	1.19	1.23	2.4	15	1.77	0.60	2.4
7	1.15	0.69	1.8	16	1.07	0.40	1.5
8	0.54	0.27	0.8	17	0.97	0.40	1.4
9	0.72	0.31	1.0	18	1.31	0.20	1.5
				19	0.73	0	0.7
				20	0.42	0	0.4
Total[b]	15.3	37.3	52.6		20.1	22.9	43.0
Total[c]	29.1%	70.9%	100%		46.7%	53.3%	100%

[a]The single-dosed animals were redosed with 20 mg/kg on the 9th day.

[b]Percent administered dose.

[c]Percent recovered dose expressed as 100%.

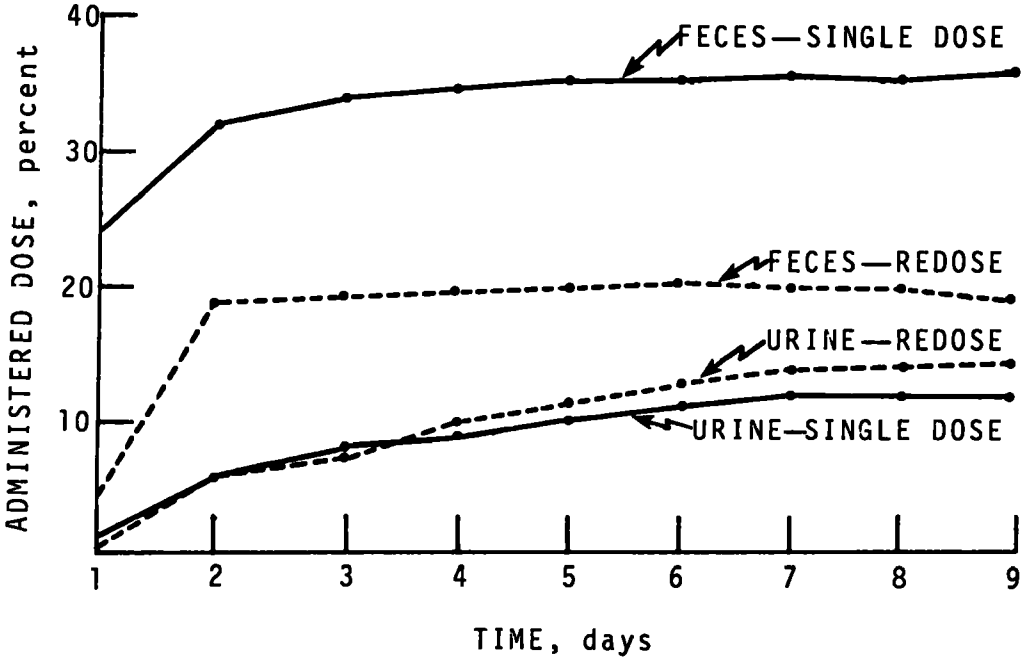

Figure 2. Accumulative excretion of radioactivity in rats administered a single dose and redose of ^{36}Cl-toxaphene.

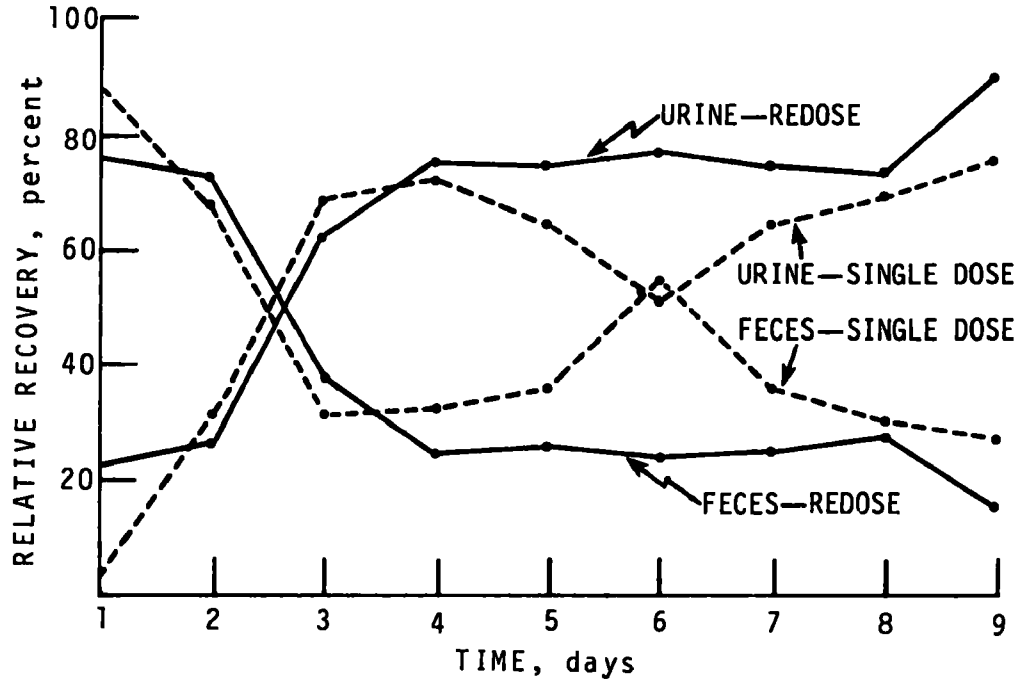

Figure 3. Relative percent recovery of ^{36}Cl in excretion of rats following ^{36}Cl-toxaphene.

In both single-dose and redose experiments, 90% of the ^{36}Cl was recovered in water fractions of feces (Table 2). Likewise in urine, only a small amount of radioactivity was observed in the hexane fractions (Table 3).

Because radioactivity was found in the water fractions, it appeared that a considerable amount of toxaphene metabolism had resulted; therefore, the water and hexane fractions were analyzed for ionic ^{36}chloride (Tables 4 and 5). 68.2% of the radioactivity excreted by single-dosed rats via feces existed as ionic ^{36}Cl; the amount in redosed animals was somewhat less. In urine, ionic ^{36}Cl increased from 76.2% in single-dosed to 90.2% in redosed rats; this resulted from a decrease of non-ionic chloride found in urine-water fractions of redosed animals. Total ionic ^{36}Cl excretion in combined feces and urine was less in redosed rats. By comparing the individual feces and urine accumulative ionic ^{36}Cl excretion (Fig. 4), it appears that this lower amount in redosed rats resulted from a smaller excretion by feces. This anaylsis emphasizes the considerable degree of toxaphene metabolism occurring in rats following an oral dose.

Tissues and organs of rats, following a redose of ^{36}Cl-toxaphene, retained 6.0%. By comparing redosed and single-dosed rats, the manner in which the first dose was concentrated compared to the second was determined. Here it was demonstrated that redosed rats contained 0.55% less in selected tissues; that is equivalent to almost 20% less dose retention than in single-treated rats.

Uptake of radioactivity in various tissues over a period from 3 hours to 20 days is tabulated in Table 6. In almost all cases, the greatest concentration was found at 12 hours followed by a rapid decrease which is in close agreement to the 6 hour peak reported by Lamb et al.[22] for dieldrin in pheasant tissues. Blood cells exhibited a peak at 3 days. Less than 10% of the administered dose remained after 1 day (Fig. 5). This differs from that found with mirex in rats where 34% of a dose was retained in tissues and organs after 7 days (Mehendale et al.[20]). Most of the large concentration up to day 1 could be accounted for by the amount in the stomach. With respect to the nervous system, brain tissues did not concentrate an extraordinary amount of radioactivity. Fat storage appeared non-significant; this agrees with the investigation of Bateman et al.[23] for sheep and steers.

Table 2. HEXANE - WATER EXTRACTION OF RAT FECES FOLLOWING ^{36}Cl - TOXAPHENE, 20 mg/kg
(percent administered dose)

Single Dose[b]				Redose[a,b]			
Day	Hexane	Water	Total	Day	Hexane	Water	Total
1	1.55	22.4	23.95	10	0.38	5.62	6.0
2	2.15	5.3	7.45	11	1.28	10.29	11.6
3	0.30	0.95	1.25	12	0.13	1.27	1.4
4	0.13	0.97	1.10	13	0.05	1.06	1.1
5	0.08	0.98	1.06	14	0.01	1.16	1.2
6	0.01	1.22	1.23	15	0	0.01	0.6
7	0.01	0.68	0.69	16	0	0.38	0.4
8	0.08	0.19	0.27	17	0	0.36	0.4
9	0.06	0.25	0.31	18	0	0.25	0.2
				19	0	0.01	0
				20	0	0	0
Total[b]	4.37	32.9	37.3		1.85	21.0	22.9
Total[c]	11.8%	88.2%	100%		8.3%	91.7%	100%

[a]The single dose animals were redosed on the 9th day with 20 mg/kg.

[b]Expressed as % administered dose.

[c]Percent recovered dose expressed as 100%.

Table 3. HEXANE - WATER EXTRACTION OF RAT URINE FOLLOWING ^{36}Cl - TOXAPHENE, 20 mg/kg
(percent administered dose)

Single dose[b]				Redose[a,b]			
Day	Hexane	Water	Total	Day	Hexane	Water	Total
1	0.023	1.42	1.46	10	0.031	1.778	1.81
2	0.004	3.19	3.20	11	0.006	3.544	3.55
3	0.004	2.88	2.89	12	0.007	2.257	2.26
4	0.002	2.35	2.35	13	0.007	3.068	3.08
5	0.001	1.82	1.82	14	0.003	3.086	3.09
6	0.002	1.19	1.19	15	0.001	1.767	1.77
7	0.014	1.14	1.15	16	0.003	1.072	1.07
8	0.001	0.54	0.54	17	0.002	0.964	0.97
9	0.002	0.719	0.719	18	0.003	1.307	1.31
				19	0	0.733	0.73
				20	0	0.421	0.42
Total[b]	0.05	15.2	15.7		0.1	20.00	20.1
Total[c]	0.3%	96.7%	100%		0.5%	99.5%	100%

[a]Single dosed animals redosed on the 9th day with 20 mg/kg.

[b]Percent administered dose.

[c]Percent recovered dose expressed as 100%.

Table 4. ^{36}Cl - IONIC AND NON-IONIC COMPOSITION OF RAT FECES (WATER FRACTION) FOLLOWING ^{36}Cl - TOXAPHENE, 20 mg/kg
(percent administered dose)

Single Dose				Redose[a]			
Day	Ionic	Non-Ionic	Total	Day	Ionic	Non-Ionic	Total
1	13.4	3.0	16.4	10	4.5	1.1	5.6
2	7.1	3.7	10.8	11	7.0	3.3	10.3
3	1.1	0.9	2.0	12	0.4	0.9	1.3
4	1.4	0.4	1.8	13	0.7	0.4	1.1
5	1.4	0.4	1.8	14	1.1	0.1	1.2
6	2.4	0	2.4	15	0.4	0.2	0.6
7	1.2	0.2	1.4	16	0.4	0	0.4
8	0.2	0.2	0.4	17	0.1	0.3	0.4
9	0.7	0.1	0.6	18	0.3	0	0.3
				19	0	0	0
				20	0	0	0
Total[b]	28.5	8.9	37.4		14.9	6.3	21.2
Total[c]	76.2%	23.8%	100%		70.3%	29.8%	100%

[a]Single dosed rats redosed with 20 mg/kg on 9th day.

[b]Percent administered dose.

[c]Percent recovered dose expressed as 100%.

Table 5. ^{36}Cl - IONIC AND NON-IONIC COMPOSITION OF RAT URINE (WATER FRACTION) FOLLOWING ^{36}Cl - TOXAPHENE, 20 mg/kg
(percent administered dose)

Single Dose				Redose[a]			
Day	Ionic	Non-Ionic	Total	Day	Ionic	Non-Ionic	Total
1	-0.36	2.5	2.2	10	1.2	0.6	1.8
2	4.4	0	4.4	11	3.2	0.3	3.5
3	3.6	0.8	4.4	12	2.3	0	2.3
4	3.7	-0.1	3.6	13	2.9	0.2	3.1
5	2.6	0.8	3.4	14	2.9	0.2	3.1
6	1.9	0.5	2.4	15	1.5	0.3	1.8
7	0.5	0.5	1.0	16	0.9	0.2	1.1
8	0.9	0.3	1.2	17	0.9	0.1	1.0
9	1.1	0.3	1.4	18	1.3	0	1.3
				19	0.7	0	0.7
				20	-0.3	-0.1	-0.4
Total[b]	18.3	5.6	24.0		17.5	1.8	19.3
Total[c]	76.2%	23.3%	100%		90.7%	9.3%	100%

[a]Single dosed animals redosed with 20 mg/kg on 9th day.

[b]Percent administered dose.

[c]Percent recovered dose expressed as 100%.

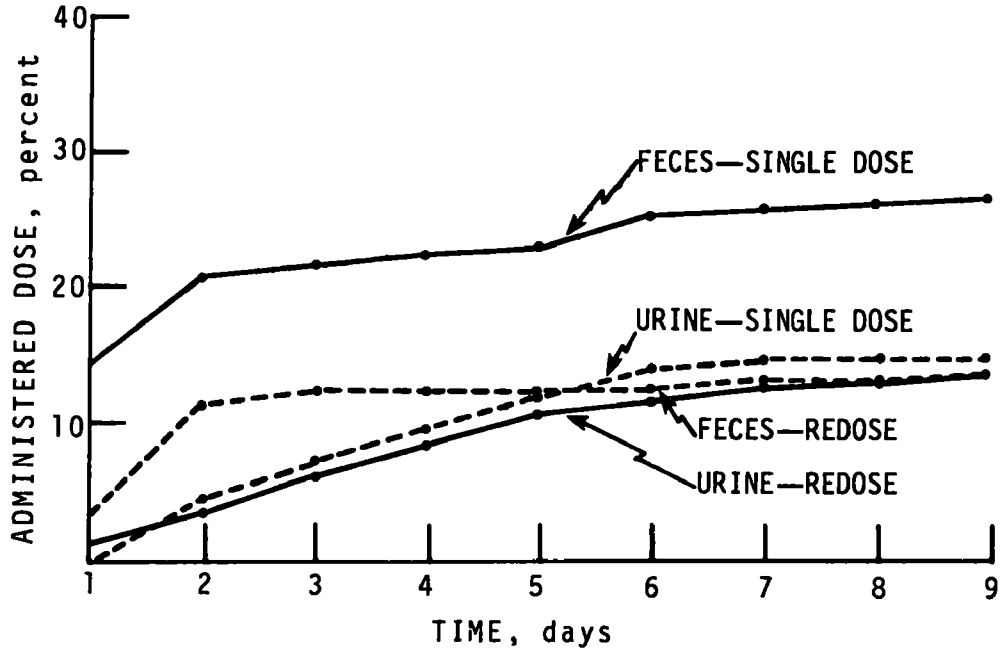

Figure 4. Ionic-^{36}Cl excretion in water fractions of the rat feces and urine following ^{36}Cl-toxaphene (20 mg/kg).

Table 6. UPTAKE OF RADIOACTIVITY IN VARIOUS RAT TISSUES AND ORGANS FOLLOWING A SINGLE-DOSE OF ^{36}Cl - TOXAPHENE, 24 mg/kg
(percent administered dose)

Tissue \ Day	3/24	6/23	12/24	1	2	3	5	7	9	20
Kidney	0.05	0.13	0.43	0.10	0.03	0.03	0.01	0	0.03	0
Muscle	0.93	1.6	5.3	1.3	0	0.65	2.4	0.40	0.14	0.81
Fat	0.14	0.15	0.86	0.57	0.31	0.18	0.18	0.02	3.65	0.03
Testes	0.02	0.08	0.28	0.06	0.04	0.03	0.03	0.02	0.06	0
Brain	0.03	0.06	0.23	0.05	0.04	0	0	0	0.01	0
Blood Cells	3.1	0	0	0.06	0.90	2.6	0	0	1.10	1.17
Blood Supernatant	0.64	1.20	2.35	1.30	0.60	0.36	0	0.18	0.09	0.06
Liver	0.33	1.10	2.33	0.50	0.31	0	0.01	0	0.48	0
First 2 cm Small Intestine	0.06	0.34	0.34	0.05	0.01	0	0	0	0.84	0.09
Last 2 cm Small Intestine	0.10	0.34	0.28	0.13	0.01	0.15	0	0	0	0
Large Intestine	0.19	0.60	1.20	0.19	0.08	0.02	0.03	0.04	0	0
Esophagus	0.04	0	0.04	0.03	0.01	0.01	0.02	0	0.03	0
Spleen	0.04	0.06	0.08	0.05	0.02	0	0.03	0.24	0.06	0
Stomach	3.70	18.6	77.20	2.00	0.63	0.61	0.39	0.16	0.12	0
Total	9.37	24.26	90.90	6.39	2.99	4.64	3.10	1.06	6.57	2.16

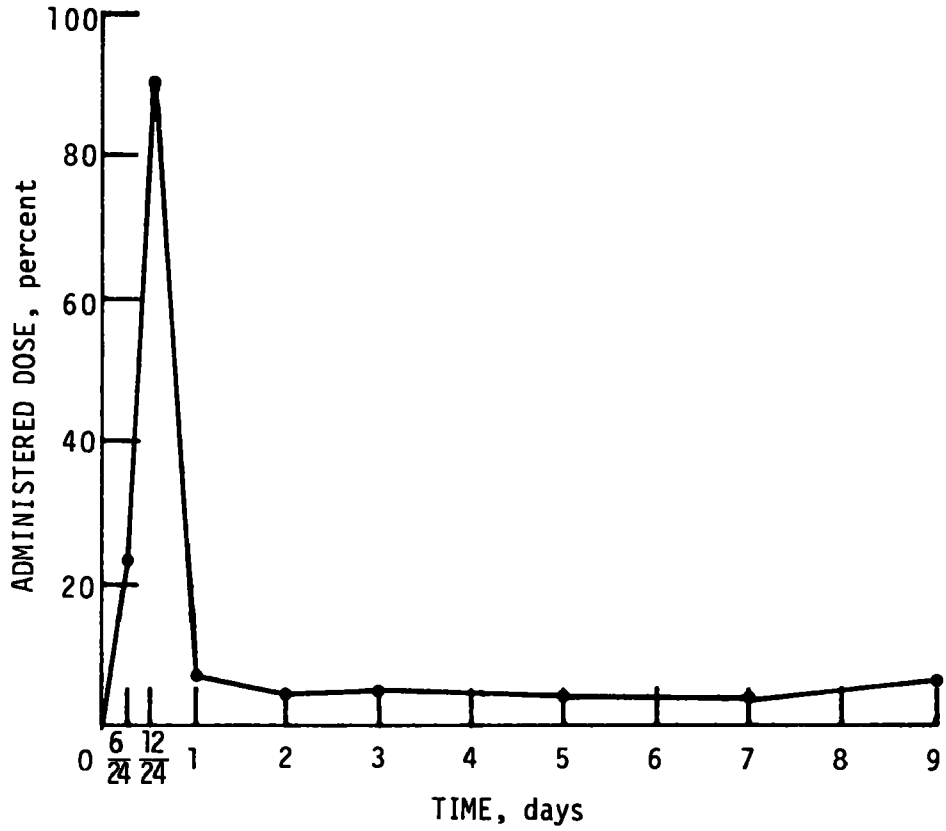

Figure 5. Total uptake of ^{36}Cl by rat tissues and organs following a single dose of ^{36}Cl-toxaphene (24 mg/kg).

Passage of ^{36}Cl through the intestinal tract required about 1 day (Fig. 6). The peak in small intestine occurred at 6 hours; no differences were noted between the first 2 cm and last 2 cm segments. At 12 hours, or an additional 6 hours, large intestine contained the bulk of ^{36}Cl. The amount passing into feces increased rapidly and peaked at 2-3 days.

2. Metabolism by the Liver

This experiment was designed to determine whether toxaphene is metabolized by rat liver. Detoxification of toxaphene by liver was reported by Conley[24] as evidenced by excretion of ethereal sulfate and glucuronate. Since the microsomal system of liver is important in detoxification and degradation of drugs, this work compared metabolism of toxaphene by a whole liver homogenate to that of a microsomal supernatant from liver.

Percent radioactivity recovered in each fraction appears in Table 7. Inferring that radioactivity found in water fractions represented toxaphene metabolism, it can be seen that whole liver homogenate metabolized about 2 1/2 times the amount metabolized by microsomal supernatant. Thus, it would seem that although the microsomal fraction was responsible for some of the toxaphene metabolism, there was another organelle(s) which may be important to the degradation of this insecticide.

B. FATE OF TOXAPHENE IN COCKROACHES

1. Uptake in the American Cockroach, Periplaneta americana

Radioisotopic studies were performed in cockroaches to observe if ^{36}Cl-toxaphene accumulated about the ventral nerve cord and in other tissues. Substantial amounts of radiolabelling associated with a single tissue might indicate a site of action. This site of action could be in the ventral nerve cord if toxaphene behaves like the cyclodienes (Sun et al.[25]). If radiolabelling were found concentrated in peripheral areas of the insect body, a possible site of action like that of DDT might be indicated (Cochran[26], Webster[27], and Weiant[28]). However, possibly due to penetration difficulties, metabolism, translocation by the hemolymph and/or excretion and storage, toxaphene may never reach its site of action as the parent molecule or an active metabolite. To this end, insects showing symptoms of poisoning must be studied along with asymptomatic animals. From this approach, plus electrophysiological studies and symptomology of poisoned insects, a well defined picture may be illustrated showing toxaphene activity in L. maderae and P. americana.

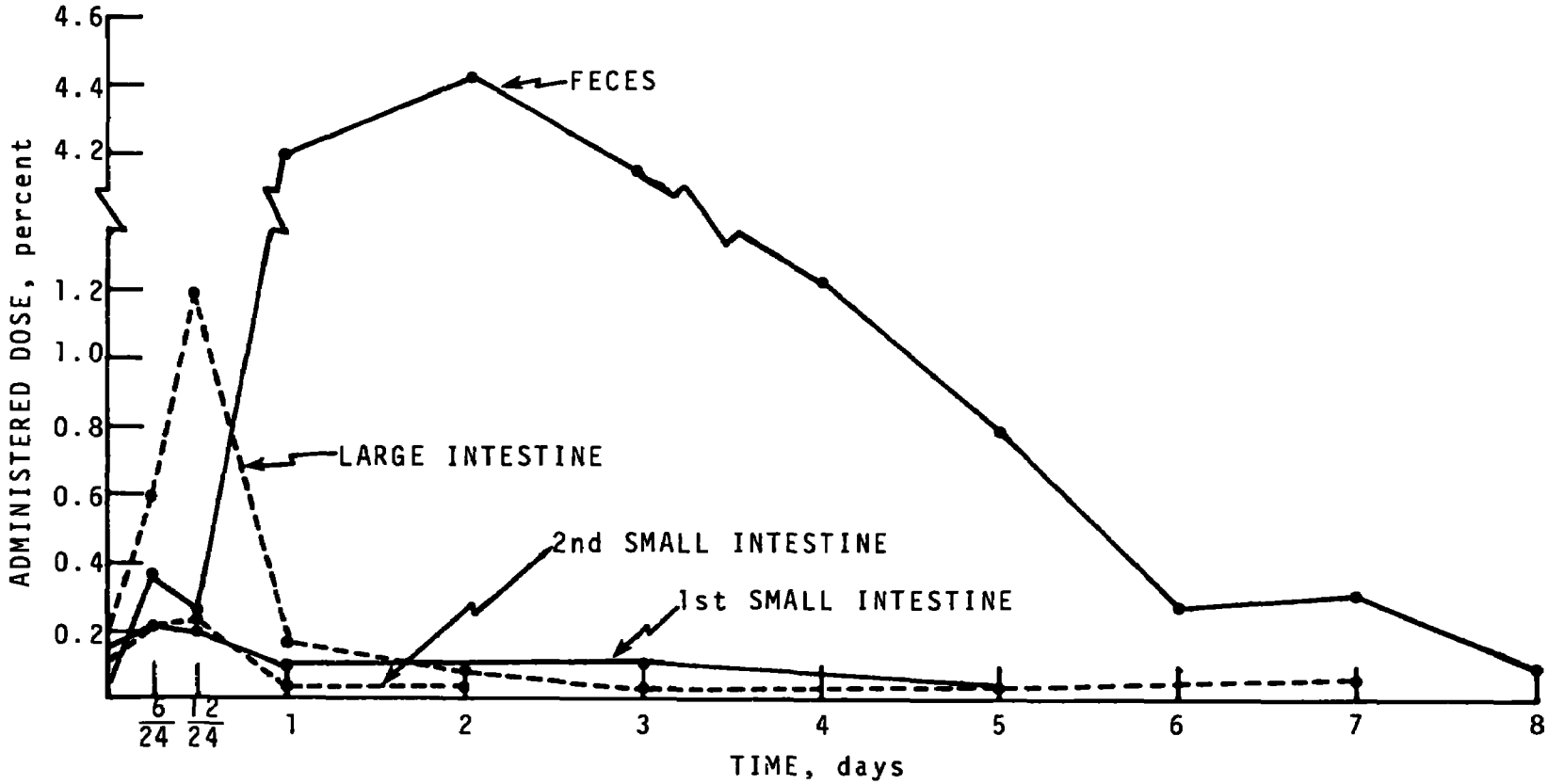

Figure 6. Passage of ^{36}Cl through the intestinal tract into the feces of rats following ^{36}Cl-toxaphene (24 mg/kg).

Table 7. RADIOACTIVITY RECOVERED FROM RAT LIVER HOMOGENATES AND SUPERNATANTS INCUBATED WITH ^{36}Cl - TOXAPHENE
(percent administered dose)

Sample \ Fraction	Hexane	Water	Precipitate	Total
Homogenate:				
Mean Recovery	32.66	9.49	42.70	84.02
% of Total Recovered	38.87	11.29	50.82	100.00
% of Total Extracted	77.49	22.51	--	100.00
Supernatant:				
Mean Recovery	45.29	4.00	34.19	83.49
% of Total Recovered	54.25	4.79	40.95	100.00
% of Total Extracted	91.88	8.12	--	100.00

Amount of toxaphene uptake up to 48 hours is reported in Table 8. Approximately 1/3 of the applied dose had penetrated by 24 hours; however, this represents only 50% of the LD_{50} dose. Cockroaches were moribund at 48 hours and contained 63 μg toxaphene, or 84% of the applied dose.

Figure 7 relates amount of penetrating dose to its occurrence in hemolymph up to 24 hours. The concentration in hemolymph appeared to plateau at ca. 4 hours while total penetration plateaued between 4 and 8 hours. Hemolymph maintained 24.09 ± S.D. 3.2% of the total toxaphene occurring throughout the 48 hour period.

2. Uptake in Leucophaea maderae

An insecticide binding mechanism associated with the membrane of nerve cells appears to be a generally recognized phenomenon applicable to the whole family of chlorinated hydrocarbons (Matsumura and Hayashi[29]). Studies have shown that dieldrin complexes with components of nerves (Matsumura and Hayashi[29, 30], Telford and Matsumura[31, 32]; Sellers and Guthrie[33], Jakubowski and Crowder[34]). These studies may be applicable to other cyclodienes.

There appeared to be uptake by the ventral nerve cord *in vitro*. ^{36}Cl-toxaphene increased as a function of concentration (Fig. 8). Rinsing did remove substantial amounts of radiolabelled material, but after 20 min nerve cords retained significant amounts (Fig. 9). The four-fold difference in radioactivity of the nerves above that found in the final rinse might indicate penetration or binding of toxaphene by the nerve cord. Radioactivity accumulated about the nerve cord of *L. maderae* incubated *in vivo* (Tables 9 and 10).

Radioactivity occurred in the greatest concentration at 48 hours (Table 9). This period of time corresponded to the symptom in the poisoning syndrome where the insects were prostrate with leg movements. At 120 hours insects which exhibited symptoms of poisoning accumulated more ^{36}Cl than did asymptomatic cockroaches (Table 10). Gas-chromatographs of nervous tissue residue showed no differences from the toxaphene standard.

Occurrence of ^{36}Cl in several other tissues of *L. maderae* is shown in Table 11. Radioactivity did not appear to accumulate in hemolymph in any regular pattern. The greatest concentration occurred at 6 hours. Radioactivity was not found in excreta until 48 hours after injection. The average amount occurring between 48 and 120 hours was 271.0 μg/g excrement.

Table 8. PENETRATION OF ^{36}Cl - TOXAPHENE INTO THE AMERICAN COCKROACH

Time, hours	Hemolymph		Carcass		Total	
	µg	Percent of applied dose[a]	µg	Percent of applied dose	µg	Percent of applied dose ± SE
½	1.11	1.48	8.84	11.78	8.37	11.16 ± 4.0
1	2.79	3.72	8.00	10.67	10.13	13.51 ± 5.8
2	2.15	2.87	5.47	7.29	6.35	8.46 ± 3.4
4	5.08	6.77	7.82	10.42	15.23	20.31 ± 7.7
8	4.57	6.09	11.75	15.66	23.67	31.56 ± 6.5
24	6.04	8.05	12.97	17.29	25.63	34.17 ± 4.3
48	11.09	14.79	51.56	68.75	62.66	83.54 ±11.7

[a] 75 µg dose.

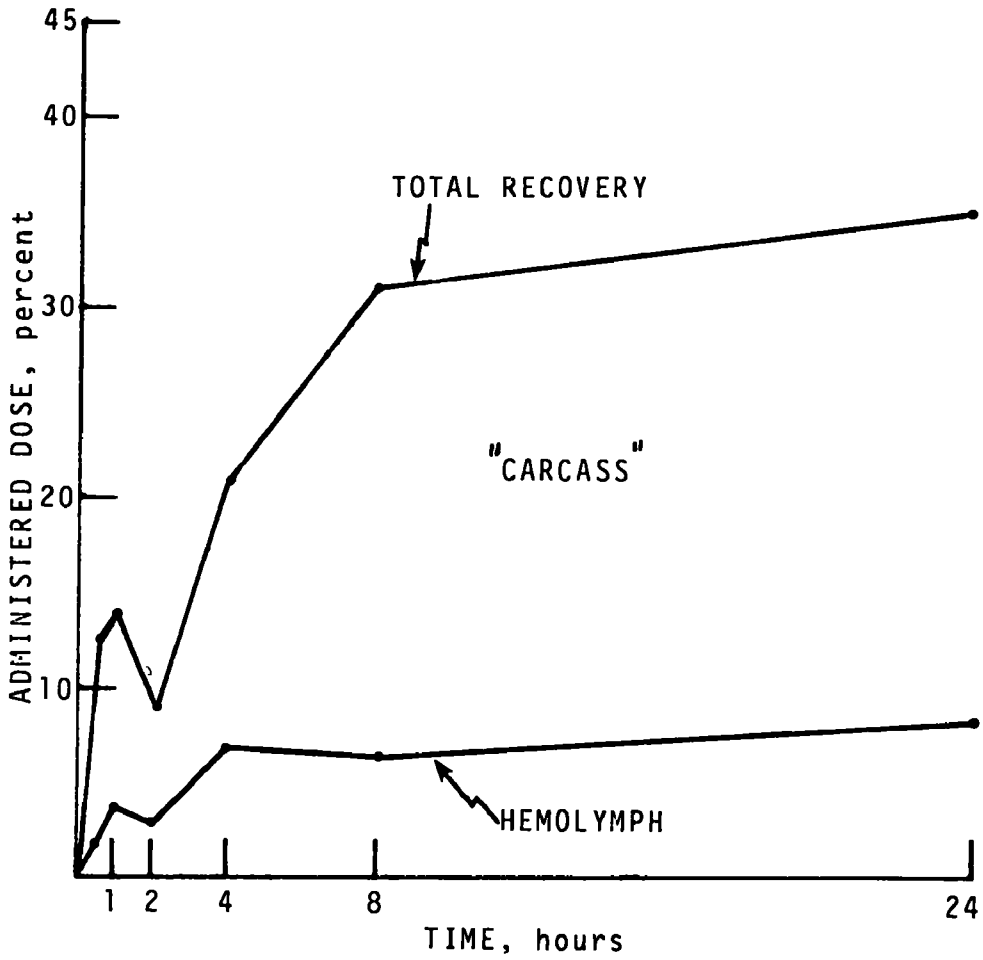

Figure 7. Penetration of ^{36}Cl-toxaphene into the American cockroach.

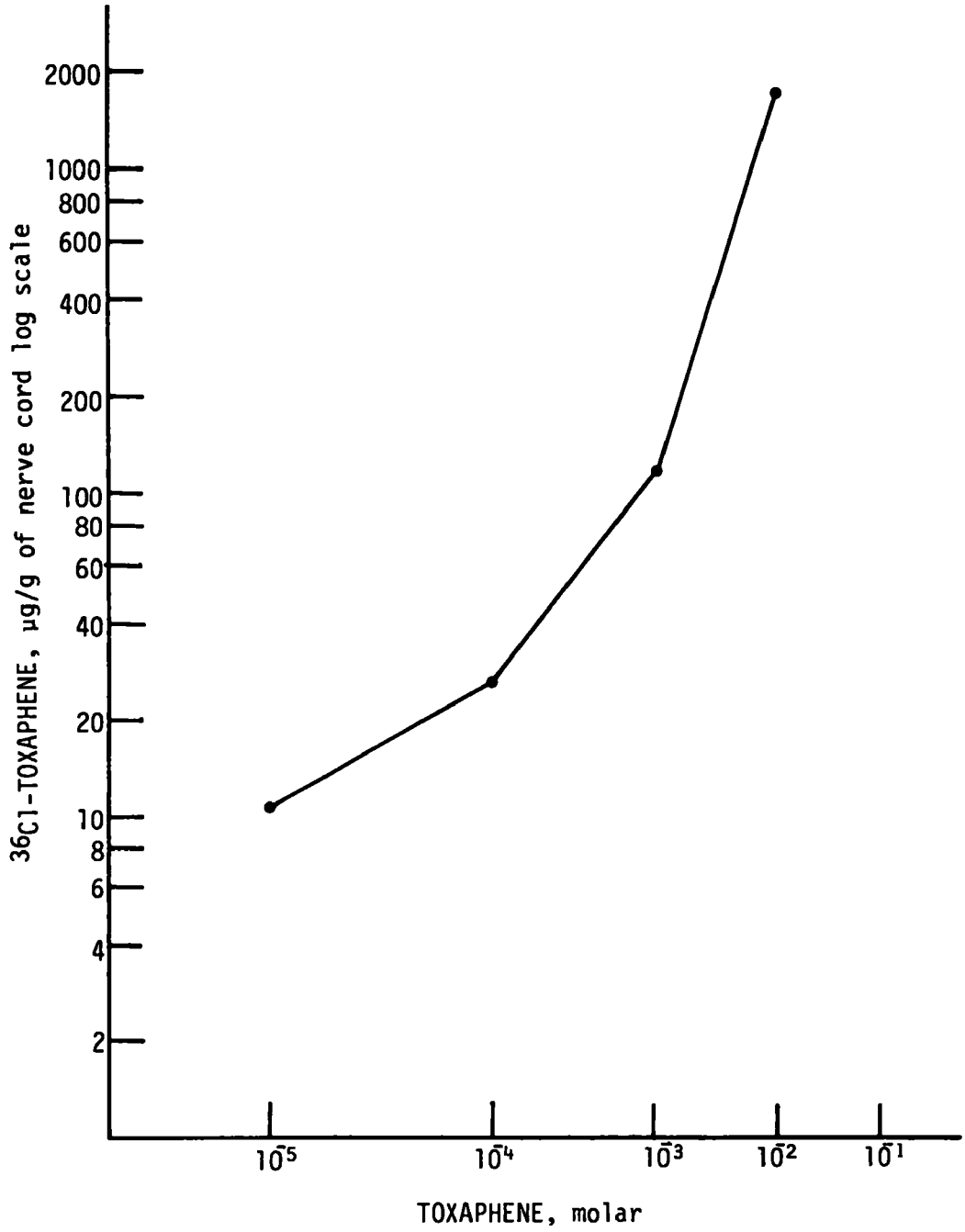

Figure 8. Uptake of ^{36}Cl-toxaphene in nerve cords of Leucophaea maderae incubated in vitro.

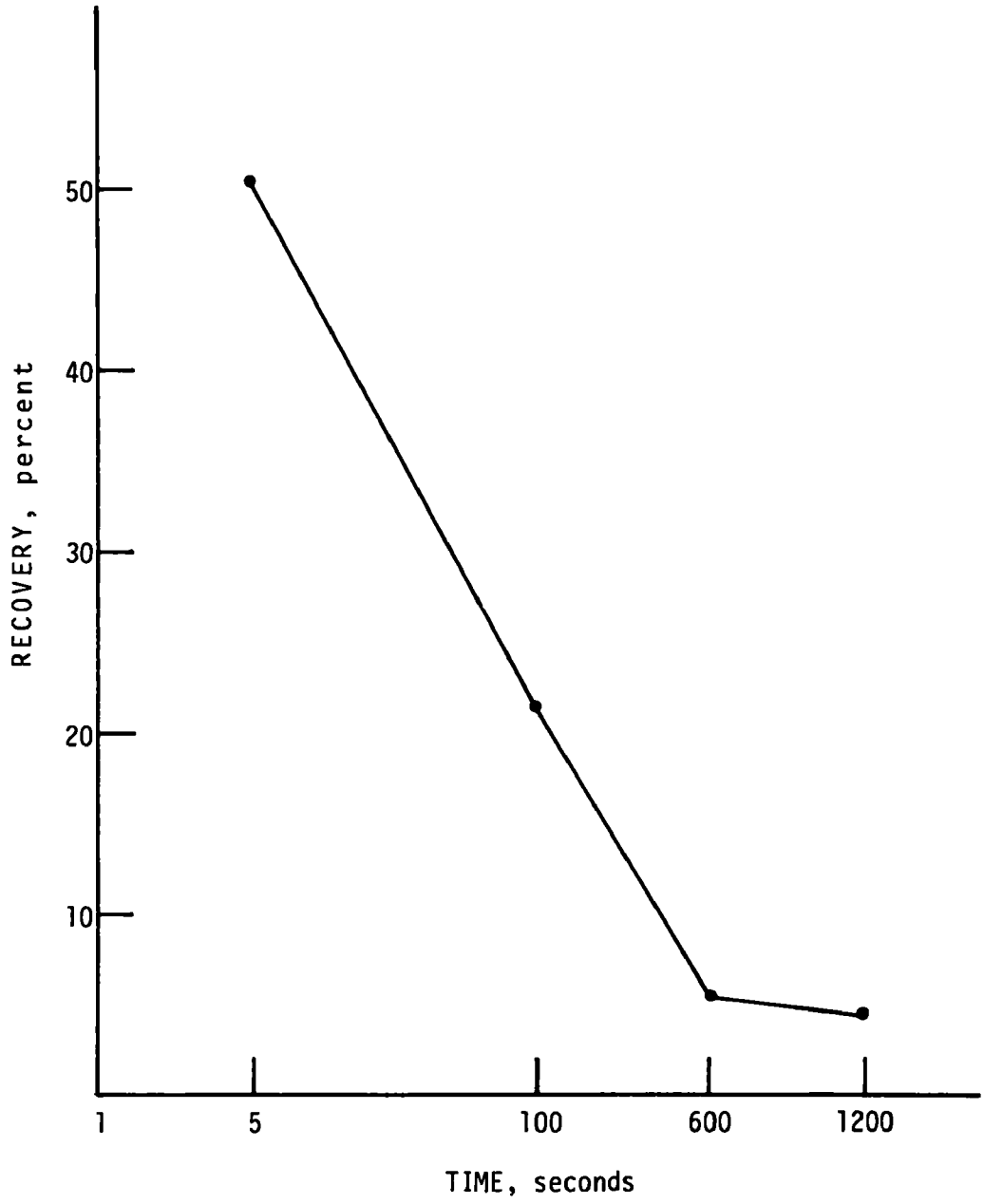

Figure 9. Percent recovery in rinse series relative to ^{36}Cl-toxaphene uptake in nerve cords of Leucophaea maderae incubated in vitro.

Table 9. UPTAKE OF ^{36}Cl IN LEUCOPHAEA MADERAE VENTRAL NERVE CORDS AFTER INJECTION WITH 175.95 µg ^{36}Cl - TOXAPHENE PER INSECT

(µg/mg tissue)

Time, hours	^{36}Cl-toxaphene
2	0.696
4	0.941
6	0.350
8	0.134
12	0.365
24	0.317
48	2.390
72	0.311
96	0.383
120	0.185

Table 10. DISTRIBUTION OF ^{36}Cl - TOXAPHENE IN TISSUES OF LEUCOPHAEA MADERAE

Tissue	No. of Insects	Disposition[a]	Hour Time	Average,[b] µg/g
Hemolymph	75	A	<120	193.88
	21	A	120	67.02
	12	S	120	563.50
Nerve cord	126	A	<120	654.00
	27	A	120	184.59
	12	S	120	268.71
Fat body	75	A	<120	263.89
	21	A	120	309.62
	12	S	120	299.03
Alimentary canal	75	A	<120	272.97
	21	A	120	260.72
	6	S	120	305.02

a A = Asymptomatic, S = Symptomatic

b Average of values recorded at 2, 4, 6, 8, 12, 24, 48, 72, and 96 hours.

Table 11. TOXAPHENE RECOVERED FROM LEUCOPHAEA MADERAE INJECTED WITH 175.95 μg ^{36}Cl - TOXAPHENE PER COCKROACH

(μg/g tissue)

Tissue \ Time, hours	2	4	6	8	12	24	48	72	96	120
Hemolymph	6.88	126.75	908.75	2.15	245.40	147.75	5.84	259.17	42.20	67.02
Fat body	190.65	155.80	18.09	1120.57	110.00	162.99	196.70	303.82	114.40	309.62
Gut	234.75	453.70	238.15	246.75	109.70	181.12	115.40	565.60	311.60	260.72

Binding patterns of toxaphene to subcellular components of insect nerve and brain were studied essentially by the methods of Telford and Matsumura[31]. Percentages of the total amount of ^{36}Cl-toxaphene added, and found in each fraction, are given in Table 12. Fractions were identified as A_1, A_2, A_3, A_4, A_5, A_6 and MIC. A_1 through A_6 were identified by the following descriptions given by Telford and Matsumura[31] with MIC referring to microsomes:

> A_1 consisted of cell membranes, and smaller particles of ca. 12 μ in diameter. A_2 also had cell membranes, but larger and more electron dense than those found in the A_1 layer. Small pieces of nerve ending particles containing synaptic vesicles were also observed. A_3 was primarily pinched-off nerve endings containing synaptic vesicles, and a few mitochondria. The A_4 layer had nerve endings of a more electron dense nature than the two previous layers, and more free mitochondria occurred here. Fragments of nerve sheath appeared in this layer from the nerve cord homogenate. Fraction A_5 had some mitochondria, nuclei, and fragments of sheath from the nerve cord homogenate. The A_6 layer contained nuclei, large pieces of tissue, and large pieces of nerve sheath from the nerve cord homogenate.

Results showed that most of the toxaphene was found associated with the heaviest (nuclei and nerve sheath) fraction and the lightest (microsomes) of both tissues. It is interesting to note that sub-fractions of the brain homogenate showed higher amounts of toxaphene than those of nerve tissue, except for the A_1 (cell membrane) and microsomal fractions. It may indicate that subcellular fractions of brain showed a higher binding affinity for toxaphene than those of nerve tissues.

C. UPTAKE OF ^{36}Cl-TOXAPHENE BY MOSQUITOFISH

High susceptibility of fish and discovery of resistant strains could aid the study of toxaphene's mode of action. Although there is information on toxicity and residues, there has been little work done to quantify actual uptake of toxaphene from the aquatic medium.

Uptake and excretion of related insecticides have been investigated in mosquitofish. Ferguson et al.[35] demonstrated existence of processes of uptake and excretion for endrin, but did not quantify these processes. The major source of endrin uptake was contaminated water rather than accumulation through the food chain. Endrin was also released into water by contaminated fish which indicated some type of excretion mechanism.

Table 12. RECOVERY OF ^{36}Cl - TOXAPHENE IN NERVE TISSUE OF LEUCOPHAEA MADERAE
(percent administered dose)

Tissue \ Fraction[a]	A_1	A_2	A_3	A_4	A_5	A_6	Microsomes
Nerve	1.6	0.4	0.3	0.8	0.6	5.0	17.3
Brain	1.1	0.8	1.3	1.9	1.3	9.7	10.3

[a]Description of fractions given in text.

Wells and Yarbrough[36, 37] and Yarbrough and Wells[38] studied retention of DDT, aldrin, dieldrin, and endrin in resistant and susceptible mosquitofish. Using radioactive tagging, they demonstrated that cell membranes of resistant fish bind more insecticide than membranes of susceptible fish. These results suggested that resistance is in part the result of a membrane barrier in resistant fish.

Quantification of the amount of toxicant actually absorbed is important for investigations into the mode of action of these insecticides. The present research was undertaken to quantify uptake of toxaphene by mosquitofish and relate these data to the toxicity syndrome.

A total of 100 fish was used to determine the 24 hour LC_{50} (Fig. 10). Since future experiments were to be conducted over short time periods, the purpose of this testing was to determine appropriate experimental concentrations rather than to define the lethal concentration of a particular population of fish. Thus the LC_{50} represents a 24 hour time period. The LC_{50} as determined by a best fit line is approximately 860 ppb. Extrapolating from this curve, 2 ppm was selected as the appropriate experimental concentration for testing fish. Fig. 11 illustrates the LT_{50} for oxidation pond Gambusia at 2 ppm of toxaphene to be 12 hours. Since both uptake and excretion were to be studied, 8 hours was selected as the optimum period for absorption of this concentration level. Fig. 12 shows that at periods longer than 8 hours mortality increases rapidly, making excretion results impossible to interpret in moribund fish.

Fig. 13 represents results of the uptake experiments with mean concentration of ^{36}Cl-toxaphene and Tr residues for each trial plotted over time. Regression analysis of each trial resulted in linear uptake equations (Table 13). These were significant at the 0.01 level.

Total recovery was calculated on the basis of uptake experiments. In each case, 9.67 mg of ^{36}Cl-toxaphene was added to the test solutions. For the 2 uptake experiments, average total recovery was 0.046 mg, or 0.47%. Presumably the remainder was left in the water or adsorbed on the test containers. The computer program used in these experiments calculated the number of mg of toxaphene represented by the scintillation counts per sample and also per g of fish. This value is misleading as it represents not only toxaphene present but also any metabolites of toxaphene which contain radioactive chlorine.

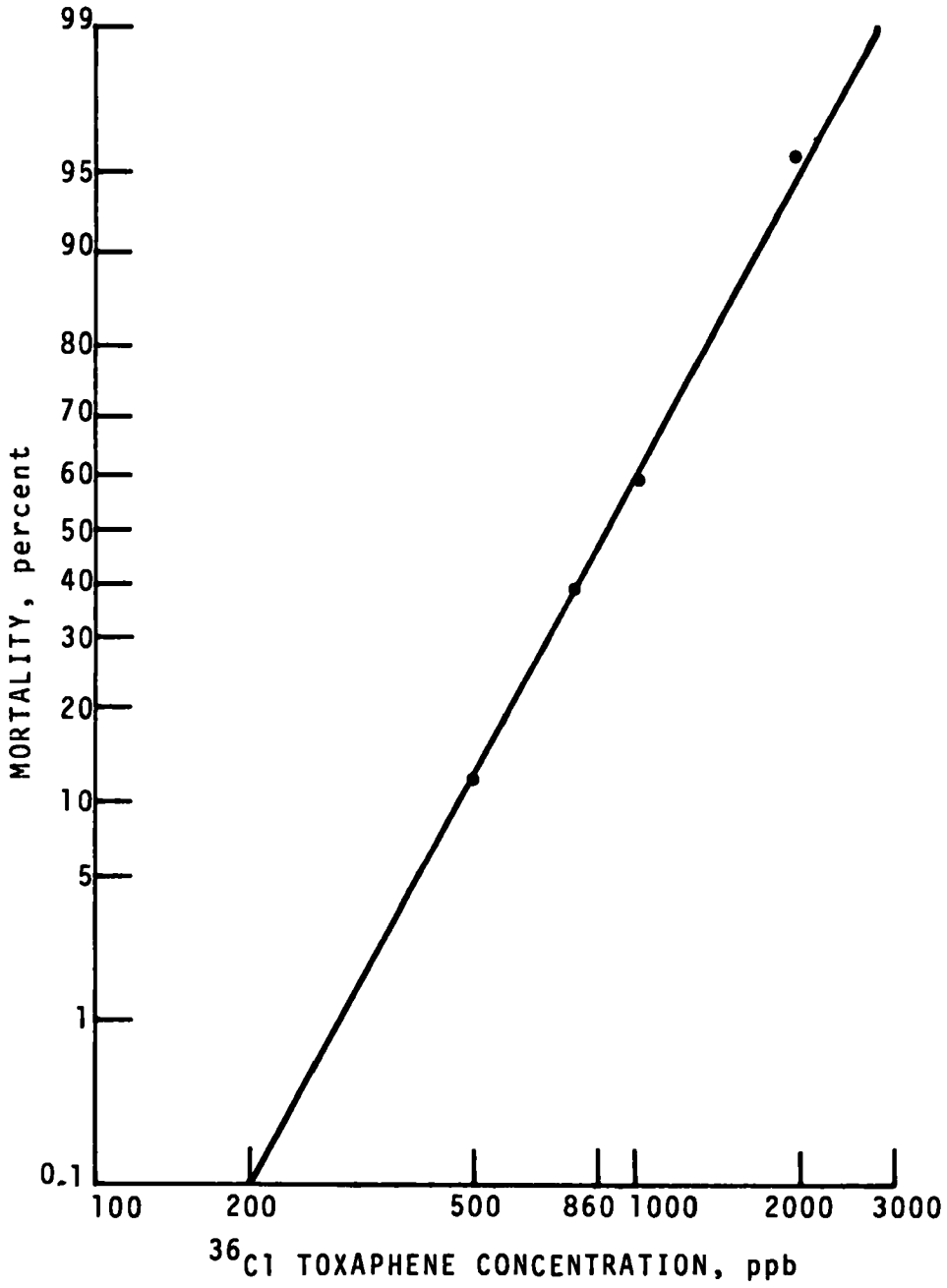

Figure 10. LC_{50} determination for Gambusia affinis after 20 hours exposure.

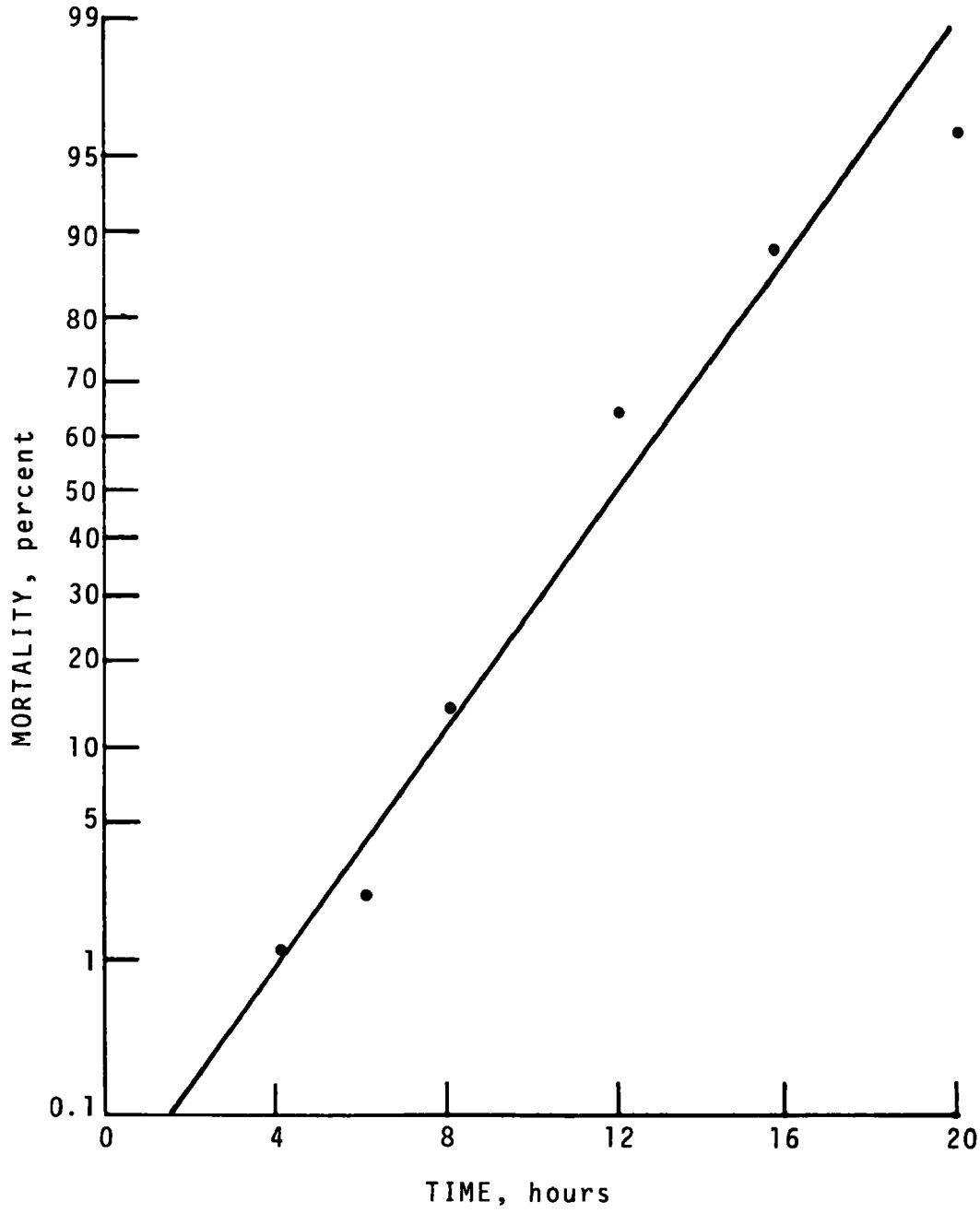

Figure 11. LT_{50} determination for Gambusia affinis at 2000 ppb ^{36}Cl-toxaphene.

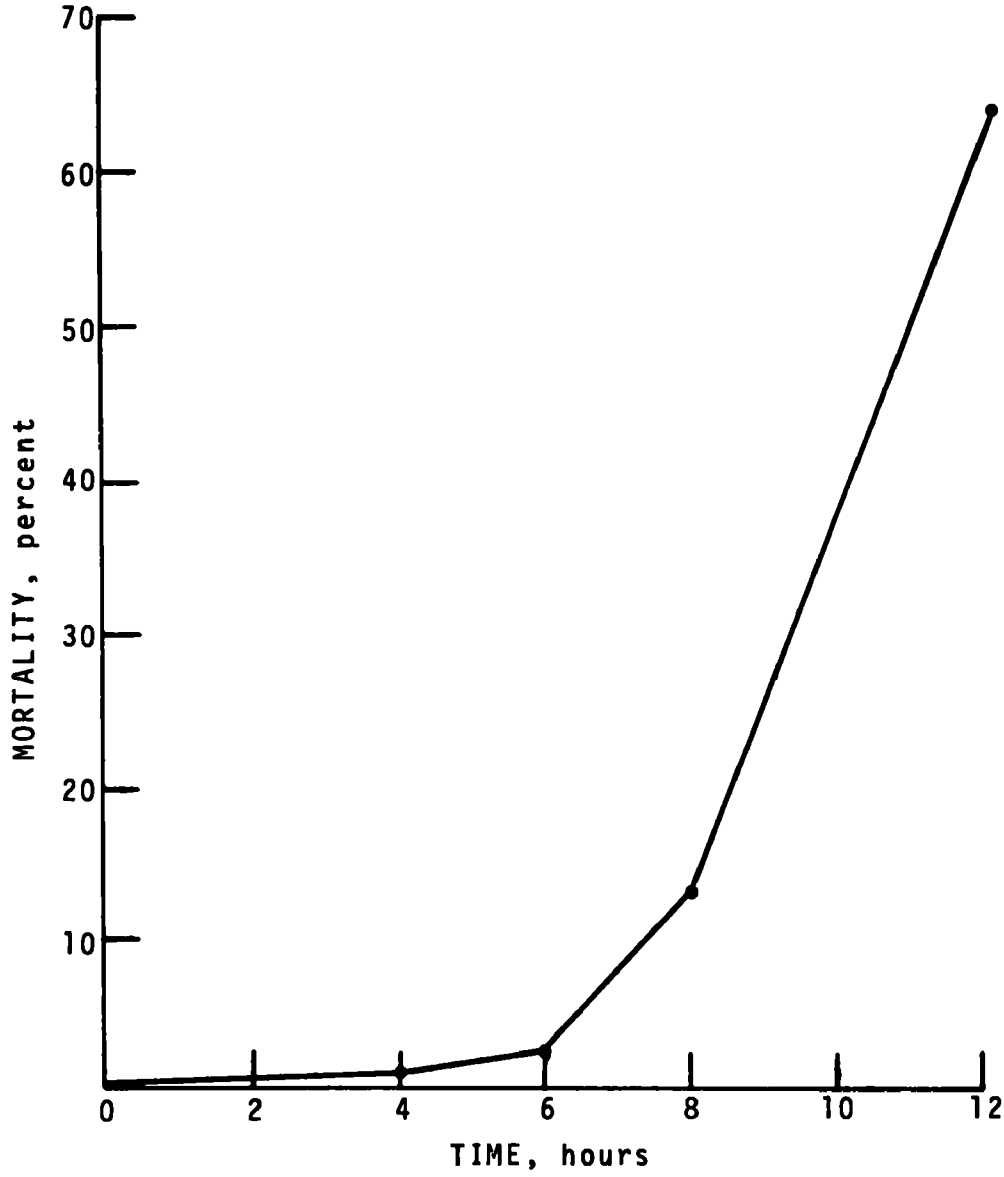

Figure 12. Percent mortality of Gambusia affinis as a function of exposure to 2000 ppb toxaphene

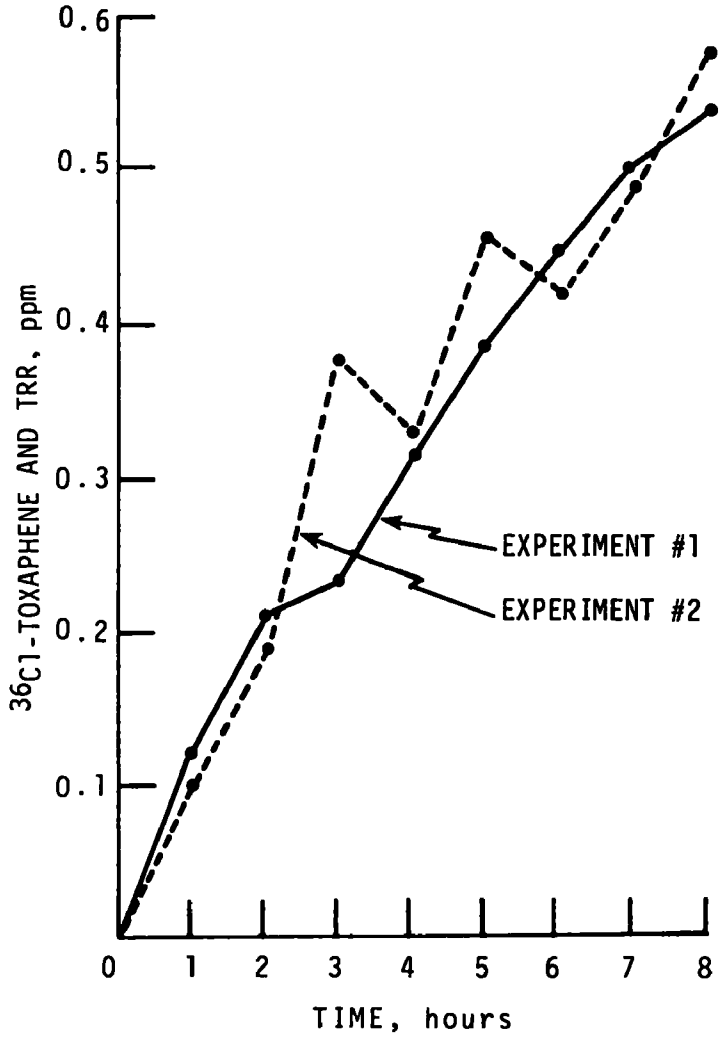

Figure 13. Uptake of ^{36}Cl-toxaphene in *Gambusia affinis* as a function of exposure to 2 ppm ^{36}Cl-toxaphene.

Table 13. RESULTS OF REGRESSION ANALYSIS FOR THE UPTAKE AND EXCRETION OF ^{36}Cl - TOXAPHENE BY GAMBUSIA AFFINIS

Experimental group	Regression equation	r
Uptake A	Y = 0.00068 + 0.00058 (X)	0.76266 *
Uptake B	Y = 0.00071 + 0.00052 (X)	0.86208 *
Excretion A	Y = 0.00601 + 0.00004 (X)	0.04808
Excretion B	Y = 0.00471 + 0.00002 (X)	0.04089

*Significant at the 0.01 level, Student t test.

On the basis of these results, several observations could be made concerning dynamics of toxaphene uptake by G. affinis. Uptake was a linear function and was directly proportional to length of exposure. Fig. 14 plots uptake by 2 different weight groups of fish, less than 800 mg and more than 1200 mg. The graph shows that at every point, small fish contained more ^{36}Cl-toxaphene per g of tissue than large fish. Mean body load of fish weighing between 800 and 1200 mg in every case fell between the plots shown in Fig. 14. Comparison between paired points revealed that fish weighing less than 800 mg acquired between 10-42% more residue than fish weighing more than 1200 mg. Mean concentration of ^{36}Cl-toxaphene and TR residue for each trial of the excretion experiment was plotted over time (Fig. 15). Regression analysis of data resulted in statistically nonsignificant excretion equations (Table 13). Calculation of confidence intervals about individual points indicated that at the 0.05 level, there was no significant difference in body load over time in either trial. Therefore, from these data, there was no indication of excretion during the first 6 hours following exposure.

Table 14 represents partitioning of fish extracts into water and hexane fractions. This revealed that 88.7% of the radioactive chlorine was soluble in the nonpolar phase.

Observation of the toxicity syndrome and characterization into 5 stages were subjective processes. Interpretation of toxicity symptoms without a knowledge of the mode of action of toxaphene was impossible although certain behavior might suggest physiological correlates. The first stage of the toxicity syndrome, and the most difficult to assess, was when fish began to swim at the surface against the side of test containers. Swimming at the surface is normal in water with low oxygen content and is also the normal feeding position for G. affinis. Normal fish, however, retreated from the surface when the aquarium was approached whereas poisoned fish remained at the surface. Subsequent toxicity stages were all marked by rapid gill ventilation which further suggested respiratory involvement. The third toxicity stage was characterized by fish swimming against the side of aquaria, but with some loss of equilibrium which was evidenced by sinking of the posterior end so that fish attempted to swim up towards the surface. Finally, fish lost their ability to maintain normal dorsal-ventral orientation and rolled to the side. At stage 4 fish sank to the bottom and were prostrate with rapidly ventilating gills. At this stage, there was occasional

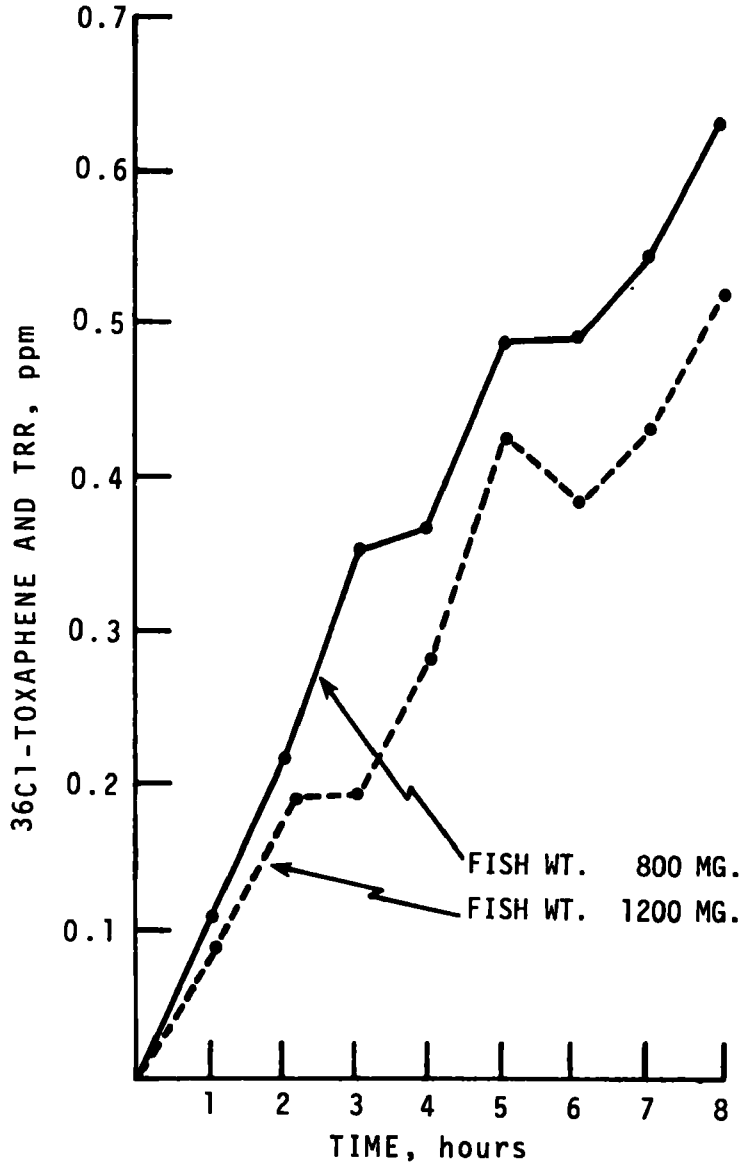

Figure 14. Uptake of ^{36}Cl-toxaphene for large and small Gambusia affinis as a function of time.

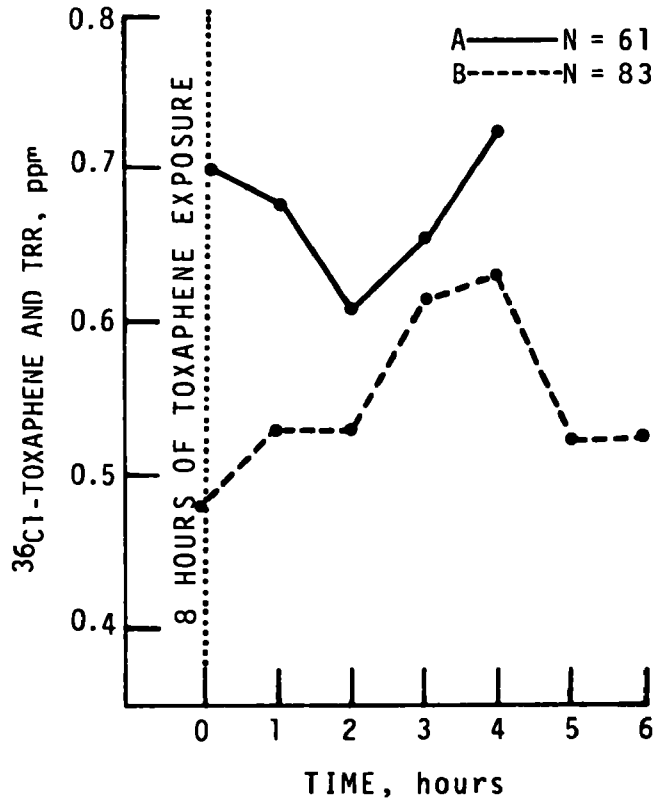

Figure 15. Body load of ^{36}Cl-toxaphene and TR residues in Gambusia affinis as a function of time in fresh water.

Table 14. METABOLITE PARTITIONING OF TOXAPHENE IN GAMBUSIA AFFINIS[a] (μg/g tissue)

	Hexane fraction	Water fraction	Total
^{36}Cl-Toxaphene and TR per sample	0.676	0.084	0.760
Percent of total	88.700	11.300	100.000
^{36}Cl-Toxaphene and TR/g of fish	0.586	0.072	0.658

[a]Average ^{36}Cl-Toxaphene and TR residues control = 0.68 ± 0.01 μg.

darting behavior, until death which was identified by the cessation of gill movements.

Fig. 16 indicates average body load of ^{36}Cl-toxaphene and TR residues at each toxicity symptom. By the time fish exhibited the first toxicity response to toxaphene, rising to the surface of the water, they had already sorbed 90.3% of the average fatal residue. Fish which were characterized as normal had accumulated 35% of the fatal residue. In stages 3, 4, and 5, fish showed obvious signs of toxicity.

Average residue per g of fish and toxicity symptom exhibited at each hour are shown in Table 15. Fish progressed through toxicity symptoms at approximately the same rate until the eighth hour. This was the point on the mortality curve (Fig. 12) where mortality increased rapidly. At this point it was possible to examine differences in body load between fish with identical exposure times, but which exhibited different toxicity symptoms. The onset of a particular toxicity stage was directly proportional to body load.

Since fish were processed whole in these experiments, it was impossible to determine what portion of these residues had been absorbed into particular organs and what portion was simply adsorbed to scales and fins. Information concerning toxicity symptoms is perhaps most important when considering *in vitro* studies into the mode of action of toxaphene in fish. Data show that earlies toxicity symptoms were visible when toxaphene content was as low as 0.2 ppm and this was in a toxaphene resistant population. In non-resistant populations, where the LC_{50} is around 30 ppb, toxic symptoms would presumably be apparent with even lower body loads.

Compared to 36 hour LD_{50}s reported by Boyde and Ferguson[17], *G. affinis* used in these experiments were highly resistant. The highest resistance reported by these workers was 480 ppb while nonresistant populations had LD_{50}s around 10 ppb.

Due to virtual insolubility of toxaphene in water, its presence in any aquatic medium is either as a suspension or else adheres to particles in the water. Thus under experimental conditions, uptake by fish may be due to 1) adsorption of toxaphene to the body of fish, 2) simple diffusion of toxaphene into fish, and 3) active processes of absorption into fish. On the basis of adsorption alone, it would be expected that toxaphene residues of whole fish would

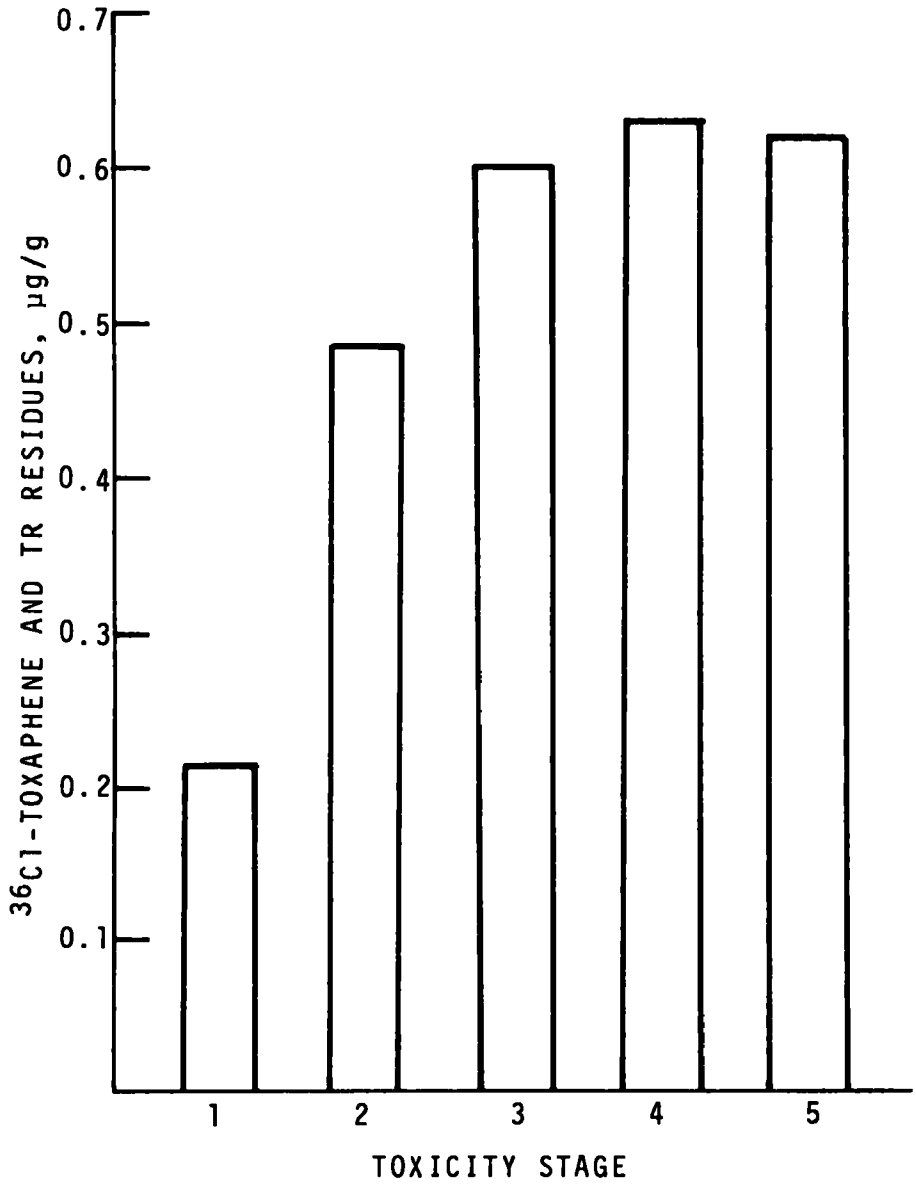

Figure 16. Mean body burden of ^{36}Cl-toxaphene and TR residues in Gambusia affinis at each stage of the toxicity syndrome.

Table 15. ^{36}Cl - TOXAPHENE AND TR RESIDUES AND TOXICITY SYMPTOMS EXHIBITED AT EACH HOUR IN GAMBUSIA AFFINIS

($\mu g/g$ fish)

Toxicity Stage	Hours													
	-------Exposure to 2 ppm ^{36}Cl-toxaphene---------													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
5								0.680						
4												0.750		
3								0.610			0.660	0.705	0.527	0.523
2					0.420	0.435	0.497	0.537	0.605	0.569	0.631	0.649		
1	0.109	0.202	0.254											

increase as a function of time. All fish of the same weight or body surface would theoretically have the same body load at any given time. Table 15 shows, however, that any time when more than one toxic symptom was exhibited, mean body load was different for fish showing different symptoms, and that amount of residue present was consistent with severity of the symptom. Since exposure time was equal for all fish, different body loads at various toxicity stages appeared to reflect differences in rates of uptake. If adsorption or diffusion were solely responsible for uptake, there should be no differences in body load at any given time regardless of toxic symptom; thus, active processes of absorption appeared to be implicated in the uptake process. This agrees with the conclusions of Ferguson et al.[35] concerning endrin uptake in G. affinis.

Partitioning of fish extracts revealed that 88.7% of the radioactive chlorine was recovered from the nonpolar fraction. Recovery rates, however, for the hexane/water partitioning procedure similarly averaged 87.62%. Thus, there was apparently minimal metabolic alteration in the toxicant after a period of 8 hours. Dehydrochlorination has been reported as a method of toxaphene metabolism in rats (Ohsawa et al.[39]). If this were the case in fish, one would also expect fish to excrete chloride. The excretion data showed no evidence of excretion within 8 hours; this correlated with lack of toxaphene metabolism during that period. Likewise, Ferguson et al.[35] found no evidence of metabolic or chemical alteration of endrin in their studies with G. affinis.

Insecticide resistance in fish is not completely understood at this time. Ferguson et al.[35] attributed resistance to increased physiological tolerance rather than differences in rates of uptake. Data presented here showed that onset of toxicity symptoms varied within any given population, and that onset of particular toxicity symptoms was directly proportional to body load. Thus within this population, differences in toxic response were due to different body loads rather than different tolerances of a particular toxicant level.

Observations of the progression of toxicity symptoms in G. affinis suggested that intoxication involved an increased oxygen requirement. The first stage of toxicity was marked by fish swimming at the surface of water which is typical for these fish in water of low oxygen content. Swimming toward the

surface, coupled with decreased activity before finally sinking to the bottom, suggested a depletion of energy. This was in spite of an apparent increasing need for oxygen which was evidenced by rapid gill ventilation which continued until death. Ferguson et al.[35] noted increased oxygen requirements for endrin-poisoned fish, but related them to increased activity that characterized endrin poisoning. With toxaphene, activity decreased with onset of toxicity symptoms while the requirement for oxygen appeared to increase.

The very sharp rise in mortality at 8 hours observed in the mortality curve (Fig. 12) coupled with similarity of body loads at toxicity stages 3-5 suggested that mortality was due to a very critical level of toxaphene at the site of action. If several different sites of action were involved, one would expect each to be affected at slightly different concentrations. This would tend to flatten the mortality curve. It was suggested, therefore, that the response of G. affinis to toxaphene was due primarily to toxicity mechanisms acting at one site.

D. EFFECT OF TOXAPHENE ON THE NERVOUS SYSTEM

1. Electrophysiological Studies of Cockroach Nerves

LD_{50} studies indicated poor insecticidal activity of toxaphene toward the cockroach L. maderae (Fig. 17). As the dose was increased, symptoms of poisoning occurred more rapidly.

Symptoms appeared similar to those of other chlorinated hydrocarbon insecticides (e.g., dieldrin, DDT, and lindane). Following injection of toxaphene at high concentrations (10^{-3} - 10^{-1}M), insects would run in circles at the bottom of their circular confine. Later, they would become prostrate on their dorsum with legs and other appendages still active. Animals observed displaying this behavior were recorded as "prostrate and kicking". This sort of activity was followed by a purely prostrate condition with only maxillary palps and antennae active. Death was recorded when the insect was prostrate, totally inactive, and would not respond to stimuli.

When examined, insects considered dead did have active hearts with irregular and faint beats. Insects dissected during the "prostrate and kicking" phase possessed hearts with rhythmic normal beating rates. Those cockroaches observed as prostrate had irregular heart beats but at a nearly normal rate.

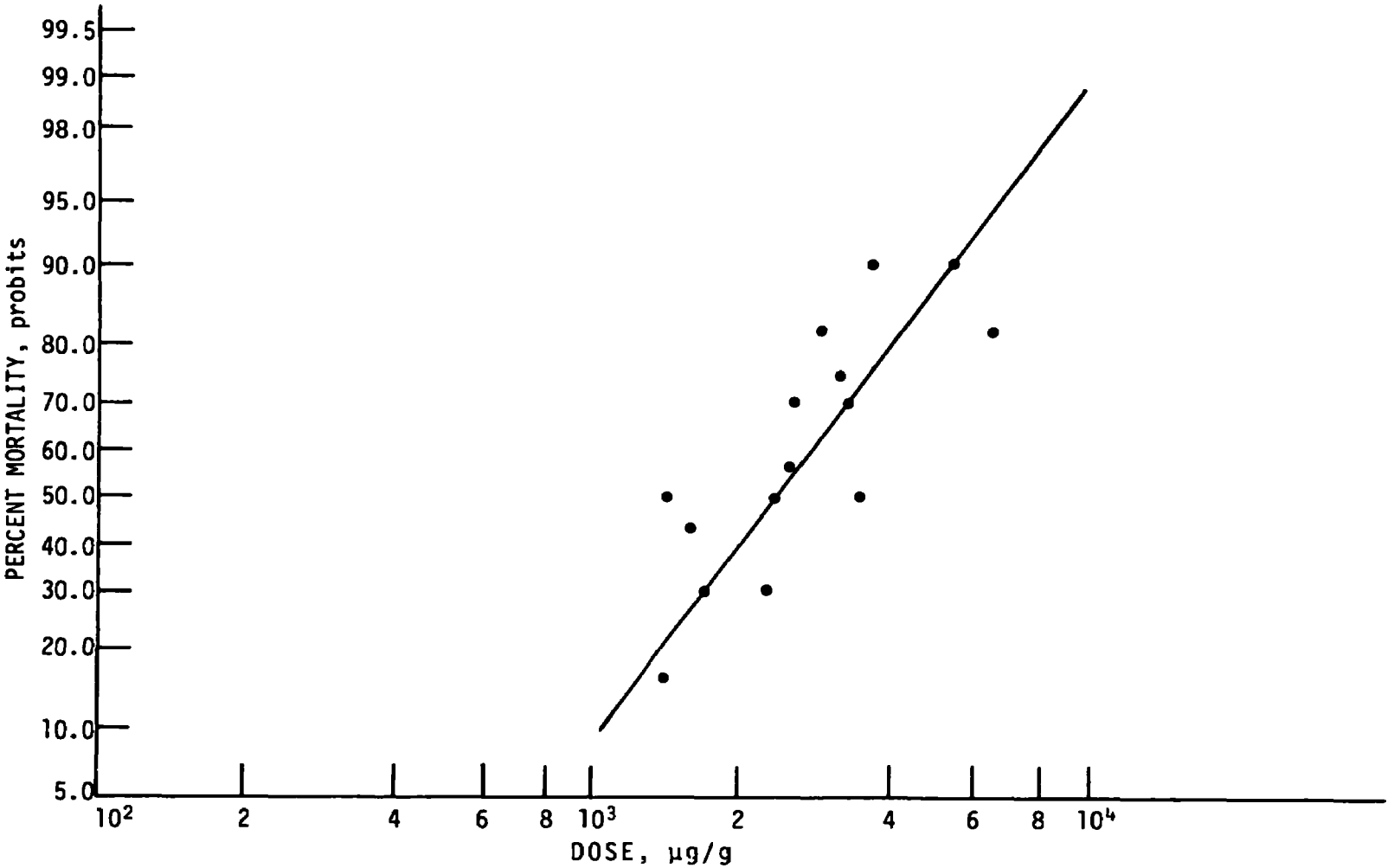

Figure 17. LD_{50} (48 hour) of toxaphene on Leucopheae maderae

Control cockroaches showed no symptoms of poisoning and all specimens survived. Dissected insects showed normal heart activity.

In electrophysiological studies of P. americana, there appeared to be 3 phases of nervous activity following dissection. The first phase was characterized by bursts of high intensity spikes that were irregular in rhythm and varying in spike amplitudes and frequency (Fig. 18). This first phase of spontaneous activity appeared to be a form of dissection shock lasting an average of 67 sec from the time of contact with the electrode. The second phase was a period of recovery from dissection. This period showed activity of an irregular form. Spikes of varying amplitude could be observed throughout this period. These spikes ranged in amplitude from 0.02 volts to 0.12 volts. No bursts of spikes were observed. The third phase was a period of stabilization usually occurring 2 hours from contact with the electrode. It was marked by strictly baseline activity where nerve impulses were too weak to be seen over the interference at 0.1 volt/division. This period was where testing of pharmacological agents began.

Mineral oil (0.05 ml) used as a control appeared to have little effect on nerve activity. When applied directly on the nerve, it covered the nerve making good contact, but produced little effect.

Toxaphene in mineral oil at doses of 10^{-3} - 10^{-1}M had immediate effects on the nerve (Fig. 19). There appeared to be "buzz saw" effects similar to DDT, spikes started at low amplitudes (0.02 volts) and frequency and then built to high amplitudes and very high frequency. This was unlike dissection shock in that these bursts occurred rhythmically. Each individual nerve appeared to react differently; some appeared very susceptible, others resistant. Toxaphene at lower concentrations (10^{-4} and 10^{-5}M) had little effect.

2. Effect on Ion Fluxes in Cockroach Nerves

Effect of chlorinated hydrocarbons on the central nervous system may be that of disrupting normal fluctuations of ions across the neural membrane. In particular, they may disrupt sodium and potassium ion exchanges. For instance, DDT seems to accelerate the rate of potassium efflux (Matsumura and O'Brien[12]), whereas dieldrin accelerates sodium influx (Hayashi and Matsumura[40]). Although both eventually lead to an accumulation of sodium ions within nerve, the way in which this is done is different, thus suggesting

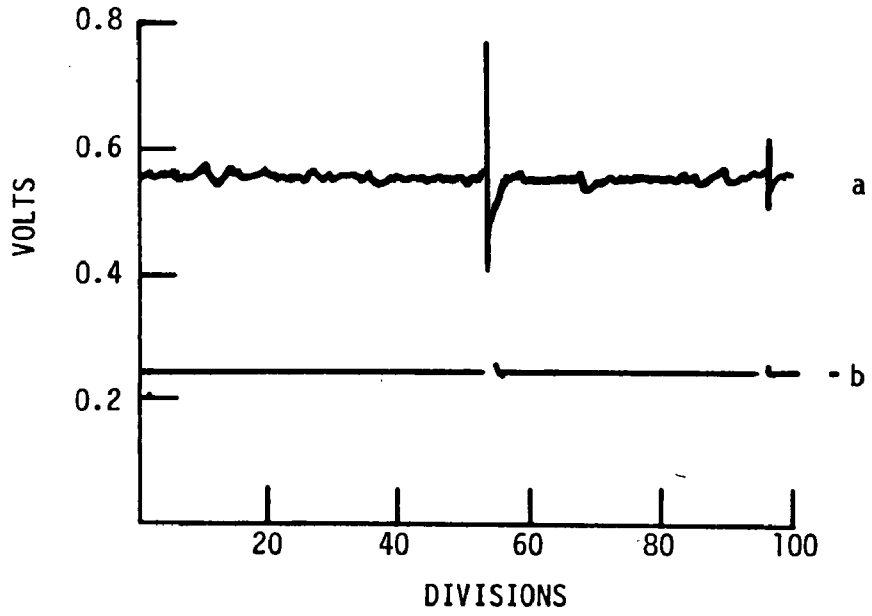

Figure 18. Oscillograph of spontaneous nerve activity from ventral nerve cord of Periplaneta americana.

a. Trace showing spontaneous nerve activity.

b. Trace showing frequency recording of spikes with discriminator setting of 0.1 volt amplitude.

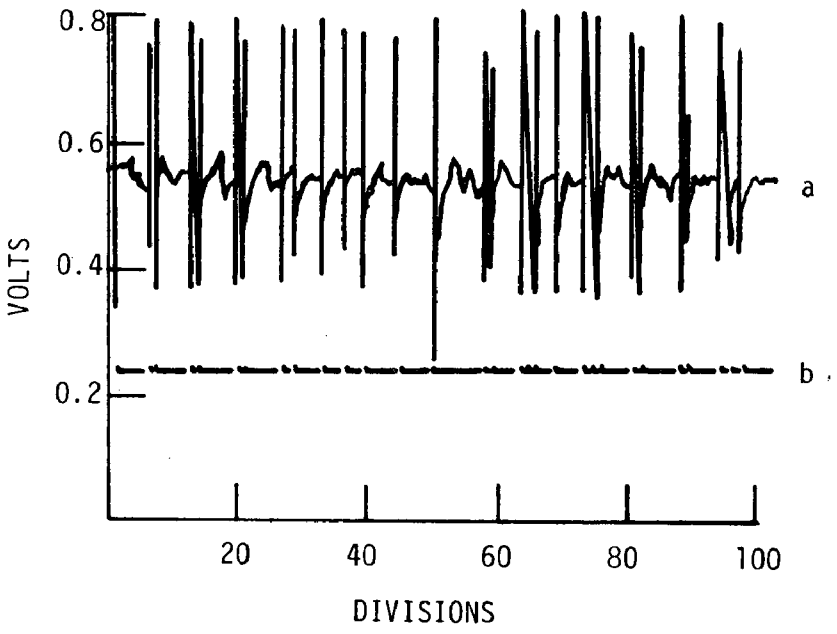

Figure 19. Oscillograph of nerve activity immediately following a dose of 2.1 mg toxaphene on the ventral nerve cord of Periplaneta americana.

a. Trace showing activity following toxaphene treatment.

b. Trace showing frequency recording of spikes with a discriminator setting of 0.1 volt amplitude.

slight differences in their mode of action. In this light, a study of the effect of toxaphene on ionic fluxes of the insect central nervous system was undertaken.

Preliminary work with chloride ion is reported herein. In order to find a concentration of toxaphene and incubation times to use for these investigations, 10^{-7}, 10^{-5}, and 10^{-4}M toxaphene have been used. At each concentration, rate of uptake of chloride ion was measured by incubating nerve segments from one minute to one hour, and plotting rate versus that of the control (Fig. 20-25). Although the difference between control and treated in Fig. 20 appeared significant, extended incubation times (e.g., 2 and 3 hours) will be necessary for other differences to become evident.

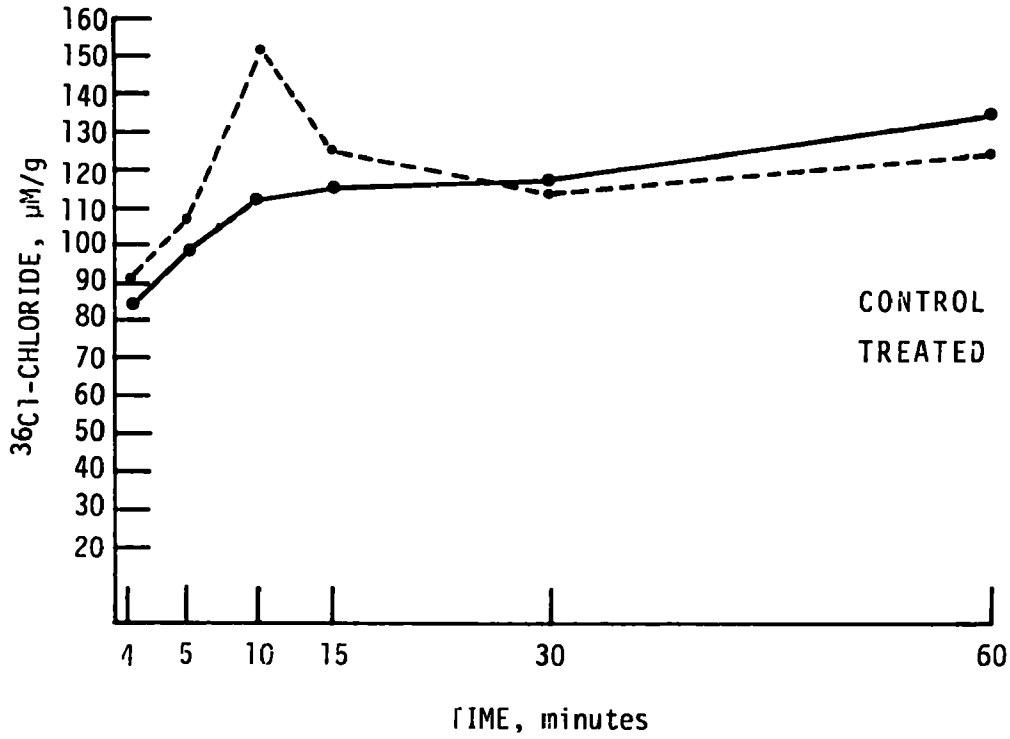

Figure 20. Uptake of ^{36}Cl in abdominal segment of ventral nerve cord of Periplaneta americana exposed to 10^{-7}M toxaphene

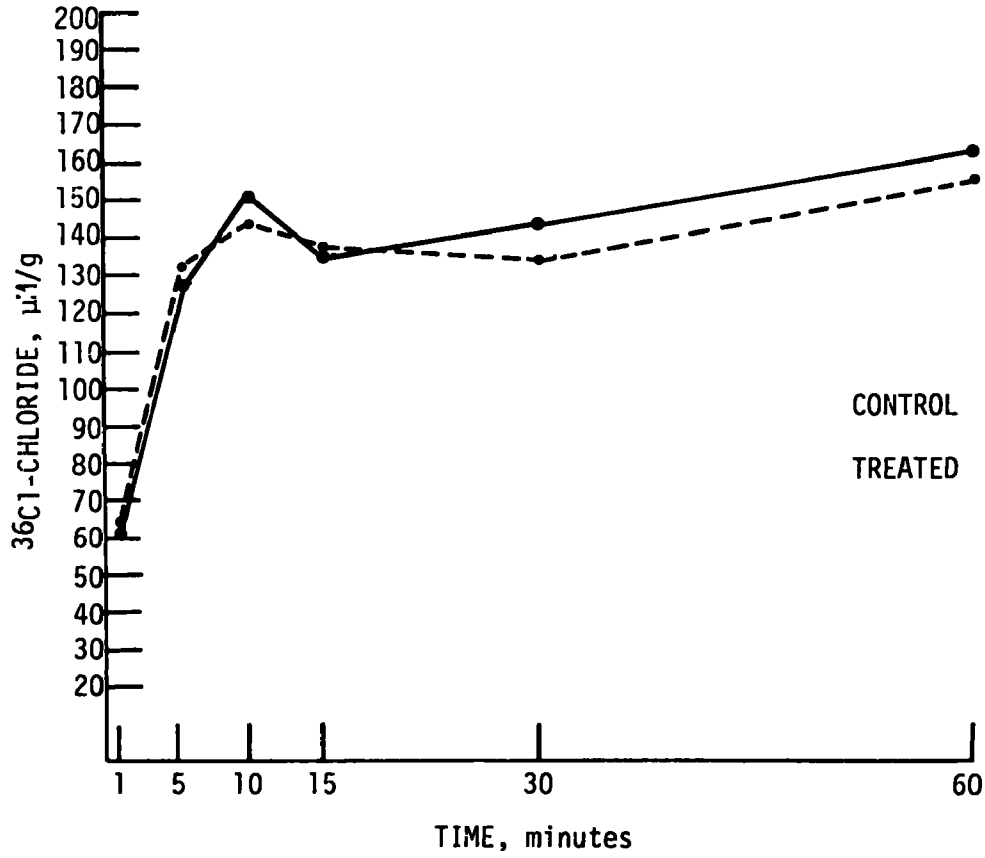

Figure 21. Uptake of ^{36}Cl in thoracic segment of ventral nerve cord of Periplaneta americana exposed to 10^{-7}M toxaphene.

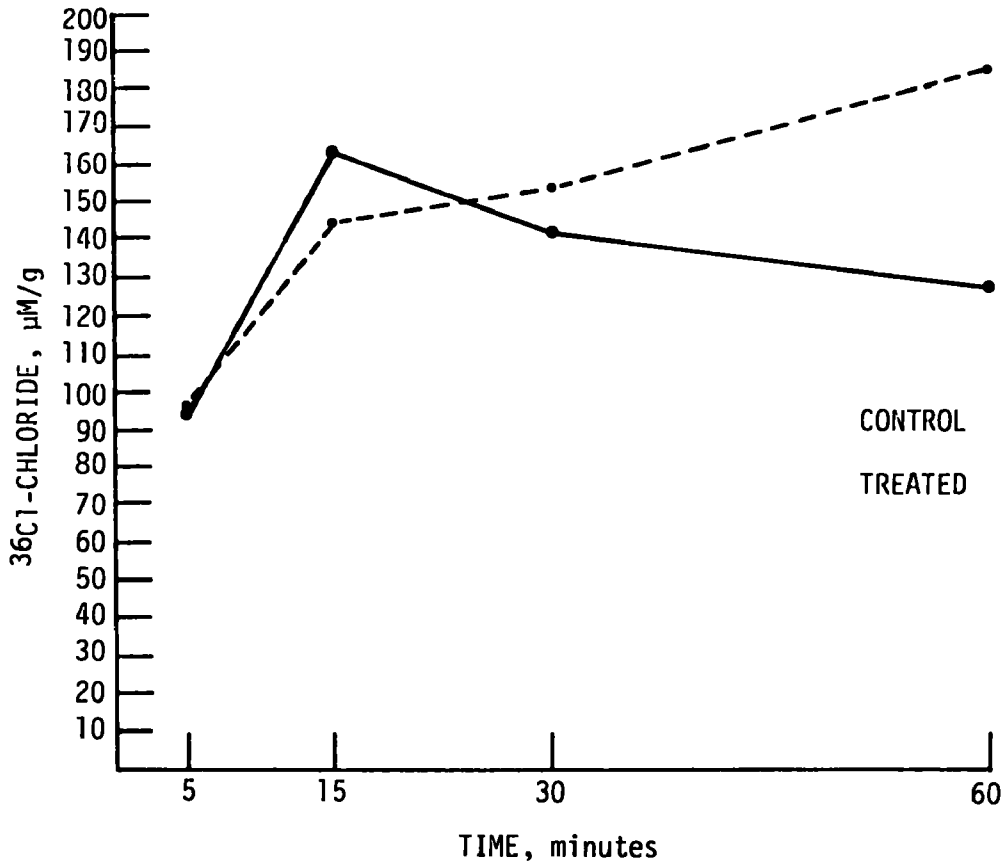

Figure 22. Uptake of ^{36}Cl in abdominal segment of the ventral nerve cord of Periplaneta americana, exposed to $10^{-5}M$ toxaphene.

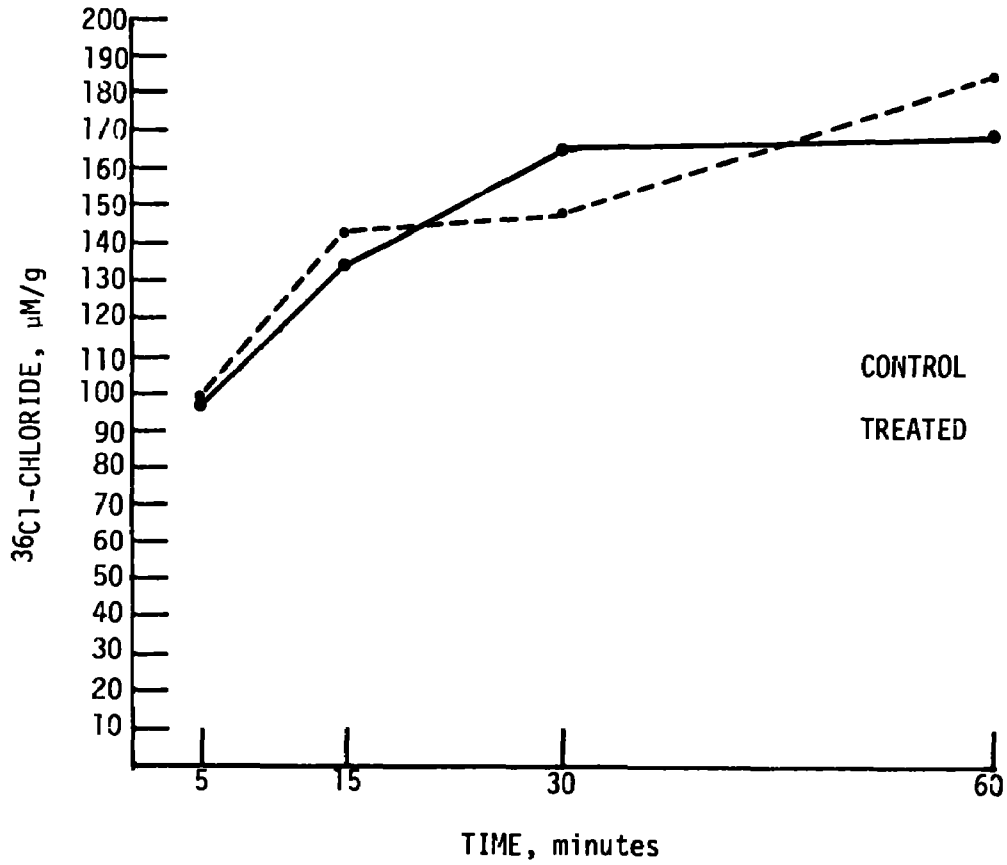

Figure 23. Uptake of ^{36}Cl in thoracic segment of the ventral nerve of Periplaneta americana exposed to $10^{-5}M$ toxaphene.

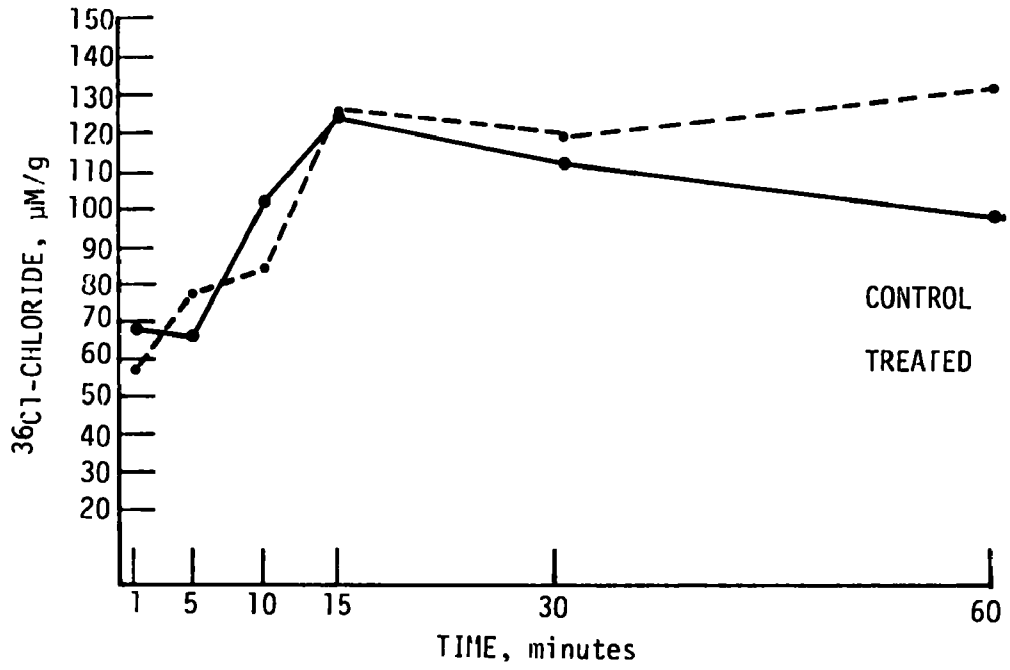

Figure 24. Uptake of ^{36}Cl in abdominal segment of the ventral nerve cord of Periplaneta americana exposed to 10^{-4}M toxaphene.

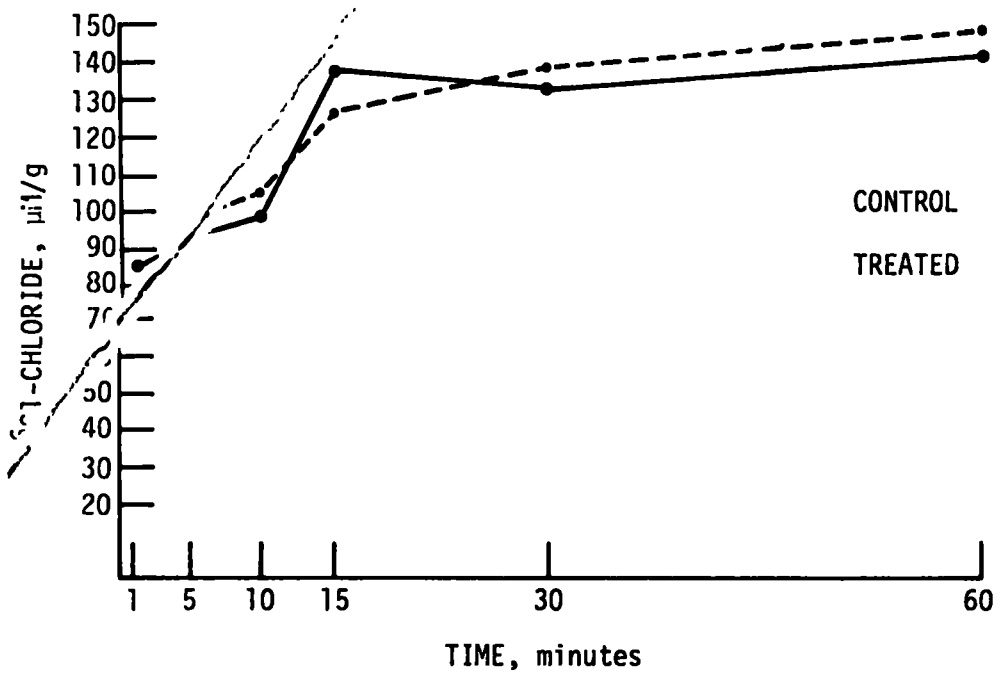

Figure 25. Uptake of ^{36}Cl in thoracic segment of the ventral nerve cord of Periplaneta americana exposed to $10^{-4}M$ toxaphene.

SECTION VI

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SECTION VII

LIST OF PUBLICATIONS

Crowder, L. A., and E. F. Dindal. Fate of ^{36}Cl-Toxaphene in Rats. Bull Enviorn Contam Tox. 12:320-327, March 1974.

Schaper, R. A., and L. A. Crowder. Uptake of ^{36}Cl-Toxaphene in Mosquitofish, Gambusia affinis. Bull Environ Contam Tox. In Press.

SECTION VIII

GLOSSARY

Abdominal Nerve Cord - The central nervous system of insects, which runs along the ventral or abdominal surface.

Absorption - Take into the body.

Adsorption - Adhere to the body.

Ag - AgCl - Silver-silver chloride; used for fabricating electrodes employed in electrophysiology.

$AgNO_3$ - Silver nitrate.

Aldrin - 1, 2, 3, 4, 10, 10-hexachloro-1, 4, 4a, 5, 8, 8a-hexahydro-endo-exo-1, 4:5, 8 - dimethanonaphthalene.

Alimentary Canal - Internal tube from mouth to anus involved in digestion of food.

Anesthesia - Agent causing loss of sensation, with or without loss of consciousness.

Anti-Enzyme - Any compound which can inhibit or destroy an enzyme.

Cell Membrane - Extremely thin membrane which covers the surface of animal cells.

Centrifuge - Machine using centrifugal force for separating substances of different densities.

Chlordimeform - N' - (4-chloro-o-tolyl)-N, N-dimethylformamidine.

^{36}Cl - Radioactive chlorine.

CO_2 - Carbon dioxide.

CPM - Counts per minute; unit of radioactivity measurement.

Cyclodiene Insecticide - Any one of a group of compounds, derived by the Diels-Alder reaction in which hexachlorocyclopentadiene is one of the reactants.

Cytosol - Background fluid in which organelles are suspended in a cell.

DDT - 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane.

Dehydrochlorination - Removal of chlorine and hydrogen from a molecule.

Dieldrin - 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-endo-exo-1,4:5,8-dimethanonaphthalene.

Diffusion - Passing through the body or medium.

Efflux - Diffusion outward.

Electrode - Conductor used to establish electrical contact with a non-metallic part of a circuit.

Electrophysiology - Area of physiology where irritable tissue, e.g. muscle and nerve, is studied usually with electrical recording instrumentation.

Endrin - 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-endo-endo-1,4:5,8-dimethanonaphthalene.

Excretion - Removal of waste products from the body.

Fat Body - Diffuse "tissue" in insects below the epidermis and around the gut, which serves as a store for fat, glycogen, protein, and plays an active part in metabolism.

Fluor - Solution of phosphorescent compounds used for liquid scintillation.

Gas Chromatography - Method of separating compounds based upon their volatility and movement on a stationary phase.

Hemolymph - Blood of insects.

HNO_3 - Nitric acid.

Influx - Diffusion inward.

In Vitro - Experimentation on a tissue or organ removed from the animal.

In Vivo - Experimentation on the whole living animal.

Ion - Atom or group of atoms that carries a positive or negative charge.

Ionic Flux - Movement of ions across a membrane.

KCl - Potassium chloride.

LC_{50} - Concentration at which 50% mortality in a population occurs.

LD_{50} - Dose at which 50% mortality in a population occurs.

Lipophilic - Readily dissolvable in non-polar solvents.

LT_{50} - Time at which 50% mortality in a population occurs.

M - Molar; a concentration in grams per liter in which one mole is equivalent to one molecular weight of the substance.

mM - Millimolar; 10^{-3}M.

Metabolism - The breaking-down or building-up of compounds in the biological system.

Methyl-parathion - 0,0-Dimethyl 0-p-nitrophenyl phosphorothioate.

uCi - Microcurie; unit of radioactivity containing 10^{-6} curies or 37,000 disintegrations per second.

Microsomes - Vesicles with attached ribosomes formed from the disrupted endoplasmic reticulum.

Mirex - Dodecachlorooctahydro-1,3,4-metheno-2H-cyclobuto[cd] pentalene.

Mitochondria - Microscopic bodies occurring in the cytoplasm of a cell, and responsible for energy production.

Mode of Action - Method by which a compound exerts its effects.

Moribund - Dying and usually motionless.

Mortality - Subject to death.

NADP - Nicotinamide adenine dinucleotide phosphate.

NCS® - Tissue solubilizer for liquid scintillation.

Neurotoxicant - A compound which adversely affects the function of nerve activity.

Nerve Impulse - Progressive alteration in the charges around a nerve fiber that follows stimulation, and responsible for transmission.

Nerve Sheath - Structural, non-neural covering of the central nervous system.

Non-Polar - Without any positive or negative charges.

Nuclei - (pl) Part of the cell which contains the chromosomes or genetic material.

Oscillograph - A recording from an instrument which exhibits alternating current wave forms or other electrical oscillations.

Partitioning - Separation of substances into various phases or solvents.

pH - Hydrogen ion concentration expressed as the negative logarithm.

Polar - Possessing a positive or negative charge.

POPOP - p-bis- 2-(5-phenyloxazolyl) -benzene.

PPB - Parts per billion.

PPM - Parts per million.

PPO - 2-5-Diphenyloxazole.

Quench - Suppression of the flashes of light during scintillation.

Radiolabelled - Compound in which one or more of the elements of it's structure are radioactive.

Resistant - Animal or population not affected by, or tolerable to, a poison, either partly or entirely so.

Saline - Solution of salts, often times used for the bathing of animal tissues.

Scintillation - Method of quantifying radioactivity by counting the flashes of light given off by a phosphor excited by ionizing radiation.

Site of Action - Location in a tissue or organ where a compound exerts it's effect.

Spike - Long narrow peak observed on an oscilloscope corresponding to a nerve impulse.

Spontaneous Activity - Nervous activity which occurs without stimulation.

Sorption - Process of taking-up or holding, including both absorbtion and adsorption.

Synaptic Vesicle - Spheres found within the nerve endings, which contain the chemical transmitter.

Tarsi - Segment (fifth to the base) of an insect leg.

Tergite - Thickened plate of cuticle on dorsal side of a segment of an arthropod.

Topical Dose - A dose which is applied externally.

Toxaphene - Substance which results when camphene is chlorinated to contain 67-69% chlorine; average molecular weight - $C_{10}H_{10}Cl_8$.

Toxicity Syndrome - Sequence of symptoms following exposure to a poison.

TR - Toxaphene related.

Trachea - Cuticle lined tube conveying air from the spiracles to the tissues in insects and other arthropods.

Triton X-100 - Emulsifier; alkyl phenoxy polyethoxy ethanol.

Uptake - Incorporate, or absorb into the body.

Viscera - Collective term for the organs of the body.

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15. SUPPLEMENTARY NOTES

16. ABSTRACT

This report contains information concerning the mode of action, excretion, and metabolism of the cyclodiene insecticides. Toxaphene was the primary candidate for investigation with major emphasis on the mammalian system.

Excretion of ^{36}Cl-toxaphene was studied in the laboratory rat. Upon extraction, most of the radioactivity occurred in the water fractions of urine and feces as ionic chloride, indicating considerable metabolism of toxaphene. Only minimal storage appeared to occur.

Occurrence of radioactivity in several tissues of Leucophaea maderae was determined after injections of ^{36}Cl-toxaphene. Uptake of $10^{-5}M$ ^{36}Cl-toxaphene in subcellular particles of ventral nerve cord and brain was studied and showed significant levels in the larger cell fragments; microsomes were also labelled.

The toxicity syndrome of toxaphene to Gambusia affinis was divided into 5 stages, and the residue level at each stage was determined. Excretion was not observed. Metabolic alteration of toxaphene appeared to be minimal. Differences in individual mortality appeared to be due to differences in uptake rather than differences in ability to tolerate particular body loads of toxaphene.

Ventral nerve cords of Periplaneta americana and L. maderae showed increased nerve activity as viewed electrophysiologically when exposed to toxaphene. Toxaphene appeared to be a neurotoxicant.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
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