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Column Chromatographic Separation of Polychlorinated Biphenyls from Chlorinated Hydrocarbon Pesticides, and their Subsequent Gas Chromatographic Quantitation in Terms of Derivatives

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Polychlorinated biphenyls (PCB's) are universal physiologically active pollutants (1,2,3). The analytical (and bio-) chemistry of these materials is poorly developed (2). Although identification of these compounds can be achieved by GC/MS techniques, the quantitation problem has not yet been solved. The analytical difficulties have been reviewed (4,5). This paper describes a column chromatographic technique for the separation of PCB's from organochlorine pesticides and their subsequent quantitation in terms of well-defined derivatives - bicyclohexyl and decachlorobiphenyl.

This system of analysis is in routine operation in our laboratory. It has been applied to a variety of samples, mainly from the aqueous environment. For example, PCB's (roughly of the Aroclor 1254 variety) have been isolated and quantitated in samples from ducks and seagulls, porpoise blubber, sewage sediments and bottom fauna in general. A water sample from Lake Michigan, at the inlet to a water purification plant supplying a major city was found to contain 275 ppt PCB's (determined as decachlorobiphenyl but expressed in terms of Aroclor 1254).

Separation of PCB's from DDT Group of Pesticides

For the quantitative determination of PCB's by gas chromatography, it is necessary to separate them from other chlorinated pesticides. More or less successful column chromatographic separation procedures have been published: Reynolds-Florisil (4), Holden and Marsden-alumina and silica gel (6), Armour and Burke - silicagel and Celite (7). For proper separations, the adsorbents have to be partially deactivated with water. In our hands, these techniques have not been too successful. In more general terms, our past experience in other areas of column chromatography has indicated that it is extremely difficult to reproduce the activity of the adsorbents from day to day and from one laboratory to the other. Recently, Zitko (5) discussed these and other difficulties in some detail. In particular, he indicated that the commercial pesticide-grade hexane

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from various suppliers was contaminated with benzene. The separation of Aroclor 1254 from organochlorine pesticides on silicic acid was affected markedly by the addition of small (5-20 mls/l) quantities of benzene. In addition, he pointed out that several batches of commercial silica gel were contaminated with PCB's of the Aroclor 1254 type. Although the PCB's could be removed from the contaminated silica by washing with acetone, the treatment irreversibly changed the chromatographic properties of the adsorbent, and the required activities could not be restored.

In view of these difficulties, we developed a separation technique based on activated charcoal. It was based on the observation that PCB's once adsorbed onto charcoal could not be recovered by hot chloroform extraction (8). We discovered, however, that they could be removed quantitatively with cold benzene. By trial and error, it was found that the DDT group of pesticides and a number of others* could be eluted from the charcoal column with an ether-acetone mixture, while the PCB's may later be recovered with benzene. The choice of charcoal is critical. Of the several commercially available materials, only one is found to be suitable - Fisher cocoanut charcoal for "decolorizing". There is some contribution of EC materials from the adsorbent. These can be removed effectively by Florisil cleanup of the eluates. Oily or fatty extracts of up to about 100 mg of oil do not change the separations markedly. After the charcoal separations, the oils are also removed by Florisil cleanup.

It should be noted that the standard eluotropic solvent series used in chromatographic separations is reversed on charcoal (9). Thus, benzene is the strongest solvent in the series and *n*-hexane is next to it. Consequently, the sample should be added in the minimum amount of acetone or 25% acetone in ethyl ether. Wet packing of the column gives more reliable results.

One particular run gave the following recoveries: (using 90 mls of 25% acetone in diethyl ether followed by 60 mls of benzene).

	Per cent
p,p'-DDE	91
p,p'-DDD	92
p,p'-DDT	92
O,p'-DDT	94
PCB-Aroclor 1254	90

*Lindane, Aldrin, Dieldrin, Endrin, Heptachlor and Heptachlorepoxide

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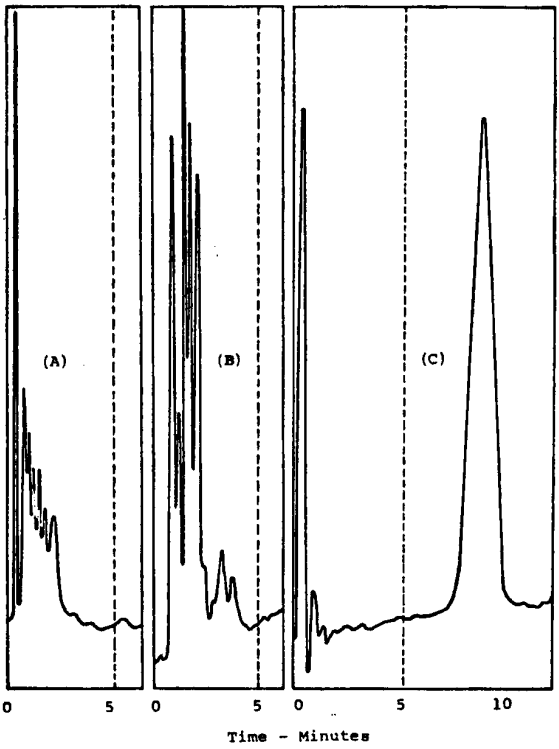
FIGURE 1.

GC/EC CHROMATOGRAM OF A GULL TISSUE SAMPLE.



A - Aroclor 1254

B - Fraction from charcoal column (PCB)

C - Fraction after chlorination and Florisil clean-up.

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The recoveries are quite consistent. The PCB's are recovered with very little alteration to overall GC peak profile. Under our experimental conditions, about 1.1 g of charcoal, the system can handle about 5 ug PCB's and about 0.5 ug each of the DDT's.

Identification and Quantitation of PCB's
In Terms of Derivatives

The identification and quantitation of PCB's by gas chromatography is a difficult matter. Although a certain amount of information can be obtained by the trial and error matching of chromatographic traces with those of commercial PCB's and measuring of prominent peak heights or total areas, the quantitative results are very uncertain.

In particular, it is difficult to determine PCB residues in biological samples. Thus, for example, Mulhern et al. found that GLC analyses of Japanese quail and eggs from mallards fed Aroclor 1254 gave peak profiles that did not match those in Aroclor 1254 (10). In view of this, it would be desirable to base the quantitation of PCB's on a single well-defined derivative. Our experimental work has indicated that PCB's can be converted quantitatively into bicyclohexyl or to decachlorobiphenyl. Outlines of our techniques have been disclosed (11, 12).

Catalytic Dechlorination of PCB's

Under suitable conditions, the PCB's can be dechlorinated quantitatively with hydrogen over Pt or Pd catalysts. The reaction product is a single compound - bicyclohexyl. Our experimental procedure followed essentially the guidelines established by Thompson et al, in the early sixties (13). The major reason for following Thompson's procedure was the discovery that fairly large, about 2.5 mls, quantities of hexane were necessary to recover the reaction product quantitatively from the reactor. Also, occasionally, with low-activity cataylsts, the conversion was not complete. In this case, the products could be recycled. A somewhat different approach was taken by Gunther et al., who used carbon-skeleton chromatography (14). The conversions were however not quantitative. Depending on the conditions, the PCB's were dechlorinated to cyclohexylbenzene, biphenyl and bicyclohexyl.

During the early stages of our work, this technique was used extensively for the identification and quantitation of PCB's. At the 10 ug levels, the conversions were good, better than 95%. The conversions were somewhat better with the palladium rather than platinum catalysts. There were occasional problems with the

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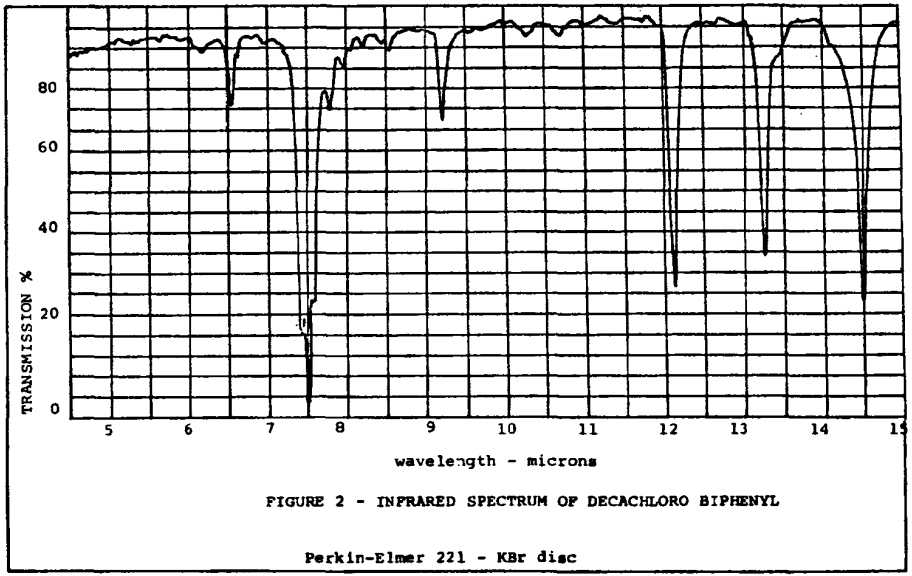

FIGURE 2 - INFRARED SPECTRUM OF DECACHLORO BIPHENYL

Perkin-Elmer 221 - KBr disc

reproducibility of catalyst activities. The main disadvantage of this procedure, however, was that the hydrocarbon had to be determined with GC/FID instrumentation at a fairly low level of sensitivity. When it was realized that PCB's could be perchlorinated on a preparative scale to another well-defined derivative - decachlorobiphenyl that could be determined with GC/EC instrumentation (15, 16), attempts were made to adapt this macro reaction to analytical problems.

Perchlorination of PCB's

The treatment of PCB's with antimony pentachloride under elevated temperatures and anhydrous conditions gives very good yields of decachlorobiphenyl. On the preparative scale (0.5 g), the yields are better than 90%, while on the analytical scale, 1 ug, the yields are somewhat lower, but consistent at 85 ± 5%. The latter procedure includes a Florisil cleanup for GC/EC determinations.

The preparative reaction is best carried out in a Teflon-lined screw-cap vial. The temperature and time do not appear to be critical. Treatment for about 5 hours at 170°C will produce a complete conversion. For analytical preparation, it was found necessary to carry out the conversion in sealed glass tubes. On occasion, the tubes were left overnight at the reaction temperature. No by-products were formed. The hydrolysis of the unreacted antimony pentachloride was carried out with 20% hydrochloric acid in order to suppress the precipitation of antimony oxychloride and to avoid possible entrainment of product. The gas chromatographic behaviour of decachlorobiphenyl is elementary. Since no separation from other materials is involved, the determination can be carried out with a short column. In our laboratory, the analysis is carried out with a 2 ft. x 1/8" stainless steel column. With the column temperature at 215°C, the retention time for decachlorobiphenyl is about 9 minutes (Figure 1). The Aroclor 1254 is eluted in about 3 minutes. The decachlorobiphenyl peak is well removed from those of common chlorinated pesticides and those of Aroclor 1254 and Aroclor 1260. This was confirmed recently by Zitko et al. (17). The relative retention time (six foot column, SE-30) in terms of p,p'-DDE=1, for decachlorobiphenyl was 8.20, while for two octachlorobiphenyls they were about 2.5. It was also noted that the relative detector response in terms of p,p'-DDE=1 for decachlorobiphenyl was 1.6.

EXPERIMENTAL

LC Separation of PCB's from DDT Group Pesticides

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Materials and Reagents

Column - 6 mm ID x 140 mm Pyrex glass tube with a 25 ml reservoir attached.

Charcoal - Fisher No. 5-690 charcoal, activated, 50-200 mesh, (Fisher Scientific Co., no substitutes have been found). The absorbent is boiled with acetone on a steambath. It is then cooled, and the solvent is removed by suction. This procedure is repeated. The filter cake in the funnel is washed with some cold acetone. The charcoal is air dried and stored at 135°C.

Ottawa Sand - acid washed.

Glass Wool - solvent extracted.

Eluting Solvent A - 25% acetone in ethyl ether (anhydrous)

Eluting Solvent B - benzene

Procedure

The tube is plugged with a small wad of solvent-washed glass wool. About 25 mm of acid-washed Ottawa sand is then added; this amount of sand is necessary to retain the fines from the charcoal. A solvent (acetone) slurry of the adsorbent is then added until the column of the charcoal is 90 mm long (1.1 gm dry weight). The sample is injected directly onto the top of the column. The DDT group pesticides are eluted with 90 mls of Solvent A and the PCB's with 60 mls of Solvent B. Heavily contaminated eluates might require cleanup on Florisil. For testing the column efficiency, the following loadings are suitable:

PCB - Aroclor 1254	- 1 ug
p,p'-DDE	- 0.5 ug
p,p'-DDD	- 1.0 ug
o,p'-DDT	- 1.0 ug
p,p'-DDT	- 1.0 ug

Catalytic Dechlorination of PCB's

Catalyst

5% by weight Pt or Pd as metal on 60-80 mesh porous glass beads (Applied Science Laboratories, State College, Pa.). An aqueous solution of chloroplatinic acid or palladium chloride is evaporated gently in contact with the glass beads. The catalyst is reduced in situ.

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Reactor and Procedure

12 x 0.25 inch stainless steel tubing fitted with a modified Swagelok tee; one arm of the tee is fitted with a silicon rubber septum, the other one is inlet for hydrogen gas. The other end of the reactor is fitted with a 2 ft. length of 1/8 inch Teflon spaghetti tubing. The coiled tubing is cooled in ice water. The products are collected in a cooled 15 ml centrifuge tube. The reactor is kept at 180°C. The PCB is injected in hexane. After a few minutes, 5-6 o.5 ml quantities of hexane are injected in order to purge the reaction products. The conversion is about 95% at the 10 ug level with on-catalyst injection.

Perchlorination of PCB

Macro Synthesis

A 0.5 g quantity of PCB (Aroclor 1254) was reacted with 3 mls of $SbCl_5$ in a Teflon capped glass tube. After 8 hours at 150°C the tube was cooled, opened and the contents treated with 20% hydrochloric acid (to prevent the precipitation of antimony oxychloride). The aqueous phase was extracted with warm benzene. The extract was shaken with aqueous bicarbonate solution, water and finally dried with anhydrous sodium sulphate. Evaporation gave a 95% yield of crude decachlorobiphenyl. The material was crystallized from a 50:50 mixture of benzene and pet. ether, M.P.304°C.

The white crystalline material gave one peak on GC. IR spectrum was obtained with Perkin-Elmer 221 spectrophotometer (Figure 2). It was sublimed in vacuum for elemental analysis.

MW-498.6 $C_{12}Cl_{10}$

	Calc.	Found
C	28.88	28.99
Cl	71.12	70.78

The molecular weight of the material was obtained by mass spectrometry.

Micro Synthesis

The PCB, one microgram or less, is treated with 0.2 mls of $SbCl_5$ in a sealed glass tube.

The PCB in a small quantity of a suitable solvent is transferred into a Carius tube, 8 mm OD, 6 mm ID, 160 mm long. The solvent is removed by gentle vacuum - thin Teflon tubing inserted into the glass

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tube. A quantity of 0.2 mls of $SbCl_5$ is added and the tube is sealed. After a minimum of three hours at 160-170°C, the tube is opened and the contents are worked up according to instructions given above for macro scale. With careful attention to the details, the recovery is about 85 ± 5%.

Gas Chromatographic Conditions for the Determination of:

1. Bicyclohexyl

   Instrument - Varian 1500 FI Detector

   Column - 8 ft. x 1/8" stainless steel, 10% DC-710 on Chromosorb W

   Temperature - 1. Column 90°C for 2.5 minutes then programmed at 10°C per minute

   2. Injector 220°C 3. Detector 220°C

   Gas Flow - Air 200 mls/min. Nitrogen 25 mls/min. Hydrogen 20 mls/min.

   Range - 1

   Attenuation - x8

   Recorder - 1 mv full scale

   Under these conditions, an injection of 50 ng of bicyclohexyl gives a chart response of 45 units. The retention time is 9.0 minutes.

2. Decachlorobiphenyl

   Instrument - Varian 1500, Tritium Detector

   Column - 2 ft. x 1/8" stainless steel 5% Se-30 on Chromosorb W

   Temperature - 1. Column 215°C 2. Injector 220°C
   3. Detector 215°C

   Gas Flow - Nitrogen at 50 mls/min.

   The peak is Gaussian in shape. The retention time is approximately 9 minutes (Figure 1).

ACKNOWLEDGEMENTS

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The Distribution and Excretion of Polychlorinated Biphenyls (Aroclor 1254) and their Effect on Urinary Gonadal Steroid Levels in the Boar

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Risebrough et al.(1) have reported that the environmental contaminants, polychlorinated biphenyls (PCB), have the capacity to enhance staroid-hydroxylating enzyme activity in the liver of the pigeon, thus increasing estradiol metabolism. The organochlorine pesticides of the DDT group exert a similar effect. On a weight basis, the PCB preparation has an estradiol degrading potential of about five times that of p,p'-DDE or technical DDT in liver homogenate preparations. In the rat, Street et al.(2) reported that the sleeping time induced by a standard dose of hexaberbital was reduced as the chlorine content of the various Aroclor preparations increased from 21 to 68 percent. In vitro, these preparations affected the rates of aniline hydroxylation and p-nitroanisole demethylation in a similar manner. Lincer and Peakall(3) reported an increase in the rate of liver metabolism of estradiol in vitro, and increased levels of cytoplasmic RNA, in American kestrels (Falco sparverius) after the feeding of 0.5 and 5 ppm of Aroclor in the diet. The estrogenic activity of various PCB mixtures, evaluated by the glycogen response of the immature rat uterus, has been described by Bitman and Cecil(4).

Recently, Platonow and Funnell(5) reported that the feeding of PCB resulted in anti-androgen effects, as assessed by a decrease in testicular and comb growth, in cockerels. The signs appeared as early as one week after the start of continuous feeding of 250 ppm of PCB in the diet.

Pathological lesions attributed to PCB have been described in the rat(6), Bengalese finch(7), mouse(8), chicken(9,10,11), monkey(8) and man(12). The liver was the organ most affected in mammals, whereas in birds, hydropericardium, kidney damage and reduced spleen size were most commonly observed.

The present study was carried out to determine the effect of oral administration of PCB in the adult boar. In view of the high excretory levels of dehydroepiandrosterone and estrogens by the boar(13), the levels of these testicular steroids was also examined. Since pathological lesions have been described in several species following the ingestion of PCB, an autopsy was performed on each animal at the completion of the experiment.

Methods and Materials

Four Yorkshire boars, approximately two years of age, and weighing 175 to 260 kg were used. One boar (P-10) was given a single dose of 10 mg of PCB per kg of body weight, a second boar (P-100) received a single dose of 100 mg of PCB per kg and the third (P-1x10) and fourth (P-10x10) animals received 10 daily doses consecutively of 1 and 10 mg per kg, respectively. The PCB,

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as Aroclor 1254*, were dissolved in a small volume of olive oil and mixed with the morning ration. The first two animals (P-10, P-100) were euthanized seven days after the single treatment and the other two boars (P-1x10, P-10x10) were euthanized seven days after receiving the last dose. The animals were fed a commercial hog ration twice daily and had access to tap water. During the experiment the animals were confined in a metabolism crate, modified from that described by Welch et al.(14), to facilitate the collection of urine and faces.

Urine and feces were collected once daily. The PCB extraction and subsequent determination by gas chromatography was performed using the chlorinated insecticide method of Saschenbrecker and Ecobichon(15). The quantitation of the PCB was made as described by Armour and Burke(16). Levels of dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one) in urine were determined on daily samples using a modified method of Fotherby(17), as described by Raeside(13). The results for total estrogens in the urine samples were estimated with the procedure outlined by Raeside(18).

The animals were euthanized with sodium pentobarbital intravenously and immediately subjected to gross pathological examination. Tissues from selected organs were fixed in 10 percent formol-saline for histological examination. These were embedded in paraffin, cut at 4 to 6 μ on a rotary microtome and stained with hematoxylin and eosin according to routine procedures. Specimens from various organs were removed and stored at -20°C until analyzed. The brain was removed and cut into two exact halves, of which one was homogenized prior to PCB analyses.

Results and Discussion

Tissue Distribution of PCB

The concentrations of PCB in the various tissues are indicated in Table I. The data show that the lowest levels of PCB were present in blood and the highest levels were in perirenal fat. The hepatic tissues contained the second highest level of PCB in the two animals given a single dose. In the two boars receiving multiple doses however, the three muscular tissues contained the second highest amount. In these latter animals also, the levels in the testes were similar to those measured in the liver. The levels of PCB in the kidney of all animals were low, which together with the low urinary levels measured, may indicate a low renal excretion of PCB compounds by the boar.

The ratios between the tissue concentrations for the high and low dose administration were calculated for the single and multiple dose experiments. For animals receiving a single dose, the highest ratio occurred in perirenal fat, 6:1, and the lowest ratio, 1:0.65, was present in the testes. Higher ratios resulted when multiple doses were administered; 11.5:1 in perirenal fat was highest and 1.7:1 in the brain was lowest.

* Aroclor 1254 was generously supplied by Monsanto Canada Ltd., Toronto, Ontario.

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TABLE I

Concentrations (in µg/g wet tissue) of Polychlorinated Biphenyls (Aroclor 1254) in Various Tissues of Boars Given 10 mg/kg (P-10) and 100 mg/kg (P-100) as Single Dose, Respectively, as well as Ten Consecutive Daily Doses of 1 mg/kg (P-1x10) and 10 mg/kg (P-10x10), Respectively.

Tissue	Boar P-10	Boar P-100	Boar P-1x10	Boar P-10x10
Blood	0.3	0.4	0.3	1.1
Brain	2.1	4.2	1.8	3.1
Perirenal Fat	39.8	245.1	13.7	156.9
Kidney	2.9	2.6	0.9	3.4
Liver	6.3	9.3	2.2	6.5
Testicle	5.8	3.8	1.7	5.6
Muscular Diaphragm	4.0	7.8	4.1	18.7
Psoas	3.5	5.6	3.4	11.0
Heart	-	3.5	2.3	6.9

Although animal P-10 and animal P-1x10 received the same total amount of PCB on a body weight basis, considerable variation was observed in the tissue concentrations. The single low dose resulted in higher tissue concentrations than the comparable multiple dose. The reverse occurred however in the two animals given the higher dose level.

Excretion of PCB

The amounts of PCB excreted in the urine and feces are depicted in Figure 1. The urinary excretion of PCB was substantially lower then that of the feces for all treatment regimes. In boars given a single dose the highest amount measured in urine occurred on the second day, followed by considerable daily variation. Multiple doses resulted in a trend for increasing urinary excretion during the experiment, although considerable daily variation occurred. Following the 10th or 12th day a decline in PCB concentration was noted.

The experimental design used in the present study did not permit the identification of those amounts of PCB which were unabsorbed compared to those portions excreted into the alimentary tract.

The data in Table 2, show the cumulative percentages of the PCB excreted via urine and/or feces. Boars given the low or high single dose of PCB, eliminated in the urine and feces, 12.0 and 16.3 percent respectively of the administered dose during the seven day post-treatment period. For animal P-1x10, 12.7 percent of the total administered dose was accounted for in the urine and feces during and after PCB administration. At the higher, multiple dose level only 6.4 percent was excreted during a similar collection period.

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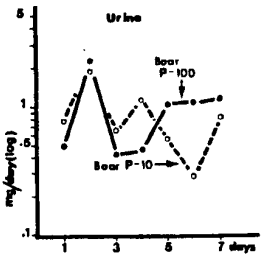
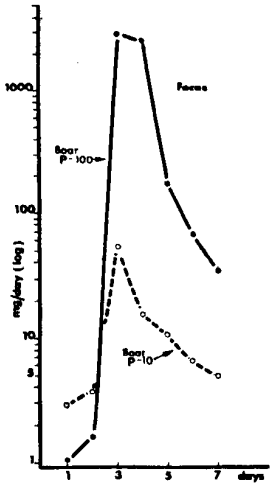
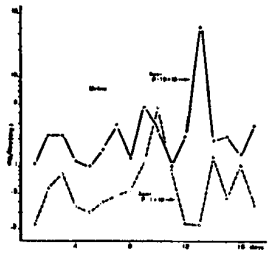
Figure 1.
The amounts of PCB excreted in the urine (upper left and lower left) and feces (upper right and lower right) of boars given high or low doses of PCB orally in single or multiple doses.







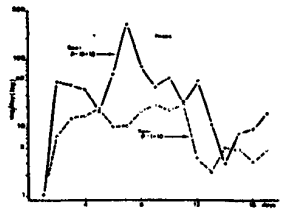


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TABLE 2

Cumulative Percentage Excretion of Polychlorinated Biphenyls (Aroclor 1254) in Boars Given 10 mg/kg (P-10) and 100 mg/kg (P-100) as Single Dose, Respectively, as well as Ten Consecutive Daily Doses of 1 mg/kg (P-1x10) and 10 mg/kg (P-10x10) Respectively.

Days	Boar P-10			Boar P-100			Boar P-1x10			Boar P-10x10		
	Urine	Feces	Urine and Feces	Urine	Feces	Urine and Feces	Urine	Feces	Urine and Feces	Urine	Feces	Urine and Feces
1	<0.1	1.5	1.5	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
2	0.1	1.7	1.8	<0.1	<0.1	<0.1	0.2	1.4	1.6	0.1	1.0	1.1
3	0.2	4.4	4.6	<0.1	7.4	7.5	0.3	3.2	3.5	0.1	1.6	1.7
4	0.2	5.2	5.4	<0.1	14.7	14.7	0.3	4.6	4.9	0.1	2.0	2.1
5	0.3	10.7	11.0	<0.1	15.7	15.7	0.4	6.0	6.4	0.2	2.1	2.3
6	0.3	11.0	11.3	<0.1	16.1	16.1	0.4	6.7	7.1	0.2	2.5	2.7
7	0.7	11.3	12.0	<0.1	16.2	16.3	0.4	7.3	7.7	0.2	4.6	4.8
8							0.4	8.1	8.5	0.2	5.1	5.2
9							0.5	9.1	9.5	0.2	5.2	5.4
10							0.7	9.7	10.4	0.2	5.5	5.7
11							0.7	10.6	11.3	0.3	5.6	5.8
12							0.7	10.7	11.4	0.3	5.8	6.0
13							0.7	10.8	11.5	0.4	5.8	6.2
14							1.2	11.0	12.2	0.4	5.8	6.2
15							1.2	11.2	12.4	0.4	5.9	6.3
16							1.3	11.3	12.6	0.4	5.9	6.3
17							1.3	11.5	12.7	0.4	6.0	6.4

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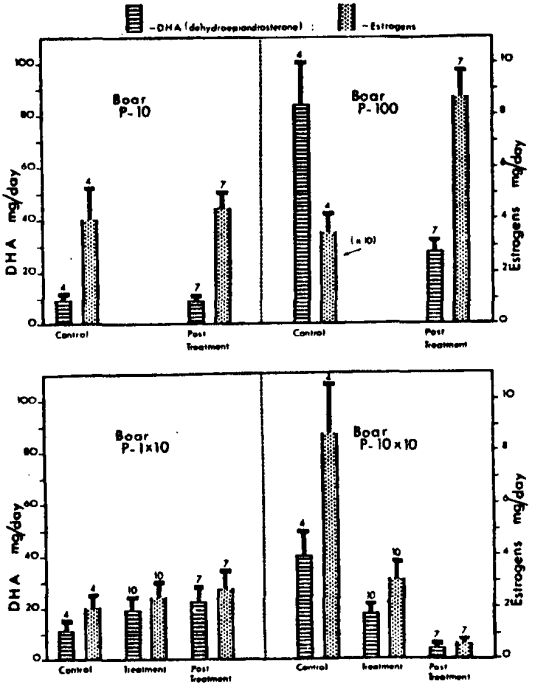

Figure 2.
Urinary excretion of estrogen and dehydroepiandrosterone (DHA) from boars before, during and after the oral administration of single or multiple doses of PCB. Vertical lines above the bar indicate the standard error of the mean. The numbers refer to the number of samples analyzed. Note, for boar P-100 the control value of estrogen shown is only 1/10th of that actually measured.

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Excretion of Estrogen and DHA

Figure 2 illustrates the urinary excretion of estrogen and dehydroepiandrosterone (DHA) before, during and after the oral administration of a single or multiple dose of PCB. The lower dosage level for both single and multiple treatments appeared to have no effect upon the urinary excretion of these gonadal steroids. The amounts of these hormones recovered after PCB treatment were not significantly different from those of the control period. However, the urinary levels of these steroids were markedly reduced following ingestion of the higher doses of PCB, in both the single and multiple treatment regimes. With the single high dose, the post-treatment levels or urinary steroids were significantly lower ($P < 0.01$) than control levels in the "t" test, while in the boar receiving the multiple high doses, the steroid levels during both the treatment and post-treatment period were significantly lower ($P < 0.01$) than those of the control period.

Organochlorine compounds such as DDT are known to have an adverse effect upon reproduction in birds, mice, rats and dogs(19). The lower levels of DHA and estrogen excreted in the urine of boars given high doses of PCB suggest that these compounds may also have a deleterious effect upon reproductive activity. The mode of action of PCB in this regard is presently unknown.

Pathology

No macroscopically visible lesions could be seen in any of the experimental animals and these findings were confirmed upon histological examination. The results suggest that although PCB administration to boars may cause significant biochemical alterations, these are not detectable with routine pathological techniques. Lesions of this slight severity may only be detectable with more sophisticated techniques such as electron microscopy.

Acknowledgements

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The animals used in the present study were donated by the Arkell Research Station of the Ontario Department of Agriculture and Food.

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Biologically Active Compounds in the Aquatic Environment: The effect of DDE on the egg viability of *Chironomus tentans*

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The widespread contamination of the aquatic environment by 1,1'-dichloro-2,2'-bis[p-chlorophenyl]ethylene (DDE) is well documented (1,2), and its biological activity has been demonstrated at subacute levels (3,4). The occurrence of this compound in surface waters and the organic substrate acting as a partitioning reservoir would mean that this compound could act as a potential biological hazard in exhibiting deleterious effects on aquatic insect populations. This study was designed to determine the effect of p,p'-DDE residues on the egg viability of the aquatic midge, *Chironomus tentans*.

METHODS AND MATERIALS

Experimental cultures of the aquatic midge, *Chironomus tentans* (Diptera:Chironomidae) were maintained in four aerated 45 ℓ all glass aquaria with a substrate. The substrate utilized was prepared by placing 50 g of paper hand towel (Nibroc Co.) in a Waring blender with 5 g of chicken feed and 1.5 ℓ of water, this mixture was then homogenized for 5 min.

To two of the aquaria, a solution of p,p'-DDE (99.9%, Aldrich Chemical Co., Milwaukee, Wis.) in ethanol was added to give a resultant concentration of 30 ppb (parts per billion) in the water. The remaining two aquaria received an equivalent amount of ethanol and served as a control source of eggs. The ethanol concentration never exceeded 5 ppm. Three egg masses of *C. tentans* were placed in each of the four aquaria. After a developmental time of approximately one month (28-35 days) the adults emerging from these four experimental aquaria were collected separately and allowed to mate in 250 ml flasks. The egg masses from these DDE exposed and control females were collected and observed. These experimental egg masses were then incorporated into four different experimental treatments:

1) H/E°-water that contained no p,p'-DDE and one egg mass that was obtained from a DDE exposed female (contaminated egg mass).

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2) H*/E-water that contained 20 ppb p,p'-DDE and one egg mass that was obtained from a control female.

3) H*/E*-water that contained 20 ppb p,p'-DDE and one egg mass that was obtained from a DDE exposed female (contaminated egg mass).

4) H/E-control-water that contained no p,p'-DDE and one egg mass obtained from a control female.

These experimental egg masses were placed in aerated 4 ℓ all glass aquaria with the described substrate. Each treatment was replicated three times in a completely randomized design and the experiment repeated twice. The number of adults emerging from the treatment was used as a measure of egg viability.

RESULTS

There was a significant reduction in the number of C. *tentans* adults emerging from aquaria that contained DDE contaminated egg masses (Table 1.).

However, the presence of 20 ppb p,p'-DDE in the water with control (uncontaminated) eggs did not show a significant reduction in the number of adults emerging. The combination of DDE treated water and DDE contaminated eggs also demonstrated no significant difference from DDE contaminated eggs alone.

TABLE 1.

Summary of the Effect of p,p'-DDE on the Egg Viability of Chironomus tentans.

Treatment	No. of Emergent Adults	
	Experiment No. 1	2
No DDE in water-DDE contaminated egg mass (H/E*)	167 a[1]	209 a
DDE in water-uncontaminated egg mass (H*/E)	385 b	402 b
DDE in water-DDE contaminated egg mass (H*/E*)	190 a	228 a
No DDE in water-uncontaminated egg mass (H/E)	432 b	408 b

[1] Means followed by unlike letters are significantly different at the 5% level by Tukey's W procedure.

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The egg masses obtained from DDE exposed females were of a less gelatinous consistency and had a shriveled appearance compared to that of the control eggs. The number of individual eggs in both DDE contaminated and uncontaminated (control) egg masses appeared to be the same. From a previous study (5) it was shown that approximately 30-34% of an adult female burden of DDE residue is lost to the extruded egg mass, thus a significantly high amount of DDE residue is being transferred to these eggs when an adult emerged from exposure of 30 ppb in the water.

DISCUSSION

The results obtained suggest that sublethal concentrations of p,p'-DDE in the aquatic environment could have deleterious effects on Chironomidae populations. Although the initial concentration of p,p'-DDE that the stock culture of C. *tentans* was exposed to was higher (30 ppb) than found in the aquatic environment the idea that egg viability is related to pesticide burden is demonstrated. These findings emphasize the need for further study on the subacute exposure of biologically active compounds to aquatic insects in relation to their reproductive physiology.

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A General Method for the Preparation of Tritiated Polychlorobiphenyls of High Specific Activity: 2,2',5,5'-Tetrachlorobiphenyl-[^{3}H] and 2,2',4,4',5,5'-Hexachlorobiphenyl-[^{3}H][1]

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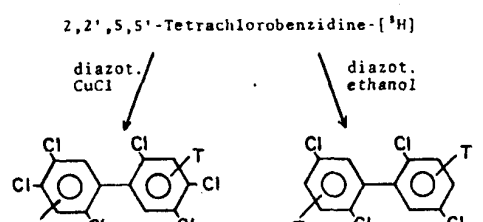
We wish to report a general method for preparing tritiated chlorobiphenyls of high specific activity. Incorporation of hydrogen isotope was low, as shown by mass spectrometry, when acid catalyzed deuterium exchange was attempted on hexachlorobiphenyls. Chlorobiphenyls in which amino groups activated this electrophilic exchange process, on the other hand, gave good incorporation. This method is useful for preparing other tritiated chlorobiphenyls since some aminochlorobiphenyls are available commercially (Pfalz & Bauer) and others are easily accessible by simple synthetic procedures such as benzidine rearrangement (e.g. 1,2) or nitration of chlorobiphenyls and subsequent reduction (e.g. 3,4,5). For each labelled aminochlorobiphenyl two chlorobiphenyls can easily be obtained by either removing the amino groups or Sandmeyer reaction.



Synthetic Procedures: Samples prepared from unlabelled material by identical procedures were analytically pure and showed only one peak on gas chromatography (3). Merck silica gel HF-254 and hexane were used for t.l.c.

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2,2',5,5'-Tetrachlorobenzidine-dihydrochloride-[^{3}H]: 2,2',5,5'-Tetrachlorobenzidine 2 HCl (50 mg), prepared from the free base (Pfalz & Bauer) and hydrogen chloride, was exchanged (New England Nuclear Corp.) by stirring with trifluoroacetic acid (^{3}H) (0.5 ml) and platinum catalyst (25 mg) at 60° overnight. Specific activity: 5.48 mCi/mg.

Preparation of Diazonium Solution: 2,2',5,5'-Tetrachlorobenzidine (100 mg) was stirred and heated to ca. 70° with conc. hydrochloric acid (3 ml), the mixture was cooled and 40 mg of the tritiated material added and diazotized, at 0°, with sodium nitrite (100 mg) in water (0.5 ml). Thirty minutes after the addition was complete, the diazonium solution was filtered and used as described below.

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2,2',4,4',5,5'-Hexachlorobiphenyl-[^{3}H]: One half of the diazonium solution described above was slowly added to a solution of cuprous chloride (50 mg) in conc. hydrochloric acid (1 ml). The mixture was stirred for 1 hour at room temperature, the crude hexachlorobiphenyl removed by filtration and washed with conc. HCl and water. The crude material was purified by preparative t.l.c. (Rf = 0.72). Yield 30 mg; m.p. 101-102°; specific activity, 1.9 mCi/mmole.

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2,2',5,5'-Tetrachlorobiphenyl-[^{3}H]: One half of the clear diazonium solution described above was added to 20 ml boiling ethanol and boiling was continued for 15 minutes. The mixture was concentrated to ca. 2 ml, extracted with benzene and the benzene layer, after drying and evaporation purified by preparative t.l.c. (Rf = 0.65). Yield 29.7 mg; m.p. 84-85°; specific activity, 1.9 mCi/mmole.

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Novel Photoproducts of Heptachlor Epoxide, *Trans*-Chlordane, and *Trans*-Nonachlor

by Glen W. Ivie[1], John R. Knox, Safy Khalifa, Izuru Yamamoto[2], and John E. Casida

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The single major photoproduct which has been obtained on exposure of heptachlor epoxide [I, Fig. 1] in acetone solution to ultraviolet light or as thin films to sunlight or a germicidal lamp (1,2) is reported to be the half-cage isomer [IIIA](2), although the isomeric structure [IIIB] also merits consideration (1). Under the same conditions, cis-chlordane [IV] yields a photoproduct which is considered to be the half-cage isomer [VA](2) or one of the two isomeric structures [VA or VB](1). In contrast, no half-cage photoisomers have been isolated following exposure of trans-chlordane [VI] or trans-nonachlor [VIII] to photolytic conditions (1,2,3,4). With these trans compounds, the orientation of the chlorine atom on the center carbon of the cyclopentane ring towards the double bond apparently precludes bridging at this position (1).

This paper describes the nature and toxicity of three novel photoisomers obtained when heptachlor epoxide, trans-chlordane, and trans-nonachlor are exposed to sunlight as deposits on bean foliage, in the presence of rotenone (5), and to ultraviolet light as solutions in acetone. The conversion of heptachlor epoxide to the half-cage isomer [IIIA or IIIB] involves an intermediate [II] which has been isolated and characterized. A new photoisomer of a novel type has also been obtained from each of trans-chlordane and trans-nonachlor.

Materials and Methods

Reference chemicals, analytical procedures, and toxicity tests. Reference samples of compounds I, III, IV, V, VI, and VIII were provided by P. B. Polen (Velsicol Chemical Corp., Chicago, Ill.). The formation of photoproducts was monitored by thin-layer chromatography (TLC) on silica gel F_{254} chromatoplates, using petroleum ether-chloroform mixture (8:1) for heptachlor epoxide and its photoproducts, petroleum ether for cis-chlordane and photo-cis-chlordane, hexane-ether mixture (4:1) for trans-

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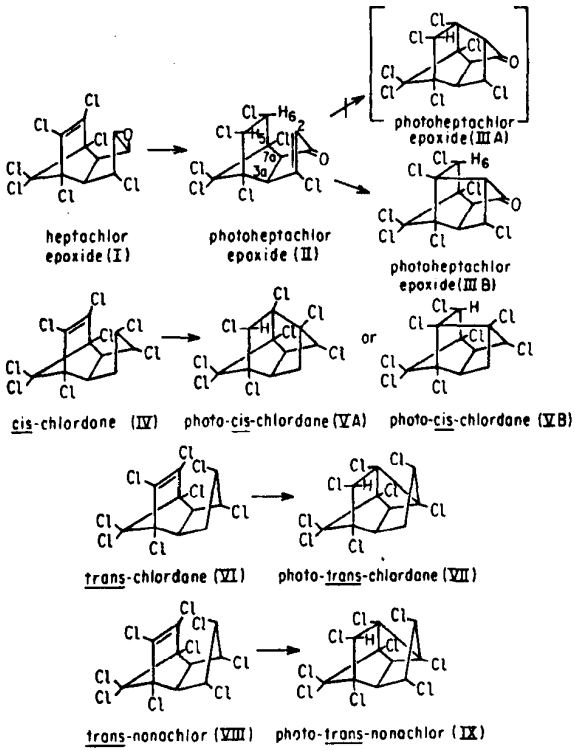
Fig. 1. Structures of heptachlor epoxide [I], cis-chlordane [IV], trans-chlordane [VI], trans-nonachlor [VIII] and some of their possible photoproducts



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chlordane and its photoproduct, and hexane-ethyl acetate mixture (20:1) for trans-nonachlor and its photoproduct. Gas-liquid chromatography (GLC) studies were made using a 6-ft x 1/8-in ID glass column packed with 10% DC-200 (viscosity grade 12,500) on 80/100-mesh Gas-Chrom Q with an injection temperature of 225°C and a column and detector temperature of 200°C. Retention times, in minutes, with a nitrogen flow rate of 60 ml/min for the designated compounds were: I - 9.1; II - >120; III - 12.3; IV - 12.0; V - 19.4; VI - 10.7; VII - 24.2; VIII - 12.7; IX - 20.8. Hexadeuterodimethylsulfoxide was the primary solvent used for the proton magnetic resonance (PMR) and ^{13}carbon nuclear magnetic resonance (NMR) studies; however, pentadeuteropyridine and deuterochloroform were also used. The toxicity of the compounds was determined on adult female houseflies of the SCR insecticide-susceptible strain and on male white mice of the Swiss-Webster strain. The flies were used 2-5 days after emergence and they were treated topically, on the thorax, with 1.0 µl of an acetone solution of the test compound; mortality determinations were made 48-hours after treatment. Also, toxicity assays were made with flies pretreated with 5 µg of sesamex synergist dissolved in 1 µl of acetone and applied to the thorax 1 hour before application of the test compound. The mice, weighing approximately 20 g, were treated intraperitoneally with 50 µl of dimethylsulfoxide solution of the test compound; mortality determinations were made 48-hours later.

Preparation and isolation of photoproducts. The photochemical apparatus consisted of a 450-watt, high-pressure, mercury-vapor lamp placed in a water-cooled, double-walled, clear, fused-quartz immersion well. The radiant energy reaching the sample was restricted by a pyrex absorption sleeve which blocked radiation below 280 nm. The cyclodienes (about 4 g in 165 ml of acetone) were irradiated for the respective times necessary to achieve reasonable yields of the photoproducts, which were isolated by chromatography on Florisil columns. With heptachlor epoxide, the irradiation was for 2 hours, followed by chromatographic removal of photoproduct II [eluted with chloroform-hexane mixture (2:1)] and recovery of a mixture of unreacted material and photoproduct III [eluted first with hexane-chloroform mixture (4:1)]. The mixture of heptachlor epoxide and photoproduct III was irradiated again for 2 hours, followed by removal of photoproduct II; this overall procedure was repeated two more times in order to accumulate photoproduct II (an intermediate in formation of photoproduct III). Finally, heptachlor epoxide [I] was separated from photoproduct III by taking advantage of the low solubility of photoproduct III in cold ether. [The final yields were: 13% unreacted heptachlor epoxide; 49% photoproduct II (m.p. 178.5 - 181.5°C, from chloroform-hexane); 26% photoproduct III (m.p. 208 - 209°C, from chloroform-hexane).] cis-Chlordane solutions were irradiated for 48 hours and the photoproduct was separated on Florisil, using petroleum ether for elution and repeating the column purification to obtain pure material (76%, m.p. 148.5 - 151°C, from hexane) and unreacted cis-chlordane (18%). trans-Chlordane was converted to its photoderivative by the same

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general procedure and the pure product was obtained after one passage through Florisil with petroleum ether (12%, m.p. 168.5 - 170°C, from hexane). (Recovery of unreacted trans-chlordane was 74%.) trans-Nonachlor was photodecomposed by the same general procedure but three passages through the Florisil column were required to separate unreacted trans-nonachlor (70%) from photoproduct IX (6%, m.p. 154 - 155.5°C, from hexane).

Groups of young bean plants were sprayed to run off with a methanolic solution of the cyclodiene (1% w/v) with or without added rotenone (1% w/v), a potential photosensitizer (5,6). After exposure to sunlight for 4 hours, the plants were rinsed with ether and the rinses were subjected to TLC and GLC analyses.

Results

Photoalteration on plant surfaces. The conversion efficiencies of the designated cyclodienes, exposed on rotenone-treated bean leaves to sunlight for 4 hours, were estimated from GLC and TLC analyses to be as follows: 50 - 60% II and 1% III from I; 70 - 80% V from IV; 15 - 20% VII from VI; 15 - 20% IX from VIII. The photoproducts from the plant surfaces are identical (TLC, GLC, IR, and MS) with those formed in acetone solutions exposed to ultraviolet light. No detectable photoproducts were formed in the absence of rotenone as a photosensitizer; so, the reactions on plant foliage are dependent on the presence of the photosensitizer.

Nature of two photoproducts derived from heptachlor epoxide. One of the heptachlor epoxide photoproducts [II] is a precursor for the other one [III] because photoproduct II converts to photoproduct III (identified by TLC, GLC, IR, and MS) in 70% yield when it is exposed in acetone solution to ultraviolet light for 2 hours. On bean leaves exposed to sunlight, rotenone sensitizes the conversion of heptachlor epoxide to photoproduct II but not the conversion of II to photoproduct III; thus, there is a rapid build-up of photoproduct II from heptachlor epoxide in the presence of rotenone, whereas photoproduct III is formed in significant amounts only after prolonged exposure. Direct exposure of photoproduct II to sunlight on plant foliage results in its slow conversion to photoproduct III, but a small amount of photoproduct II remains, even after 7 days.

Photoproduct II contains a ketone group (IR) and it is isomeric with heptachlor epoxide and photoproduct III (elemental analysis, MS). One possible isomeric structure [X] is not appropriate because neither of two epimeric chloro compounds formed on reduction of photoproduct II to an alcohol, followed by chlorination, is the same as δ-chlordane [XI]. However, the spectral properties (IR, PMR, ^{13}carbon-NMR, MS), of each of these derivatives and of the photoproduct itself, are consistent with the designated structure for photoproduct II. In particular, three protons (H_2, H_{3a}, and H_{7a}) of photoproduct II have similar chemical shifts and splittings in the PMR spectrum to those of

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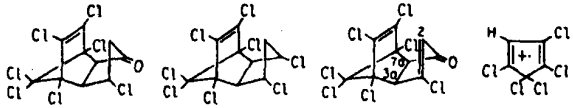

X 8-chlordone(XI) XII XIII

the corresponding protons of the related ketone [XII] of known structure (7); also, the IR and UV spectral absorptions due to the α,β-unsaturated ketone grouping are similar for the two compounds. The hexachlorocyclopentane moiety in photoproduct II is clearly indicated by an AB pattern in the PMR spectrum due to two protons (H_5 and H_6) and by a prominent pentachloro-ion [XIII](m/e 236) in the MS of the alcohol and two chloro derivatives. The ^{13}carbon NMR spectrum of photoproduct II is also consistent with the structural assignment; it shows resonances at the following δ values downfield from tetramethylsilane: 57.7 and 62.3 ppm, each resulting from two ^{13}carbon nuclei; 75.7, 76.2, 99.9, 138.2, 165.3, and 198.6 ppm, each due to single ^{13}carbon nuclei.

Photoproduct III is identical to the compound previously described (1,2), for which structure IIIA (1,2) or structure IIIB (1) has been proposed. Structure IIIB is preferred for photoproduct III because proton H_6 resonates at relatively higher field in the PMR spectrum of the photoproduct than in spectra of related half-cage structures whereas the corresponding signal for the alcohol derived by $NaBH_4$ reduction is at lower field. This is in accord with the anisotropies of the carbonyl and hydroxyl groups. The structure designated [IIIB] for the photoproduct is also preferred to the alternative structure [IIIA] on the basis of a possible intramolecular hydrogen transfer mechanism in the conversion of photoproduct II to photoproduct III.

Nature of photo-trans-chlordane [VII] and photo-trans-nonachlor [IX]. Photo-trans-chlordane is an isomer of trans-chlordane and photo-trans-nonachlor is an isomer of trans-nonachlor (elemental analysis, MS). In each case, a typically low field singlet in the PMR spectrum and the lack of IR absorption due to a disubstituted dichloroethylene grouping indicate that the photoisomers have half-cage structures. Formation of these two photoisomers proceeds slowly relative to the rate of photobridging encountered with cis-chlordane, possibly because a less-accessible hydrogen needs to be abstracted for bridging with the trans compounds; bridging to the central carbon of the cyclopentane ring is precluded by the endo-configuration of the chlorine substituent (1). The PMR spectrum of photo-trans-nonachlor shows it to have structure [IX]. Photo-trans-chlordane gives a closely related PMR spectrum which establishes the similarity of these two photoproducts and, in addition, shows the presence of a methylene group; so, in photo-trans-chlordane, the bridging occurs as shown

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in structure [VII].

Toxicity of the photoisomers. The two photoisomers [II and IIIB] of heptachlor epoxide differ markedly in their toxicity to houseflies and, to a lesser extent, mice (Table 1). It is

TABLE 1

Toxicity of Cyclodienes and Photoisomers

Compound (See Fig. 1)	LD_{50}, Mg/Kg		
	Housefly		
	Without Synergist	With Sesamex Pretreatment	Mouse
Heptachlor epoxide [I]	7	7	18
Photo-heptachlor epoxide [II]	>2500	>2500	36
Photo-heptachlor epoxide [IIIB]	2	1	6
cis-Chlordane [IV]	15	15	30
Photo-cis-chlordane [V]	32	28	20
trans-Chlordane [VI]	225	250	130
Photo-trans-chlordane [VII]	>2500	>2500	>1000
trans-Nonachlor [VIII]	75	90	>500
Photo-trans-nonachlor [IX]	550	425	-

interesting that the two-step pathway for heptachlor epoxide photoisomerization shown in Fig. 1 initially involves conversion of the toxicant to an intermediate [II], which has a reduced toxicity to mice and which is completely nontoxic to houseflies, and then to the end product [IIIB], which is more toxic to flies and mice than the parent heptachlor epoxide [I]. Photo-cis-chlordane [V] is approximately half as toxic to flies as the parent cyclodiene but it is slightly more toxic than cis-chlordane to mice. Photo-trans-chlordane [VII] and photo-trans-nonachlor [IX] are essentially nontoxic. Pretreatment with sesamex has little or no effect on the toxicity of these compounds to houseflies (Table 1).

Discussion

The results reported here establish several novel types of

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phototransformations for methano-bridged cyclodiene insecticide chemicals. Heptachlor epoxide converts to a compound with an α,β-unsaturated ketone grouping and a dichloroethane moiety, possibly by intramolecular transfer of two hydrogen atoms. The two new functional groups generated allow bridge formation through transfer of one hydrogen atom to yield the half-cage structure. To the best of our knowledge, comparable photochemical reactions have not been reported previously for related compounds. A portion of the final photoisomer yield may arise from photoreaction(s) of heptachlor epoxide not involving the identified intermediate, but the studies were not designed to test this possibility. The transformations occuring with trans-chlordane and trans-nonachlor appear to be novel, also, because the half-cage products contain a four-membered ring system rather than the five-membered ring system present in the half-cage products derived from related compounds.

The photoisomers of heptachlor epoxide, cis-chlordane, trans-chlordane, and trans-nonachlor are probably of little importance in evaluating the significance of environmental residues associated with the use of heptachlor and chlordane as insecticide chemicals. The photoisomers are not expected to form in appreciable amounts in the environment unless a potent photosensitizer is present. Further, these studies suggest that the small quantities of photoisomer residues that might occur do not pose a hazard, from a toxicological standpoint.

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