tained in body tissues than either mirex or its more nonpolar photoproducts. Thus, any environmental degradation of mirex to these derivatives might be expected to result in a lessening of potential ecological hazards.

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Isolation of 2,2,5-*endo*,6-*exo*,8,9,10-Heptachlorobornane and an Octachloro Toxicant from Technical Toxaphene

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Technical toxaphene was subjected to the following sequence of chromatographic steps to isolate those components which are of the highest acute toxicity to mice treated intraperitoneally: liquid-liquid partition column, adsorption column, liquid-liquid partition column, adsorption column, and preparative gas-liquid chromatography. Two crystalline toxicants were isolated, one a $C_{10}H_{10}Cl_8$ component 14-fold more toxic to mice than toxaphene and the other a $C_{10}H_{11}Cl_7$ component with a sixfold greater toxicity than toxaphene. These two compounds are four- and twofold more toxic, respectively, than toxaphene to houseflies treated topically. The $C_{10}H_{11}Cl_7$ component is identified as 2,2,5-*endo*,6-*exo*,8,9,10-heptachlorobornane. The $C_{10}H_{10}Cl_8$ and $C_{10}H_{11}Cl_7$ toxicants, which make up about 6 and 3%, respectively, of technical toxaphene, appear to contribute significantly to its mammalian toxicity. These chromatographic procedures should be appropriate to isolate any individual component of toxaphene provided it is stable under the chromatographic conditions employed and a suitable monitoring technique is available.

Toxaphene produced by chlorination of camphene to an overall average composition of $C_{10}H_{10}Cl_8$ (Buntin, 1951) is extensively used in insect control even though its chemical composition is poorly understood (Guyer *et al.*, 1971). Examination of technical toxaphene by silica gel column chromatography followed by coupled gas-liquid chromatography (glc)-mass spectroscopy reveals a complex mixture of at least 175 C_{10} polychloro compounds made up of $C_{10}H_8Cl_{10}$, $C_{10}H_9Cl_9$, $C_{10}H_7Cl_9$, $C_{10}H_{10}Cl_8$, $C_{10}H_8Cl_8$, $C_{10}H_{11}Cl_7$, $C_{10}H_9Cl_7$, $C_{10}H_{12}Cl_6$, and $C_{10}H_{10}Cl_6$ derivatives (Casida *et al.*, 1974). A procedure is needed for isolation of individual toxaphene components to permit determination of their structure, residual persistence, metabolic fate, and toxicity. The difficulties in achieving such isolations are evident on considering the number of components in technical toxaphene and the likelihood that many of them are closely related compounds.

This report describes the isolation and properties of two components of technical toxaphene. A mouse intraperitoneal (ip) acute toxicity assay is used to monitor the purification since the toxaphene components which are most toxic to mammals warrant special attention in evaluating the hazards associated with toxaphene residues.

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MATERIALS AND METHODS

Chemicals. Technical toxaphene (reference standard, sample X 16189-49) and [^{14}C]toxaphene (1.35 mCi/g; from chlorination of [8-^{14}C]camphene; sample X-19098-4-2R) were provided by Hercules Incorporated, Wilmington, Del. Seven toxaphene fractions (I–VII) were obtained by chromatographing the technical material on a silica gel column with hexane as the developer (Knox *et al.*, 1974); this chromatographic procedure is similar to one referred to later as adsorption chromatography system II. The eluted materials were combined in the order of elution such that each fraction contained one-seventh of the total chlorine content of technical toxaphene (Knox *et al.*, 1974).

Chromatography. *Thin-Layer Chromatography (Tlc).* Silica gel 60 F-254 tlc chromatoplates (20 × 20 cm, 0.25 mm layer thickness, EM Laboratories Inc., Elmsford, N. Y.) were used either without treatment or after coating the gel by dipping the chromatoplates for 5 sec into a 20% (v/v) solution of β-methoxypropionitrile (MPN) in dichloromethane or a 15% (v/v) solution of dimethylformamide (DMF) in acetone and then placing the plates in a horizontal position for solvent evaporation. The chromatoplates after spotting with technical toxaphene or fractions thereof (10–100 μg) were developed for 17 cm with one of the following solvent systems: pentane with a first development for 9 cm and drying before a second development in the same direction for 17 cm; hexane; hexane saturated with formic acid;

MPN-saturated hexane for the MPN-treated chromatoplates; DMF-saturated hexane for the DMF-treated chromatoplates. Organochlorine compounds were detected by spraying 10% (w/v) diphenylamine in acetone and then exposing to ultraviolet light yielding gray spots on a white background. This new chromogenic reagent is better for visualizing the toxaphene components than the $AgNO_3$–ultraviolet method.

Column Chromatography. Liquid–liquid partition chromatography columns were prepared by adding MPN in dichloromethane (500 ml) to silica gel 60 (30–70 mesh; EM Laboratories Inc.) (160 g) and then completely evaporating the dichloromethane with thorough mixing on a vacuum rotary evaporator. The stationary phase of partition column I contained 80 g of MPN while that of partition column II contained 32 g of MPN. The columns (1.9 cm i.d. × 93 cm length) were packed from slurries in MPN-saturated heptane, topping the column with a filter paper disk and a 1-cm layer of white sand. Technical toxaphene (10 g) in 15 ml of MPN-saturated heptane was introduced into partition column I and toxaphene fractions (560–700 mg) in 2 ml of MPN-saturated heptane were introduced into partition column II. The columns were developed with 600 ml of MPN-saturated heptane collecting 10-ml fractions at 20 drops/min.

Adsorption chromatographic columns (1.9 cm i.d. × 93 cm length) were prepared from two grades of silica gel packed from slurries in hexane and topped with a filter paper disk and 1 cm of white sand. Adsorption column I used 160 g of silica gel 60 while adsorption column II used 130 g of MN silica gel (70–120 mesh; Machery, Nagel and Co., Düren, Germany). Toxaphene fractions (295 mg–2.5 g) dissolved in hexane (1–2 ml) were introduced into the column followed by development with 2 and 3.5 l., respectively, of hexane (Pesticide Grade hexanes, Fisher Scientific Co., Fair Lawn, N. J.) collecting 10-ml fractions at 20 drops/min. Each batch of hexane should be checked prior to use for column chromatography by determining its resolving power for technical toxaphene on tlc; appropriate batches give a streak of partially resolved components from R_f 0.00 to 0.65. Variations in resolving power may be due to small amounts of benzene contaminating the hexane (Berg *et al.*, 1972).

Gas–Liquid Chromatography. Routine glc analyses involved the use of a Varian Aerograph Model 1400 instrument with a ^{63}Ni electron capture detector and a 2 mm i.d. × 180 cm length glass column containing 3% SE-30 on Gas Chromosorb Q (80–100 mesh). The operating conditions were as follows: injection, column, and detector temperatures of 225, 170, and 245°, respectively; nitrogen flow rate, 75 ml/min; samples of 5–150 ng injected in 2 μl of hexane or acetone.

The purity of certain fractions was examined by capillary column glc using the Beckman Thermotrac instrument and a coiled glass column (0.76 mm i.d. × 41 m length). The column was coated by the method of German *et al.* (1973) with a 19:1 mixture of Poly-I 110 and Igepal CO-880 [nonylphenoxypoly(ethylenoxy)ethanol] and operated at a column temperature of 175° with helium at a flow rate of 40 ml/min and using a flame ionization detector.

Preparative glc involved the use of the Beckman Thermotrac instrument with a thermal conductivity detector and either one of two stationary phases in 3 mm i.d. × 180 cm length glass columns. The specific conditions were as follows: for the preparative glc I system, 4% (w/w) SF96-50 on Chromosorb G (80–100 mesh) at 200° with helium at 30 ml/min; for the preparative glc II system, 3% (w/w) SE-30 on Gas Chromosorb Q (80–100 mesh) at 175° with helium at 50 ml/min. Other conditions, the same for both columns, were: injection temperature, 225°; detector at 220°; 3–5-mg samples of toxaphene fractions injected in 10 μl of acetone or hexane. Fractions were collected in Teflon tubes of 1 mm i.d. and 3 cm exposed length resulting in the deposition of white residues of the eluting compound. The desired compounds were recovered by washing the Teflon tubing with hexane for preparative glc I separation and acetone for the II system; prior to use, the Teflon tubes were thoroughly washed with the respective solvent to minimize interfering extractives.

Spectroscopy and Other Procedures. Infrared (ir) spectra were obtained with KBr disks or 10% (w/v) solutions of the sample in carbon tetrachloride and carbon disulfide using a Cary-White 90 instrument. Mass spectra were determined using the Finnigan 1015D instrument with a chemical ionization source. Elemental analyses were carried out at the Department of Chemistry, University of California, Berkeley, after the samples were held 2 hr at 80° under reduced pressure (0.1 mm); this sample preparation procedure removes interfering solvent. Melting points determined on the Fisher-Johns instrument are uncorrected. Crystals were observed with the Dialux-POL polarizing microscope (E. Leitz, Inc., Rockleigh, N. J.).

Bioassays. Male albino white mice (18–20 g, Horton Laboratories Inc., Oakland, Calif.) were treated ip with the test compounds dissolved in dimethyl sulfoxide, using 100 μl of dimethyl sulfoxide per mouse. Adult female houseflies (*Musca domestica* L., SCR susceptible strain, 4 days after emergence, 20 mg average weight) were treated topically on the dorsum of the abdomen with acetone solutions of the test compounds, using 1 μl of acetone per fly. All LD_{50} values are based on 24-hr determinations with several replications on separate days. The mouse assay is particularly convenient because of the large change in mortality resulting from a relatively small change in dose. An increase in dose by a factor of only 1.4-fold usually increases the mortality from a value of 16% to one of 84% when assaying technical toxaphene, purified fractions, and the toxicants ultimately isolated.

PRELIMINARY STUDIES ON CHROMATOGRAPHIC PROPERTIES OF TOXAPHENE COMPONENTS

Preliminary studies on fractionation of toxaphene by crystallization, tlc, adsorption and partition column chromatography, and glc revealed that the complexity of the mixture precludes isolation of individual components by the use of a single separation method. Crystallization of technical toxaphene from isopropyl alcohol with four subsequent recrystallizations does not simplify the mixture to any great extent, as monitored by tlc or glc analysis; this confirms a previous observation with ir monitoring (Kenyon, 1952).

The tlc characteristics of the seven toxaphene fractions (I–VII) separated originally on a silica gel–hexane column were examined under a number of conditions to provide the necessary information to develop column chromatographic procedures for isolation of individual components. The best tlc resolution is obtained with silica gel chromatoplates, aluminum oxide being less suitable. On silica gel chromatoplates developed with hexane, the seven toxaphene fractions are of decreasing R_f value with some overlapping of the components of lower R_f value in one fraction with those of higher R_f value in the succeeding fraction (Figure 1). Development with pentane gives a similar pattern to that obtained with hexane but with higher R_f values (Table I) whereas heptane and isooctane are less satisfactory giving lower R_f values. Saturation of the hexane developer with formic acid gives higher R_f values than hexane alone (Table I) and results in a slight shift in the chromatographic characteristics of some components. The MPN- and DMF–hexane partition systems on tlc separate the toxaphene components on a different basis than the silica gel–hexane adsorption tlc system. Partition tlc results in long streaks for the fractions that appear more homogeneous on adsorption tlc (Figure 1). The use of pentane, heptane, or isooctane saturated with MPN or DMF does not improve the separation. A series of fractions sep-

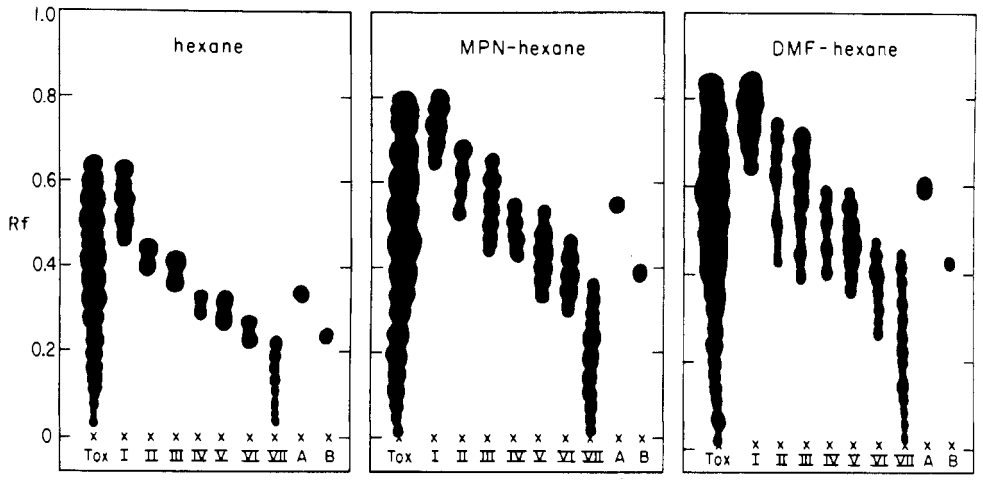


Figure 1. Comparison of the thin-layer chromatographic properties of technical toxaphene (Tox), seven fractions (I–VII) of equal total chlorine content as originally fractionated on a silica gel column with hexane, and of toxicants A and B. The chromatographic systems are as follows: hexane development of a silica gel chromatoplate; β-methoxypropionitrile (MPN) saturated hexane development of a MPN-treated silica gel chromatoplate; dimethylformamide (DMF) saturated hexane development of a DMF-treated silica gel chromatoplate.

arated as distinct spots by adsorption tlc often produces overlapping spots on partition tlc and *vice versa*.

The tlc observations indicate that both adsorption and partition chromatography are useful in separating toxaphene components and that components not separated by one system may be separated by the other system. The tlc conditions were adapted for column chromatography in order to process 10-g batches of technical toxaphene, a scale that should permit isolation of milligram amounts of the individual components.

Partition columns prepared with MPN or DMF give similar resolution of toxaphene components on development with MPN- or DMF-saturated heptane, respectively. The MPN column was selected arbitrarily for use in the isolations described later. Partition column I has a higher capacity but less resolving power than partition column II.

The adsorption columns used in this study provide better separation than the partition columns for the toxaphene components. The most useful combination for column adsorption chromatography is the silica gel–hexane system; other adsorbants (silica gel, silica gel activated by heating under different conditions, aluminum oxide, and poly(vinyl chloride)) with other alkanes are less satisfactory. Two grades of silica gel were selected for use in the isolations described later, first a 30–70 mesh (adsorption chromatography system I) and second, for greater resolving power, a 70–120 mesh (adsorption chromatography system II). When technical toxaphene is chromatographed by these procedures, over 85% of the total chlorine content is eluted with hexane and the materials remaining on the column, when subsequently eluted with ether, are of very low toxicity to mice (less than one-fourth that of technical toxaphene on a milligram per kilogram basis) and they are of lower chlorine content.

Gas–liquid chromatography is the most effective single procedure of the techniques examined for separating toxaphene components, the order of elution of the components being greatly different than that obtained with partition or adsorption columns. The components of lower chlorine content generally elute first on glc, whereas on partition and adsorption columns the elution position is less related

Table I. Comparison of Properties of Toxaphene with Those of Toxicants A and B

Property	Mixture or chemical		
	Toxaphene	Toxicant A	Toxicant B
Mp, °C	70–90	134–136	166–167
Empirical formula, mass spectral	(Mixture)	$C_{10}H_{10}Cl_8$	$C_{10}H_{11}Cl_7$
Chlorine, %			
Calcd	68.54	68.54	65.50
Found	68.57	68.24	65.18
Recrystallization solvent		Isopropyl alcohol	Hexane–acetone (5:1)
Crystal type		Hexagonal	Orthorhombic
Tlc, R_f[a]			
Pentane	0.00–0.75	0.51	0.38
Hexane	0.00–0.65	0.34	0.24
Hexane–formic acid	0.14–0.73	0.54	0.45
MPN–hexane	0.00–0.82	0.55	0.39
DMF–hexane	0.00–0.86	0.59	0.42
Glc, T_r, min[a]			
3% SE-30 on Gas Chromosorb Q	1–42	17.2	12.6
Capillary column		125	60
Preparative glc system I		45	
Preparative glc system II			28
Approximate amount in toxaphene, %	(100)	6	3
LD_{50}, mg/kg			
Mouse, ip	42	3.1	6.6
Housefly, topical	70	16	32

[a] For conditions, see text.

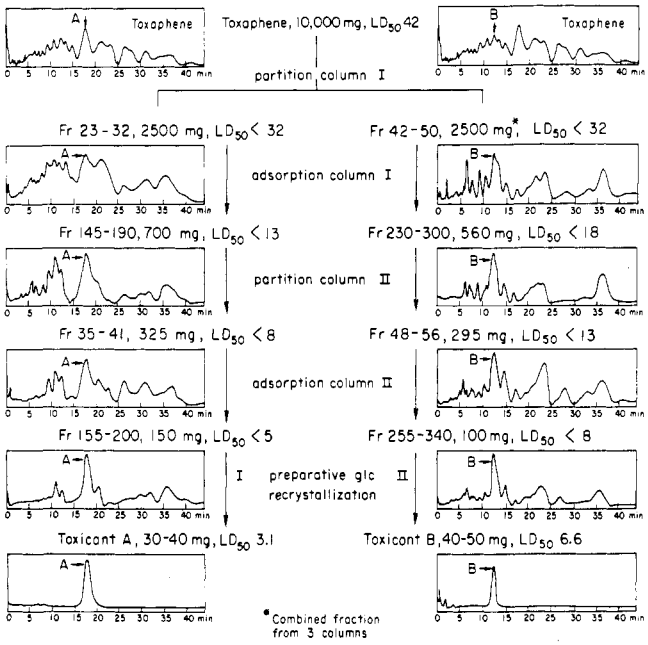


Figure 2. Chromatographic steps in isolation of toxicants A and B from technical toxaphene showing the yields, gas–liquid chromatographic patterns, and mouse LD_{50} values at each stage of purification.

to the degree of chlorination. The resolution of components obtained on columns of 3% SE-30 on Gas Chromosorb Q at 170° proved to be equivalent to or better than that obtained on any of the following liquid phases on Gas Chromosorb Q at appropriate temperatures: 10% DC-200, 3% Dexsil 300, 3% OV-17, and 3% OV-17 plus 1% QF 1. Isolation of components by preparative glc at 200° is equally satisfactory using 4% SF96-50, 3% SE-30, 3% Dexsil 300, or 3% OV-17. Some evidence is available that the toxic components do not decompose during preparative glc. The recovered fractions give the anticipated glc patterns with respect to both retention times and number of components and the toxicity to mice of the recovered fractions is consistent with that of the material prior to glc.

PROCEDURE FOR ISOLATION OF TOXICANTS A AND B FROM TECHNICAL TOXAPHENE BY COLUMN CHROMATOGRAPHY

Figure 2 shows the steps used to isolate toxicants A and B from technical toxaphene and the results of glc and mouse toxicity monitoring of the isolations. Technical toxaphene is used as the starting material instead of a recrystallized sample to rule out the possibility that toxicants may be discarded in the noncrystalline material or appear in both crystalline and noncrystalline fractions. The sequence of steps takes full advantage of the resolving potential of the various chromatographic systems, alternating partition and adsorption columns and using columns of higher capacity in early stages and of greater resolving power at later stages. The fractions from each step carried forward to the next step are selected on the basis of mouse ip toxicity assays, using progressively lower discriminating doses to assay the fractions from each successive column.

Partition column I yields only two regions within which each fraction has a mouse LD_{50} value below 32 mg/kg. The first of these regions, fractions 23–32, is designated as A and the second, fractions 42–50, as B. Further fractionation of components in these regions leads ultimately to the isolation of toxicants A and B. The recovery and degree of purification at each step depend on the resolving power of the column and the discriminating dose used for toxicity assays. Preparative glc system I is used for final isolation of toxicant A and preparative glc system II for toxicant B. The products from preparative glc were subjected to recrystallization (Table I) with or without prior sublimation to obtain the chromatographically pure materials referred to as toxicants A and B. These components are pure based on tlc in five different solvent systems and on glc with either 3% SE-30 on Gas Chromosorb Q or with capillary columns (Table I). Toxicant A also gives a single glc peak when examined on columns with five packings other than the standard SE-30; these include SF96-50 and cyclohexane dimethanol succinate, each with and without the addition of Igepal CO-880, and Dexsil 300 alone. Additional evidence that toxicant B is a single component is available from nuclear magnetic resonance (nmr) and X-ray structure determinations (Casida *et al.*, 1974). Toxicant A appears to be a mixture of at least two components based on nmr examination.

This sequence of chromatographic steps should permit, with appropriate monitoring, the isolation of any individual component of toxaphene, provided it is stable under the chromatographic conditions employed.

PROCEDURE FOR ISOLATION OF ^{14}C-LABELED TOXICANTS A AND B FROM [^{14}C]TOXAPHENE BY THIN-LAYER CHROMATOGRAPHY

An alternative tlc method for the isolation of ^{14}C-labeled toxicants A and B involves the spotting of [^{14}C]toxaphene from hexane in 15 spots of 4 mm diameter and 50 μg amount, each positioned 1 cm apart at their center across the origin of a silica gel chromatoplate containing on both sides spots of the authentic unlabeled standards for toxicants A and B. The chromatoplate is developed in the pentane system; then the unlabeled standards are detected without decomposing the labeled compounds by covering the radioactive portion of the plate with aluminum foil and glass and subjecting the exposed edges to the diphenylamine–ultraviolet procedure. The radioactive products corresponding to the markers are then scraped free of the glass support and extracted with hexane and a hexane–acetone mixture to recover ^{14}C-labeled toxicants A and B, respectively, in impure form. The toxicant B region is rechromatographed on tlc by the same procedure but using hexane saturated with formic acid for development; this solvent system gives higher R_f values than the pentane system and is particularly useful in the isolation of toxicant B. Preparative glc, under the conditions used with the unlabeled materials, then gives pure ^{14}C-labeled toxicants A and B in yields, based on radioactivity, of 6 and 3%, respectively. It appears likely that these values based on [^{14}C]toxaphene also approximate the amount of unlabeled toxicants A and B in technical toxaphene.

PROPERTIES OF TOXICANTS A AND B

The ir spectra (Figure 3) show the complexity of technical toxaphene relative to toxicants A and B and indicate the presence of chloromethyl groups (1305 cm^{-1}) in each case. This ir band has been used for identification and analysis of technical toxaphene (Clark, 1962), toxaphene fractions (Knox *et al.*, 1974), and related materials (Donev and Nikolov, 1965).

Table I compares many properties of toxaphene with those of toxicants A and B.

Toxicant A was obtained as colorless hexagonal crystals from isopropyl alcohol but the crystals were too small or too fragile for X-ray structure determination. This crystallization behavior is probably a characteristic of the type of compound (McCrone, 1957) although it may also result from impurities remaining in the sample. The same type of crystals are obtained from toxicant A on crystallization from methanol solutions on cooling with or without addition of a seed crystal and on very slow solvent evaporation at 25°. They are also obtained with these procedures carried out prior to or after sublimation of toxicant A.

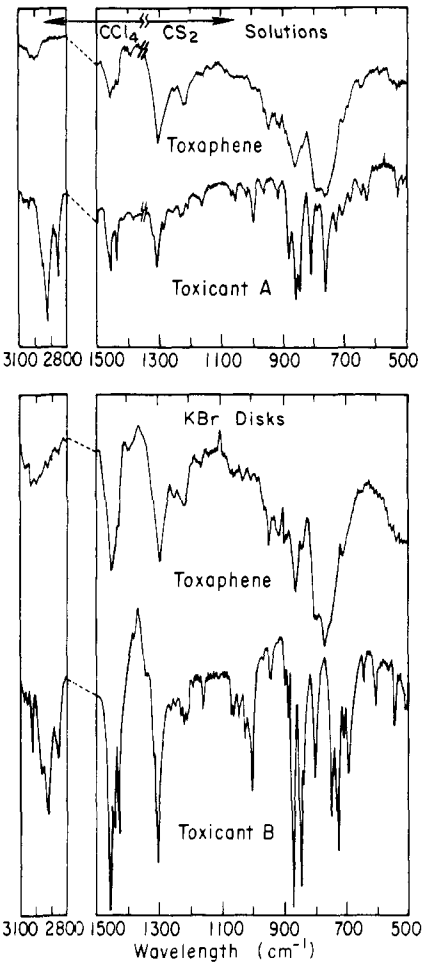
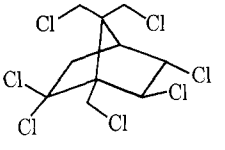


Figure 3. Comparison of the infrared spectra of toxaphene and those of toxicants A and B either as carbon disulfide and carbon tetrachloride solutions or as KBr disks.

Toxicant B is identified by X-ray examination of the colorless orthorhombic crystals as 2,2,5-*endo*,6-*exo*,8,9,10-heptachlorobornane (Casida *et al.*, 1974).



2, 2, 5-*endo*, 6-*exo*, 8, 9, 10-heptachlorobornane (toxicant B)

The toxicants are isolated from toxaphene on the basis of mouse ip acute toxicity assays and so they are of greatly increased toxicity to mice relative to technical toxaphene, 14-fold for A and sixfold for B (Table I). The potency of toxicants A and B is such that they may contribute significantly or almost entirely to the acute toxicity of toxaphene to mammals, assuming that the estimates of their content in toxaphene are reasonably accurate and that there are no synergistic or antagonistic effects between toxaphene components in their toxicity. It is known from available information not considered in detail here that at least one other component of toxaphene is more than twofold more toxic to mice than technical toxaphene and that many of the components are of relatively low toxicity. The toxicity of A and B to houseflies relative to their amount in toxaphene (Table I) indicates that they cannot account for the toxicity of the technical material. Therefore, other potent insecticidal materials must also be present in technical toxaphene or there are interactions between components in the toxicity.

There is a need to isolate other biologically active components from technical toxaphene. Components of the highest acute toxicity to mammals may not be those of the greatest chronic toxicity, as exemplified by the insecticide benzene hexachloride (1,2,3,4,5,6-hexachlorocyclohexane) where the γ and β isomers are most potent in acute and chronic tests, respectively (Metcalf, 1955). It is also likely that the components will vary in their specificity for control of different pest insect species, this being the case with other closely related chemical series, such as DDT and cyclodiene analogs (Metcalf, 1955). The problem of isolation of toxaphene components with high acute toxicity to mammals has been solved. These components, A and B, should be useful in studies on analysis of toxaphene residues and in evaluating the metabolic fate and environmental persistence of toxaphene constituents.

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