Toxaphene Degradation by Iron(II) Protoporphyrin Systems

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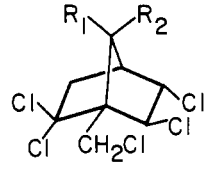
Toxaphene reacts with reduced hematin in neutral aqueous medium to cleave about half of the C–Cl bonds, yielding derivatives of shorter retention times on gas chromatography and of reduced sensitivity for detection by electron capture. This system converts toxicants A and B, two of its most toxic components, to products formed by reductive dechlorination, dehydrochlorination, and a combination of these reactions. Extensive metabolism of toxaphene and toxicants A and B by rat liver microsomes requires both NADPH and anaerobic conditions, thereby suggesting that reduced cytochrome P-450 acts as the reducing agent. These iron(II) protoporphyrin systems may serve as models for understanding how toxaphene degradation proceeds under certain metabolic and environmental conditions.

Toxaphene, the major chlorinated insecticide used in the United States, is a mixture of more than 177 polychlorinated derivatives evident on analysis by chromatography and mass spectrometry (MS); no constituent makes up more than a few percent of the technical mixture and most of the components are probably isomeric hepta-, octa-, and nonachlorobornanes (Casida et al., 1974, 1976; Holmstead et al., 1974). The toxaphene components of greatest concern are those most toxic to mammals and fish such as toxicant A (a mixture of A-1 and A-2) and toxicant B of the indicated structures (Casida et al., 1974; Palmer et al., 1975; Turner et al., 1975):



R_1	R_2	Designation
CH_2Cl	$CHCl_2$	toxicant A-1
$CHCl_2$	CH_2Cl	toxicant A-2
CH_2Cl	CH_2Cl	toxicant B

These toxic components are not currently available in amounts adequate for routine studies on their metabolic and environmental fate.

The use of appropriate model systems is one way to simplify the problem of examining the possible biological modes of degradation of such a complex mixture as toxaphene. Toxaphene undergoes extensive dechlorination in rats (Casida et al., 1974; Crowder and Dindal, 1974; Ohsawa et al., 1975), and toxicants A and B are converted under similar conditions to metabolites of shorter gas chromatography (GC) t_R values (Ohsawa et al., 1975). These findings indicate that toxaphene may undergo metabolic dechlorination or dehydrochlorination or both.

Two iron(II) protoporphyrin systems reductively dechlorinate the trichloroethane, DDT, to form the corresponding dichloroethane, DDD: these are hematin reduced with $Na_2S_2O_4$ (Miskus et al., 1965; Zoro et al., 1974) and liver microsomes containing cytochrome P-450 at their active site with reduced nicotinamide adenine dinucleotide phosphate (NADPH) serving indirectly as the reducing agent (Walker, 1969). These two systems and other iron(II) porphyrins (Castro, 1964; Wade and Castro, 1973) serve as models for the conversion of DDT to DDD in bacteria, yeast, and other microorganisms (including those in bovine rumen fluid, in sewage sludge, and possibly in lake water) under anaerobic conditions, and in invertebrate animals and in the blood and liver of birds and mammals following death. Furthermore, Mirex undergoes reductive dechlorination at a geminal dichloro group in sewage sludge (Andrade et al., 1974; Ivie et al., 1974), and its decomposition in the hematin–$Na_2S_2O_4$ system is initiated by this type of reaction (Holmstead, 1976).

The present study concerns the reactions of toxaphene and toxicants A and B with two iron(II) protoporphyrin systems, hematin reduced by $Na_2S_2O_4$ and microsomes in the presence of NADPH.

MATERIALS AND METHODS

Chemicals. Toxaphene (technical grade and this material fortified with tracer levels of [^{14}C]- or [^{36}Cl]-toxaphene), toxicants A and B (unlabeled and ^{14}C labeled), and Na^{36}Cl were obtained or isolated as previously described (Khalifa et al., 1974; Ohsawa et al., 1975) with additional purification of ^{14}C-labeled toxicant A by thin-layer chromatography (TLC) in the dimethylformamide–pentane system (Ohsawa et al., 1975) to remove minor impurities. Hematin (ferriprotoporphyrin hydroxide) from bovine blood was from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals and solvents used were of reagent grade quality.

Analyses. GC analyses utilized glass columns (180 cm × 2 mm i.d.) of 3% SE-30 on Gas-Chrom Q (80–100 mesh) either at 170 °C with a N_2 flow rate of 115 ml/min and an electron capture (ec) detector (Khalifa et al., 1974) or at 210–245 °C (temperature program, 6 °C/min) with methane at a flow rate of 20 ml/min and coupling to a computerized chemical ionization (CI)–MS system with a source pressure of ~1.5 Torr (Holmstead et al., 1974). For TLC, silica gel 60 F-254 chromatoplates (20 × 20 cm, 0.25 mm layer thickness) were developed with one or more of the following solvent systems: pentane; benzene; chloroform–methanol (10:1 or 1:1). ^{14}C-Labeled products were detected by radioautography.

Radiolabeled materials were quantitated by liquid scintillation counting (lsc) with quench correction by internal standards of [^{14}C]toluene or Na^{36}Cl.

Reaction of Toxaphene and Toxicants A and B with Reduced Hematin. In standard reactions, the components were added in the indicated order to 25-ml Erlenmeyer flasks to obtain solutions of pH 6.5–7.5: hematin (0–16 μmol) dissolved in 0.1 M Na_2CO_3 (0.5 ml); H_2O (5 ml); ethanol (1 ml); unlabeled-, [^{14}C]-, or [^{36}Cl]toxaphene (0–16 μmol, calculated as $C_{10}H_{10}Cl_8$), or unlabeled toxicant A or B (8 μmol) in ethanol (0.2 ml), or Na^{36}Cl (32 μmol) in H_2O (0.2 ml). Immediately after the last addition, the gas phase of the flask was replaced with argon, $Na_2S_2O_4$ (0 or 25 mg; 0 or 144 μmol) was added, the argon flush was

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continued, and a gas-tight rubber stopper with double seal septum was inserted into the flask. The reaction mixtures were incubated with rapid shaking for up to 4 h at 37 °C. The color of the reduced hematin-containing solutions remained bright red and there was no evidence that toxaphene or its derivatives precipitated from solution during the incubation. Appropriate controls were run in all cases to ascertain that the products detected were due to the interaction of hematin and toxaphene or its components in the $Na_2S_2O_4$-containing medium. All products that are reported were formed only in the presence of the toxaphene component, hematin, and $Na_2S_2O_4$—deletion of any one of these components yielded no product.

Analysis for Dechlorination of [^{36}Cl]Toxaphene by Reduced Hematin. Each reaction mixture containing 8 μmol of [^{36}Cl]toxaphene (experimental samples), or 8 μmol of [^{14}C]toxaphene or 32 μmol of Na^{36}Cl (control samples), was treated in a manner appropriate to obtain three fractions: the insoluble hematin derivative with some precipitated toxaphene derivatives, a soluble fraction not precipitable by $AgNO_3$, and an AgCl precipitate. The hematin derivative was precipitated by first adding concentrated HNO_3 (10 drops), then holding 15 min at 25 °C and centrifuging, followed by one H_2O wash (0.5 ml) of the precipitate and the addition of this wash to the supernatant fraction. The combined supernatant fraction was treated, in sequence, with unlabeled NaCl (86 μmol with toxaphene systems and none with Na^{36}Cl systems) and $AgNO_3$ (600 μmol) in H_2O (2 ml), holding 1 h at 25 °C, for AgCl precipitation prior to centrifugation. For quantitation by lsc, the three fractions were treated as follows: the hematin fraction, sometimes with H_2O-insoluble toxaphene derivatives, was dissolved in acetone (1 ml), and a 10-μl aliquot was counted; the supernatant fraction, after $AgNO_3$ treatment, was counted using a 0.1-ml aliquot; the AgCl precipitate was dissolved by addition of concentrated NH_4OH (6 ml) and H_2O (4 ml), and a 0.2-ml aliquot was counted. The level of radioactivity in each fraction was compared with the initial amount, recording that portion not accounted for as "loss".

Analysis of Chlorinated Hydrocarbon Products from the Reaction of [^{14}C]Toxaphene and Unlabeled Toxicants A and B with Reduced Hematin. For GC–ec analysis, each reaction mixture was extracted with hexane (10 ml), acidified to pH 0.9 with HCl, allowed a 15-min holding period, and then extracted with ether (2 × 10 ml). Analysis by GC involved a 1-μl aliquot of the hexane extract and a 2-μl aliquot of the combined ether extracts, when the starting chlorinated hydrocarbon level was 8 μmol, or proportionately larger aliquots when smaller amounts of toxaphene or its components were used initially. The GC–ec data are presented as actual chromatograms since attempts to quantitate on the basis of peak heights or areas did not yield proportionality with amount through the region of eluting compounds. The elemental composition of hexane-soluble products from toxicants A and B was determined by GC–CI–MS from appropriate $[M - Cl]^+$ fragment ions. With the [^{14}C]toxaphene reactions, aliquots of each of the hexane and combined ether extracts were also used for radiocarbon analysis; that portion of the radiocarbon not recovered in the organic extracts was assigned to the H_2O phase.

An alternative analytical procedure for products from [^{14}C]toxaphene involved evaporation (<30 °C, N_2 stream) of the H_2O and ethanol from reaction mixtures, dissolving the residue in methanol, and TLC separation of the methanol-soluble products using radioautography for product detection on the TLC plate and GC–ec for analysis of products recovered from appropriate radioactive gel regions by methanol extraction.

Table I. Fractionation of Labeled Products from Incubation Mixtures Containing 32 μmol of Na^{36}Cl, 8 μmol of [^{36}Cl]Toxaphene, or 8 μmol of [^{14}C]Toxaphene and Various Amounts of Reduced Hematin after 4-h Incubation[a]

Fraction	Radioact. recovd, % of initial amount, in the presence of the indicated hematin level, μmol				
	0	0.125	0.5	2	16
	Na^{36}Cl				
AgCl frac.	100	100	99	100	99
Hematin frac.	0	0	0	0	0
Sol. deriv.	0	0	0	0	0
Loss	0	0	1	0	1
	[^{36}Cl]Toxaphene				
AgCl frac.	2[b]	37	49	48	55
Insol. tox. deriv. with hematin frac.	97[b]	52	40	41	36
Sol. tox. deriv.	1[b]	11	11	10	7
Loss	0[b]	0	0	1	2
	[^{14}C]Toxaphene				
AgCl frac.	0	0	0	0	0
Insol. tox. deriv. with hematin frac.	98	72	61	68	68
Sol. tox. deriv.	2	27	37	26	24
Loss	0	1	2	6	8

[a] The reaction volume in each case was 6.7 ml (see Materials and Methods). [b] Similar results were obtained with no $Na_2S_2O_4$ at hematin levels of 0, 0.125, and 16 μmol.

Metabolism of [^{14}C]Toxaphene and ^{14}C-Labeled Toxicants A and B by Liver Microsome–NADPH Systems. Reaction mixtures in 0.1 M pH 7.4 phosphate buffer (2 ml) consisted of: rat liver microsomes (0 or 5 mg of protein); NADPH (0 or 5 μmol); and [^{14}C]toxaphene, -toxicant A, or -toxicant B (30 nmol) added last in ethanol (50 μl). After a 2-h incubation at 37 °C in air or N_2 with shaking, the mixtures were extracted with hexane (3 × 5 ml) for GC–ec analysis or with ether (3 × 5 ml) and then sometimes followed by ether-ethanol (2:1 mixture, 3 × 5 ml) for TLC analysis.

RESULTS

Dechlorination of [^{36}Cl]Toxaphene by Reduced Hematin. Toxaphene undergoes extensive dechlorination in the presence of even small amounts of reduced hematin. This is evidenced by the formation of labeled products from [^{36}Cl]toxaphene that precipitate with $AgNO_3$ under conditions in which there is complete precipitation of ^{36}Cl-labeled chloride but no precipitation of labeled products formed from [^{14}C]toxaphene (Table I). The dechlorination of toxaphene requires the presence of both hematin and $Na_2S_2O_4$, i.e. reduced hematin, since almost no radioactivity from [^{36}Cl]toxaphene appears in the AgCl fraction in the presence of only hematin or $Na_2S_2O_4$. The extent of dechlorination appears to reach a plateau with increasing hematin levels such that approximately half of the C–Cl bonds and no more are cleaved in the system.

The major portion of the radiocarbon from [^{14}C]toxaphene is precipitated along with the hematin derivative on acidification of the reaction mixture. These labeled compounds can be completely separated from the hematin derivative by applying the mixture to a silicic acid column and developing with ether, a procedure which yields all of the radiocarbon in the eluate and retains all of the hematin derivative on the column. An alternative method to separate the ^{14}C-labeled products from the hematin derivative involves evaporation of the solvents from the

Table II. Recovery of ^{14}C-Labeled Products in Hexane and Ether Extracts of Incubation Mixtures Containing [^{14}C]Toxaphene and Various Molar Ratios of Reduced Hematin after 4-h Incubation

Molar ratio, toxaphene/ red. hematin[a]	Radiocarbon recovd, % of initial amount, in indicated fraction[b]		
	Hexane	Ether	H_2O
1:0[c]	84	11	5
64:1	50	41	9
16:1	50	37	13
4:1	35	38	27
1:2	27	28	45
1:16	23	26	51

[a] Eight micromoles of toxaphene was used except at the 1:16 molar ratio where 1 μmol of toxaphene was used. The reaction volume in each case was 6.7 ml (see Materials and Methods). [b] Average of results from two experiments. Recovery in the H_2O fraction was determined as the difference between the total radiocarbon used and that recovered in the organic extracts. With toxaphene-reduced hematin molar ratios intermediate to those tabulated, there was a progressive shift in product extractability falling into the same pattern. [c] Average of results obtained with either $Na_2S_2O_4$ or hematin alone and at toxaphene levels of 1 and 8 μmol.

reaction mixture, dissolving the residue in methanol, and TLC of the methanol-soluble products with chloroform–methanol (1:1)—which moves all of the labeled compounds but none of the hematin derivative free from the origin.

Some of the toxaphene degradation products, other than chloride ion, appear in the soluble fraction. These are relatively polar compounds since they were not recovered in organic solvent on extraction of the soluble fraction with ether.

Chlorinated Hydrocarbon Products from the Reaction of [^{14}C]Toxaphene with Reduced Hematin. [^{14}C]Toxaphene incubated with either hematin or $Na_2S_2O_4$ is recovered almost completely in hexane and ether extracts of the incubation mixtures. The addition of $Na_2S_2O_4$ to a mixture of toxaphene having increasing levels of hematin results in a progressive decrease in hexane-extractable radiocarbon from neutral solution, an increase and then a decrease in ether-extractable radiocarbon from acidic medium, and a progressive increase in H_2O-soluble radioactivity (Table II). In these analyses, the iron protoporphyrin (probably chlorohemin) was precipitated by addition of HCl prior to the ether extraction, a procedure that yielded much higher overall recoveries than without acidification. A portion of the toxaphene and its products appears to bind with the reduced hematin in a manner such that it is not recovered on hexane extraction; however, on conversion to chlorohemin some of these toxaphene-derived products are recovered on ether extraction. The proportion of labeled material remaining in the H_2O phase increases with hematin level indicating the formation of polar derivatives or of products that bind tenaciously to the iron protoporphyrin. These observations were confirmed by TLC analysis of the methanol-soluble products following evaporation of the reaction mixtures. In the presence of $Na_2S_2O_4$, but not in its absence, increasing the hematin level gave the following TLC results: on development with pentane or benzene there was an increase in radioactive products retained at the origin along with the hematin derivative; on development with either benzene or chloroform–methanol (10:1) there was an increase in products chromatographing between the origin and R_f 0.77 (toxaphene position); on development with chloroform–methanol (10:1) there was an increase in

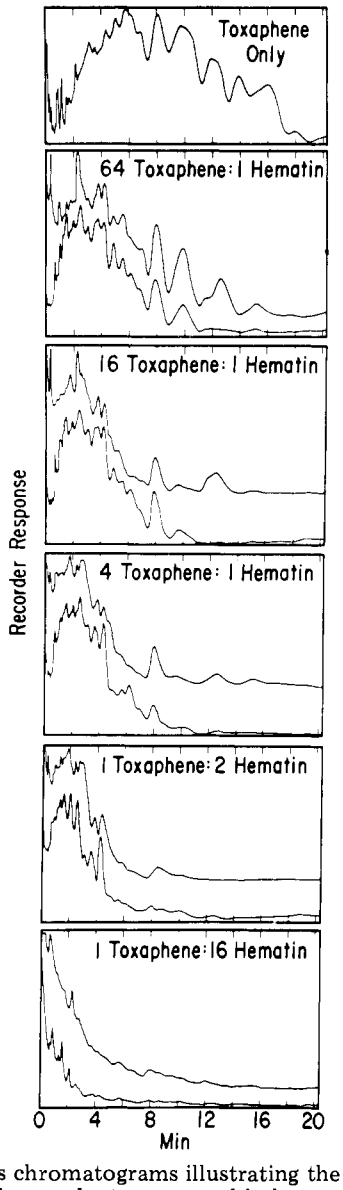

Figure 1. Gas chromatograms illustrating the electron-capture sensitive products recovered in hexane and ether extracts (lower and upper traces, respectively, where two chromatograms are shown) from incubation mixtures containing toxaphene and various molar ratios of reduced hematin. Two additional peaks from toxaphene with t_R > 20 min are not detected with the toxaphene-reduced hematin incubations.

amounts of at least five polar derivatives of low R_f but having R_f 0.5–1.0 on subsequent development with chloroform–methanol (1:1).

The dramatic degradation of toxaphene components in the hematin–$Na_2S_2O_4$ system is most readily apparent on considering the GC–ec patterns of the mixture of toxaphene components and the reaction products recovered on hexane and ether extractions of the incubation mixtures (Figure 1). With either hematin or $Na_2S_2O_4$ alone, the chlorinated components in the hexane extract (and also in the ether extract) give an unaltered GC–ec pattern for toxaphene itself. On progressively increasing the amount of hematin in the presence of $Na_2S_2O_4$, the long t_R peaks of toxaphene disappear first, and there is a progressive increase in short t_R peaks. No further change takes place when the toxaphene:hematin molar ratio is reduced beyond 1:16, but at this stage only short t_R compounds remain, and they give only a small proportion of the ec sensitivity of the original toxaphene starting material. The GC–ec

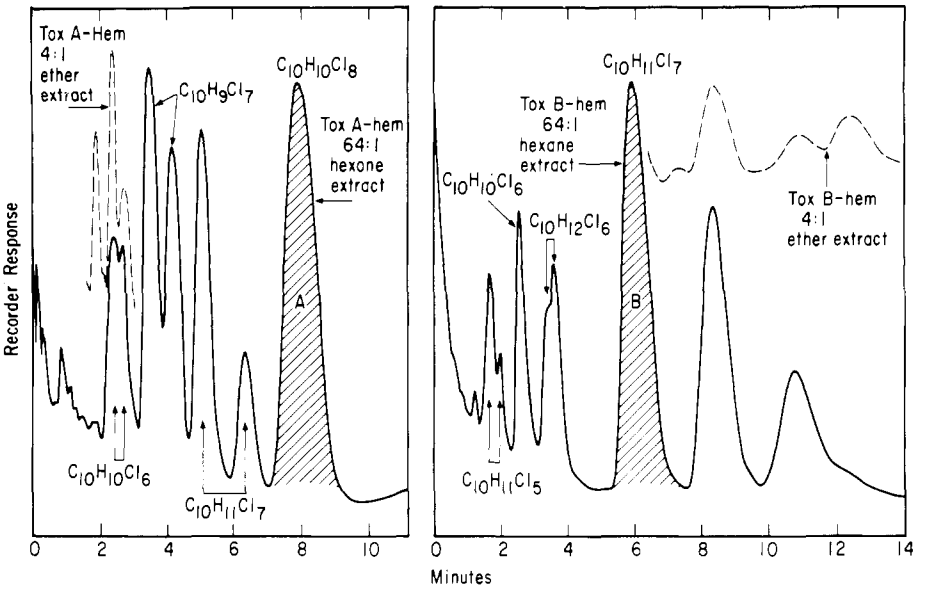

Figure 2. Gas chromatograms illustrating the electron-capture sensitive products recovered in hexane and ether extracts from incubation mixtures containing toxicant A or toxicant B and various molar ratios of reduced hematin with designations of the elemental composition of certain components.

patterns are very similar but not identical for the products recovered on hexane and ether extractions. In further studies, not detailed here, it was found that even very low levels of hematin, i.e. a toxaphene:hematin molar ratio of 256:1, cause significant changes in the product extraction and GC–ec patterns. Thus, the reaction of toxaphene with reduced hematin generally yields organosoluble products of shorter t_R values and ones that lack the original sensitivity for detection by ec. These same phenomena are apparent on fractionating the toxaphene–hematin reaction products by TLC with chloroform–methanol (10:1) development prior to GC–ec analysis; compounds from the TLC position of toxaphene give GC–ec patterns dominated by short t_R peaks, and products chromatographing on TLC below the toxaphene region give only very short t_R peaks for the ec-sensitive compounds eluting from the GC column.

In the studies discussed above, the incubation time was standardized at 4 h, and the hematin level varied. Similar results were obtained on holding the hematin level constant (e.g. toxaphene:hematin molar ratio of 64:1) and varying the time of incubation. These reactions were terminated by bubbling air through the deep red solutions, resulting in an immediate color change to brown and a slow precipitation of the brown material. The toxaphene products undergo no further degradation after this aeration treatment. These studies established that, even with high toxaphene:hematin molar ratios, the reaction is essentially complete after incubation for 15 min.

This type of system was also applied with similar results to large amounts of toxaphene, e.g. 1.0 g of toxaphene with a toxaphene:hematin molar ratio of 3:1, a total reaction volume of 1.2 l., and 4-h incubation under N_2. Extraction with hexane (2 × 1 vol) and ether (2 × 1 vol) gave a 50% recovery by weight of organosoluble materials with a GC–ec pattern very similar to that shown in Figure 1 for a toxaphene:hematin molar ratio of 16:1 on the small scale. A prominent peak at t_R = 8.0 min cochromatographed on GC with toxicant A and was found by GC–CI–MS to consist mainly of a $C_{10}H_{10}Cl_8$ component(s); however, this material did not give the same fragmentation ion intensities as obtained with toxicant A under the same conditions. The mouse intraperitoneal LD_{50} value (determined according to Khalifa et al., 1974) of the product mixture recovered from the toxaphene-reduced hematin reaction was 80 mg/kg vs. 42 mg/kg for toxaphene itself.

The above studies suggest that, on reaction with this iron(II) protoporphyrin, toxaphene undergoes extensive dechlorination and conversion to products of shorter t_R values on GC–ec and lower R_f values on TLC. Further interpretation of the results is severely hampered by the complexity of the GC–ec pattern of toxaphene itself, so that products from reaction of reduced hematin with individual components within toxaphene cannot be discerned from the background of other toxaphene components and their reaction products.

Products from Reaction of Toxicants A and B with Reduced Hematin. Some of the complications encountered above were overcome by replacing toxaphene with a single compound (toxicant B) or a mixture of compounds of defined chemical structure that give one GC peak (toxicant A). Toxicants A and B (8 μmol) were treated with reduced hematin at polychlorobornane:hematin molar ratios of 64:1, 4:1, and 1:2, analyzing the hexane- and ether-soluble products by GC–ec. Both hematin and $Na_2S_2O_4$ were necessary to generate degradation products of toxicants A and B. Figure 2 shows in its major portions the hexane-extractable products from reaction of toxicants A and B with reduced hematin at the 64:1 molar ratio. The proportion of products varies with increasing hematin levels and in the hexane and ether extracts allowing preliminary considerations on the sequence of product formation.

Toxicant A ($C_{10}H_{10}Cl_8$) gives, in order of decreasing t_R values, two reductive dechlorination products ($C_{10}H_{11}Cl_7$), two dehydrochlorination products ($C_{10}H_9Cl_7$), and two products corresponding to a molecular formula of $C_{10}H_{10}Cl_6$. The $C_{10}H_{11}Cl_7$ product of longest t_R value appeared only with the toxicant A:hematin molar ratio of 64:1, indicating that it rapidly undergoes further reaction. The other $C_{10}H_{11}Cl_7$ product and the $C_{10}H_9Cl_7$ products are prominent at the 64:1 and 4:1 but not at the 1:2 molar ratios. The $C_{10}H_{10}Cl_6$ products and a t_R 1.9 min material (shown in the insert of Figure 2) reach maximum levels with the largest amounts of hematin. Thus, the $C_{10}H_{10}Cl_6$ products may be formed from the $C_{10}H_{11}Cl_7$ compounds by dehydrochlorination, from the $C_{10}H_9Cl_7$ material by reductive dechlorination, or from the starting $C_{10}H_{10}Cl_8$

compound by vicinal halide elimination. Toxicant B ($C_{10}H_{11}Cl_7$) gives, again in order of decreasing t_R values starting from toxicant B itself, two reductive dechlorination products ($C_{10}H_{12}Cl_6$), one dehydrochlorination product ($C_{10}H_{10}Cl_6$), and two products corresponding in molecular formula to $C_{10}H_{11}Cl_5$. The $C_{10}H_{12}Cl_6$ and $C_{10}H_{10}Cl_6$ products are prominent at the 64:1 and 4:1 but not at the 1:2 molar ratios of toxicant B:hematin. The $C_{10}H_{11}Cl_5$ materials increase in proportion and amount as the hematin level increases; these products may be formed directly from toxicant B by vicinal halide elimination or from the $C_{10}H_{12}Cl_6$ and $C_{10}H_{10}Cl_6$ compounds as intermediates. Three additional products appear with toxicant B, each with a longer t_R value than the starting material and the one of longest t_R value appearing in major amounts only in ether extracts. The ratios of these materials to other products considered above suggest that they are formed directly from toxicant B rather than from any of the components of shorter t_R values. Although not identified, the t_R 8.4 and 10.9 min materials give identical CI–mass spectra with large distinguishable ion clusters at m/e 334 and 335 (four chlorines) and 370 and 371 (five chlorines), and smaller clusters centered at m/e 401, 413, and 447. The large ion clusters at m/e 370 and 371 may be $C_{10}H_{11}Cl_5SO_2^+$ and $C_{10}H_{12}Cl_5SO_2^+$, respectively. However, since these ions are not observed under electron impact conditions, an accurate mass measurement was not possible. Other derivatives of toxicants A and B, if present, are either minor or are not detected under the analytical conditions used.

Metabolites of Toxaphene and Toxicants A and B in Microsome–NADPH Systems. [^{14}C]Toxaphene and ^{14}C-labeled toxicants A and B are each converted to products of lower TLC R_f values when incubated with rat liver microsome–NADPH systems—the metabolism in each case requiring the presence of NADPH and proceeding to a greater extent in N_2 (where reductive dechlorination is expected) than in air (where hydroxylation is the most likely reaction). The substrates and products are almost completely (97–98%) recovered on extracting the incubation mixtures with ether, the ether-extractable radiocarbon from ^{14}C-labeled toxicants A and B reactions yielding TLC patterns as shown in Figure 3. Microsomal metabolism of each of toxicants A and B in anaerobic conditions gives two major metabolites which chromatograph just below the original substrates. There are also three to seven additional metabolites of lower R_f values. [^{14}C]Toxaphene yields at least five metabolite regions similar in chromatographic positions but more diffuse than those from toxicant A, the unmetabolized toxaphene components appearing as a long streak on pentane development. On incubation in air, the ether-extractable metabolite yields were always lower than in N_2, and fewer metabolites were detected from ^{14}C-labeled toxicants A and B and labeled toxaphene. Some of the ether-extractable metabolites of each substrate may be similar or the same when formed on incubation in air or N_2 but no attempt was made to establish their individual identity by cochromatography. Subsequent ether–ethanol extraction of the mixtures with [^{14}C]toxaphene and ^{14}C-labeled toxicant A incubated in air yielded at least six new metabolites (totaling less than 3% of the radiocarbon) of low R_f values resolved on TLC development with chloroform–methanol (1:1). Thus, on considering all TLC spots for NADPH-dependent metabolites formed on incubation in air and N_2, each of toxaphene and toxicants A and B gives at least 9 to 11 distinct metabolite regions.

Microsomal metabolites of toxicants A and B are also

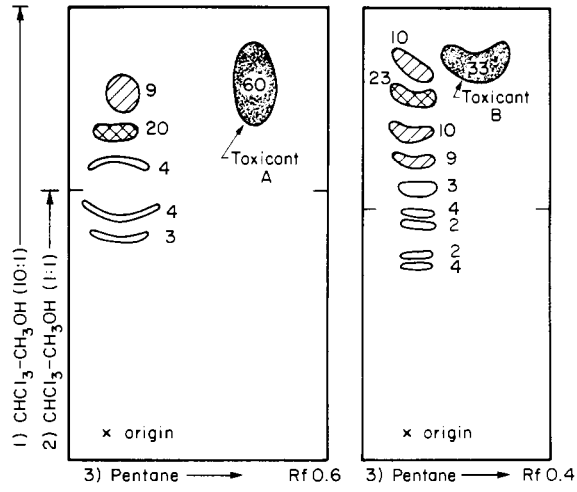

Figure 3. Thin-layer chromatographic patterns and percentage amounts of ether-extractable ^{14}C-labeled metabolites of ^{14}C-labeled toxicants A and B formed on incubation with rat liver microsomes and NADPH in an N_2 atmosphere. Only a portion of each chromatogram is shown in the direction of pentane development (i.e., the regions up to R_f 0.6 and 0.4) since no product of higher R_f value was detected in any case.

detected on GC–ec, using hexane extracts of incubation mixtures to minimize interfering materials. Thus, toxicant A (t_R = 8.0 min) incubation in the microsome–NADPH–air system yields a new peak at t_R = 3.6 min, while toxicant B (t_R = 6.0 min) incubated in the microsome–NADPH–N_2 system gives a new peak at t_R = 5.3 min, each of these metabolites being dependent on cofactor addition to the incubation mixture. It appears that only a portion of the metabolites detected by TLC of ether extracts are evident on GC–ec of hexane extracts.

On varying the substrate level (3, 10, 30, or 100 nmol) with ^{14}C-labeled toxicant B, the percentage metabolism detected by TLC or GC–ec decreased markedly as the substrate level was increased, possibly due to the relatively low solubility of this substrate. On this basis, the microsomal system is not a convenient source for large amounts of metabolites.

DISCUSSION

Studies on the metabolism and environmental degradation of toxaphene components pose unusual difficulties because of the complexity of the mixture making up the commercial insecticide, the small amounts of toxic components available in pure form, and the formation of metabolites and degradation products that are not separated from initial components in routine GC–ec analyses. To overcome some of these difficulties, iron(II) protoporphyrin systems (hematin reduced with $Na_2S_2O_4$ and microsomal cytochrome P-450 reduced with NADPH) were examined with toxaphene and two of its most toxic components, toxicants A and B. From the results presented here, it is clear that many toxaphene components, including octa- and heptachlorobornanes such as toxicants A and B, are converted by one or both of these iron(II) protoporphyrins to more polar derivatives, based on TLC, and products that are usually of shorter GC t_R values. Studies with reduced hematin establish that toxaphene undergoes extensive dechlorination and that the products from toxicants A and B determined by GC–CI–MS are formed by two or more of these processes: reductive dechlorination, dehydrochlorination, and vicinal chloride elimination. Many of the reaction products can be obtained in microgram amounts in the anaerobic microsome–NADPH system and in milligram or potentially even

in gram amounts in the reduced hematin system. Thus, the model systems provide a means of generating degradation products of toxaphene and its components under very mild conditions.

The utility of model systems is dependent, in part, on their similarity, both mechanistically and in the products formed, to metabolism and environmental degradation conditions. The proposed mechanism of reductive dehalogenation of alkyl halides by iron(II) porphyrins (Wade and Castro, 1973) is shown in reactions 1 and 2. Following

$$RCl + \overset{|}{Fe^{II}} \rightleftharpoons [(RCl)\overset{|}{Fe^{II}}] \rightleftharpoons R\cdot + Cl\overset{|}{Fe^{III}} \quad (1)$$

$$R\cdot + \overset{|}{Fe^{II}} + H_2O \rightarrow RH + \overset{|}{Fe^{III}} + OH^- \quad (2)$$

initial formation of an alkyl halide–iron(II) porphyrin complex, the alkyl halide undergoes scission of the carbon–halogen bond generating the alkyl radical (reaction 1). Further reaction of the alkyl radical involves hydrogenolysis by a suitable proton source during the attack by a second iron(II) porphyrin (reaction 2). The iron(III) porphyrins in reactions 1 and 2 are then reduced and the cycle continues, limited eventually by the available level of reductant. Geminal and vicinal halides are particularly reactive in this type of system. Thus, toxicants A and B and probably many other toxaphene components contain substituents readily attacked by iron(II) protoporphyrins. The products formed with toxicants A and B are those expected, i.e. reductive dechlorination products and unsaturated compounds from elimination of two chlorines. Each toxaphene component contains many possible sites for initial attack and further degradation. The pathway of breakdown should be primarily determined by the ease of C–Cl bond scission at each stage as well as stereochemical selectivity. Thus, the initial products are likely to be those of reductive dechlorination at geminal dichloro groups and of elimination of vicinal chlorines. The remarkable effectiveness of the hematin–$Na_2S_2O_4$ system results not only from the reactivity of the polychlorobornanes but also from continuous regeneration of the Fe(II) protoporphyrin in the presence of a large excess of $Na_2S_2O_4$.

There is some similarity in the overall metabolic reactions to those taking place in the iron(II) protoporphyrin system since (1) about half of the C–Cl bonds of toxaphene are cleaved both on metabolism in rats (Ohsawa et al., 1975) and on incubation with reduced hematin and (2) products of greater polarity and shorter GC–ec t_R values are formed from toxicants A and B in both rats and the reduced hematin system. It is important in this context to determine in future studies if some of the microsomal products and those formed by reduced hematin are identical with in vivo metabolites of toxaphene or its components in mammals.

Extensive studies on the conversion of DDT to its reductive dechlorination product, DDD, show that findings from anaerobic microsome–NADPH and reduced hematin systems may have relevance to in vivo situations (see introductory statement). If this is also true for toxaphene, then extensive dechlorination of its polychlorobornane components might be expected to occur in microbial systems under anaerobic conditions (such as bovine rumen fluid and sewage sludge), in postmortem blood and liver samples during storage prior to analysis, and in general on death of animals containing toxaphene residues. The model systems utilized in the present study provide the basis for these speculations and for subsequent investigations of their relevance.

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