Bull. Environm. Contam. Toxicol. 21,539-547 (1979)

Metabolism of Toxicant B and Toxicant C of Toxaphene in Rats

P. S. Chandurkar and F. Matsumura
Department of Entomology, University of Wisconsin, Madison, Wis. 53706

Toxaphene is the most widely used chlorinated hydrocarbon insecticide in the United States today. It is a very complex mixture of more than 177 polychlorobornane compounds containing 6 to 11 chlorine atoms (HOLMSTEAD et al. 1974). Only a few of these compounds have been chemically characterized (ANAGNOSTOPOULOS et al. 1974, BLACK 1974, TURNER et al. 1975, MATSUMURA et al. 1975, LANDRUM et al. 1976, CHANDURKAR 1977), and very little is known about the mode of action, and metabolic fate of its components. CASIDA et al. (1974), CROWDER and DINDAL (1974), OHSAWA et al. (1975), and POLLOCK and KILGORE (1976) have studied the metabolism of toxaphene in rats. They reported that toxaphene components are metabolized via dechlorination reactions. Toxic fraction A and toxicant B are also shown to be metabolized to several dechlorination and/or dehydrochlorination products by the rat (OHSAWA et al. 1975) and by the iron (II) protoporphyrin systems (KHALIFA et al. 1976). However, besides chloride ion, no metabolite of toxaphene components has ever been identified.

In the previous report (CHANDURKAR 1977) from this laboratory it was shown that toxaphene is metabolized to polar hydroxyl and acidic compounds as well as to water-soluble conjugates by NADPH-dependent mixed-function oxidase system in rats. This report gives further details of the results on the chemical nature of *in vitro* metabolites of toxicant B and toxicant C (2-endo, 3,3,5,6-exo, 8,9,10,10-nonachlorobornane).

MATERIALS AND METHODS

Standard toxaphene (sample X-16189-49) was provided by Hercules Incorporated, Wilmington, Delaware. Toxicants B and C were isolated from four-times recrystallized toxaphene using the method of NELSON and MATSUMURA (1975). Tetramethylsilane nmr grade and o-phenylenediamine were purchased from Aldrich Chemical Co. Chromium trioxide was obtained from Matheson,

0007-4861/79/0021-0539 $01.80

Coleman and Bell Manufacturing Chemists. Other chemicals used in this study have been described previously (CHANDURKAR 1977).

Preparation of 20,000 g supernatant from rat liver and its incubation with toxicants B and C were carried out as before (CHANDURKAR 1977): 3.5 µg of toxicant B or C in 4 to 7 µl of absolute ethanol were added to the assay tube containing 0.5 ml of enzyme preparation, 0.3 ml of standard buffer (i.e., 0.2 M sodium phosphate buffer at pH 7.4), and 0.2 ml of standard buffer with cofactor. The reaction mixture was incubated at 37°C for 2 hrs. in a Lab-line metabolic shaker. At the end of the incubation period the reaction mixture was extracted twice with 3 ml of solvent mixture ($CHCl_3$: MeOH, 5:1) each time.

Synthesis and spectroscopic identification of the dechlorination product of toxicant C.

The dechlorination method used was that of FIESER (1963) with some modifications. To 200 µg of toxicant C in one ml of absolute diethyl ether in a small test tube, 0.2 ml of glacial acetic acid was added and mixed well. Five mg of zinc powder was then added and the reaction mixture was stirred continuously at room temperature. The progress of the reaction was monitored by checking the product intermittently on gas chromatography (GLC). When the reaction was complete after 35 minutes, 0.5 ml of distilled water was added. The diethyl ether phase was then removed and washed three times with 0.5 ml of water each time. The combined water phase was extracted with four ml of diethyl ether. All the diethyl ether phase was combined, concentrated and dried over anhydrous sodium sulfate. The product was purified on thin-layer chromatography (TLC) with four times development in heptane. This is the only TLC system used.

The dechlorination product was identified by proton magnetic resonance (PMR) and Mass spectroscopy. PMR spectrum in CCl_4 (with a d_6-acetone capillary as an internal lock) with tetramethylsilane as standard was obtained on a 90 MHZ Bruker Fourier Transform NMR Spectrometer. Mass spectrum was taken on an AEI MS 9 Mass Spectrometer equipped with AEI DS-50 data system. The electron energy used was 70 eV.

The analysis of polar metabolites was carried out by chemical derivitization methods as follows. Thirty µg of each toxicant were incubated with the standard enzyme system in the presence of added NADPH. After the extraction of reaction mixture with regular solvent

mixture of $CHCl_3$:MeOH, the aqueous phase was reextracted with three ml of ethyl acetate per ml of aqueous phase. The ethyl acetate extract and the $CHCl_3$:MeOH extract were combined. The combined extract was then evaporated to dryness, and the residue was picked up in a small volume of diethyl ether. It was

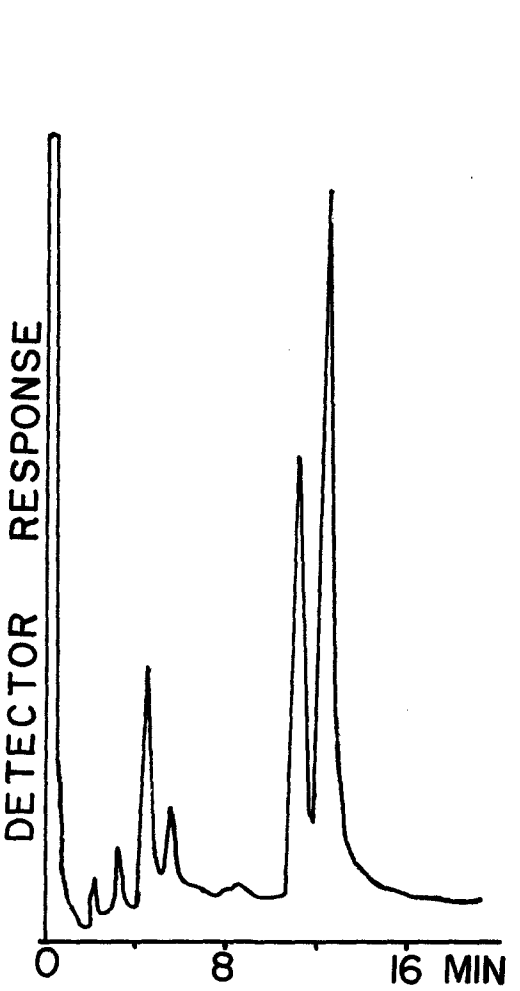


Fig. 1a: Gas chromatogram of the solvent extractable metabolites from NADPH fortified incubates of toxicant B.

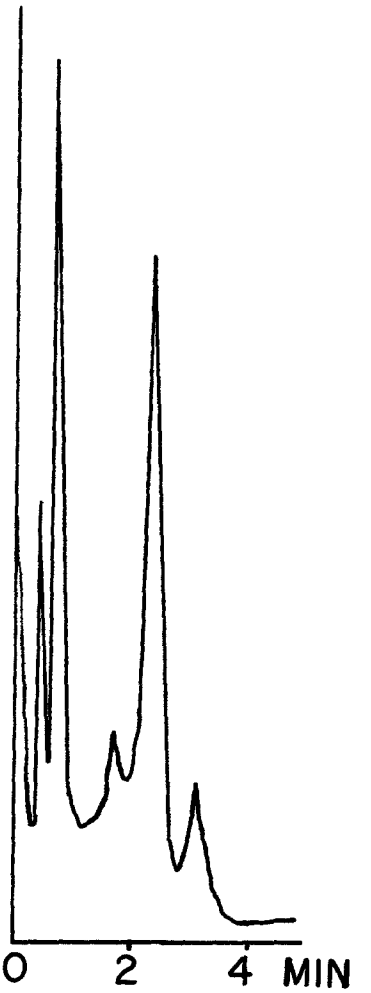


Fig. 1b: Gas chromatogram of the oxidation-methylation products of toxicant C metabolites from NADPH fortified incubates.

spotted on TLC plate and the chromatogram developed. The silica gel from TLC area corresponding to Rf 0.0 to 0.33 was scraped and extracted with diethyl ether. This extract was concentrated to a small volume. A part of it was used for GLC analysis, and the rest was used for an oxidation reaction according to FIEL et al. (1970) with slight modifications. The metabolites were dissolved in five ml of diethyl ether in a fifty ml round bottom flask. To this 0.29 ml of chromic acid reagent was added with continuous stirring. The chromic acid reagent was prepared by adding one g of chromium trioxide to five ml of distilled deionized water, containing 0.75 ml of concentrated sulfuric acid and stirred well. After seven hours another 0.29 ml-aliquot of chromic acid reagent was added, and the reaction mixture further stirred for sixteen hours. The diethyl ether phase was transferred to another container and the aqueous phase was extracted twice with three ml each of diethyl ether. All the ether phase was combined and washed with three ml of water. It was then concentrated and analyzed by GLC. Part of the oxidation product was further treated with a trimethylsilyl (TMS)-derivatization reagent, Tri-Sil'Z', and diazomethane, separately, and the products were analyzed by GLC. In all cases blank samples (=0 min control) were prepared by carrying out all the above biochemical and chemical reactions without toxaphene components. These blank samples were used for GLC analyses to ascertain the absence of any artifacts in the resulting chromatograms.

An attempt was made to further derivatize the oxidation products using o-phenylenediamine. The method used was that of MORRISON (1954) with some modifications. To fifteen µg of o-phenylenediamine in 0.25 ml of 10% acetic acid, the oxidation products (3 µg) of toxicant C were slowly added in 50 µl of ethanol, with continuous stirring at 37°C. After 15 minutes of reaction, the product was extracted three times with one to two ml each of diethyl ether. The diethyl ether extract was concentrated, and analyzed by GLC.

All GLC analyses were performed on an F&M Scientific, Hewlett Packard, Research Chromatograph 5750 equipped with a ^{63}Ni electron-capture detector (EC). The column used was 3% SE30 on varaport 30 (100-120 mesh) packed in a glass column of 1.8 m long and 3 mm I.D. The operating conditions were: injector temperature 250°C, column temperature 200°C, detector temperature 280°C, argon-methane (95:5) carrier gas at a flow rate of 14 ml/min., and purge gas flow rate of 17 ml/min.

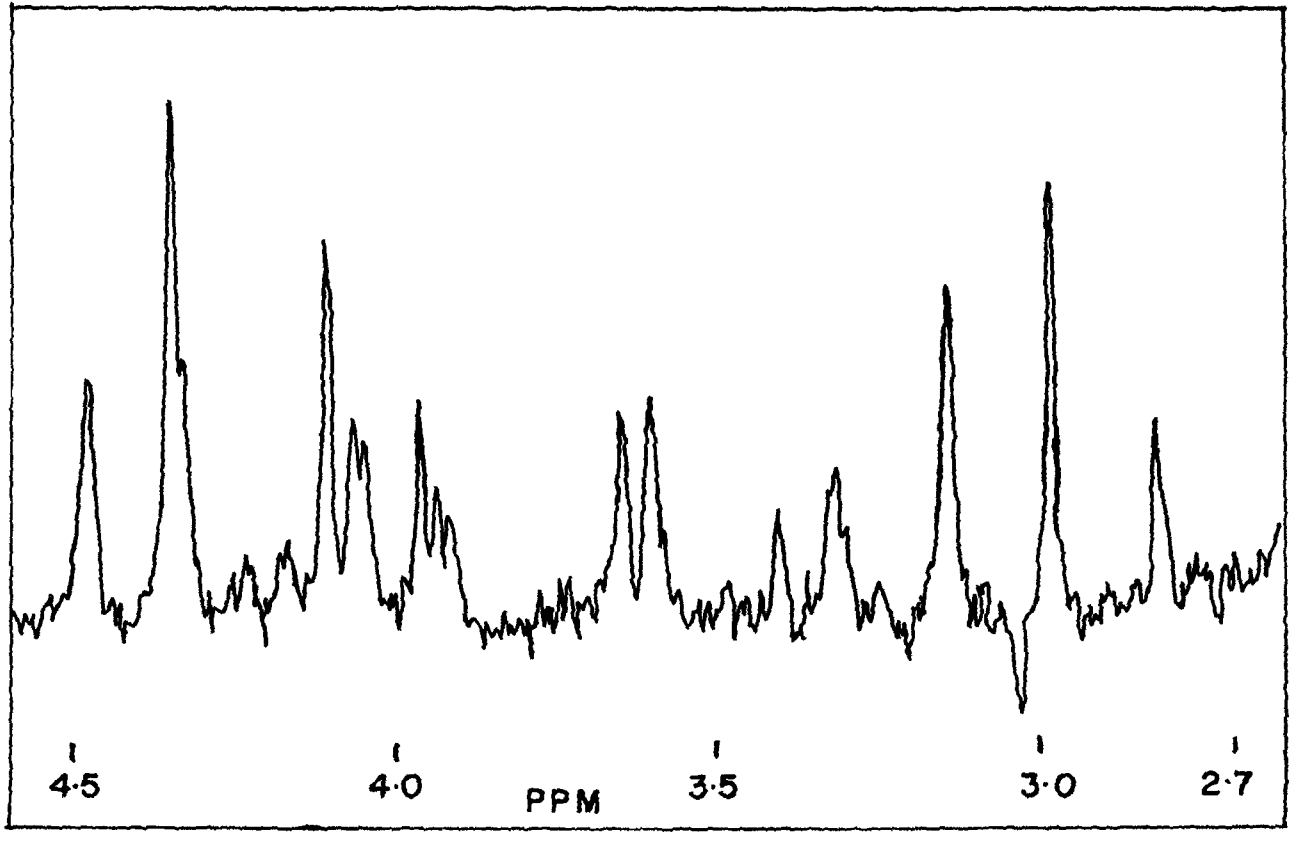


Fig. 2: PMR spectrum of the dechlorination product of toxicant C. The resonance for a singlet proton at 6.37 is not shown.

TABLE 1

Chemical shift data for toxicant C and its metabolite (ppm)

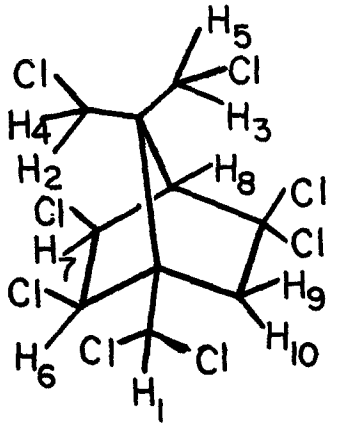
Proton	Metabolite	Toxicant C
H_1	6.37 s	6.83 s
H_2	4.40 broad d	5.08 dd
H_3	3.99 dd	4.20 dd
H_4	4.03 d	4.20 d
H_5	4.41 d	4.65 d
H_6	3.65 s	3.66 s
H_7	3.61 s	3.66 s
H_8	3.14 s	3.20 s
H_9	2.90 d	5.70 s
H_{10}	3.23 d	

s = singlet, d = doublet, dd = doublet of doublet.

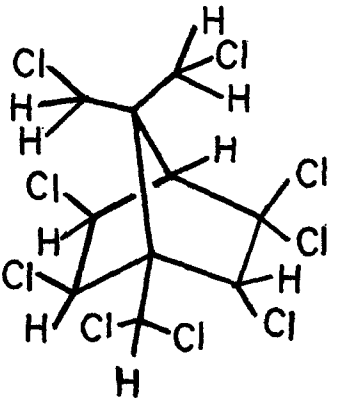
TABLE 2

Coupling constant data for toxicant C and its metabolite (Hz)

Compound	J2,3	J2,4	J3,5	J9,10
Toxicant	3.0	13.0	12.5	-
Metabolite	1.75	12.75	12.00	15.5



TOXICANT C METABOLITE



TOXICANT C

RESULTS AND DISCUSSION

The incubation mixtures of toxicants with rat liver preparation were extracted twice with 3 ml each of $CHCl_3$:MeOH (5:1) solvent mixture. The GLC analysis of solvent extractable metabolites of toxicant B from NADPH fortified incubates shows (Figure 1a) the presence of five peaks due to the appearance of metabolites: t_R 2.1, 3.25, 4.5, 5.5 and 11.0 min. The *in vitro* incubate with toxicant B in the absence of NADPH (= control) produced only a very minor peak at t_R 11.0 min. besides the parent compound at t_R 12.4 min.

The gas chromatogram of the solvent extract of NADPH fortified incubates of toxicant C showed a major metabolite at t_R 9.0 min., and a minor metabolite at t_R 14.55 min. besides the original compound at t_R 23.8 min. The nature of the major metabolite at t_R 9.0 min., was then studied. Toxicant C was chemically dechlorinated using zinc powder and acetic acid as outlined in the methods section. As a result of careful matching on GLC systems it was concluded that the major chemical reaction product was identical to the major metabolic product of toxicant C. The reaction product was isolated using TLC in a sufficient quantity for further analyses by spectroscopy.

Mass spectroscopic data showed that the empirical formula of the product is $C_{10}H_{10}Cl_8$. The PMR spectrum (Figure 2) was obtained in CCl_4 (d_6-acetone capillary as the internal lock). Table 1 shows the chemical shift data, and Table 2 shows the coupling constant data for toxicant C metabolite and toxicant C. On the basis of the spectral difference between these two compounds the structure for the metabolite has been determined as shown in Table 2.

The nature of the polar metabolites of toxicants B and C was studied by preparing their derivatives using chromic acid oxidation, trimethylsilane (TMS) and diazomethane as outlined in the methods section. The GLC-EC analysis of these metabolites was carried out before and after the derivatization. Before the oxidation, the diethyl ether extract of toxicant C metabolites showed no peaks on GLC, but the diethyl ether extract of toxicant B metabolites showed two peaks; a metabolite at t_R 11.0 min., and original compound at 12.4 min. The result of GLC-EC analysis of oxidation products of toxicant B metabolites showed no peaks in addition to those already present before oxidation, but the oxidation products of toxicant C metabolites showed four new peaks, t_R 0.47, 1.73, 2.35 and 3.06 min. (Figure 1b). The theory behind the

oxidation attempt is that primary alcohols are converted to carboxylic acids, secondary alcohols are converted to ketones and the tertiary alcohols do not undergo oxidation. Therefore, such a conversion should tell the nature of the hydroxyl compounds. A part of the oxidation products was TMS-derivatized, and their GLC-EC analysis showed no additional peaks in case of both toxicants B and C metabolites, indicating that there are no tertiary hydroxyl metabolites. Since carboxylic acids formed from primary alcohols upon oxidation cannot be detected on GLC using SE-30 column, a part of the oxidation products was treated with diazomethane to form methyl esters of the acids. The result of GLC-EC analysis of methylation products of toxicant C metabolites showed no new peaks, but those of toxicant C metabolites showed one prominent peak at t_R 0.75 min. in addition to the already mentioned four peaks which must be due to ketones (Figure 1b). This means that there is one primary hydroxyl metabolite of toxicant C in the NADPH incubate. There are only three possible positions, C_8, C_9 and C_{10} in toxicant C for the formation of primary hydroxyl metabolite, C_8 and C_9 being equivalent.

TABLE 3

Percent recovery of toxicants B and C in solvent extracts of in vitro incubates.[a]

Toxicant	Control	Control + NADPH[b]
Toxicant B	53.60	27.30
Toxicant C	46.68	43.90

[a]Percent recovery of toxicants was determined by quantitative gas-liquid chromatography equipped with electron-capture detector.

[b]Concentration of NADPH was 10^{-3} M.

An attempt was made to further react the oxidation products of toxicant C metabolites with o-phenylenediamine, a reagent which reacts with diones. The GLC-EC analysis of such derivatives showed that none of the ketone peaks disappeared, nor were any new peaks seen, indicating that probably none of the secondary hydroxyl products is a diol.

The results show that toxicant C is metabolized to, in addition to a dechlorination product, five hydroxyl compounds, one of which is at the bridge head position and four hydroxylation products each with a -OH at C_2, C_3, C_5 or C_6 positions by the rat liver enzyme preparation supplemented with NADPH. Strantely enough,

the addition of NADPH to the incubation mixture had a greater stimulatory effect on the metabolism of toxicant B than that of toxicant C (Table 3). Yet, the above analysis shows no hydroxyl metabolites of toxicant B have been formed as a result. It deems important, therefore, to ask at this stage, a question as to how important the oxidative degradation processes are as compared to well known reductive degradation processes in toxaphene metabolism.

Supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison and by a research grant R801060 from the Environmental Protection Agency, Washington, D.C.

REFERENCES

ANAGNOSTOPOULOS, M. L., H. PARLAR, and F. KORTE: Chemosphere 2. 65 (1974).

BLACK, D. K.: Paper presented to the Division of Pesticide Chemistry, 168th American Chemical Society National Meeting, Atlantic City, NJ, Sept. 8-13 (1974).

CASIDA, J. E., R. L. HOLMSTEAD, S. KHALIFA, J. R. KNOX, T. OHSAWA, K. J. PALMER, and R. Y. WONG: Science 183, 620 (1974).

CHANDURKAR, P. S.: Ph.D. Thesis, University of Wisconsin, Madison (1977).

CROWDER, L. A. and E. F. DINDAL: Bull. Environ. Contam. Toxicol. 12, 320 (1974).

FEIL, V. J., R. D. HEEDE, R. G. ZAYLSKIE, and C. H. ZACHRISON: J. Agric. Food Chem. 18, 120 (1970).

FIESER, L. F.: Organic Synthesis Coll. Vol. 4, 195 (1963).

HOLMSTEAD, R. L., S. KHALIFA, and J. E. CASIDA: J. Agric. Food Chem. 22, 939 (1974).

KHALIFA, S., R. L. HOLMSTEAD, and J. E. CASIDA: J. Agric. Food Chem. 24, 277 (1976).

LANDRUM, P. F., G. A. POLLOCK, and J. N. SEIBER: Chemosphere 2, 63 (1976).

MATSUMURA, F., R. W. HOWARD, and J. O. NELSON: Chemosphere 5, 271 (1975).

MORRISON, D. C.: J. Am. Chem. Soc. 76, 4483 (1954).

NELSON, J. O. and F. MATSUMURA: J. Agric. Food Chem. 23, 984 (1975).

OHSAWA, T., J. R. KNOX, S. KHALIFA, and J. E. CASIDA: J. Agric. Food Chem. 23, 98 (1975).

POLLOCK, G. A. and W. W. KILGORE: Paper presented to the 15th Annual Meeting of the Society of Toxicology, Atlanta, Georgia. March 14-18 (1976).

TURNER, W. V., S. KHALIFA, and J. E. CASIDA: J. Agric. Food Chem. 23, 991 (1975).