The Identification of Five Unreported Lindane Metabolites Recovered from Rat Urine

by R. W. CHADWICK and J. J. FREAL
Environmental Protection Agency
Perrine Primate Laboratory
P. O. Box 490
Perrine, Fla. 33157

Lindane (γ-hexachlorocyclohexane) has found wide application as an insecticide since the second world war. The metabolic fate of this insecticide in mammals however, is still unresolved. Grover and Sims (1) have shown that rats metabolize both lindane and γ-2,3,4,5, 6-pentachlorocyclohex-1-ene, (γ-PCCH), to 2,3,5-and 2,4,5-trichlorophenol which are excreted in the urine as free phenols, sulfates, and glucuronic acid conjugates. The metabolite, 2,4-dichlorophenylmercapturic acid, was also identified in this study (1). Because these metabolites resembled those from the degradation of 1,2,4-trichlorobenzene in rabbits (2) the authors speculated that the metabolism of lindane in rats proceeds via γ-2,3,4,5,6-pentachlorocyclohex-1-ene to 1,2, 4-trichlorobenzene. However two isomers of pentachlorocyclohexene have since been obtained from the exposure of houseflies to lindane (3). The isomer whose configuration is uncertain but is referred to as Iso-PCCH, is metabolized more slowly but to a greater extent than γ-PCCH (4). Furthermore, a recent report (5) indicates that rabbits metabolize lindane to o-dichlorobenzene, 1,2,4-trichlorobenzene, the three isomers of tetrachlorobenzene, pentachlorobenzene, 2,3-, 2,4- and 2,5-dichlorophenol, 2,3,5-, 2,4,5- and 2,4,6-trichlorophenol 2,3,4,5- and 2,3,4,6-tetrachlorophenol and pentachlorophenol. These data indicate that the metabolism in mammals is considerably more complex than was previously realized. Through an improved analytical method based on gas liquid chromatography and Coulson electrolytic conductivity detection, it has been possible to characterize five previously unreported metabolites from the urine of rats fed lindane. This paper concerns the isolation and identification of these metabolites.

EXPERIMENTAL

Twenty-four weanling female Sprague-Dawley rats, individually housed in metabolism cages were fed diets containing 400 ppm lindane (obtained from the Perrine Primate Laboratory repository). After the first week

Bulletin of Environmental Contamination & Toxicology,
Vol. 7. No. 2/3. 1972 © Springer-Verlag New York Inc.

of treatment, GLC analysis of the benzene extracts of acidified urine samples (pH=2) indicated the presence of at least five unknown metabolites in addition to the 2,3,5- and 2,4,5-trichlorophenols reported by Grover and Sims (1). For the next month, the urine samples were pooled daily, acidified to pH=2 and extracted 3 times with equal volumes of benzene. The combined benzene extracts were then partitioned with a volume of 0.1 N sodium hydroxide equivalent to 20% that of the organic phase. Neutral metabolites remained in the benzene while the polar metabolites were extracted in the aqueous phase. The 0.1 N NaOH was acidified and extracted 3 times with equal volumes of benzene.

The neutral benzene soluble metabolite fraction was concentrated to about 3 ml and chromatographed on an activated silicic acid column under vacuum. The column, previously described in the literature (6), is 36 cm high, 2 cm in diameter and equipped with a 24/40 ground glass joint that fits the collection flasks used in this procedure. The column was packed with 2 cm of anhydrous sodium sulfate at the bottom, 20 cm of silicic acid (activated at 170°C overnight) above this, and another 2 cm of anhydrous sodium sulfate at the top. The column was first washed with 200 ml of hexane. Then the concentrated metabolite fraction was added and the column was developed with an additional 500 ml of hexane. This was followed by 700 ml of benzene, collected in 100 ml fractions. The chlorobenzenes were isolated in the hexane fraction while a compound tentatively identified as a configurational isomer of 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol, (PCCOL), was eluted in the fourth and fifth benzene fractions. This metabolite was further purified by collection of the effluent, at the appropriate retention time, from the gas chromatographic separation of the components of the benzene fraction. The PCCOL was collected in a sufficient quantity and purity to prepare the acetate derivative and obtain unadulterated ultraviolet, infrared, NMR and mass spectra.

Similarly, the polar metabolites were purified by collection of the individual peaks resulting from gas chromatography of the mixture. The polar metabolites were identified as chlorophenols by comparison of the retention times of both the free phenol and the ethyl ether derivative (7) with that of the corresponding standard chlorophenol. In addition mass spectra of these chlorophenols confirmed the identification.

All standard chlorophenols were obtained from Aldrich Chemical Co., Inc. and were purified by recrystallization where necessary.

The gas chromatograph employed was a Tracor MT-220 equipped with a Coulson electrolytic conductivity detector operated in the oxidative mode. The column employed in the analysis and the purification of the chlorophenols consisted of a 6'x1/4" U-tube glass column packed with 5% DEGS on 80/100 mesh Gas Chrom Q isothermally maintained at 180°C. The column used for the collection of the 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol contained 5% OV-210 on 100/120 mesh Gas Chrom Q and was isothermally maintained at 140°C. Nitrogen flow was regulated at 90 cc/minute.

Other instruments employed for identification included a model 270 Perkin-Elmer GC mass spectrometer, a Perkin-Elmer 621 and a Perkin-Elmer 257 Infrared spectrophotometer, a Varian HA-100 NMR spectrometer and a Cary-14 recording spectrophotometer.

RESULTS AND DISCUSSION

Rats receiving lindane in their diet were found to excrete metabolites identified as 3,4-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,5- and 2,3,4,6-tetrachlorophenol as well as the previously reported 2,3,5- and 2,4,5-trichlorophenols.

TABLE 1

Molecular Masses and Retention Times (Relative to 2,4,5-trichlorophenol) of the Metabolites

Metabolite Identification	m/e (Molecular Ion) (d)	RRT (e)	RRT (f)
2,4,6-TCP (a)	196	0.61	0.40
2,3,5-TCP	-	0.79	0.99
2,4,5-TCP	-	1.00	1.00
2,3,4,6-TTCP (b)	230	1.66	0.98
2,3,4,5-TTCP	230	2.48	2.82
3,4-DCP (c)	162	3.14	1.51 (g)

(a) TCP=trichlorophenol
(b) TTCP=tetrachlorophenol
(c) DCP=dichlorophenol
(d) Molecular mass as determined by low resolution mass spectrometry
(e) Retention times of the free chlorophenols (relative to 2,4,5-TCP) on 5% DEGS at 180°C
(f) Retention times of the chlorophenyl ethyl ethers (relative to 2,4,5-trichlorophenyl ethyl ether) on 5% DEGS at 140°C.
(g) Retention time of the ethyl ether of the unknown identified as 3,4-dichlorophenol (relative to free 2,4,5-TCP) on 5% DEGS at 160°C.

In the identification of these compounds mass spectra of the unknowns indicated that these metabolites were di-, tri- and tetrachlorophenols respectively with appropriate molecular masses (based on ^{35}Cl) (Table 1). Also, in table 1, the retention times of the unknown chlorophenols relative to 2,4,5-trichlorophenol are recorded. These retention times are identical to those of the corresponding standard chlorophenols. The retention times of the ethyl ether derivatives of the unknown relative to the ethyl ether of 2,4,5-trichlorophenol are also recorded. Again the relative retention times of the unknown derivatives are identical to those of the ethyl ethers of the standard chlorophenols.

In addition to the identification of the chlorophenols, a neutral organic-soluble metabolite with the empirical formula $C_6 H_5 Cl_5 O$ and a molecular mass of 267.878 (based on ^{35}Cl) was characterized as PCCOL.

The empirical formula and molecular mass of this unknown was determined by high resolution mass spectrometry. The low resolution mass spectrum (figure 1) had major ion peaks at m/e 268 (M^+); 233 (M-Cl, Base peak); 197 (233-HCl); 169 (197-CO) and 162 (197-Cl).

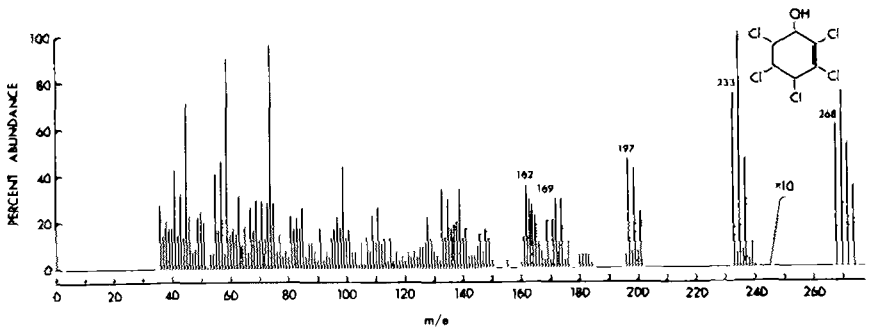
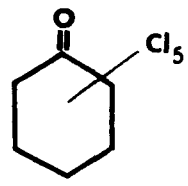
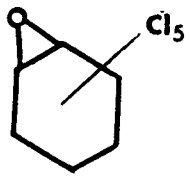


Figure 1. Mass spectrum of PCCOL

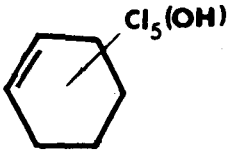
Any of the following three structures would accommodate the empirical formula.



I



II



III

The infrared spectrum of PCCOL, as a KBr pellet, had a broad complex absorption band in the 3400 - 3600 cm^{-1} region (figure 2). That this band indicated the presence of an alcohol was confirmed by a second spectrum of the PCCOL in chloroform (figure 2 insert). In this second spectrum the absorption of the free alcohol at 3600 cm^{-1} is readily apparent. The strong absorption

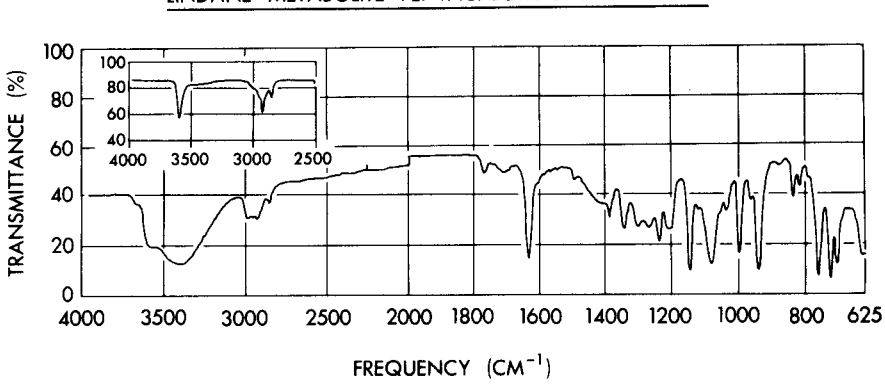


Figure 2. Infrared spectra of PCCOL as a KBr pellet and in $CHCl_3$ (insert)

peak at 1074 cm-1 is indicative of a secondary alcohol with $\alpha\beta$-unsaturation (8). The presence of a 1629 cm^{-1} band and the absence of a C-H stretch band above 3000 cm^{-1} suggests a chlorine substituted double bond. There was no stretching vibration which could be attributed to a carbonyl and the 1629 cm^{-1} band could not be accounted for by the epoxide isomer (III).

The NMR spectrum showed a pair of doublets with the first doublet at 473.7 Hz. and the second at 453.9 Hz. The second doublet had an area 3 times greater than the first and each doublet was split by a coupling constant of 4 Hz. These data indicate two chemically inequivalent protons present in a ratio of three to one. This may be interpreted as indicating the occurrence of one proton on each of three halogenated ring carbons and one proton on the carbinol carbon.

Ultraviolet spectrophotometry lent additional support to the suggested occurrence of a double bond with a maximum absorption band at 220 mμ. Furthermore this absorption maximum supports the argument for conjugation between the double bond and the hydroxyl group since cyclohexene absorbs at 208 mμ (9) and dieldrin at 215 mμ. (10). The further shift of the λ max to 220 mμ in the metabolite could be the result of conjugation.

The unknown was reacted with MOX (2% methoxyamine HCl in pyridine) (11,12). This reaction converts keto groups to a methoxime derivative suitable for GLC analysis. That no reaction occurred was indicated by 100% recovery of the parent peak in the GLC analysis. Refluxing the unknown metabolite in ethanol-hydrochloric acid for 4 hours (13) failed to produce a new product based on GLC analysis. This suggested that the compound was not an epoxide. Finally the compound was successfully acetylated (14) and a new product was obtained which had a GLC retention time of 1.68 on 5% OV-210 at 140°C relative to the unknown metabolite. The mass spectrum of the acetylated metabolite gave a parent ion at m/e 310 which would be expected if an alcohol were acetylated. In the infrared spectrum of the derivatized metabolite, the hydroxyl band at 3500 - 3600 cm^{-1} was no longer present while two new bands appeared at 1770 cm^{-1} and 1200 cm^{-1} representing respectively the C=O and C-O stretching frequencies of an ester.

The total amassed data were considered sufficiently conclusive to permit the tentative identification of the neutral metabolite as a configurational isomer of 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol.

Figure 3a illustrates the excretion pattern of the lindane metabolites from a pooled urine sample. The benzene extract of the acidified pooled urine was chromatographed on the 5% DEGS column at 180°C. All the metabolites identified in this paper are readily detected except 3,4-dichlorophenol which is present in trace quantities. The 3,4-dichlorophenol is a minor metabolite found in small concentrations even after extended exposure of rats to lindane. However, after one week of treatment the 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol, 2,4,6-trichlorophenol, 2,3,4,5- and 2,3,4,6-tetrachlorophenol are excreted in greater quantities than either 2,3,5- or 2,4,5-trichlorophenol, the metabolites reported by Grover and Sims in 1965. Figure 3b is the gas chromatogram of the extract used in 3a after partitioning it into sodium hydroxide as described in the experimental section. The polar chlorophenols all reappear in 3b while the less polar PCCOL remains behind in the benzene residue.

The excretion of the metabolites reported here indicate that major revisions are necessary in the currently accepted scheme of lindane metabolism. For example, one would not anticipate the excretion of tetrachlorophenols via the metabolic pathway proposed by Grover and Sims (figure 4). Perhaps the answer may lie in the formation of more than one isomer of the intermediate metabolite γ-PCCH. For example, the second

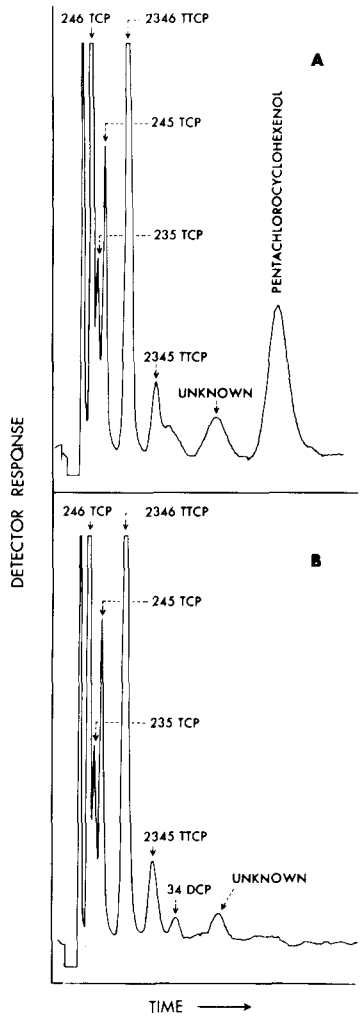


Figure 3. Chromatograms of the benzene extract of a pooled rat urine sample A) Before NaOH partition B) The polar metabolites partitioned into the NaOH.

PCCH (ISO-PCCH) isolated by Reed and Forgash yields 1,2,4- and 1,2,3-trichlorobenzene, 1,2,3,4- and 1,2,4,5-tetrachlorobenzene and pentachlorobenzene upon application to houseflies while the previously reported intermediate, γ-PCCH produced only 1,2,4-trichlorobenzene and 1,2,4,5-tetrachlorobenzene (15). In mammals, the chlorobenzenes are further altered to form the corresponsing chlorophenols. Thus the excretion of

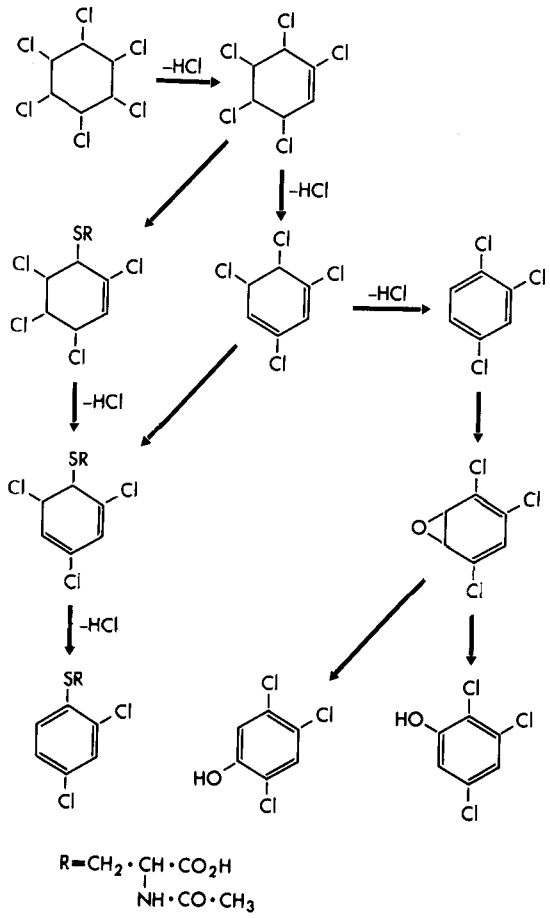


Figure 4. The lindane metabolic pathway proposed by Grover and Sims. Lindane is dehydrochlorinated to γ-PCCH which is further metabolized to either 2,4-dichlorophenyl mercapturic acid or 2,3,5- and 2,4,5-trichlorophenol.

tetrachlorophenols could be accounted for if mammals metabolize lindane via such an intermediate. However, even though γ-PCCH has been identified as a lindane metabolite in flies (16,17,18,19,20,21,22,23,24) plants (25,26), and microorganisms (27) there is no evidence that mammals produce this intermediate. Indeed it would be difficult to account for the formation of the PCCOL with its chlorine substituted double bond, by an initial step involving dehydrochlorination. Perhaps the metabolic mechanism in mammals involves additional pathways or a more efficient pathway than dehydrochlorination to γ-PCCH. Bradbury and Standen state that the first step

in the metabolism of lindane in flies is the removal of one chlorine atom and the formation of a C-S bond (25). Such a route may also occur in mammals, or alternately, the removal of the chlorine atom may be followed by the formation of a C-OH bond. In any case, to be acceptable, a proposed mechanism will have to account for the formation of 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol, an unusual lindane metabolite.

SUMMARY

Previously unreported metabolites from the urine of rats fed lindane have been identified as 3,4-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,5-tetrachlorophenol, 2,3,4,6-tetrachlorophenol and 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol. While the 3,4-dichlorophenol is a minor metabolite, the others are excreted in greater quantities than either 2,3,5- or 2,4,5-trichlorophenol, previously identified lindane metabolites. The excretion of tetrachlorophenols and PCCOL require a revision in the currently accepted theory regarding the metabolism of lindane by mammals.

ACKNOWLEDGEMENT

We wish to thank the following individuals for valuable assistance: Dr. P. L. Grover of the Chester Beatty Research Institute, London, England, for samples of the lindane metabolites, 2,3,5-trichlorophenol and 2,4-dichlorophenyl mercapturic acid; Dr. Norio Kurihara from the Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan, who supplied several isomers of the tetrachlorocyclohexane epoxides. These compounds aided in the rejection of an epoxide configuration for the metabolite eventually identified as 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol; Dr. R. C. Dougherty of the High Resolution Mass Spectrometry Laboratory at Florida State University for the high resolution mass spectra of PCCOL; Dr. Frank Biros of this laboratory for the infrared, NMR and low resolution mass spectra and aid in their interpretation.

REFERENCES

1. GROVER, P.L. and SIMS, P., Biochem. J. 96, 521 (1965).
2. JONDORF, W.R., PARKE, D.V. and WILLIAMS, R.T., Biochem. J. 61, 512 (1955).
3. REED, W.T. and FORGASH, A.J., Science 160, 1232 (1968).
4. REED, W.T. and FORGASH, A.J., J. Agr. Food Chem. 18, 475 (1970).
5. KARAPALLY, J.C., SAHA, J.G. and LEE, Y.W., Abstracts, 162nd National Meeting of the American Chemical Society, Washington, D.C., PEST NO. 41, (1971).
6. MOATS, W.A., J. Ass. Off. Anal. Chem, 46, 172 (1963)
7. CRANMER, M. and FREAL, J., Life Sci. 9, 121 (1970).
8. BELLAMY, L.J., The Infra-red Spectra of Complex Molecules, p. 109 (1958) John Wiley and Sons Inc., New York.
9. WHEELER, O.H. and MATEOS, J.L., J. Org. Chem. 21, 1110 (1956).
10. GORE, R.C., HANNAH, R.W., J. Ass. Off. Anal. Chem. 54, 1040 (1971).
11. GARDNER, W.L., HORNING, E.C., Acta Biochimi Biophys. 115, 425 (1966).
12. HORNING, M.G., MOSS, A.M. and HORNING, E.C., Anal. Biochem. 22, 284 (1968).
13. KURIHARA, N., SONEMUTSU, Y., KIMURA, T., KOBAYASHI, M. and NAKAJIMA, M., Agr. Biol. Chem. 34, 784 (1970).
14. CHERONIS, N.D. and MA, T.S., Organic Functional Group Analysis by Micro and Semimicro Methods, p. 493 (1964), Interscience Publishers, New York
15. REED, W.T. and FORGASH, A.J., J. Agr. Food Chem. 17, 896 (1969).
16. BRADBURY, F.R., J. Sci. Food Agr. 8, 90 (1957).
17. BRADBURY, F.R. and NIELD, P., Nature, 172, 1052 (1953)
18. BRADBURY, F.R. and STANDEN, H., J. Sci. Food Agr. 6, 90 (1955).
19. BRADBURY, F.R. and STANDEN, H., J. Sci. Food Agr. 7, 389 (1956).
20. BRADBURY, F.R. and STANDEN, H., J. Sci. Food Agr. 9, 203 (1958).
21. BRIDGES, R.G., Nature, 184, 1337 (1959).
22. ISHIDA, M. and DAHM, P.A., J. of Econ. Entomol. 58, 602 (1965).
23. OPPENOORTH, F.J., Nature, 173, 1000, (1954).
24. STERNBURG, J. and KEARNS, C.W., J. Econ. Entomol 49, 548 (1956).
25. BOGDARINA, A.A., Translations of Plant Physiology A.I.B.S., (Fiziol. Rast. 4, 254 (1957).
26. SAN ANTONIO, J.P., J. Agr. Food Chem. 7, 322 (1959).
27. YULE, W.N., CHIBA, M., and MORLEY, H.V., J. Agr. Food Chem. 15, 1000, (1967).
28. BRADBURY, F.R. and STANDEN, H., Nature 183, 983 (1959).