Metabolism of Chlorobiphenyls in the Goat and Cow

Stephen Safe,* Nicholas Platonow, and Otto Hutzinger

4-Chlorobiphenyl and 4,4′-dichlorobiphenyl were administered to goats by intravenous injection and mass spectrometric analysis indicated the presence of oxygenated chlorobiphenyl metabolites in the urine. The urinary metabolites of 4-chlorobiphenyl were isolated and their structures shown to be 4′-chloro-4-biphenylol and 4′-chloro-3,4-biphenyldiol. The former metabolite was also identified in urine from a cow which was orally fed 4-chlorobiphenyl. The only urinary metabolite of 4,4′-dichlorobiphenyl was identified as 4,4′-dichloro-3-biphenylol. The structures of all the metabolites were confirmed by nuclear magnetic resonance spectrometry and by comparison with samples of the metabolites which have been identified in the rat and confirmed by synthesis. Analysis of extracts from the feces and several organs of the goats did not reveal any of the metabolites which were identified in the urine. The results thus for the first time indicate a metabolic pathway for PCB's in domestic animals and the excretion of these metabolites in the urine.

Polychlorinated biphenyls (PCB's) are now generally recognized as among the most persistent and widespread environmental pollutants (Hutzinger *et al.*, 1974b; Risebrough *et al.*, 1968). The commercial PCB preparations as a mixture of isomeric chlorinated biphenyls and analysis by gas-liquid chromatography (glc) gives a complex but characteristic pattern of peaks. Glc analysis of PCB extracted from wild mammals (Bailey and Bunyan, 1972; Reiman *et al.*, 1969; Risebrough and de Lappe, 1972) and man animals (Fries, 1972; Platonow and Chen, 1973; Platonow and Karstad, 1973; Platonow and Meads, 1975) showed the disappearance or the diminished intensity of certain characteristic peaks which suggested an *in vivo* metabolism of these compounds. Recent work has indicated that individual PCB isomers are metabolized by such diverse organisms as bacteria (Ahmed and Focht, 1973), monkeys (Greb *et al.*, 1973), pigeons (Hutzinger *et al.*, 1972), fungi (Wallnofer *et al.*, 1973), lichens (Mass *et al.*, 1974), rats (Hutzinger *et al.*, 1972; Safe *et al.*, 1974a,b, 1975; Yoshimura and Yamomoto, 1973; Goto *et al.*, 1973), and rabbits (Gardner *et al.*, 1973; Block and Corbett, 1959) and in most cases a monohydroxylated chlorobiphenyl was identified as the major metabolic product. One of the major metabolites of 2,2′,5,5′-tetrachlorobiphenyl fed to rabbits was identified as *trans*-3,4-dihydro-2,2′,5,5′-tetrachloro-3,4-biphenyldiol and the two phenolic compounds, 2,2′,5,5′-tetrachloro-3-biphenylol and 2,2′,5,5′-tetrachloro-4-biphenylol, were also formed. These data suggested the intermediacy of an arene epoxide in the oxidation of the PCB isomer (Daly *et al.*, 1972).

High-resolution photoplate mass spectrometric analysis of urine extracts from goats fed commercial PCB mixtures and 4-chlorobiphenyl isomers and urine extract from a cow fed 4-chlorobiphenyl clearly showed the presence of oxygenated metabolites (Safe *et al.*, 1974b). The purpose of this investigation was to isolate and determine the structure of these oxygenated products.

MATERIALS AND METHODS

Animal Feeding Experiments. 4-Chlorobiphenyl and 4,4′-dichlorobiphenyl were emulsified in oil and intravenously administered (5 mg/kg) to two goats (average weight 50 kg). Urine and feces were collected for 5 days after administration of the chlorobiphenyl; on the sixth day the goats were killed and then exsanguinated as in commercial slaughter procedures. Samples from various organs and tissue were removed and stored at −20° until analyzed. A lactating cow (*ca.* 350 kg) was orally fed 4-chlorobiphenyl (50 mg/kg) in a gelatin capsule. The urine and feces were collected as described above and 8 days after feeding the PCB the cow was slaughtered and treated as described (Platonow and Meads, 1975).

Extraction and Analysis. The urine samples were diluted with sufficient concentrated sulfuric acid to make a 6 *N* acid solution and then heated for 1 hr at 100°. The solution was then diluted with an equal volume of distilled water and extracted with ether. The feces samples were treated with 6 *N* sulfuric acid solution at 100° for 1 hr and then diluted with water and extracted with ether. The tissue and organ samples (5 g) were macerated with distilled water in a Waring Blendor and then diluted with concentrated sulfuric acid until the solution was 6 *N* with respect to the acid. The mixture was heated for 1 hr at 100°, diluted with an equal volume of water, and extracted with ether.

The ethereal extracts obtained from the urine extract were purified by preparative thin-layer chromatography (tlc) on silica gel HF_{254} (Merck) using chloroform as the eluting solvent. A band with an R_f value similar to 4-biphenylol and 4-chlorobiphenylol (Safe *et al.*, 1974a) (R_f 0.30–0.50) was removed from the plate and extracted with ether to give a hydroxy fraction and the remainder of the plate was also removed and extracted with ether-methanol (98:2) to give a polar fraction. Further cleanup of these fractions was obtained by treating the residues isolated from the above fractions with acetic anhydride (10 ml) and sodium acetate (0.1 g) for 90 min at 110°. The mixture was then cooled, diluted with methanol (40 ml), and allowed to stand for 1 hr. The solution was evaporated to dryness and the residue thoroughly extracted with ether and water. The ether layer was dried and concentrated and the residue further purified by preparative tlc (benzene–hexane, 1:1, as solvent). The urine extract obtained from the goat injected with 4-chlorobiphenyl gave two main fluorescent bands, bands 1 and 2; the corresponding extract from the goat injected with 4,4′-dichlorobiphenyl was also purified as above to give a band (band 3) which had an R_f value similar to band 1. The chemical nature of these purified acetate derivatives was further investigated using spectroscopic techniques.

The acetate fractions (*i.e.*, bands 1 and 3) isolated from the tissue, organ, and fecal extracts were analyzed by gas–liquid chromatography (glc) on a ⅛ in. × 6 ft glass column packed with 2% OV-17 (100–120 mesh). Gas chromatographic conditions were: oven temperature, 190°; inlet

Department of Chemistry (S.S.) and Department of Biomedical Sciences (N.P.), University of Guelph, Guelph, Ontario, Canada, and Laboratory of Environmental Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, Amsterdam, The Netherlands (O.H.).

DSW 025992

STLCOPCB4009954

temperature, 220°; detector temperature, 250°; helium carrier gas flow, 40–50 ml/min. The samples were analyzed on a Hewlett-Packard 700 gas chromatograph equipped with a flame ionization detector. The quantitation of the material in bands 1 and 3 was determined by comparison with accurately weighed samples of the acetate metabolites isolated from these bands.

Spectroscopic Methods. Mass spectra were obtained using a DuPont CEC 21-110B double-focusing high-resolution mass spectrometer at 70 eV and equipped with electrical detection. The samples were inserted into the ion source using a temperature controlled probe at temperatures between 20 and 90°. Nuclear magnetic resonance spectra (nmr) were obtained using a Varian HR220 spectrometer using deuteriochloroform as solvent.

RESULTS

Goat Metabolism. Tlc purification of the acetylated urine extracts from the goat intravenously given 4-chlorobiphenyl gave several fluorescent bands which were removed from the plate and extracted with ether; the extracts were subjected to mass spectrometric analysis. Using this technique the presence of any chlorine-containing metabolites could readily be ascertained by their characteristic isotope distribution pattern (*i.e.*, 75.8% ^{35}Cl and 24.2% ^{37}Cl). The acetylated urine extract yielded only two bands (bands 1 and 2) which exhibited ions containing chlorine; examination of the mass spectrum of the extract from the goat injected with 4,4′-dichlorobiphenyl also revealed a band (band 3) which contained a dichloro component.

The mass spectrum of the extract from band 1 gave a molecular ion at *m/e* 246 with a major fragment ion at *m/e* 204. This fragmentation is typical of aryl acetates and is consistent with a chlorobiphenyl acetate metabolite. The 220-MHz nmr spectrum of this component gave the following results: δ (ppm) 7.15 (d, J = 8.2 Hz), 7.42 (d, J = 8.2 Hz), 7.48 (d, J = 8.2 Hz), 7.55 (d, J = 8.2 Hz); it was identical with the spectrum of 4′-chloro-4-biphenylyl acetate.

The mass spectrum of the extract from band 2 gave a molecular ion at *m/e* 304 with abundant fragment ions at *m/e* 262 and 220. The data suggested the presence of a chlorobiphenyl diacetate metabolite. Hydrolysis of the diacetate with 10% aqueous methanolic potassium hydroxide solution gave a diol with the following spectral properties: M^+ = 220; δ (ppm) 7.43 (d, J = 8.2 Hz), 7.37 (d, J = 8.2 Hz), 7.08 (d, J = 1.9 Hz), 7.01 (d, d, J = 8.2, 1.9 Hz), and 6.93 (d, J = 8.2 Hz). The spectral data were identical with the results obtained from 4′-chloro-3,4-biphenyldiol, the major metabolite of 4′-chloro-4-biphenylol fed to rats (Safe *et al.*, 1975).

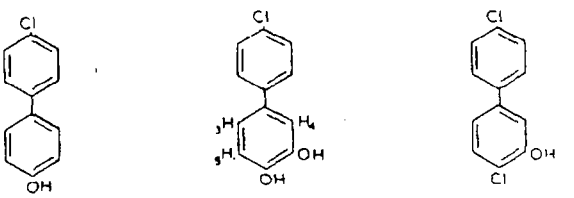
The mass spectrum of the band 3 extract gave a molecular ion at *m/e* 280 and was consistent with a dichlorobiphenyl acetate metabolite. The nmr spectrum of the acetate gave the following resonances [δ (ppm) 7.52 (d, J = 8.2 Hz), 7.49 (d, J = 8.2 Hz), 7.41 (d, J = 8.2 Hz), 7.39 (d, d, J = 8.2, 1.9 Hz), and 7.34 (d, J = 1.9 Hz)] and was identical with the spectrum of 4,4′-dichloro-3-biphenylyl acetate (Safe *et al.*, 1974a).

Analysis of the goat feces did not yield any isolable metabolites. In addition glc analysis of heart, lung, kidney, and liver samples also did not detect any appreciable quantity (<0.1 ppm) of the hydroxylated PCB's in these organs.

Cow Metabolism. Mass spectral analysis of the acetylated urine extract from a cow fed 4-chlorobiphenyl showed only one band which contained a chlorinated metabolite (M^+ 246). The mass and nmr spectra were identical with the corresponding spectra of 4′-chloro-4-biphenylyl acetate.

DISCUSSION

The mass spectra of the two major metabolites of 4-chlorobiphenyl in the goat gave molecular ions at *m/e* 201 and 220 suggesting the introduction of one and two atoms of oxygen, respectively, into the biphenyl nucleus. The spectral properties of the mono-ol were identical with the spectra of 4′-chloro-4-biphenylol (1), the major rat metabolite of 4-chlorobiphenyl (Safe *et al.*, 1974a). The nmr spectrum of the *m/e* 220 compound was consistent with the catechol-like structure 2. The chemical shifts at 7.43 and 7.37 ppm (d, J = 8.2 Hz) corresponded to the AA′BB′ system of the *p*-chlorophenyl ring while the resonances (and coupling constants) at 7.08, 7.01, and 6.93 ppm are consistent for the ABX system represented by protons H_4, H_5, and H_6, respectively in 2 (Safe *et al.*, 1975). The goat injected with 4,4′-dichlorobiphenyl gave a single mono-oxygenated metabolite (M^+ 238) which was identical with a synthetic sample of 4,4′-dichloro-3-biphenylol (3) (Hutzinger *et al.*, 1974a), a compound which is also the major urinary metabolite of 4,4′-dichlorobiphenyl in the rat (Safe *et al.*, 1974a).



1 2 3

The PCB metabolites identified in these experiments were identified exclusively in the urine and analysis of the feces, liver, kidney, heart, and brain extracts failed to detect any of these compounds. Previous work with the rat (Hutzinger *et al.*, 1972; Safe *et al.*, 1974a) has shown that intraperitoneal administration of PCB isomers also only gave urinary metabolites, whereas oral feeding experiments (Yoshimura and Yamomoto, 1973; Goto *et al.*, 1973) gave both urinary and fecal metabolites. Although the results do not indicate a single excretion pathway it is now clear that PCB's are not just absorbed into animal tissue but they are hydroxylated and excreted presumably in a hydrolyzed form.

Platonow and Meads (1975) reported the uptake and distribution of 4-chlorobiphenyl and decachlorobiphenyl in cows and the former isomer was only observed in the feces but not in urine or in various organ and tissue samples. Reexamination of the urine extract yielded a metabolite which was identified as 4′-chloro-4-biphenol (1), the same mono-ol observed in the urine of both rats and goats to which 4-chlorobiphenyl had been administered.

The mechanism of aryl hydroxylation in many cases proceeds *via* an arene epoxide intermediate which can rearrange to give a phenol or hydrolyze to give a dihydro dihydroxy compound which in turn readily dehydrogenates to give a vicinal aromatic catechol (Daly *et al.*, 1972). The presence of the dihydro intermediate was not observed in the goat urine extract since the acid hydrolysis work-up would dehydrate this compound. Recent studies (Safe *et al.*, 1975) with rats have shown that both 4-chlorobiphenyl and 4′-chloro-4-biphenylol (1) are converted into the catechol (2) so that the hydroxylation reactions might proceed *via* an epoxide intermediate or by direct hydroxylation. Similarly, the results reported herein do not distinguish between the two pathways and experiments with labeled PCB substrates are currently in progress in order to elucidate the mechanism of the hydroxylation reactions.

DSW 025993

STLCOPCB4009955

...is clear from the above experiments that PCB's are ...ceptible to metabolic degradation in the goat and cow ... are excreted in the urine. Since the toxicology and ...gical properties of hydroxylated PCB's are unknown ...is, therefore, an area of potential environmental haz... nd concern.

...ERATURE CITED

...ed, M., Focht, D. D., *Bull. Environ. Contam. Toxicol.* 10, 70 ...173).
...ev, S., Bunyan, P. J., *Nature (London)* 236, 34 (1972).
..., W. D., Cornish, H. H., *J. Biol. Chem.* 234, 3301 (1959).
..., J. W., Jerina, D. M., Witkop, W. T., *Experientia* 28, 1129 ...972).
..., G. F., *Environ. Health Perspect.* 1, 55 (1972).
...ner, A. M., Chen, J. T., Roach, J. A. G., Ragelis, E. P., *Bio...m. Biophys. Res. Commun.* 55, 1377 (1973).
..., M., Sugiura, K., Hattori, M., Miyagawa, T., Okamura, K., ...New Methods in Environmental Chemistry and Toxicology," ...ulston, F., Korte, F., Goto, M., Ed., International Printing ..., 1973, p 219.
..., W., Klein, W., Coulston, F., Goldberg, L., Korte, F., *Che...sphere*, 143 (1973).
...inger, O., Nash, D. M., Safe, S., DeFreitas, A. S. W., Nor...m, R. J., Wildish, D. J., Zitko, V., *Science* 178, 312 (1972).
...inger, O., Safe, S., Zitko, V., *J. Ass. Offic. Anal. Chem.* 57, ...81 (1974a).
...inger, O., Safe, S., Zitko, V., "The Chemistry of PCB's," ...emical Rubber Publishing Co., Cleveland, Ohio, 1974b.
Koeman, J. H., Ten Noever de Brauw, M. C., deVos, R. H., *Nature (London)* 221, 1126 (1969).
Maas, W. S. G., Safe, S., Hutzinger, O., Proceedings of the Third International Congress of Pesticide Chemistry, Helsinki, Finland, 1974.
Platonow, N. S., Chen, N. Y., *Vet. Rec.* 92, 69 (1973).
Platonow, N. S., Karstad, L. H., *Can. J. Comp. Med.* 37, 391 (1973).
Platonow, N. S., Meads, E. B., *Can. J. Comp. Med.*, in press (1975).
Risebrough, R. W., de Lappe, B., *Environ. Health Perspect.* 1, 39 (1972).
Risebrough, R. W., Reiche, P., Peakall, D. B., Herman, S. G., Kirven, M. N., *Nature (London)* 220, 1098 (1968).
Safe, S., Hutzinger, O., Ecobichon, D., *Experientia* 30, 720 (1974a).
Safe, S., Hutzinger, O., Ecobichon, D., Grey, A. A., *Can. J. Biochem.*, submitted for publication (1975).
Safe, S., Platonow, N. S., Jamieson, W. D., Hutzinger, O., Proceedings of the Third International Congress of Pesticide Chemistry, Helsinki, Finland, 1974b.
Wallnöfer, P. R., Engelhardt, G., Safe, S., Hutzinger, O., *Chemosphere*, 69 (1973).
Yoshimura, H., and Yamomoto, H., *Chem. Pharm. Bull.* 21, 1168 (1973).

Received for review September 16, 1974. Accepted November 20, 1974. The financial assistance of the Health and Welfare Canada Research Programs Directorate and the National Research Council of Canada is gratefully acknowledged.

Metabolism and Effects of Hexachlorobenzene on Hepatic Microsomal Enzymes in the Rat

Harihara M. Mehendale,* Minerva Fields, and Hazel B. Matthews

Seven days following a single oral dose of hexachlorobenzene (HCB-^{14}C) to adult male rats, approximately 16% of the dose was excreted in the feces and less than 1% in urine. Metabolites of HCB included pentachlorobenzene, tetrachlorobenzene, pentachlorophenol, and four unknowns; all were abstracted from urine and none was detected in the feces. Seventy per cent of the total dose remained in the body 7 days after administration, fat being the major depot. This residue consisted mainly of HCB with traces of the dechlorinated metabolites. Reductive dechlorination of HCB was catalyzed by an enzyme located in the microsomal fraction of liver, lung, kidney, and intestine. Added NADPH was required for the formation of pentachlorophenol. Hepatic glucuronyl transferase, aniline hydroxylase, ethylmophine, and *p*-nitroanisol demethylases and cytochromes P-450 and b_5 were induced by HCB pretreatment. Urinary coproporphyrin levels more than doubled by the fourth day of treatment and remained relatively constant thereafter, although apparent signs of porphyria were not observed in these animals.

Hexachlorobenzene (HCB) is a selective fungicide for bunt of wheat and has been used as a general seed coating for cereal crops. It was introduced for agricultural use in 1945 (Yersin) and has since been reported to be a by-product of certain industrial processes (EPA, 1973). HCB has a low acute toxicity to mammals (Davis *et al.*, 1959; Savitzkii, 1964), but is known to cause porphyria cutanea tarda, characterized by skin rashes and extreme sensitivity to sunlight (Schmid, 1960; Cam and Nigogosyan, 1963). A massive outbreak of human porphyria occurred in Turkey following consumption of wheat precoated with HCB (Schmid, 1960). There is some evidence for ecological magnification of residues of HCB (Metcalfe *et al.*, 1973) and residues have been reported in wild birds in the Netherlands (Vos *et al.*, 1968; Koeman *et al.*, 1969) and in North America (Gilbertson and Reynolds, 1973), in beef cattle in the U.S. (EPA, 1973), and in humans in Germany (Acker and Schulte, 1970), Japan (Carley *et al.*, 1973), and Australia (Brady and Siyali, 1972; Siyali, 1972). In the first reported study of HCB metabolism Parke and Williams (1960) administered a relatively large dose (0.4 g/kg) of unlabeled HCB to rabbits and reported that 75% remained in the gastrointestinal tract with only 6% excreted in feces. No HCB was detected in the urine and no metabolites were found. Since that time little has been reported on the metabolism of HCB. In view of the ubiquitous nature of the HCB residues and the forementioned distressing effects of its chronic ingestion by humans, it was of interest to study the distribution, metabolism, and excretion of a small dose of HCB. Such a study was carried out in rats using HCB-^{14}C. In addition, the effect of HCB pretreatment on some hepatic enzyme systems and

National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709.

DSW 025994

STLCOPCB4009956