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Metabolic Studies on Polychlorinated Biphenyls. II.[1] Metabolic Fate of 2,4,3′,4′-Tetrachlorobiphenyl in Rats[2]

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In order to understand the toxic nature of Kanechlor-400 (KC-400, a commercial preparation of polychlorinated biphenyls) and establish the treatment of the patients of this KC-400 intoxication (so-called Yusho), metabolic fate of 2,4,3′,4′-tetrachlorobiphenyl (2,4,3′,4′-TCB), a major component of KC-400, was investigated using rats. It was found that at least four metabolites having phenolic nature were excreted exclusively into the feces together with a large amount of unchanged 2,4,3′,4′-TCB. Among these, a major metabolite (M-A_2), mp 155—156°, and a minor metabolite (M-A_1), mp 92—98°, were isolated from the feces and characterized to be monohydroxylated TCB by ultraviolet, infrared, nuclear magnetic resonance, and mass spectral analyses.

After 2,4,3′,4′-TCB was orally administered at a single dose of 25 mg/body to the rat, a little less than one half of the dose was excreted as unchanged 2,4,3′,4′-TCB during 12 days, most of which were eliminated in the first day. The excretion of major metabolite reached maximum at the second day, and total M-A_2 was accounted for about 10% of dose during 12 days. Both 2,4,3′,4′-TCB and its major metabolite were still excreted in a small but significant amount on 12th day.

In addition to a widespread contamination of environment with polychlorinated biphenyls (PCB), recent outbreaks of serious PCB intoxication in the southwest part of Japan prompted us to investigate the metabolic fate of PCB, particularly of Kanechlor-400 (KC-400), a commercial PCB preparation of about 48% chlorine content. Because, this PCB intoxication was caused by ingestion of the specific lots of Kanemi Rice Oil which was accidentally contaminated with a large amount of KC-400 during the course of manufacture of the rice bran oil.[4] Therefore metabolic study of this KC-400 is very important to understand its toxic nature and also to establish the treatment of the intoxicated patients.

In our previous study[5] on the elimination of KC-400 in rats using ^{3}H-labeled preparation, it was found that the urinary excretion of the radioactivity was very limited (only about 2% of the dose) while about 70% of the dose was excreted into the feces during 4 weeks after oral administration. The radioactivity excreted in the feces was further analyzed to be mostly composed of unchanged KC-400, but in part, of phenolic metabolites.[5] More detailed informations on either qualitative or quantitative aspects of metabolites, however, seemed very difficult to obtain unless individual components were utilized for this investigation. Therefore, an attempt was made to isolate major components of KC-400. Four tetrachlorobiphenyls and one pentachlorobiphenyl were then isolated by a preparative gas chromatography and identified by ultraviolet (UV) and mass spectrometry and by chemical synthesis.[6] Subsequently, a series of metabolic studies on individual isomers was initiated using firstly

1) Part I: H. Yoshimura and H. Yamamoto, *Chem. Pharm. Bull.* (Tokyo), **21**, 1168 (1973).
2) This work was presented at the 4th Symposium on Drug Metabolism and Action, Sendai, Sept. 1972; Abstracts of papers, p. 63.
3) Location: *Katakasu, Higashi-ku, Fukuoka.*
4) H. Tsukamoto, *et al.*, *Fukuoka Acta Med.*, **60**, 403 (1969).
5) H. Yoshimura, H. Yamamoto, J. Nagai, Y. Yae, H. Uzawa, Y. Ito, A. Notomi, S. Minakami, A. Ito, K. Kato, and H. Tsuji, *Fukuoka Acta Med.*, **62**, 12 (1971).
6) S. Saeki, A. Tsutsui, K. Oguri, H. Yoshimura, and M. Hamana, *Fukuoka Acta Med.*, **62**, 20 (1971).

3,4,3′,4′-tetrachlorobiphenyl (3,4,3′,4′- TCB), one of the major components of KC-400, and the results were communicated shortly.[1)]

The present paper will describe the another metabolic study on PCB isomers in rats using 2,4,3′,4′-tetrachlorobiphenyl (2,4,3′,4′-TCB), a most abundant component of KC-400.

Method

Administration of Compound——2,4,3′,4′-TCB, which was prepared according to the method described in the previous paper,[6)] was dissolved in soybean oil to make a 2.5% (w/v) solution. One ml of this solution, which contained 25 mg of 2,4,3′,4′-TCB, was orally administered into adult male rat of Wistar King strain anesthetized with ether. For isolation of metabolites, repeated doses of 2,4,3′,4′-TCB (25 mg/body) were administered into 26 rats weighing 180—250 g every third day for 9 days, while for the determination study a single dose was administered into 3 rats weighing about 120 g. Each animal was housed in an individual metabolic cage giving free access to food and water, and the urine and feces were separately collected for 12 days after the first administration.

Extraction of Metabolites——The urine samples described above were adjusted to pH 2.0 with conc. HCl and extracted continuously with $CHCl_3$ for 6 hr. The extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated. A part of the residue obtained was dissolved in MeOH for submitting to thin-layer chromatographic examination. Another part was dissolved in dry pyridine and trimethylsilylated with N,O-bis(trimethylsilyl)acetamide for gas chromatographic analysis.

To the urine remained after $CHCl_3$ extraction was added conc. HCl to make 10% HCl concentration, and it was heated for 1 hr on a boiling water bath. The hydrolyzed urine was extracted continuously with $CHCl_3$ and treated same as above.

The feces, after dried in a desicator (P_2O_5) and powdered with mortar, were extracted with $CHCl_3$ by Soxhlet extractor for 14 hr. The feces remained after $CHCl_3$ extraction was heated with 10% HCl for 1 hr on a boiling water bath and this hydrolyzed sample was continuously extracted with $CHCl_3$ for 6 hr. Thin-layer and gas chromatographic samples of both extracts from unhydrolyzed and hydrolyzed feces were prepared similarly as described in the urinary extracts.

Thin-Layer Chromatography (TLC)——TLC was carried out using silica gel plates (Wakogel B-5UA containing fluorescent indicators, 0.25 mm thick, activated at 105° for 30 min). Solvent systems used were (A) hexane–$CHCl_3$ (1:2), (B) hexane–$CHCl_3$–MeOH–28% NH_4OH (8:28:6:1) and (C) hexane–AcOEt–AcOH (40:10:1). 2,4,3′,4′-TCB and its metabolites were visualized as fluorescent spots by ultraviolet lamp (Manasulu Light, short wavelength). Phenolic metabolites were also revealed as blue or red spots by spraying with Folin–Ciocalteu reagent or diazotized benzidine reagent, respectively.

Gas-Liquid Chromatography (GLC)——The instrument used was a Shimadzu GC-3AE gas chromatograph equipped with electron capture detector. The column was a glass spiral tube (4 mm × 2.5 m) and the column packing was 1.5% SE-30 on Chromosorb W (60—80 mesh). The column temperature was maintained at 200°. Nitrogen was used as a carrier gas with the flow rate of 60 ml/min (1.5 kg/cm²).

Result

Detection of Metabolites in the Urine

The $CHCl_3$ extracts of urine samples collected every day for 12 days after administration of 2,4,3′,4′-TCB were submitted to TLC and GLC examinations according to the procedure described in Method, and the chromatograms were compared with those of control urine sample which was collected before administration of the compound. No difference, however, could be observed between chromatograms of test and control. In addition, no evidence was obtained for the excretion of conjugated metabolites by examinations on hydrolyzed urine samples.

Detection of Metabolites in the Feces

The $CHCl_3$ extracts of the feces collected every day for 12 days after administration of 2,4,3′,4′-TCB were examined by TLC and GLC, comparing with those of control feces. The result indicated that these extracts contained at least 4 metabolites along with unchanged 2,4,3′,4′-TCB. A typical thin-layer chromatogram of the extract of the third day feces after the administration is shown in Fig. 1.

As can be seen in Fig. 1, two spots having *Rf* values of 0.32 (M-a) and 0.66 (M-b) were only detected in the test extract with the solvent system of hexane–$CHCl_3$ (1: 2). Among these, M-a was revealed as a blue or a red spot with Folin–Ciocalteu reagent or diazotized benzidine reagent, respectively, while M-b was detected as a red-violet fluorecent spot under exposure of ultraviolet lamp showing the same *Rf* value as that of unchanged compound. From these facts, M-a and M-b were assumed to be phenolic metabolites and unchanged 2,4,3′,4′-TCB, respectively.

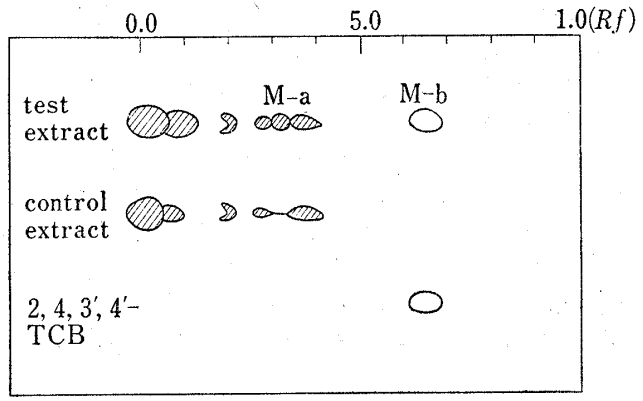

Fig. 1. Thin-Layer Chromatogram of Extract of the Third Day Feces and 2,4,3′,4′-TCB

solvent system: hexane–$CHCl_3$ (1: 2)
▨: positive to Folin–Ciocalteu reagent and UV lamp
○: positive to UV lamp

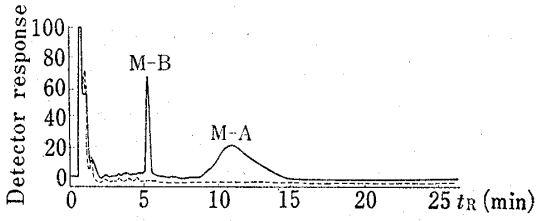

Fig. 2-A. Gas Chromatogram of Extract of the Third Day Feces

——: test ------: control

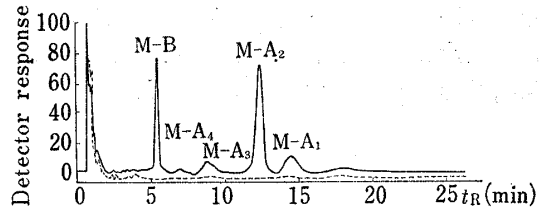

Fig. 2-B. Gas Chromatogram of Trimethylsilylated Extract of the Third Day Feces

——: test ------: control

Gas chromatograms of the extract of the third day feces, same as described above and of its trimethylsilylated product are shown in Fig. 2-A and 2-B, respectively. As indicated in Fig. 2-A, M-B was detected at t_R 5.5 min, by which it was also identified to be unchanged 2,4,3′,4′-TCB, and M-A was revealed as a broad peak at t_R 11.0 min. As shown in Fig. 2-B, the peak of M-B remained unchanged after trimethylsilylation of the extract, while a broad peak of M-A were separated into 4 sharp peaks of $M\text{-}A_1$, $M\text{-}A_2$, $M\text{-}A_3$ and $M\text{-}A_4$ on this treatment. From these results it was concluded that M-A should be a mixture of phenolic metabolites, among of which $M\text{-}A_2$ was a major one, and M-B should be unchanged compound. Possible excretion of conjugated metabolites in the feces was negated by TLC and GLC of hydrolyzed fecal samples.

Isolation and Purification of Metabolites in the Feces

The $CHCl_3$ extract of 12 days feces of 26 rats administered with repeated doses of 2,4,-3′,4′-TCB was dissolved in hexane and chromatographed through a column packed with 10 volumes of silisic acid (Mallinckrodt, 100 mesh), using hexane and hexane–$CHCl_3$ (1: 1 and 1: 2) as effluent solvents. Elution of unchanged compound and its metabolites were examined by GLC. As the result, M-B was isolated as colorless needles, mp 123—124°, from the hexane eluate after recrystallization from MeOH and the metabolites (M-A) were obtained as oily mixture from the subsequent fractions with hexane–$CHCl_3$ (1: 1 and 1: 2). The fractions containing metabolites were combined and further purified by preparative TLC using a solvent system of hexane–$CHCl_3$ (1: 2). By this procedure, $M\text{-}A_1$ (*Rf* 0.40) was separated from other three metabolites ($M\text{-}A_2$, $M\text{-}A_3$ and $M\text{-}A_4$) showing *Rf* 0.32 and isolated as colorless needles, mp 92—98°, after recrystallization from MeOH–H_2O (2: 1). Separation of a major metabolite, $M\text{-}A_2$, from $M\text{-}A_3$ and $M\text{-}A_4$ was finally conducted by preparative TLC using solvent

system of hexane–$CHCl_3$–MeOH–28% NH_4OH (8: 28: 6: 1). M-A_2 located at *Rf* 0.50 was thus isolated as crystals which were recrystallized from MeOH–H_2O (2: 1) to colorless needles, mp 155—156°. However, two minor metabolites, M-A_3 (*Rf* 0.32) and M-A_4 (*Rf* 0.38), were failed to obtain as crystalline forms because of their very limited amounts.

Characterization of Metabolites

M-B, mp 123—124°, has the same melting point and also the same *Rf* and t_R values in TLC and GLC as those of unchanged 2,4,3′,4′-TCB. The complete identy of M-B with 2,4,-3′,4′-TCB was further proved by comparison of various spectra of UV (λ_{max}^{EtOH}: 253 mμ), IR (ν_{max}^{KBr} cm^{-1}: 1587, 1460, 1360, 1022, 890, 868, 834, 805, 778, 725) and mass spectrum [*m/e*: 290 (M^+), 292 (M+2)]. In the mass spectrum, M+2 peak showed approximately 130% of the intensity of the parent ion peak, and this is a characteristic of tetrachloro-compound.[7]

M-A_1, mp 92—98°, colored blue with Folin–Ciocalteu reagent being suggested to be a phenolic metabolite. The mass spectrum of M-A_1 showed M^+ and M+2 ion peaks at *m/e* 306 and 308, respectively, in which M+2 peak was about 130% of the intensity of M^+ peak. These facts indicated that a single oxygen atom was introduced to the parent 2,4,3′,4′-TCB molecule [*m/e*: 290 (M^+), 292 (M+2)] to form monohydroxylated 2,4,3′,4′-TCB. This assumption was further supported by existence of a sharp band around 3500 cm^{-1} due to hydroxyl group in the infrared (IR) absorption spectrum (Fig. 3) and also by occurence of bathochromic shift with a change from neutral to basic condition in the UV absorption spectra (UV λ_{max}^{EtOH}: 253, 290 mμ; $\lambda_{max}^{0.1N\ NaOH}$: 320 mμ).

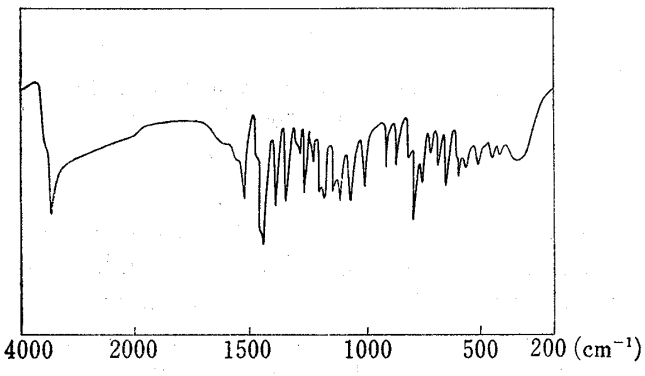

Fig. 3. IR Spectrum of M-A_1 (KBr)

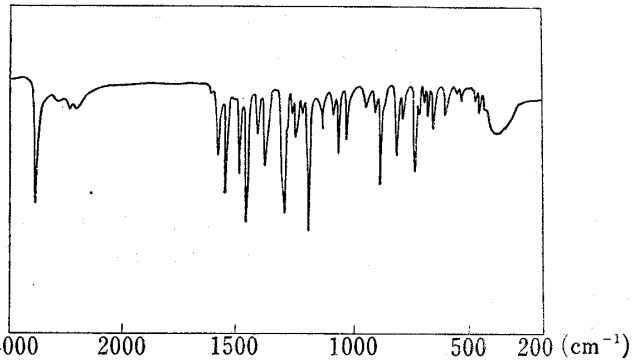

Fig. 4. IR Spectrum of M-A_2 (KBr)

A major metabolite, M-A_2, mp 155—156°, was also found to be a monohydroxy-metabolite same as M-A_1 by its spectral characteristic of mass [*m/e*: 306 (M^+), 308 (M+2)], IR (ν_{max}^{KBr}: 3560 cm^{-1}, see Fig. 4) and UV (λ_{max}^{EtOH}: 255 (shoulder), 300 mμ; $\lambda_{max}^{0.1N\ NaOH}$: 325 mμ). Thel ocation of hydroxyl group, however, could not be determined by these spectral analyses and decided by synthetic works which will be described in the following paper.[8]

Excretion Rate of 2,4,3′,4′-TCB and Its Major Metabolite, M-A_2

Excretion rate of unchanged compound and M-A_2 in the rat feces was determined by GLC during a period of 12 days after oral administration of 2,4,3′,4′-TCB at a single dose of 25 mg/body. The results are summarized in Table I.

A little less than one half of the dose was excreted as unchanged compound during 12 days, most of which were eliminated in the first day. On the other hand, the excretion of M-A_2 in the feces reached maximum at the second day, and total M-A_2 was accounted for about 10% of the dose during 12 days after the treatment. It is rather surprising that a little

7) R.M. Silverstein and G.C. Bassler, "Spectrometric Identification of Organic Compounds," 2nd ed., John Wiley and Sons, Inc., New York, 1967, p. 29.
8) H. Yamamoto and H. Yoshimura, *Chem. Pharm. Bull.* (Tokyo), **21**, 2237 (1973).

Table I. Excretion Rate of Unchanged 2,4,3′,4′-TCB and M-A_2 in the Rat Feees

Days after administration	Excretion rate (% of dose)	
	Unchanged	M-A_2
1	37.8	0.9
2	1.8	2.5
3	0.4	1.7
4	0.4	1.3
5—6	0.8	0.7
7—8	0.8	1.7
9—12	1.3	1.8
Total	43.3	10.6

Values in Table represent mean of 3 rats.

but significant amount of both unchanged compound and its major metabolite, M-A_2, still continued excreting on 12th day after a single oral administration.

Discussion

Commercial PCB preparations such as KC-400 are complex mixture containing a large number of chlorobiphenyls and therefore unsuitable for metabolic study. In order to overcome this difficulty to obtain general aspects of PCB metabolism by use of KC-400, we have started working on the metabolic fate of 3,4,3′,4′-TCB followed by the present study on 2,4,-3′,4′-TCB, since both compounds were representative components of KC-400.[6] By these studies, orally administered tetrachlorobiphenyls were found to be excreted together with their metabolites exclusively in the feces of rats. It was also elucidated that metabolites of these tetrachlorobiphenyls detectable in the feces of rats were all monohydroxylated derivatives although metabolic rate were seemed different between 2,4,3′,4′- and 3,4,3′,4′-TCB, since excretion rate of the major metabolite of 2,4,3′,4′-TCB was about 10% of the dose during a period of 12 days after the administration, while in 3,4,3′,4′-TCB the major metabolite excreted during 14 days was only accounted for about 3.3% of the dose.[1]

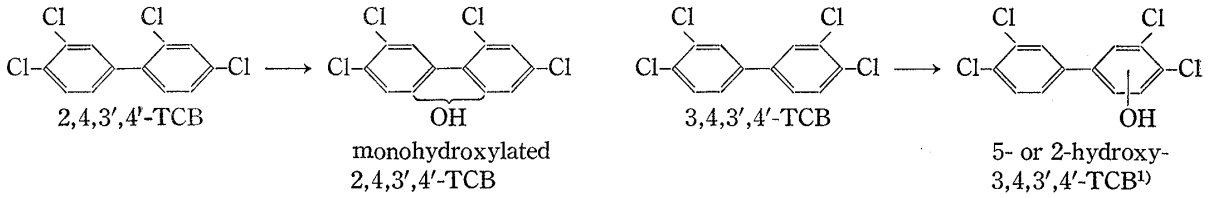

Fig. 5. Biotransformation of 2,4,3′,4′- and 3,4,3′,4′-TCB in the Rat

These findings were well coincident with results obtained in the previous study using ^{3}H-KC-400 that the radioactivity orally administered to rats was excreted mostly into the feces and only a little part was eliminated in the urine, and this excretion continued for a long period of time.[5] The present investigation also provided strong support for the previous assumptions obtained by studies on elimination of PCB from the tissues of mice[9] and rats[10] that all components were not metabolized at the same rate.

In general, a major metabolic reaction of aromatic compound has been recognized to be

9) H. Yoshimura and M. Ôshima, *Fukuoka Acta Med.*, **62**, 5 (1971).
10) D.L. Grant, W.E.J. Phillips, and D.C. Villeneuve, *Bull. Environ. Contam. Toxicol.*, **6**, 102 (1971).

hydroxylation. In agreement with this, earlier studies on metabolism of biphenyl[11] and 4-chlorobiphenyl[12] in various animals indicated that they were mainly hydroxylated at the *para* position and then conjugated with glucuronic acid being excreted into the urine. Using gas chromatographic and mass spectrometric techniques Hutzinger, *et al.*[13] reported quite recently that 2,5,2′,5′-TCB injected intraperitoneally into rats was largely excreted as unchanged in the feces, and only a part was detected as a monohydroxylated metabolite in the urine. This result was essentially same as those obtained in the studies using isomeric 3,4,-3′,4′- and 2,4,3′,4′-TCB. However, complete difference was observed in the elimination route of the metabolites. In the case of either 3,4,3′,4′-or 2,4,3′,4′-TCB, monohydroxylated metabolites were exclusively excreted into rat feces, while a monohydroxy-metabolite of 2,5,2′,5′-TCB was only eliminated in rat urine. The reason for this difference is uncertain at present.

As can be seen in Table I, excretion of unchanged compound was highly concentrated in the first day feces, suggesting that they were probably those unabsorbed from the gastrointestinal tract rather than those excreted through biliary system. In other words, absorption of 2,4,3′,4′-TCB from the gastrointestinal tract was not so good in the dose level of 25 mg/body. On the contrary, M-A_2 was excreted very slowly in rat feces and assumed to be excreted through the bile after metabolized in the liver, the major site of aromatic hydroxylation of foreign compounds. Concerning with this assumption Williams, *et al.*[14] reported that for extensive biliary excretion in the rat the compound or its metabolite should have a molecular weight of 325 $\pm$50 or more and a polar anionic group. It is, therefore, very probable to assume that the primary metabolites of 2,4,3′,4′-TCB might be glucuronides which could be hydrolyzed by gut bacteria. However, this possibility was ruled out by careful examination of rat bile.[15]

One more thing to be discussed is that either unchanged compound or the metabolite was excreted rather constantly from 3 or 4th day to 12th day after the treatment. The reason for such a little but constant excretion for a long period of time is as yet not at all clear, however, similar results have also been reported on polychlorobenzenes by Parke and Williams.[16] One of the possible explanations could be provided by our preliminary experiment,[15] in which a significant and constant excretion of 2,4,3′,4′-TCB into the small intestine of rats from the blood stream could be observed for a long period of time after intraperitoneal or intravenous injection.

Acknowledgement This work was supported partially by a Grant-inlAid for Scientific Research provided by the Ministry of Education and also by a research grant provided by the Ministry of Health and Welfare to which the authors are greatly indebted.

11) H.D. West, J.R. Lawson, I.H. Miller, and G.R. Mathura, *Arch. Biochem. Biophys.*, **60**, 14 (1956); P.J. Creaven, D.V. Parke, and R.T. Williams, *Biochem. J.*, **96**, 879 (1965); P.J. Creaven and D.V. Parke, *Biochem. Pharmacol.*, **15**, 7 (1966).
12) W.D. Block and H.H. Cornish, *J. Biol. Chem.*, **234**, 3301 (1959).
13) O. Hutzinger, D.M. Nash, S. Safe, A.S.W. DeFreitas, R.J. Norstrom, D.J. Wildish, and V. Zitko, *Science*, **178**, 312 (1972).
14) M.M. Abou-El-Makarem, P. Millburn, R.L. Smith, and R.T. Williams, *Biochem. J.*, **105**, 1289 (1967).
15) H. Yoshimura and H. Yamamoto, unpublished data.
16) D.V. Parke and R.T. Williams, *Biochem. J.*, **74**, 5 (1960).