Effect of Chlorine Substitution on the Bacterial Metabolism of Various Polychlorinated Biphenyls

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Of 36 pure isomers (chlorine numbers 1 to 5) of polychlorinated biphenyls examined, 23 compounds were metabolized by *Alcaligenes* sp. strain Y42, and 33 compounds were metabolized by *Acinetobacter* sp. strain P6. The major pathway of many polychlorinated biphenyl isomers examined was considered to proceed through $2',3'$-dihydro-$2',3'$-diol compounds, concomitant dehydrogenated $2',3'$-dihydroxy compounds, subsequently the $1',2'$-meta-cleavage compounds (chlorinated derivatives of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acids), and then chlorobenzoic acids. The meta-cleavage products were usually converted to chlorobenzoic acids upon further incubation in many polychlorinated biphenyls, but they accumulated specifically in the metabolism of 2,4′-, 2,4,4′-, and 2,5,4′-chlorobiphenyls, which are all chlorinated at the 2,4′-position in the molecules in common. Dihydroxy compounds accumulated mainly in the metabolism of 2,6-, 2,3,6-, 2,4,2′,5′-, 2,5,2′,5′-, and 2,4,5,2′,5′-chlorobiphenyls by *Acinetobacter* sp. P6. The 2,3,2′,3′-, 2,3,2′,5′-, and 2,4,5,2′,3′-chlorobiphenyls, which are chlorinated at the 2,3-position of one of the rings, were metabolized in a different fashion. Two major metabolites of a chlorobenzoic acid and an unknown compound accumulated always in the metabolism of this group of polychlorinated biphenyls. 2,4,6-Trichlorobiphenyl was metabolized quite differently between the two organisms. *Alcaligenes* sp. Y42 metabolized this compound very slowly to trichlorobenzoic acid by the major oxidative route. In contrast, *Acinetobacter* sp. P6 metabolized it to a trihydroxy compound via a dihydroxy compound.

Polychlorinated biphenyls (PCBs) have been recognized as almost universally distributed pollutants which are generally considered to be quite resistant to degradation. Recently, it has been shown that some PCB isomers have been converted to hydroxylated products in such various mammals as rats (13, 14, 24), monkeys (12), rabbits (19), and pigs (20). Some studies have also been conducted on microbial degradation of PCBs. Ahmed and Focht (1) studied the degradation of several mono- and dichlorobiphenyls by two species of *Achromobacter* and found that chlorinated benzoic acids were formed as a result of oxidative degradation. Ballschmiter et al. (3) also detected chlorinated benzoic acids as the metabolites of several PCB isomers with mixed culture of soil bacteria. Wallnöffer et al. (23) observed that 4- and 4,4′-chlorobiphenyls were converted to mono- or dihydroxylated compounds or both by using a soil fungus, *Rhizopus japonicus*. Tucker et al. (22) demonstrated that commercial PCB mixtures, which contain predominantly mono- and dichlorobiphenyls, readily underwent primary biodegradation by activated sludge microorganisms and that as the

levels of tri-, tetra-, and pentachlorobiphenyls increased, the biodegradation rates decreased accordingly. Sayler et al. (21) reported that a *Pseudomonas* sp. was found to degrade PCB mixtures (Aroclor 1254) and 2,4,5,2′,4′,5′-hexachlorobiphenyl and that PCBs stimulated the bacterial growth and oxygen uptake.

In our previous studies (7–9), we have attempted to correlate structural features to microbial degradation of PCBs. The number and position of substituted chlorines governed the rate of the degradation as follows. (i) Degradation decreased as chlorine substitution increased. PCB isomers containing more than four chlorines were less susceptible to degradation. (ii) PCBs containing two chlorines on either the ortho position of a single ring (i.e., 2,6-) or both rings (i.e., 2,2′-) showed striking resistance to degradation. (iii) PCBs containing all chlorine atoms on only single ring were generally degraded faster than those containing the same number on both rings. (iv) Preferential ring fission of molecules occurred with nonchlorinated or lesser chlorinated rings. (v) The formation and stable accumulation of a yellow, meta-cleav-

age product were always observed in 4'-chloro-substituted PCBs such as 2,4'-, 4,4'-, 2,4,4'-, and 2,5,4'-chlorobiphenyls. (vi) Significant differences between *Alcaligenes* sp. Y42 and *Acinetobacter* sp. P6 with respect to biodegradability of PCBs were not observed except with 2,4,6-trichlorobiphenyl.

Metabolism of 2,4,4'-trichlorobiphenyl by *Acinetobacter* sp. P6 has been reported separately (K. Furukawa, K. Tonomura, and A. Kamibayashi, Agric. Biol. Chem., submitted for publication). The major pathway of 2,4,4'-trichlorobiphenyl was considered to proceed oxidatively through the 2',3'-dihydro-2',3'-diol compound [1-chloro-2,3-dihydroxy-4-(2,4-dichlorophenyl)hexa-4,6-diene], the concomitant dehydrogenated 2',3'-dihydroxy compound (2,4,4'-trichloro-2',3'-dihydroxybiphenyl), and then the 1',2'-meta-cleavage compound [3-chloro-2-hydroxy-6-oxo-6(2,4-dichlorophenyl)hexa-2,4-dienoic acid], which accumulated predominantly in the reaction mixture upon further incubation with a small amount of dichlorobenzoic acid. From these results, our efforts were focused on the correlation between chlorine substitution and metabolic sequence for a variety of PCBs.

There are many reports of co-metabolic turnover of chlorinated aromatic compounds by microorganisms. The biochemical problems concerning microbial degradation of these compounds that arise from environmental pollution are extensively reviewed by Bollag (4) and Pfister (17). However, only a few investigators have varied the position of halogen substitution during their studies of co-metabolism of halogenated aromatic compounds. By studying the co-metabolism of chlorinated compounds of methoxylated benzoic acids, Crawford et al. (5) demonstrated the blocking effect of a chlorine atom ortho but not meta to the ring position involved in the next catabolic reaction. MacRae and Alexander (16) reported that the position rather than the number of halogens governs susceptibility to decomposition in halogenated compounds of phenol and phenoxy compounds.

Chlorinated biphenyls have as many as 210 different components containing 0 to 10 chlorine atoms per biphenyl molecule. PCBs, in this sense, are good materials for studies on chemical structure versus microbial metabolism. The present paper describes metabolic fates of 36 pure isomers of PCB by two bacterial strains of *Alcaligenes* sp. Y42 and *Acinetobacter* sp. P6.

MATERIALS AND METHODS

Microorganisms and cultivation. *Alcaligenes* sp. strain Y42 and *Acinetobacter* sp. strain P6 were used throughout the experiment. Cultivation was carried out in the following mineral medium supplemented with biphenyl (1 g) for *Alcaligenes* sp. Y42 and 4-chlorobiphenyl (1 g) for *Acinetobacter* sp. P6 as a sole source of carbon and energy: $(NH_4)_2SO_4$, 1 g; KH_2PO_4, 0.2 g; K_2HPO_4, 1.6 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 0.1 g; $FeSO_4.7H_2O$, 0.01 g; $CaCl_2.2H_2O$, 0.02 g; deionized water, 1 liter (pH 7.5).

Incubation and extraction. The organisms were grown for 3 to 4 days, and the culture fluid was filtered once to remove remaining solid biphenyl or 4-chlorobiphenyl. The cells were then harvested by centrifugation and washed twice in 0.05 M phosphate buffer (pH 7.5). The washed cells were resuspended in 10 ml of phosphate buffer to which 500 nmol of pure PCB isomers was added. The final absorbance of the cell was adjusted to 1.0 at 660 nm (2.0 × 10⁹ cells per ml for *Alcaligenes* sp. Y42 and 4.4 × 10⁸ cells per ml for *Acinetobacter* sp. P6). Incubation was carried out under consideration to obtain a variety of metabolic intermediates from parent PCB components, so that easily degradable PCBs were incubated for a short time without shaking at 15°C. Some highly chlorinated or refractile PCBs were shaken on a rotary shaker at 30°C for a long period. The incubation mixtures were then extracted with 10 ml of ethylacetate after acidification to pH 1 with concentrated HCl.

GC-MS. The ethylacetate layer was removed and evaporated once a gentle stream of nitrogen gas and dissolved in a small amount of ethylacetate. Bistrimethylsilyl acetamide was added to obtain trimethylsilyl derivatives of certain PCB metabolites. The samples were analyzed with a gas-liquid chromatographmass spectrometer (GC-MS) (JEOL Ltd., model JMS D-300) with a coiled glass column (1 m by 4 mm internal diameter) packed with silicon OV 1 (at 2% on 80- to 100-mesh Chromosorb G). Helium was used as a carrier gas at a flow rate of 20 ml/min. The column temperature on GC was increased from 140 to 250°C at a rate of 8°C/min. The electron impact MSs were measured at a 20 eV ionization potential, 300-μA trap current, and 200°C ion source temperature. The GC-MS condition for chemical ionization MS was as follows. The MSs were obtained at 250 eV of ionization potential. The pressure in ionization source was 0.5 to 1 torr. Methane was used as the reagent gas. In this study, mass data of PCB metabolites are obtained as trimethylsilyl derivatives at electron impact mode except otherwise mentioned.

Chemicals. 4-Chlorobiphenyl was purchased from Aldrich Chemical Co. All other pure isomers of PCB were obtained from Analabs Inc., in which no significant impurity was observed by GC-MS analysis. Bistrimethylsilyl acetamide was obtained from Merck Inc.

RESULTS

The data obtained from GC-MS runs were analyzed by computer treatment. Metabolites containing chlorines were easily detected by their isotopic distribution in the mass spectra. The results obtained in this experiment are summarized in Table 1 (*Alcaligenes* sp. Y42) and Table 2 (*Acinetobacter* sp. P6). For monochlorobiphenyls (group A), incubation was carried out without shaking at 15°C for 30 min. Tri-

methylsilyl derivatives of monochlorobenzoic acids (M$^+$, electronic mass (m/e) 228, M$^+$ −Me, m/e 213 as a base peak) were detected in 2-, 3-, and 4-chlorobiphenyl metabolites in the two organisms. A yellow, meta-cleavage product (λ_{max}, 394 nm in neutral and alkaline pH) was

TABLE 1. *Metabolism of various PCBs by Alcaligenes sp. Y42*[a]

Isomer group	PCB chlorine position	Metabolites in major pathway				Other metabolites
		Dihydrodiol compound	Dihydroxy compound	Meta-cleavage compound	Chlorobenzoate	
A	2-			++	++++	
	3-		+		++++	
	4-				++++	
B	2,3-		+++		+++	
	2,4-			++	++++	
	2,5-			+++	++++	
	2,6-[b]					
	3,4-			++	++++	
	3,5-			++++	++++	
C	2,2'-				++	
	2,4'-	+		++++	+++	
	3,3'-				++++	
	4,4'-			++	++++	
D	2,3,4-				++++	
	2,3,6-[b]					
	2,4,5-		+	++++	++++	
	2,4,6-	++		+	+	
E	2,5,2'-		++	+	+++	
	2,5,3'-		+++		+++	
	2,5,4'-			++++	+	
	2,4,4'-			++++	+	
	3,4,2'-	+		++	+++	Monohydroxy compound (++)
F	2,3,4,5-			++++	++++	
	2,3,5,6-[b]					
G	2,3,2',3'-				+++	mol wt 300 Cl$_2$ compound (++++)
	2,3,2',5'-				++	mol wt 300 Cl$_2$ compound (++)
	2,4,2',4'-[b]					
	2,4,2',5'-[b]					
	2,5,2',5'-[b]					
	2,6,2',6'-[b]					
	2,4,3',4'-[b]					
	2,5,3',4'-[b]					
	3,4,3',4'-[b]					
H	2,3,4,5,6-[b]					
I	2,4,5,2',3'-[b]					
	2,4,5,2',5'-[b]					

[a] The incubation and analytical methods are presented in the text. The relative amount of each metabolite in the incubation mixture is roughly expressed by plus signs according to the total ion chromatogram on GC-MS at the electron impact mode. The structures of metabolites in the major pathway are presented in Fig. 4: dihydrodiol compound (II in Fig. 4), dihydroxy compound (III), metacleavage compound (IV), and chlorobenzoic acid (V).

[b] No metabolite was observed.

TABLE 2. *Metabolism of various PCBs by Acinetobacter sp. P6*

Isomer group	PCB chlorine position	Metabolites in major pathway				Other metabolites
		Dihydrodiol compound	Dihydroxy compound	Meta-cleavage compound	Chlorobenzoate	
A	2-			++	++++	
	3-				++++	
	4-				++++	
B	2,3-		+++		++++	Trihydroxy compound (+)
	2,4-				++++	
	2,5-		++	++	++++	
	2,6-		++			Trihydroxy compound (+)
	3,4-				++++	
	3,5-				++++	
C	2,2'-		++		++	
	2,4'-	++	+	+++	+++	Monohydroxy compound (+++)
	3,3'-		+		+++	Monohydroxy compound (+)
	4,4'-	+		++	+++	
D	2,3,4-				++++	
	2,3,6-		+			Trihydroxy compound (+)
	2,4,5-			+++	++++	Trihydroxy compound (+)
	2,4,6-	+	++++			Trihydroxy compound (++++)
E	2,5,2'-	+	+++		++++	Monohydroxy compound (+)
	2,5,3'-		+++		++	Dichlorodihydroxy compound (++)
	2,5,4'-			++++	+	Monohydroxy compound (+) Dichlorodihydroxy compound (+)
	2,4,4'-	++	+	++++	+	Monohydroxy compound (++) Dichlorodihydroxy compound (++)
	3,4,2'-	+			++++	Dichlorodihydroxy compound (+)
F	2,3,4,5- 2,3,5,6-[b]		+++	++++	++++	Trihydroxy compound (+)
G	2,3,2',3'-		+		+++	mol wt 300 Cl_2 compound (++++)
	2,3,2',5'-		++		+++	mol wt 300 Cl_2 compound (++++)
	2,4,2',4'-			+	+++	
	2,4,2',5'-		+++	+	+	
	2,5,2',5'-		+++			
	2,6,2',6'-[b]					
	2,4,3',4'-				+	Monohydroxy compound (+)
	2,5,3',4'-		+		+	
	3,4,3',4'-				++	
H	2,3,4,5,6-[b]					
I	2,4,5,2',3'-				++	mol wt 334 Cl_3 compound (++++)
	2,4,5,2',5'-		++			Monohydroxy compound (+)

[a] See footnote to Table 1.
[b] No metabolite observed.

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observed in 2-chlorobiphenyl metabolism. The electron impact MS indicated the molecular ion peak at m/e 396, M^+ −Me at m/e 381, and M^+ − $SiMe_3O_2C$ at m/e 279 as a base peak. The chemical ionization MS of the same compound is presented in Fig. 1. Typical quasi-molecular ions (QM^+) appeared at m/e 397 (M^+ + 1) as a base peak, m/e 425 (M^+ + 29) and m/e 437 (M^+ + 41) with some fragment ion peaks such as m/e 307 (M^+ − $SiMe_3O$) and m/e 279 (M^+ − $SiMe_3O_2C$). The meta-cleavage product was considered to be 2-hydroxy-6-oxo-6(2-chlorophenyl)hexa-2,4-dienoic acid.

Dichlorobiphenyls with two chlorines substituted on the single ring (group B) except 2,6-dichlorobiphenyl were readily metabolized to dichlorobenzoic acids by the two organisms. To-

tal ion chromatogram of 2,5-dichlorobiphenyl metabolites by *Acinetobacter* sp. P6 is shown in Fig. 2. A large amount of 2,5-dichlorobenzoic acid (peak no. 1 in the figure, M^+, m/e 262; M^+-Me, m/e 247 as a base peak) was readily produced after 1 h of incubation without shaking at 15°C. 2,5-Dichloro-2',3'-dihydroxy biphenyl (peak no. 3, M^+, m/e 398 as a base peak) and a meta-cleavage product (peak no. 4, M^+, m/e 430; M^+ − $SiMe_3O_2C$, m/e 313 as a base peak) were also detected in this reaction mixture. The yellow, meta-cleavage intermediates were also observed in the metabolism of 2,4-, 3,4-, and 3,5-dichlorobiphenyls by *Alcaligenes* sp. Y42. These yellow compounds disappeared soon to yield dichlorobenzoic acids upon further incubation. On the other hand, 2,6-dichlorobiphenyl was
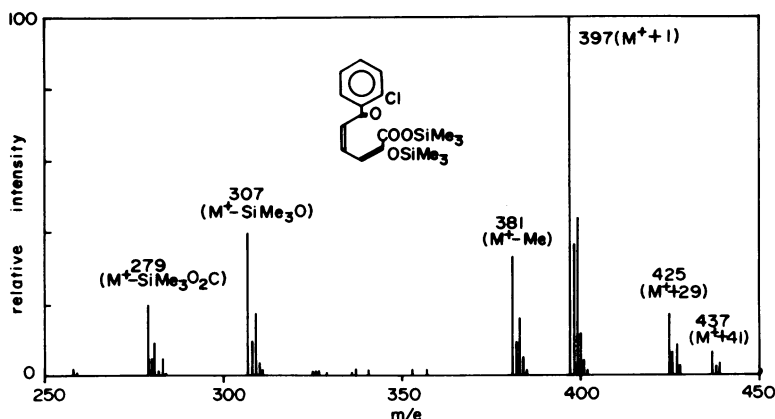

FIG. 1. *Chemical ionization MS of the meta-cleavage product from 2-chlorobipenyl by Alcaligenes sp. Y42. The product is proposed to be 2-hydroxy-6-oxo-6(2-chlorophenyl)hexa-2,4-dienoic acid.*
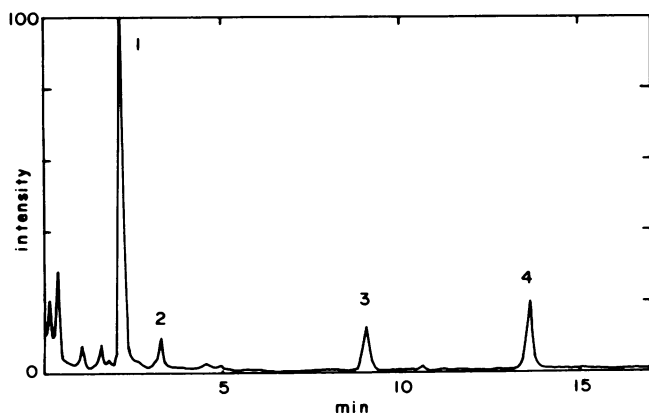

FIG. 2. *Total ion chromatogram on GC–electron impact–MS of 2,5-dichlorobiphenyl metabolites by Acinetobacter sp. P6. The incubation was carried out without shaking for 1 h at 15°C. The entire reaction mixture was extracted with ethylacetate, to which bistrimethylsilyl acetamide was added after concentration. The sample was subjected to GC-MS at the electron impact mode. Peak no. 1, 2,5-dichlorobenzoic acid; no. 2, 2,5-dichlorobiphenyl; no. 3, 2,5-dichloro-2',3'-dihydroxy biphenyl; no. 4, 2-hydroxy-6-oxo-6(2,5-dichlorophenyl)hexa-2,4-dienoic acid (meta-cleavage product).*

refractile and was hardly metabolized by *Alcaligenes* sp. Y42 even after 2 days of incubation. *Acinetobacter* sp. P6, however, converted this compound very slowly to a dihydroxy compound (M^+, m/e 398 as a base peak).

Dichlorobiphenyls with one chlorine substituted on each ring (group C) gave some intermediates, as shown in Tables 1 and 2. When 2,4-dichlorobiphenyl was incubated with *Acinetobacter* sp. P6 for 1 h without shaking at 15°C, seven compounds containing chlorine atoms in the molecules were detected in the reaction mixture. The GC–MS data of these compounds are presented in Table 3. Two isomeric monohydroxyl compounds, a dihydrodiol compound, a dihydroxy compound, a meta-cleavage compound, and a monochlorobenzoic acid were demonstrated together with the unchanged parent compound. Monochlorobenzoic acids were readily produced from 2,2'-, 3,3'-, and 4,4'-dichlorobiphenyls in the two organisms.

Trichlorobiphenyls with three chlorines on a single ring (group D) were of great interest. 2,3,4-Trichlorobiphenyl was readily metabolized to 2,3,4-trichlorobenzoic acid (M^+, m/e 296; $M^+ -$ Me, m/e 281 as a base peak) in the two organisms. From 2,4,5-trichlorobiphenyl, meta-cleavage product (M^+, m/e 464; $M^+ -$ SiMe$_3$O$_2$C, m/e 347 as a base peak) and 2,4,5-trichlorobenzoic acid were demonstrated with some minor metabolites. 2,4,6-Trichlorobiphenyl was striking. *Alcaligenes* sp. Y42 metabolized this compound very slowly to 2,4,6-trichlorobenzoic acid with some intermediates such as a dihydroxy compound (M^+, m/e 432 as a base peak) and a meta-cleavage compound (M^+, m/e 464; $M^+ -$ SiMe$_3$O$_2$C, m/e 347 as a base peak) after 2 days of incubation. In contrast, *Acinetobacter* sp. P6 rapidly metabolized this compound in a different fashion from that of *Alcaligenes* sp. Y42. In an early stage of the incubation, a large amount of dihydroxy compound (M^+, m/e 432 as a base peak) appeared. Then the dihydroxy compound was subsequently metabolized to a trihydroxy compound (M^+, m/e 520 as a base peak) which accumulated in the reaction mixture upon further incubation. 2,3,6-Trichlorobiphenyl was refractile and hardly metabolized by *Alcaligenes* sp. Y42. *Acinetobacter* sp. P6, however, could slowly convert this compound to a dihydroxy compound and a trihydroxy compound to some extent as in the case with 2,6-dichlorobiphenyl.

Trichlorobiphenyls with two chlorines on one ring and one chlorine on another ring (group E) were incubated with or without shaking. Among this group of PCBs, a variety of metabolic intermediates was obtained from 2,4,4'-trichlorobiphenyl. When the compound was incubated without shaking at 15°C with *Acinetobacter* sp. P6, two isomeric monohydroxy compounds (M^+, m/e 344), a dihydrodiol compound (M^+, m/e 434 as a base peak), a dihydroxy compound (M^+, m/e 432 as a base peak), a meta-cleavage compound (M^+, m/e 464; $M^+ -$ SiMe$_3$O$_2$C, m/e 347 as a base peak), and small amount of dichlorobenzoic acid (M^+, m/e 262; $M^+ -$ Me, m/e 247 as a base peak) were demonstrated. As an additional metabolite of 2,4,4'-trichlorobiphenyl during degradation with *Acinetobacter* sp. P6, dichlorodihydroxy biphenyl (dechlorination-hydroxylation product) was also observed. The MS of this product indicated a compound with a molecular ion peak at m/e 398 as a base peak and with a typical isotope distribution at m/e 398, 400, and 402, indicating two chlorine atoms in the molecule. Such dechlorination-hydroxylation products were also observed as a minor product in the metabolism of 2,5,3'-, 2,5,4'-, and 3,4,2'-trichlorobiphenyls by *Acinetobacter* sp. P6. 2,5,4'-Trichlorobiphenyl was metabolized

TABLE 3. *GC-EI-MS data of 2,4'-dichlorobiphenyl and its trimethylsilated metabolites*[a]

Peak no.[b]	M^+ of TMS derivative[c] (m/e)	Relative intensity for the base peak (100) of typical fragment peaks					Estimated structure
		M^+	M^+-Me	M^+-Cl	M^+-SiMe$_3$O	M^+-SiMe$_3$O$_2$C	
1	228	6	100				2-(or 4-) chlorobenzoic acid
2	222	100		2			2,4'-dichlorobiphenyl
3	310	100	27				2,4'-dichloro-hydroxybiphenyl
4	310	67	100				2,4'-dichloro-hydroxybiphenyl
5	400	71		100	4		1-(or 5-) chloro-2,3-dihydroxy-4(2- or 4-chlorophenyl)hexa-4,6-diene
6	398	100	7		12		2,4'-dichloro-2,3-(or 2',3'-)dihydroxybiphenyl
7	430	2	5	6		100	3-(or 5-)chloro-2-hydroxy 6-oxo-6(2- or 4-chlorophenyl)hexa-2,4-dienoate

[a] Calculation based on fragment containing ^{35}Cl only.

[b] The peak number corresponds to the elution order on GC–MS total ion chromatogram.

[c] TMS, Trimethylsilyl.

quite similarly to 2,4,4'-trichlorobiphenyl. A meta-cleavage product was readily formed and accumulated with a small amount of dichlorobenzoic acid in the two organisms. Dichlorobenzoic acids appeared always as the major products in the metabolism of 2,5,2'-, 2,5,3'-, and 3,4,2'-trichlorobiphenyls with some unknown products.

Tetrachlorobiphenyls with four chlorines on a single ring (group F) were of interest. 2,3,4,5-Tetrachlorobiphenyl was rapidly degraded to 2,3,4,5-tetrachlorobenzoic acid through the major oxidative route in the two organisms. Figure 3 shows the total ion chromatogram of the supernatant extract (Fig. 3a) and the cell extract (Fig. 3b) when 2,3,4,5-tetrachlorobiphenyl was incubated for 2 h with *Acinetobacter* sp. P6 without shaking at 15°C. A large amount of dihydroxy compound (peak no. 3 in Fig. 3; M^+, m/e 466), a yellow, meta-cleavage compound (peak no. 5, M^+, m/e 498; $M^+ - SiMe_3O_2C$, m/e 381) were demonstrated in the two organisms. A small amount of trihydroxy compound (peak no. 4, M^+, m/e 554) was also observed in *Acinetobacter* sp. P6. In contrast, 2,3,5,6-tetrachlorobiphenyl was not metabolized at all for 4 days of incubation with shaking at 30°C in the two organisms.

Tetrachlorobiphenyls with two chlorines on each ring (group G) were shaken with the organisms for 2 to 4 days at 30°C. Among nine isomers of this group, only two isomers of 2,3,2',3'- and 2,3,2',5'-tetrachlorobiphenyls were metabolized in quite similar fashion by *Alcaligenes* sp. Y42 to a dichlorobenzoic acid and an unknown metabolite (M^+, m/e 444; $M^+ - Me$, m/e 429 as a base peak). The unknown compound contained two chlorines in the molecule and the molecular weight was determined to be 300 from the chemical ionization MS of the purified sample. On the other hand, *Acinetobacter* sp. P6 was capable of metabolizing eight isomers of this group. For 2,3,2',3'- and 2,3,2',5'-tetrachlorobiphenyls, a dichlorobenzoic acid and a large amount of unknown compound accumulated in the reaction mixture as observed with *Alcaligenes* sp. Y42. From 2,4,2',5'- and 2,5,2',5'-tetrachlorobiphenyls, dihydroxy compounds (M^+, m/e 466) were mostly observed in the cell extract of *Acinetobacter* sp. P6. Dichlorobenzoic acids were produced from 2,4,2',4'-, 2,4,3',4'-, and 3,4,3',4'-tetrachlorobiphenyls.

2,3,4,5,6-Pentachlorobiphenyl with five chlorines substituted on a single ring (group H) was not metabolized at all even after 4 days of incubation by the two organisms. Two pentachlorobiphenyls with three chlorines on one ring and two chlorines on another ring (group I) were not
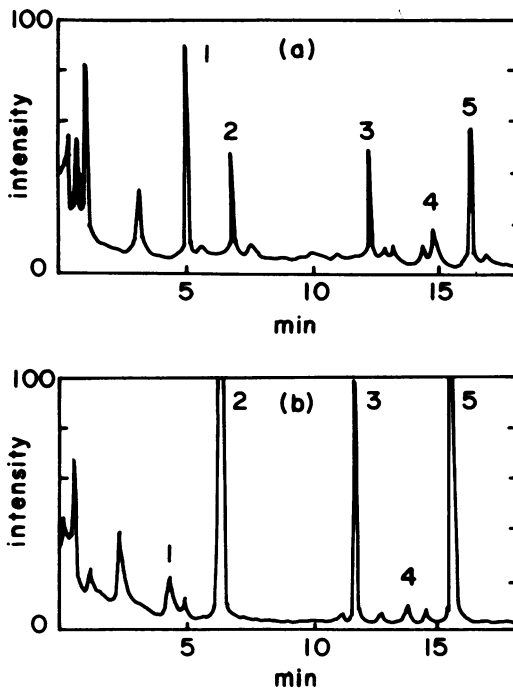

FIG. 3. *Total ion chromatograms on GC–electron impact–MS of the supernatant extract (a) and the cell extract (b) from 2,3,4,5-tetrachlorobiphenyl metabolism by Acinetobacter sp. P6. The incubation was carried out without shaking for 4 h at 15°C and centrifuged. The supernatant and the cells were extracted separately with ethylacetate, to which bistrimethylsilyl acetamide was added after concentration. The samples were subjected to GC-MS at the electron impact mode. Peak no. 1, 2,3,4,5-tetrachlorobenzoic acid; no. 2, 2,3,4,5-tetrachlorobiphenyl; no. 3, tetrachlorodihydroxy biphenyl; no. 4, tetrachlorotrihydroxy biphenyl; no. 5, 2-hydroxy-6-oxo-6(2,3,4,5-tetrachlorophenyl)hexa-2,4-dienoic acid (meta-cleavage product).*

metabolized by *Alcaligenes* sp. Y42. However, *Acinetobacter* sp. P6 always metabolized 2,4,5,2',3'-pentachlorobiphenyl to a trichlorobenzoic acid and unknown compound (M^+, m/e 478; $M^+ - Me$, m/e 463 as a base peak). The compound contained three chlorines and two active sites which react with trimethylsilyl reagent in the molecule, and the molecular weight was determined to be 334. 2,4,5,2',5'-Pentachlorobiphenyl was converted to a dihydroxy compound (M^+, m/e 500) by *Acinetobacter* sp. P6.

DISCUSSION

A number of PCB isomers examined in this experiment were metabolized to chlorobenzoic acids by an oxidative route. Bacterial oxidation

of several aromatic compounds has been considered to proceed via a dioxygenase-catalized reaction (10, 15, 18). Gibson et al. (11) demonstrated that a species of *Beijerinckia* oxidizes biphenyl to *cis*-2,3-dihydroxy-1-phenylhexa-4,6-diene, suggesting that a cyclic peroxide is initial oxidation product in the metabolism of biphenyl by this organism. In the present studies, the initial oxidation process of PCBs has not yet been investigated, however, the major metabolic pathway of PCBs may be proposed as illustrated in Fig. 4. Two atoms of molecular oxygen would most likely be incorporated at the 2',3'-position of the lesser chlorinated ring, and a *cis*-dihydrodiol compound (II in Fig. 4) might be produced via a cyclic peroxide. Then compound II will be dehydrogenated to yield a 2',3'-dihydroxy compound (III). The meta-cleavage will occur at the 1',2'-position of the compound III, since the fragment peak of dichlorobenzoyl (*m/e* 173) was specifically observed as a base peak in the chemical ionization MS of the yellow, meta-cleavage product from 2,4,4'-trichlorobiphenyl, as has been reported in a separate paper (K. Furukawa, K. Tonomura, and A. Kamibayashi, submitted for publication). The fragment peak of chlorinated benzoyl will never occur from other structures, such as the 2',3'-orthocleavage or the 3',4'-meta-cleavage product. The yellow, meta-cleavage products were usually converted rapidly to chlorinated benzoic acids in many PCB isomers. Both *Alcaligenes* sp. Y42 and *Acinetobacter* sp. P6 were not capable of metabolizing chlorobenzoic acids formed except 3-chlorobenzoic acid.

The 36 PCB isomers examined can be divided into the following six groups according to the pattern of metabolic behavior. (i) PCBs which chlorobenzoic acids mainly accumulate in the reaction mixture through the major oxidative route as illustrated in Fig. 4 are as follows: 2-, 3-, 4-, 2,3-, 2,5-, 3,4-, 3,5-, 2,2'-, 3,3'-, 4,4'-, 2,3,4-, 2,4,5-, 2,5,2'-, 3,4,2'-, and 2,3,4,5-chlorobiphenyls in the two organisms. 2,4,6-Trichlorobiphenyl by *Alcaligenes* sp. Y42, 2,4,2',4'- and 3,4,3',4'-tetrachlorobiphenyls by *Acinetobacter* sp. P6 are also considered to be metabolized to

chlorobenzoic acids in this manner. (ii) PCBs which yellow, meta-cleavage compounds accumulate predominantly through the major oxidative route are 2,4'-di-, 2,4,4'-, and 2,5,4'-trichlorobiphenyls. This group of PCBs has chlorine atoms at the 2,4'-position in the molecule in common. It is of interest why the presence of such a moiety prevents the meta-cleavage compound from being metabolized to chlorobenzoic acids. (iii) PCBs which dihydroxy compounds are mainly produced are 2,6-, 2,3,6-, 2,4,2',5'-, 2,5,2',5'-, and 2,4,5,2',5'-chlorobiphenyls. These compounds were slowly metabolized by only *Acinetobacter* sp. P6. *Alcaligenes* sp. Y42 was not capable of metabolizing this group of PCBs. The position of hydroxyl has not yet been clear, but the 2',3'-position is not likely for 2,5,2',5'- and 2,4,5,2',5'-chlorobiphenyls since all the ortho position of both rings are occupied by chlorine atoms. The 3,4-position is most likely. (iv) 2,4,6-Trichlorobiphenyl was quite differently metabolized between *Alcaligenes* sp. Y42 and *Acinetobacter* sp. P6. *Alcaligenes* sp. Y42 metabolized this compound very slowly to trichlorobenzoic acid through the major pathway. On the other hand, *Acinetobacter* sp. P6 rapidly converted the same compound to a trihydroxy compound via a dihydroxy compound. Trihydroxy compounds were observed as a minor metabolite in 2,3-, 2,6-, 2,3,6-, 2,4,5-, and 2,3,4,5-chlorobiphenyls by *Acinetobacter* sp. P6. However, the one from 2,4,6-trichlorobiphenyl was predominant in a late stage of incubation. No trihydroxy compound was observed in the PCB metabolism by *Alcaligenes* sp. Y42. Investigation is now in progress to assign the hydroxyls in the di- and trihydroxyl compounds derived from 2,4,6-trichlorobiphenyl. (v) PCBs which possess chlorine atoms at the 2,3-position in the molecule, such as 2,3,2',3'-, 2,3,2',5'-, and 2,4,5,2',3'-chlorobiphenyls, seem to be alternatively metabolized. Two metabolites were always produced and accumulated in this group of PCBs. One of them was chlorinated benzoic acid. Another metabolite has not yet been identified. They were dichloro compounds with molecular weights of 300 from
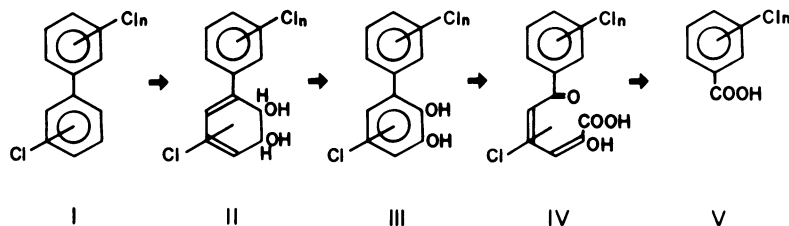
FIG. 4. *Proposed major metabolic sequence of PCBs by Alcaligenes sp. Y42 and Acinetobacter sp. P6. n = 1 to 4.*

two tetrachlorobiphenyls and a trichloro compound with a molecular weight of 334 from pentachlorobiphenyl. The fragmentation patterns of mass spectra of these compounds were quite similar, and the only difference was the chlorine number in the molecule among them. (vi) Of 36 isomers of PCB examined, *Alcaligenes* sp. Y42 was not able to metabolize 13 compounds: 2,6-, 2,3,6-, 2,3,5,6-, 2,4,2',4'-, 2,4,2',5'-, 2,5,2',5'-, 2,6,2',6'-, 2,4,3',4'-, 2,5,3',4'-, 3,4,3',4'-, 2,3,4,5,6-, 2,4,5,2',3'-, and 2,4,5,2'5'-chlorobiphenyls. On the other hand, *Acinetobacter* sp. P6 was not able to metabolize only three compounds such as 2,6,2',6'-, 2,3,5,6-, and 2,3,4,5,6-chlorobiphenyls. A number of PCB isomers examined generally seems to be metabolized through the major pathway in the two organisms shown in Fig. 4, but *Acinetobacter* sp. P6 was able to metabolize a wide variety of PCB components compared with *Alcaligenes* sp. Y42.

Introduction of substituents on a benzene ring influences its biodegradation considerably. Systematic surveys of the effect of chemical structure on the microbial degradation of substituted benzenes have their shortcomings. However, results from various studies showed that the type, the number, and the position of substitutions affect the rate of microbial decomposition of organic compounds as reviewed by Bollag (4) and Pfister (17). MacRae and Alexander (16) demonstrated that the number of chlorines on the aromatic ring determines the susceptibility of the benzoates to microbial degradation. That is contrast with the phenol and phenoxy compounds, in which the position rather than the number of halogens governs susceptibility or resistance to degradation. Alexander and Lustigman (2) reported that in studies with mixed soil microflora, meta-isomer substitution of various groups on the benzene ring was almost invariably degraded more slowly than the ortho-chloro substitution and that substituents on the methylene-carbon governed the resistance of the dichlorodiphenyltrichloroethane molecule to microbial metabolism (Focht and Alexander, 6). Crawford et al. (5) reported that chloro-substituents in the ring of methoxylated benzoic acids arrested their normal metabolism by *Nocardia*: an ortho-chloro substituent thwarted both demethylation and ring opening. However, if the chlorine is oriented meta to the methyl, demethylation does occur. Evidently an ortho atom sterically and/or electronically interferes with the demethylating enzyme system. In the previous studies (7, 9), we found that the number and the position of chlorine substitution greatly affect the transformation of PCBs. The rate of reaction must be influenced by steric and elec-tronic factors of chlorine atoms in the molecule. In the present studies, it has also revealed that metabolic behavior of PCBs is considerably affected by chlorine substitution and that several PCBs are metabolized in different fashions between *Alcaligenes* sp. Y42 and *Acinetobacter* sp. P6.

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