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Bacterial O-Methylation of Halogen-Substituted Phenols

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Two strains of bacteria capable of carrying out the O-methylation of phenolic compounds, one from the gram-positive genus *Rhodococcus* and one from the gram-negative genus *Acinetobacter*, were used to examine the O-methylation of phenols carrying fluoro-, chloro-, and bromo-substituents. Zero-order rates of O-methylation were calculated from data for the chloro- and bromophenols; there was no simple relationship between the rates of reaction and the structure of the substrates, and significant differences were observed in the responses of the two test organisms. For the gram-negative strain, the pattern of substitution was as important as the number of substituents. Hexachlorophene was resistant to O-methylation by both strains, and tetrabromobisphenol-A was O-methylated only by the gram-positive strain. It is suggested that in the natural environment, bacterial O-methylation of phenols carrying electron-attracting substituents might be a significant alternative to biodegradation.

Chlorophenols are widely used industrially and are toxic to a wide spectrum of organisms (1, 6, 30), while a range of chlorinated guaiacols are formed during production of fully bleached chemical pulp (20). A number of bromophenols may also enter the environment. Some, such as tetrabromobisphenol-A [2,2-bis-(3,5-dibromo-4-hydroxyphenyl)-propane] and tetrabromo-*o*-cresol are used as commercial products, while others are used as pesticides and herbicides or may be produced through abiotic hydrolysis of the original insecticide, e.g., bromophos [*O*,*O*-dimethyl-*O*-(4-bromo-2,5-dichlorophenyl)-phosphorothioate] (3). In addition, bromophenols may be produced during the chlorination of water containing bromide and phenolic compounds (47). By analogy with the microbial metabolism of polychlorobiphenyls (11), it is possible that polybromobiphenols are formed from polybromobiphenyls (9, 15, 36). The possible persistence in the environment of all of these compounds is therefore a matter of concern, even though phenols carrying both chloro- (13, 44) and bromo- (5, 10, 14) substituents have been identified as natural microbial and algal metabolites.

The persistence of a chemical is determined largely by its susceptibility to microbial attack. Degradation and mineralization to CO_2 may not, however, be the only processes by which a compound is removed. For example, although pentachlorophenol has been degraded in pure cultures of a number of microorganisms (7, 19, 26, 39, 43, 45, 48) and in a model stream system (33), an important alternative to biodegradation has been recognized; i.e., both bacteria (38, 46) and fungi (8) are able to bring about its O-methylation to pentachloroanisole. Indeed, pentachloroanisole has been detected in fish recovered from a stream into which pentachlorophenol had been accidentially discharged (35). Similar reactions are the O-methylation of 2,3,4,6-tetrachlorophenol by fungi (12) and that of a range of chloroguaiacols by bacteria (27).

The O-methylation of phenols is not solely of intrinsic biochemical interest; it may have significant environmental relevance for a number of reasons. For example, we showed in earlier studies (2) that at low substrate concentrations ($\leq$100 $\mu g \cdot liter^{-1}$), O-methylation of 4,5,6-trichloroguaiacol is generally quantitative and is therefore highly competitive with biodegradation. We also showed (28) that the products of O-methylation (chloroanisoles and chloroveratroles) have a high potential for bioconcentration and that in the zebra fish embryo-larva test, they are at least as toxic as their precursors. The O-methylation reaction may therefore have widespread repercussions in biological systems. For all of these reasons, it is necessary to find how commonly the O-methylation reaction occurs. We therefore used two strains of bacteria capable of O-methylating phenolic compounds to examine a structurally diverse group of phenols substituted with fluoro-, chloro-, and bromo-groups. The strains, one gram positive and one gram negative, were selected from some 20 strains available in this laboratory. The results strengthen the conclusions drawn from our earlier and more restricted study and suggest that in the natural environment, O-methylation may be a significant alternative to biodegradation. For the chloro- and bromophenols, it was shown that there was no universal relationship between the structure of the substrate and the rate at which it was O-methylated and that significant differences existed between the response of the gram-negative and gram-positive organisms. The data therefore underline important limitations in the current understanding of the O-methylation reaction.

MATERIALS AND METHODS

Substrates and metabolites. The chloro- and bromophenols were obtained from commercial suppliers or were gifts from Imperial Chemical Industries Plant Protection Division, Dow Chemical Europe, and Raschig GmbH. They were checked for purity by gas chromatography (GC) before use and were found to be >97% pure. Reference samples of the anisoles were synthesized by methylation of the phenols with dimethyl sulfate; they were purified by recrystallization three times (in methanol, methanol-water, or toluene-hexane) or, for liquids, by chromatography (neutral Al_2O_3; E. Merck AG). Samples of the acetates were prepared by acetylation of the phenols with acetic anhydride in pyridine and were purified in the same way as the anisoles.

Tetrabromocatechol was synthesized by bromination of catechol in refluxing acetic acid with an excess of bromine. The product was recrystallized from toluene-hexane as colorless needles (mp, 189 to 191°C). Tetrabromoguaiacol was prepared from this by mono-O-methylation with

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dimethyl sulfate with tetramethylammonium hydroxide as the base. After 24 h, the mixture was acidified, and the dichloromethane phase was washed extensively with water, dried (Na_2SO_4), and taken to dryness. The residue was dissolved in a small volume of toluene and chromatographed on a column of SiO_2 (Merck Kieselgel 60; 70/280 mesh). Initial fractions contained the veratrole and were followed by fractions containing only the bromoguaiacol; these were combined and shaken with 1 M NaOH, the organic phase was discarded, and the guaiacol was recovered from the alkali extract by acidification and extraction into dichloromethane (mp 155 to 157°C). Tetrabromoveratrole was similarly prepared by using an excess of dimethyl sulfate and was obtained as white needles (mp 123 to 125°C). All of the samples had a purity (GC) of >99%, and were free from interfering compounds.

Microbiological procedures. All of the experiments were carried out with strains 1395 (gram-positive *Rhodococcus* sp.) and 1678 (gram-negative *Acinetobacter* sp.). Details of the procedures have been given previously (2); the procedures involved the use of cell suspensions of ca. 10^8 organisms per ml for strain 1395 and ca. 10^9 organisms per ml for strain 1678. Experiments with the di- and trihalogenophenols, tetrabromo-*o*-cresol, and tetrabromoguaiacol were carried out in 250-ml conical flasks (2). On account of the extremely low solubility of tetrabromobisphenol-A and hexachlorophene in water, experiments with these compounds were carried out in 28-ml serum bottles containing 3 ml of cell suspension; the whole sample was used for each sample point (27). In preparative experiments to obtain sufficient material for GC–mass-spectrometric analysis, 600 ml of cell suspensions was used (experiments with pentafluorophenol were carried out in an enclosed system) and the substrate concentrations were increased to 1.0 mg $liter^{-1}$. Toxicity toward the test strains was assessed by the disk assay procedure described previously (2).

Analytical procedures. Previous analytical procedures (2) could not be used without substantial modification owing to incomplete extraction and loss of volatile components during derivatization. We were able to resolve these difficulties satisfactorily by (i) extracting both the phenols and the anisoles after acidification of the samples; (ii) carrying out all of the extractions at 25°C, which eliminated the loss of the more volatile anisoles (<5%) but did not affect the recovery of the others (results at 25 and 70°C were identical to within ca. ±5%); and (iii) for dihalogenophenols, adding a surrogate standard structurally related to the phenol being analyzed and relating concentrations of the relevant phenol to this (17). Some of the dichloroanisoles and pentafluoroanisole had extremely weak electron capture responses. Since there are fairly rigid restrictions on the relative volumes of the sample, the extraction solvent, and the solvent in the vials used for automatic injection of the samples, kinetic data for the synthesis of pentafluoroanisole cannot be given and the rates for synthesis of 3,4-dichloroanisole are less accurate than for the other anisoles. It should be emphasized that most of the compounds involved in the present study had relatively short GC retention times, so that it was necessary to be scrupulously careful in the choice of reagents and solvents. The following were used throughout: hexane and acetonitrile, pesticide analysis quality (J. T. Baker Chemical Co.) and *tert*-butyl methyl ether, distilled in glass (Burdick and Jackson Laboratories Inc.). The procedures described below summarize these developments.

Analysis of di- and trihalogenophenols and anisoles. Separate duplicate samples were used for analysis of phenols and anisoles. For phenols, a surrogate standard (100 μl of a solution of 2,6-dichlorophenol [15 $\mu g \cdot ml^{-1}$] or 2,4,6-tribromophenol [1.5 $\mu g \cdot ml^{-1}$]) was added to the sample immediately before analysis. Samples (1 ml or up to 3 ml if required) were acidified in an ice bath with 0.5 ml of H_2SO_4 (98%), acetonitrile (0.75 ml) was added, and the tube was placed in an ultrasonic cleaning bath for 30 min. Water (1 ml) was added, and the mixture of phenol and anisole was extracted for 5 min with 1.5 ml of hexane–*tert*-butyl methyl ether (3:1) containing 2,3,4,5-tetrachlorophenol and pentachloro- or hexachlorobenzene (depending on the retention time of the anisole) (0.15 $\mu g \cdot ml^{-1}$ each) as internal standards. The phases were separated by centrifugation, and the aqueous phase was extracted once again with hexane–*tert*-butyl methyl ether lacking the internal standards. For analysis of the anisole, the phenol was removed from the combined organic phase by shaking with 1 ml of NaOH (1 M). The phenol in the duplicate sample was acetylated as described previously (2), except that a final wash with 4 ml of water was included. GC analysis for the anisole and phenol acetate was carried out as described previously (2), except that a column temperature of 170°C was used.

Analysis of tetrabromo-*o*-cresol, tetrabromoguaiacol, and the corresponding *O*-methyl ethers. Analysis of tetrabromo-*o*-cresol, tetrabromoguaiacol, and their esters was carried out essentially as described above with the following minor modifications. (i) After the ultrasonic treatment, water (2 ml) was added and the mixture of phenol and anisole (veratrole) was extracted with 1.5 ml of hexane–*tert*-butyl methyl ether (3:1) containing 2,3,4,5-tetrachlorophenol and lindane (0.15 $\mu g \cdot ml^{-1}$ each) and then with hexane–*tert*-butyl methyl ether (1:1). (ii) The combined extracts were dried (Na_2SO_4), since water is appreciably soluble in the second extraction mixture, and acetylated at 70°C for 30 min. (iii) GC analysis was carried out at a column temperature of 240°C.

Analysis of hexachlorophene and tetrabromobisphenol-A and their di-*O*-methyl ethers. H_2SO_4 (98%; 1.5 ml) was added with cooling to the sample (3 ml) followed by 2 ml of acetonitrile. The serum bottle was placed in an ultrasonic cleaning bath for 30 min, and the contents were then transferred to a screw-cap tube. Water (3 ml) was added to the serum bottle followed by 1.5 ml of cyclohexane–*tert*-butyl methyl ether (1:1) containing dieldrin and tetrachloroguaiacol (0.1 $\mu g \cdot ml^{-1}$ each) as internal standards, and the contents were placed in the ultrasonic bath for 5 min. The contents of the serum bottle were then transferred to the screw-cap tube, and the tube was shaken gently for 10 min. The phases were separated by centrifugation, and the aqueous phase was further extracted with cyclohexane–*tert*-butyl methyl ether (2 ml) lacking internal standards. Phenol was removed from the combined organic phases by extraction with 3 ml of 1 M KOH for 10 min, and the organic phase was treated as follows. Acetonitrile (1.5 ml) was added, the tube was shaken for 30 s, water (3 ml) was added, and the phases were separated by centrifugation. The organic phase was washed with 3 ml of 2 M NaCl and used for analysis of the di-*O*-methyl ether. The phenol analysis was carried out by acidifying the alkaline extract and extracting the phenol twice with 1.5 ml of cyclohexane–*tert*-butyl methyl ether for 10 min. The organic phase was dried (Na_2SO_4) for 30 min and acetylated as described previously (2). GC analysis of the dimethyl ether and the acetate was carried out as previously described, except that an injector temperature of 250°C was used.

GC–mass-spectrometric analysis. The metabolites were generally extracted from the culture fluids with *tert*-butyl

TABLE 1. GC retention times, relative to that of tetrachloroguaiacol-*O*-acetate, of the chloro- and bromophenyl-*O*-acetates and their *O*-methyl ethers

Phenol[a]	Relative retention time of:	
	O-acetate	*O*-methyl ether
Group A		
2,4-Dichlorophenol	0.23	0.20
2,6-Dichlorophenol	0.22	0.18
3,4-Dichlorophenol	0.26	0.21
2,4-Dibromophenol	0.36	0.31
2,6-Dibromophenol	0.34	0.25
2,3,4-Trichlorophenol	0.38	0.34
2,3,6-Trichlorophenol	0.32	0.25
2,4,5-Trichlorophenol	0.33	0.29
2,4,6-Trichlorophenol	0.29	0.22
3,4,5-Trichlorophenol	0.40	0.30
2,4,6-Tribromophenol	0.68	0.48
Group B		
Tetrabromo-*o*-cresol	2.02	1.69
Tetrabromoguaiacol	2.20	1.69
Group C		
Tetrabrombisphenol-A	17.52	9.92

[a] Conditions used: group A, 170°C (column temperature) and 90 kPa; group B, 240°C and 90 kPa; group C, 240°C and 190 kPa.

methyl ether. Toluene was used, however, for extraction of tetrabromo-*o*-cresol *O*-methyl ether, tetrabromoveratrole, and tetrabromobisphenol-A di-*O*-methyl ether. All of the samples were purified before analysis by chromatography on SiO_2 (Merck Kieselgel 60; 70/280 mesh). The analysis was carried out with the same instrumentation as described previously (27), with the following modifications. For 2,6-dibromoanisole, tetrabromo-*o*-cresol *O*-methyl ether, and tetrabromoveratrole, the injector temperature was 200°C, the ionization temperature was 250°C, and the following temperature program was used: isothermal for 1 min at 90°C and then increasing by 10°C · min^{-1} to 270°C. The column pressure was 70 kPa. For tetrabromobisphenol-A di-*O*-methyl ether, the column pressure was 140 kPa, the injector temperature was 250°C, and the following temperature program was used: isothermal for 1 min at 195°C and then increasing at 10°C · min^{-1} to 270°C. For pentafluoroanisole, an OV-17 column was used with the following temperature program: isothermal for 1 min at 50°C and then increasing at 16°C · min^{-1} to 100°C.

RESULTS

We previously (27) presented mass-spectrometric evidence for the synthesis of 2,4,6-trichloroanisole and pentachloroanisole from the corresponding phenols. We therefore felt justified in basing the identification of the other chloroanisoles on their GC retention times relative to those of authentic anisoles (Table 1). Unambiguous identification of the fluoroanisole, the bromoanisoles, and the bromoveratrole, which had not previously been recognized as bacterial metabolites, was based on mass-spectrometric comparison with reference compounds (Fig. 1A to E). Hexachlorophene [2,2′-methylenebis(3,4,6-trichlorophenol)] was completely resistant to transformation by either strain and was not toxic at the concentrations used. The results of a typical kinetic experiment are shown in Fig. 2. Comparable degrees of linearity in the rates were observed for all of the substrates; in some cases, a decline in the anisole concentration was observed after the maximum had been attained. Since this was most marked for the more volatile anisoles, we attribute this decline to slow volatilization rather than to biodegradation of the anisoles. The rates of synthesis of the anisoles, the yields obtained, and the toxicities in the disk assay are given in Table 2. The rates of decline in the concentrations of the phenols were essentially identical (ca. ±15%) to those of the rates of synthesis of the anisoles.

A statistical analysis was made of the correlation between the pK_a of the halogenated phenols (16, 40, 52) and the rates of O-methylation by the gram-positive strain. With $P = 0.060$, the correlation was only marginally significant.

DISCUSSION

Our results show that a range of fluoro-, chloro-, and bromophenols with widely differing degrees and patterns of substitution were O-methylated by the test organisms and that the yields generally exceeded 70%. Since we previously demonstrated O-methylation of chloroguaiacols by bacteria belonging to the genera *Rhodococcus*, *Pseudomonas*, and *Acinetobacter* (2, 27, 28), we conclude that O-methylation of phenols carrying electron-attracting substituents is an important reaction and a significant alternative to biodegradation. It is not, however, a universal response, since 3,4-dichlorophenol was not O-methylated by strain 1395 and 2,4-dinitrophenol was not O-methylated by either of the test strains (unpublished results); degradation of both of these phenols occurred without the formation of detectable metabolites.

Bacterial strains capable of carrying out O-methylation of substituted phenols are widely distributed in aquatic systems in Sweden, including both freshwater and brackish water habitats. Although the genera *Pseudomonas* (31) and *Acinetobacter* (21) have a worldwide distribution, none of our strains could be identified with clearly circumscribed taxa. At this stage, it is therefore not possible to define the quantitative significance of bacterial O-methylation in a wider geographical context.

The response of the gram-positive strain differed significantly from that of the gram-negative strain, so that for clarity, we have chosen to discuss the results of these experiments separately.

For the gram-positive strain, we observed substantial differences in the rates of O-methylation of isomeric phenols; for the dihalogenophenols, 10-fold ratios were found, and for the trihalogenophenols, up to 100-fold ratios were found. These observations emphasized that not only the degree but also the pattern of substitution is a cardinal factor. This could be rationalized partly on the basis of the differences in the degree of dissociation of the phenols, assuming that the phenolate anion was the reactive species (as in abiotic O-methylation with CH_3^+ donors such as dimethyl sulfate or biotic O-methylation with *S*-adenosyl methionine). The rates for strain 1395 ranged from ca. 5 to ca. 500×10^{-10} μg h^{-1} · (cells per ml)$^{-1}$, and the pK_a values of the phenols ranged from ca. 5 to ca. 8; there was, however, only a moderately significant correlation between the two parameters ($P = 0.06$).

Quite different results were obtained with the gram-negative strain. For phenols which were O-methylated, the rates were virtually the same, with the exception of the two dibromophenols, so that there was little discrimination between phenols carrying different substituents. These results, for what was apparently a single-step reaction, should be

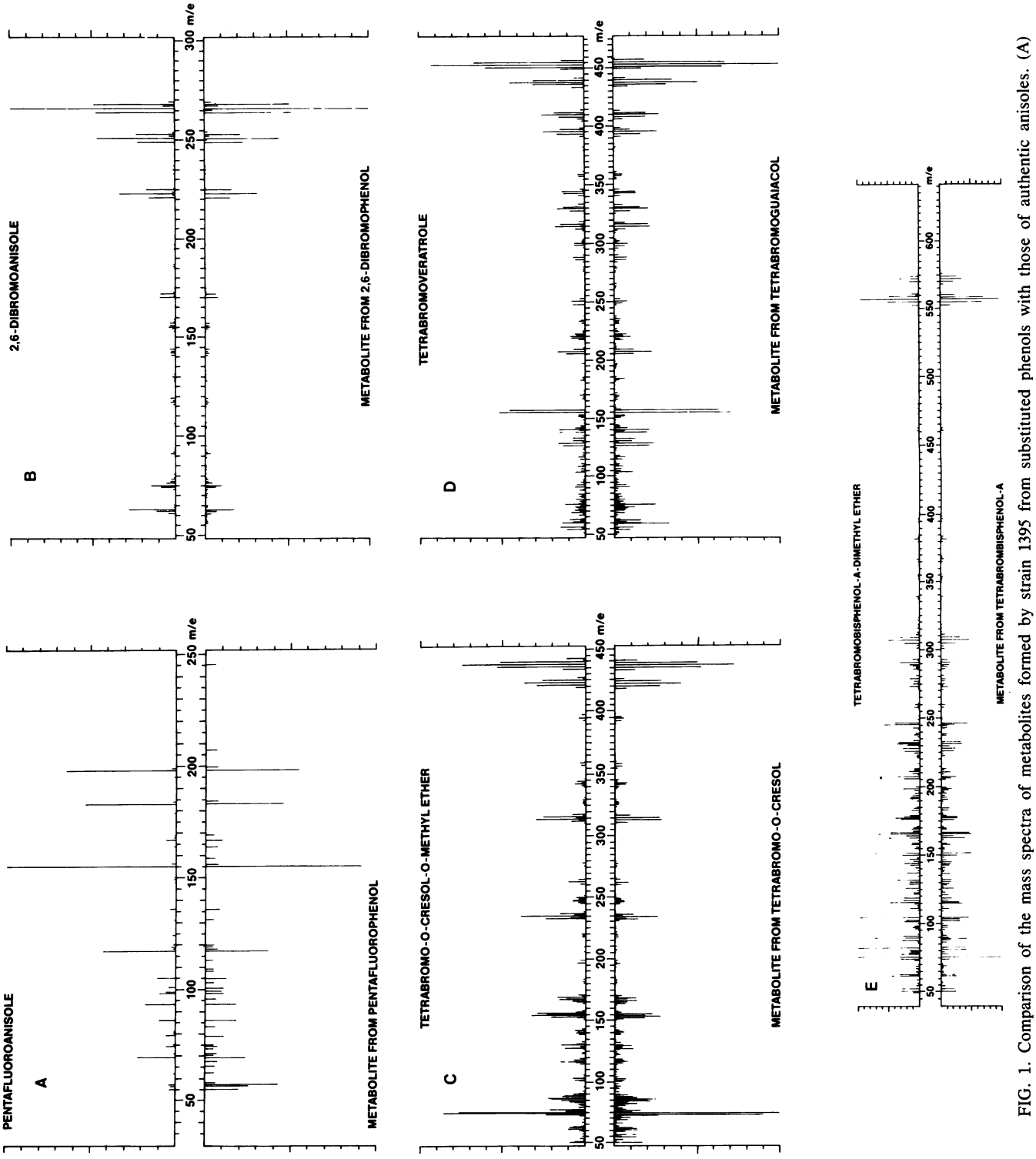

FIG. 1. Comparison of the mass spectra of metabolites formed by strain 1395 from substituted phenols with those of authentic anisoles. (A) Pentafluorophenol; (B) 2,6-dibromophenol; (C) tetrabromo-*o*-cresol; (D) tetrabromoguaiacol; (E) tetrabromobisphenol-A.

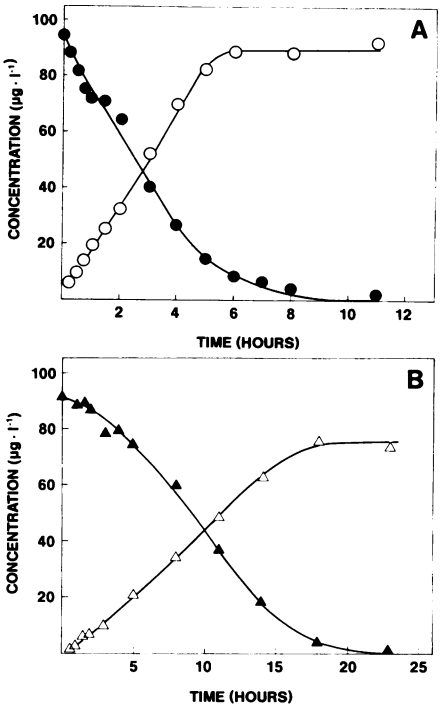

FIG. 2. Kinetics of O-methylation of 2,6-dibromophenol (●, ▲) and synthesis of 2,6-dibromoanisole (○, △) by strain 1395 (A) and strain 1678 (B).

contrasted with the success in relating the rates of another single-step reaction (hydroxylation of substituted phenols to catechols) with the van der Waals' radii of the phenols (32). Efforts to establish structure-activity relationships for complex degradations involving consecutive steps may therefore be fraught with difficulty. Quantitatively significant differences in the responses of gram-positive and gram-negative organisms which are clearly revealed from our restricted investigation (Table 2) should be taken into consideration.

TABLE 2. Rates of *O*-methylation of halogenated phenols by strains 1395 and 1678, percent product yields, and zones of inhibition[a] in disk assays

Phenol	Strain 1395		Strain 1678	
	Rate (10^{-10} μg · h^{-1} · [cells per ml]$^{-1}$) (% yield)	Toxicity[b]	Rate (10^{-10} μg · h^{-1} · [cells per ml]$^{-1}$) (% yield)	Toxicity
2,4-Dichlorophenol	16 (30)	R[c]	1.6 (100)	R
2,6-Dichlorophenol	30 (100)	R	2.4 (100)	R
3,4-Dichlorophenol	0 (0)	18	3.0 (30)	30
2,4-Dibromophenol	22 (50)	R	14 (100)	R
2,6-Dibromophenol	280 (85)	R	9.6 (75)	R
2,3,4-Trichlorophenol	140 (100)	48	4.2 (100)	24
2,3,6-Trichlorophenol	64 (100)	19	2.7 (90)	11
2,4,5-Trichlorophenol	75 (100)	30	3.6 (70)	15
2,4,6-Trichlorophenol	550 (100)	R	2.4 (50)	9
3,4,5-Trichlorophenol	5.8 (90)	64	2.0 (90)	31
2,4,6-Tribromophenol	35 (100)	24	4.0 (75)	9
Tetrabromobisphenol	4.7 (60)	8[d]	0 (0)	R
Tetrabromo-*o*-cresol	480 (100)	9[d]	1.2 (55)	R
Tetrabromoguaiacol	110 (100)	16	0 (0)	R

[a] Assays were done with 150 μg per disk.
[b] Toxicity is measured as zones of inhibition (millimeters).
[c] R, Resistant: zone diameter, ≯5.5 mm.
[d] Response not concentration dependent.

Tetrabromobisphenol-A was O-methylated only by the gram-positive strain. This observation is nonetheless significant for at least two reasons: (i) this established microbiological reaction provides a rationale for the occurrence in natural samples of the bis-*O*-methyl ether (49), and (ii) this result shows that even relatively large molecules (molecular weight, 544) may be subjected to microbial transformations even though the rate was ca. 50 times less than that found for the simple analog (2,6-dibromophenol). We have no explanation of why the bisphenol and tetrabromoguaiacol were resistant to O-methylation by the gram-negative strain, nor why hexachlorophene was resistant to both strains despite the facile O-methylation of its simpler analog (2,4,5-trichlorophenol).

All of our experiments were carried out under laboratory conditions with dense cell suspensions of pure cultures. Evidence supporting the probable ubiquity of O-methylation of phenols in the environment may be adduced, however, from two other kinds of investigations: studies on the fate of chlorophenoxyacetates in the terrestrial environment and examination of natural samples of sediments and biota. This is discussed in more detail below.

Although biodegradation of both 2,4-dichlorophenoxyacetate (see reference 4 and references therein) and 2,4,5-trichlorophenoxyacetate (18) has been demonstrated, it has been shown that 2,4-dichlorophenol (41) and 2,4,5-trichlorophenol (23) are produced in soil samples during degradation of the corresponding phenoxyacetates and are subsequently O-methylated to the anisoles, probably through microbial activity. The corresponding reaction did not, however, take place with mecoprop [2-(4-chloro-2-methylphenoxy)propionate] (42).

Chemical analyses of a number of environmental samples have revealed the presence of halogenoanisoles which have no known source (24, 50, 51). In some cases, the anisoles may originate from the corresponding phenols, which are commercial products [e.g., from the flame retardant tetrabromobisphenol-A (49) or from the antibacterial agent 5-chloro-2-(2,4-dichlorophenoxy)phenol (25)], while in others, the phenols may be intermediates in microbial degradation (e.g., of 2,4-dichlorophenoxyacetate [22] and 2,4,5-trichlorophenoxyacetate [37]).

It should be emphasized that all of our experiments were carried out under aerobic conditions; investigations with chloroguaiacols have shown that O-methylation dominates only under such conditions and that de-O-methylation dominates in anaerobic sediments (34). A final analysis of the fate of substituted phenols in the environment should therefore take into account the cardinal role of oxygen tension in soils and sediments.

On the basis of the collective evidence, we conclude (i) that bacterial O-methylation of halogen-substituted phenols is a ubiquitous reaction; (ii) that the rate of the reaction is a function of both the degree and the pattern of substitution and that significant differences may exist in the response of gram-positive and gram-negative organisms; (iii) that the lipophilic metabolites have a potential for bioconcentration and should not be overlooked in the analysis of fatty biological tissue; and (iv) that the O-methylation reaction provides a plausible hypothesis for the formation of halogenated anisoles having no known anthropogenic source. These conclusions lend additional support to our contention (29) that in environmental-hazard assessments, attention should be directed not only to the compounds discharged but

also to those which may be produced in the environment by natural microbial processes.

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