Transformations of Chloroguaiacols, Chloroveratroles, and Chlorocatechol: by Stable Consortia of Anaerobic Bacteria

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Metabolically stable consortia of anaerobic bacteria obtained by enrichment of sediment samples with 3,4,5-trimethoxybenzoate (TMBA), 3,4,5-trihydroxybenzoate (gallate [GA]), or 5-chlorovanillin (CV) were used to study the anaerobic transformation of a series of chloroveratroles, chloroguaiacols, and chlorocatechols used as cosubstrates. Experiments were carried out with growing cultures, and the following pathways were demonstrated for metabolism of the growth substrates: (i) TMBA produced GA, which was further degraded without the formation of aromatic intermediates; (ii) GA was degraded to 3-chlorocatechol before ring cleavage. Mono-de-O-methylation of the cosubstrates occurred rapidly in the order 4,5,6-chloroguaiacol > 3,4,5-trichloroveratrole > tetrachloroguaiacol and was concomitant with degradation of the growth substrates. For the polymethoxy compounds—chloroveratroles, 1,2,3-trichloro-4,5,6-trimethoxybenzene, and 4,5,6-trichlorosyringol—de-O-methylation took place sequentially. The resulting chlorocatechols were stable to further transformation until the cultures had exhausted the growth substrates; selective dechlorination then occurred with the formation of 3,5-dichlorocatechol from 3,4,5-trichlorocatechol and of 3,4,6-trichlorocatechol from tetrachlorocatechol. 2,4,5-, 2,4,6-, and 3,4,5-trichloroanisole and 2,3,4,5-tetrachloroanisole were de-O-methylated, but the resulting chlorophenols were resistant to dechlorination. These results extend those of a previous study with spiked sediment samples and their endogenous microflora and illustrate some of the transformations of chloroguaiacols and chlorocatechols which may be expected to occur in anaerobic sediments.

The biodegradability of an organic compound discharged into the aquatic environment is an important factor in determining its environmental impact. We have been concerned with developing laboratory procedures for assessing biodegradability and have illustrated their application to chloroguaiacols and chlorocatechols which are formed during the production of fully bleached chemical pulp (23).

We have previously shown that, under aerobic conditions, bacterial O-methylation of chloroguaiacols occurs with the formation of chloroveratroles (32, 34) and that these metabolites produced by environmentally mediated reactions could be identified in samples of wild fish captured from areas subject to bleachery discharge (33). Although chlorocatechols, chloroguaiacols, and chloroveratroles are readily bound to sediments (40), only the first two groups have been recovered from natural sediment samples (40, 30). In experiments designed to elucidate microbial processes taking place in the sediment phase, we showed (40) that in spiked samples, anaerobic de-O-methylation occurred for both chloroguaiacols and chloroveratroles with the formation of the corresponding chlorocatechols. These appeared to be further degraded slowly but their ultimate fate could not be satisfactorily determined from these experiments, which took advantage of the endogenous bacterial flora and the relatively large amounts of organic carbon in the sediments.

We wanted to examine the bacterial transformation of these chlorinated compounds under laboratory conditions simulating as closely as possible those in natural anaerobic sediments rich in organic carbon. Our approach attempted to take into account the following aspects: (i) application of the enrichment methodology using substrates structurally related to those which might occur in natural sediments, (ii) study of metabolic transformations under growth conditions with carbon sources at relatively high concentrations and simultaneous exposure to the xenobiotics at concentrations low enough to simulate those which could occur in a natural aquatic system, and (iii) analysis throughout the experiments of the concentrations of the growth substrates and the xenobiotics together with their principal metabolites. We made no attempt to produce material balances for all of the components of the system and emphasized the delineation of the anaerobic transformations of the xenobiotics.

By anaerobic enrichment of sediment samples with a variety of carbon sources, we have isolated metabolically stable consortia of anaerobic bacteria and have used these to study in detail the transformations of chloroveratroles, chloroguaiacols, and chlorocatechols. Experiments were carried out using concentrations of the growth substrates of ca. 500 mg liter⁻¹, which supported visible growth. The xenobiotics investigated, however, were used at initial concentrations of ca. 100 µg liter⁻¹, which was clearly inadequate to sustain growth. We have shown that under these conditions a rapid de-O-methylation of chloroveratroles and chloroguaiacols took place with the formation of chlorocatechols and that these chlorocatechols were subsequently dechlorinated with the production of isomers which were apparently stable under the experimental conditions.

MATERIALS AND METHODS

Synthesis of substrates. The synthesis of 3,4,5- and 4,5,6-trichloroguaiacol, tetrachloroguaiacol, 3,4,5-trichlorosyringol, 3,4,5-trichloro- and tetrachlorocatechol, and 3,4,5-tri-

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chloro- and tetrachloroveratroles has been described previously (32). Reference samples of 3-chloro-, 3,5-dichloro-, and 3,4,6-trichlorocatechol were kindly provided by J. Knuutinen, University of Jyväskylä, Jyväskylä, Finland. 4,5,6-Trichloropyrogallol was synthesized by refluxing a solution of pyrogallol (1 mol) in diethyl ether with sulfuryl chloride (4 mol) added in portions for 5 h. Water saturated with NaCl was carefully added to the cooled mixture, the ether extract was dried (Na2SO4), and the solvent was removed to give a solid which was recrystallized several times from toluene. The mass spectrum of the trimethylsilyl ether had parent ions (m/e, 444) corresponding to C14H13Cl3O5Si, with the pattern expected (ca. 100:97:32:3) for molecules containing three chlorine atoms.

5-Chlorovanillin (CV) was synthesized from vanillin by chlorination at room temperature with sulfuryl chloride in dichloromethane. The product crystallized from the reaction mixture after ca. 30 min and was recovered by filtration and recrystallized several times from acetic acid. This mixture was contaminated with low concentrations (ca. 0.5%) of 4,5,6-trichloroguaiacol formed by chlorination of guaiacol existing as a minor impurity in the vanillin. A product completely free of 4,5,6-trichloroguaiacol was obtained by converting the CV to its tri-O-acetate (37), crystallizing this to purity from methanol and hydrolyzing the product with methanolic KOH. The free phenol was liberated by acidification and recrystallized from acetic acid. The mass spectrum of the trimethylsilyl ether had a parent ion (m/e, 258) corresponding to C14H12Cl3O5Si.

3-Chloro-4,5-dihydroxybenzaldehyde was prepared by refluxing a solution of CV in acetic acid with an equal volume of 48% hydrobromic acid for 4 h. The reaction mixture was poured into water to yield a black powder, which was dried and extracted exhaustively (Sosklet) with toluene. The solvent was removed, and the product was crystallized from acetic acid as buff-colored needles. The mass spectrum of the trimethylsilyl ether had a parent ion (m/e, 316) corresponding to C14H13Cl3O5Si2.

5-Chlorovanillic acid was prepared by oxidizing CV with freshly precipitated Ag2O under alkaline conditions (35). The product was precipitated in aqueous NaOH solution upon the addition of a chilled solution of NaCl and dried in vacuo. A product which was thought to be a mixture of 5-chloro- and 5-hydroxyvanillin was obtained. The mixture of the trimethylsilyl ether of the methyl ester had a parent ion (m/e, 258) corresponding to C14H12Cl3O5Si.

3-Chloro-4,5-dihydroxybenzoic acid was prepared by refluxing a solution of 5-chlorovanillic acid in acetic acid with an equal volume of 48% hydrobromic acid for 7 h. Solvent was removed in vacuo, and the thick gum was set aside to yield chunky crystals. These were recrystallized from acetic acid. The mass spectrum of the trimethylsilyl ether of the methyl ester had a parent ion (m/e, 346) corresponding to C14H13Cl3O5Si2.

Analytical procedures. We estimate that the analytical procedures gave a repeatability of ca. ±10%. In addition, a large number of sampling points were used in the kinetic experiments. We therefore feel that the level of experimental error was acceptable.

Analysis of 3,4,5-trimethoxybenzoate (TMB), 3,4,5-trihydroxybenzoate (GA), and pyrogallol was carried out by acidifying the sample (0.5 ml) with 0.25 ml 1 M HCl, adding ca. 100 mg of NaCl and 50 μl of the surrogate standard (4-hydroxybenzoate, 1 mg ml−1), and extracting three times (1.5, 1.5, and 1.0 ml) with t-butylmethyl ether or ethyl acetate. The combined extracts were dried (Na2SO4), toluene (0.5 ml) was added, and the solvent was removed under N2 at ca. 40°C. Complete desiccation of the sample was accomplished by adding an additional 200 μl of toluene and evaporating to dryness. The silylating reagent (50 μl of Tri-SiPZ; Pierce Chemical Co., Rockford, Ill.) was added, the mixture was heated at 100°C for 30 min and diluted with 0.5 to 1.0 ml of hexane dried over molecular sieves, and a 1-μl portion was used for analysis by gas chromatography (GC) on a DB-5 (J & W Scientific, Inc., Rancho Cordova, Calif.) megabore column (15 m) with N2 (40 kPa) as the carrier gas and the following temperature program: column, 130°C isothermal for 2 min, increasing at a rate of 15°C min−1 to 210°C; injector, 220°C; and detector, 300°C. A flame ionization detector system was used.

Analysis of chlorocatechols, chlorophenols, chloroguaicols, chloroanisoles, and chloroveratroles was carried out as described previously (1) except that extracts were dried (Na2SO4) before acetylation for 20 min at 75°C, and the 0.05 M K2CO3 in the final extraction was replaced with 0.1 M phosphate buffer (pH 8.0).

Analysis of CV and 3-chloro-4,5-dihydroxybenzaldehyde was carried out as follows. Since acetonitrile reacts with these aldehydes, this solvent could not be used in the extraction procedure, and therefore the following procedure was adopted. The sample (10 to 50 μl) was first acidified with 0.5 ml of 1 M HCl, then ascorbic acid (0.2 ml of a 1 M solution) and ca. 100 mg of NaCl were added. The mixture was extractable with 1.5 ml of hexane—r-butylmethyl ether (1:1) containing pentachlorobenzene and 2,3,4,5-tetrachlorophenol (0.15 μg ml−1 each) as internal standards and then again with the solvent mixture lacking internal standards. The combined extracts were dried (Na2SO4) and acetylated at 75°C for 20 min. After cooling, the organic phase was washed with a 0.8 M K2CO3 solution (4 ml) and the organic phase was used directly for GC analysis using an electronic capture detector as described previously (1).

Analysis of 3-chloro-4,5-dihydroxybenzoate was done by drying the acidified sample (10 to 50 μl) in a desiccator over P2O5 overnight and esterification with BF3 in methanol (14% [wt/wt]) for 30 min at 100°C. A solution of NaCl (2 ml, 10%) was added, and the ester was extracted, acetylated, and analyzed (GC) as described above for CV and its metabolite.

Identification of metabolites. (I) GC methods. Identification of the chloroguaicols (4,5,6-tri- and tetrachloroguaicols), 4,5,6-trichloropyrogallol, the de-O-methylation products from 1,2,3-trichloro-4,5,6-trimethoxybenzene, and the chloroanisoles (2,4-5-, 2,4,6-, 3,4,5-tri-, and 2,3,4,5-tetra chloroanisoles) was carried out by conversion of the metabolites into three independent derivatives and the comparison of the retention times with those of authentic samples. We used the O-acetates and O-trimethylsilyl ethers as described previously (1). Use of the heptafluorobutyrate was not practicable due to interference from products formed by reaction of this highly reactive reagent with amino acids in the growth medium. We therefore converted the chloroguaicols, chlorocatechols, and chlorophenols into their O-ethyl ethers by reaction with diethyl sulfate and tetramethylammonium hydroxide in dichloromethane (38) or, for chlorocatechols and trichloropyrogallol, with diazooethane prepared from N-nitroso-β-ethylaminosobutylmethy ketone by the method of Redemann et al. (39). If necessary, colored material was removed by chromatography on short columns of silica gel (Kieselgel 60, 70/280 mesh; E. Merck AG, Darmstadt, Federal Republic of Germany). Reference
samples of the authentic O-ethyl ethers were prepared in the same way from the appropriate starting materials.

(ii) GC-mass spectrometry methods. Samples (10 to 50 ml) were acidified, extracted with t-butylmethyl ether, dried, and derivatized. Carboxylic acids were first esterified with methanolic BF3 as described above, and phenolic compounds were converted into the trimethylsilyl ethers. Some of the trimethylsilyl ethers were somewhat prone to hydrolysis, so samples were prepared immediately before analysis. Some samples were chromatographed on short columns of silica gel as described above, and partial hydrolysis then necessitated a second trimethylsilylation. Instrumentation and operating conditions were as described previously (32).

Microbiological procedures. All of the experiments were set up in an anaerobe chamber (model 800; Anaerobe Systems, Santa Clara, Calif.) which was continuously connected to a gas mixture of N2:H2:CO2 (90:5:5). Anaerobic conditions were consistently maintained by frequent change of the catalyst (at least twice per week and even more frequently with heavy chamber loading). The prereduced base medium was that designed by Tschech and Pfennig (48) with the addition of Na2SO4 (0.2 g liter⁻¹) and the SL9 trace element mixture was supplemented with selenite, tungstate, and 1 ml of a vitamin mixture per liter that contained thiamine hydrochloride (60 mg liter⁻¹), nicotinic acid (100 mg liter⁻¹), pantothenic acid (100 mg liter⁻¹), riboflavin (10 mg liter⁻¹), pyridoxal (40 mg liter⁻¹), inositol (1,000 mg liter⁻¹), biotin (1 mg liter⁻¹), p-aminobenzoic acid (10 mg liter⁻¹), folic acid (40 mg liter⁻¹), and vitamin B12 (0.4 mg liter⁻¹). Casamino Acids (Difco Laboratories, Detroit, Mich.; 1.0 g liter⁻¹) was added to all of the media except those used for the initial enrichment.

The pH of the base medium containing the trace elements, vitamins, and Casamino Acids, but excluding sulfide and bicarbonate, was adjusted to 3.5 (2.5 in the absence of Casamino Acids), sterilized by autoclaving, cooled to ca. 55°C, and transferred to the chamber. Stock solutions of Na2S · 9H2O (120 g liter⁻¹) and NaHCO3 (64 g liter⁻¹) were prepared in distilled water which had been boiled for at least 10 min and then cooled under a stream of N2. The solutions were sterilized by membrane filtration and transferred to the chamber. Fresh solutions were prepared at least once a week. Growth substrates were prepared as concentrated stock solutions in base medium, the pH was adjusted to 7.2, and the solutions were sterilized either by autoclaving or by membrane filtration. Oxygen-sensitive substrates were prepared as described for the sulfide stock solution. All of the solutions were transferred to the chamber and mixed appropriately under aseptic conditions to provide the final prereduced growth medium. In all of the kinetic experiments, resazurin (1 mg liter⁻¹) was added to indicate that anaerobic conditions had been maintained during incubation. Any colored tubes or ampoules were discarded.

Enrichment experiments were carried out as follows. Sediment samples (15 to 30 g) were transferred to sterile screw-cap bottles fitted with sterilized rubber septa and aluminum caps. The loosely capped bottles were transferred to the chamber, and 50 ml of prereduced medium supplemented with the growth substrate (0.5 g liter⁻¹) was added. The bottles were capped and allowed to stand for 1 to 6 months with periodic transfer (ca. 10% inoculum) into fresh medium. Before kinetic experiments were undertaken, metabolically stable consortia were achieved through at least 10 additional transfers (ca. 4% inoculum). The frequency of transfer depended on the growth substrate (i.e., TMBA, ca. every 10 days; GA, ca. every 14 days; CV, every 2 to 3 weeks). The consortia were characterized only on the basis of microscopy after Gram staining (28).

Kinetic experiments with growing cells. Experiments were done using the consortia described above which metabolized high concentrations (ca. 500 mg liter⁻¹) of the appropriate individual substrate (TMBA, GA, or CV): we consider these to be growth substrates because of their ability to sustain visible growth. Equal growth occurred in the absence of the low concentrations (ca. 100 µg liter⁻¹) of the xenobiotics, which we have termed cosubstrates. At the beginning of each experiment, both the growth substrate and the cosubstrate were present.

In view of the prolonged incubation times used in many of the experiments, it was imperative to establish first that anaerobic conditions were consistently maintained and second that the cultures were viable throughout the experiment. The first was inferred from the lack of visual evidence of oxidation of reduced resazurin, and the second was confirmed by streaking a loopful of the culture at the end of the experiments onto plates of the appropriate medium and demonstrating the presence of viable cells after anaerobic incubation. The concentrations of all of the cosubstrates remained unchanged in uninoculated controls during the experiments.

Experiments with nonvolatile cosubstrates were carried out in anaerobe tubes (2048-00150; Bellco Glass, Inc., Vineyard, N.J.) sealed with butyl-rubber stoppers (2048-11800; Bellco) and aluminum crimp caps. The complete anaerobic medium containing resazurin and the growth substrates (400 to 500 mg liter⁻¹) was allowed to stand in the chamber for at least 16 h after the sulfide and bicarbonate solutions were added. Inoculation was then done using a 6-day-old culture of the appropriate consortium (ca. 2% [vol/vol]), and the cosubstrate was added to yield a final concentration of ca. 100 µg liter⁻¹. Portions (5 ml) of the culture were then transferred to sterile tubes which had also been maintained in the anaerobic chamber for at least 16 h after sterilization by autoclaving. The tubes were capped, removed from the chamber, and then incubated in the dark at 22°C. Duplicate tubes were periodically removed to determine the concentrations of the growth substrate, the cosubstrate, and all of the metabolites. Samples were generally frozen immediately, and several drops of 6 M HCl were added to the frozen contents immediately after opening. Larger volumes (100 ml) were also kept in the chamber and were used to prepare the greater amounts of metabolites necessary for identification (GC and GC-mass spectrometry).

Experiments with appreciably volatile cosubstrates such as chlorinated anisoles and veratroles were carried out in glass ampoules (10 ml). These were covered loosely with aluminum foil and sterilized by autoclaving before transfer to the chamber. It was important that the foil should not sit too tightly over the necks of the ampoules so complete gas exchange could take place and that the ampoules were maintained in the chamber under anaerobic conditions for at least 48 h before use. Cosubstrates with adequate water solubility were prepared as concentrated solutions in the base medium, the pH of which was adjusted to ca. 7, and sterilized by membrane filtration. The final culture medium containing base medium, growth substrate, cosubstrate, and inoculum was prepared in the chamber as described above. For cosubstrates of insufficient water solubility, the compounds were dissolved in t-butylmethyl ether, portions were pipetted into sterile ampoules, and the ether was allowed to evaporate gently at room temperature (some loss of the most-volatile substrates was inevitable). The ampoules were
transferred to the chamber, the complete medium (5 ml) was added, and the necks were temporarily closed with a short length of sterile silicone tubing tightly blocked with a glass bead. The ampoules were removed from the chamber and immediately sealed with a fine oxygen/acetylene flame. Sealing could readily be accomplished under anaerobic conditions since there was a slight excess pressure in the ampoules which prevented the ingress of air. The ampoules were incubated in the dark at 22°C, and the samples were removed periodically as described above.

RESULTS

The TMBA consortium consisted of at least two different groups of bacteria, stout gram-positive rods and curved, thin gram-negative rods, the GA consortium included at least two different types of gram-negative rods, and the CV consortium contained at least three different groups of bacteria: stout gram-positive rods (similar morphologically to those in the TMBA consortium), gram-negative rods, and gram-negative filaments.

The metabolic pathways utilized by the consortia were established from the structures of the intermediate metabolites. These structures were based on the identity of relative GC retention times of derivatives and confirmed by comparing their mass spectra with those of authentic compounds (Fig. 1). The same metabolites were formed in the presence or absence of the cosubstrates, thus supporting our view that growth took place at the expense of the high-concentration substrate.

The kinetics of de-O-methylation of chloroguaiacols during growth of the three consortia with TMBA, GA, and CV are shown in Fig. 2, and a comparison of the rates for the three chloroguaiacols during growth of the TMBA consortium is shown in Fig. 3. Rates for the primary de-O-methylation of both chloroveratroles were virtually identical to the de-O-methylation rate of 3,4,5-trichloroguaiacol. The kinetics of sequential de-O-methylation of tetrachloroveratrole and 3,4,5-trichlorosyringol to the corresponding chlorocatechols during growth of the TMBA consortium are shown in Fig. 4. 3,4,5-Trichloroveratrole formed exclusively 4,5,6-trichloroguaiacol and then 3,4,5-trichlorocatechol, and 1,2,3-trichloro-4,5,6-trimethoxybenzene gave rise to both of the possible dimethoxy and monomethoxy compounds and finally to 4,5,6-trichloropyrogallol. The TMBA consortium was also able to de-O-methylate 2,4,5-, 2,4,6-, and 3,4,5-trichloro- and 2,3,4,5-tetrachloroanisole. The rate for 3,4,5-trichloroanisole was much less than for the other anisoles.
FIG. 2. Kinetics of de-O-methylation of 4,5,6-trichloroguaiacol during growth of consortia with TMBA (A) and GA (B) and of tetrachloroguaiacol with a CV consortium (C). The upper panels illustrate metabolism of the cosubstrates, and the lower figures present the metabolism of the growth substrates. Symbols: ●, 4,5,6-trichloroguaiacol (A and B); ○, tetrachloroguaiacol (C); □, 3,4,5-trichlorocatechol (A and B); △, tetrachlorocatechol (C); ▽, 3,4,6-trichlorocatechol (C); ▼, growth substrates; □, GA (A); □, pyrogallol (B); △, 3-chloro-4,5-dihydroxybenzaldehyde (C); ○, 3-chloro-4,5-dihydroxybenzoate (C); □, 3-chlorocatechol (C), for which the concentrations are 0.1× those on the ordinate axis.

Confirmation of the structures of these metabolites (chloroguaiacols, chlorocatechols, and chlorophenols) was based on the identity of the relative GC retention times with those of authentic samples. We used the following three derivatives for comparison: O-acetate, O-trimethylsilyl ether, and O-ethyl ether (Table 1).

The kinetics of monodechlorination of 3,4,5-trichloro- and tetrachlorocatechol by the TMBA consortium are shown in Fig. 5, and the mass spectra of the metabolites are compared with those of authentic compounds in Fig. 6. The mass spectral analysis conclusively showed the loss of a chlorine atom from the cosubstrates; but since the mass spectra of isomers are virtually identical, the spectral data did not provide evidence of the position from which loss occurred. Unambiguous proof of the structures of the metabolites was provided by the identity of the relative GC retention times of their O-acetates, O-trimethylsilyl ethers, and O-ethyl ethers with those of authentic compounds (Table 1). A comparable level of dechlorination was also demonstrated during growth of the GA consortium.

In an experiment with the TMBA consortium over 23 days, a sufficiently long period for dechlorination of the chlorocatechols, no dechlorination of 2,4,5-, 2,4,6-, or 3,4,5-trichlorophenol or 2,3,4,5-tetrachlorophenol could be demonstrated. Cells at the termination of the experiment had retained both their viability and their metabolic capability since an inoculum from the ampoules that was transferred into fresh medium produced a culture that could both de-O-methylate and dechlorinate 4,5,6-trichloroguaiacol.

DISCUSSION

The concentration of the xenobiotics used here was so low that it was experimentally unrealistic to use them as primary enrichment substrates. All of the experiments were therefore carried out with cells growing at the expense of various aromatic substrates. Transformation of the xenobiotics therefore occurred under conditions of what we have termed "concurrent metabolism" (34). We felt that, under such conditions, the contribution to cell growth from the cosubstrate was insignificant in comparison with that available from the growth substrate. Although we know that the concentrations of the growth substrates (ca. 500 mg liter⁻¹) were probably not representative of those occurring in natural habitats, we did use concentrations of the cosubstrates which more closely approximated those which might be encountered in a natural environment (±100 μg liter⁻¹).

The concurrent-metabolism conditions were adopted for two additional reasons. (i) To study anaerobic transformations, it was an attractive experimental alternative to the use of resting dense-cell suspensions, whose preparation requires centrifugation under sterile and anaerobic conditions. (ii) The procedure introduces a degree of environmental relevance since, under natural conditions, anaerobic sedi-
ments often contain substantial concentrations of organic carbon which could plausibly serve as a growth substrate during transformation of recalcitrant compounds. On the other hand, the concentrations of the anthropogenic compounds are generally substantially lower.

We were primarily interested in anaerobic transformations of substituted veratroles, guaiacols, and catechols, the last two of which have been identified (40, 50) in numerous field samples of sediments from the Baltic Sea and the Gulf of Bothnia. We therefore took advantage of enrichment substrates such as methoxy aromatic acids (2) and trihydroxybenzenes (43), which have been used successfully in studies demonstrating anaerobic de-O-methylation and catechol degradation. Use of CV was motivated by the fact that it is a component—albeit a relatively minor one—in bleaching effluents and was deemed to be susceptible to microbial degradation under anaerobic conditions. Although all of the enrichment cultures yielded metabolically stable consortia, thus far we have not been able to isolate pure strains of the individual components. A comparable situation has been encountered in studies on the methanogenic degradation of catechols and hydroquinone (47). Comparison of transformation rates cannot therefore validly be made between separate experiments. Where comparative rates have been presented (Fig. 3) and in the rates for the chloroveratroles determined here, the data were collected from simultaneous experiments with the same inoculum and carried out under identical conditions. It should also be emphasized that the experiments encompass both growth- and stationary-phase cultures and inevitably involve populations of different microorganisms.

Metabolites produced both from the growth substrates and from the cosubstrates were identified based on extensive comparison (GC and GC-mass spectrometry) with authentic reference compounds, so there can be no reasonable doubt as to their identity.

Since we used consortia in all of our experiments, it is premature to discuss metabolic aspects in detail. Some interesting contrasts emerge, however, between the catabolic pathways established in other investigations and those shown here. The TMBA consortium metabolized the growth substrate initially by the pathway found in Acetobacterium woodii (2), although the GA produced was further degraded without the formation of demonstrable intermediates (Fig. 2A). On the other hand, when GA was used as the growth substrate, the GA consortium decarboxylated this to pyrogallol, which was apparently stable to further degradation even after prolonged incubation (Fig. 2B). This is in sharp contrast to the pathway demonstrated for both Pelobacter acidigallici (42, 43) and Eubacterium oxidireducens (27), in which chlorogluconol and resorcinol were formed. Neither of these compounds was produced by our GA consortium. The metabolic capabilities of the TMBA and GA consortia were therefore different, and the transformations effected by them were distinct from those demonstrated with pure culture utilizing the same substrates. On the other hand, the metabolic pathways closely resembled those initially followed by a methanogenic consortium utilizing syringic acid (22) and by a nonmethanogenic consortium adapted to vanillate and its derivatives (22, 23).

TABLE 1. Retention times of derivatives of cosubstrates and their metabolites relative to that of tetrachloroguaiaicol-O-acetate

<table>
<thead>
<tr>
<th>Substance</th>
<th>Relative retention time</th>
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<tbody>
<tr>
<td></td>
<td>O-Acetate</td>
</tr>
<tr>
<td>4,5,6-Trichloroguiaicol</td>
<td>0.774</td>
</tr>
<tr>
<td>Tetrachloroguiaicol</td>
<td>1.000</td>
</tr>
<tr>
<td>3-Methoxy-4,5,6-trichlorocatechol</td>
<td>1.352</td>
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<tr>
<td>2-Methoxy-4,5,6-trichlororesorcinol</td>
<td>1.371</td>
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<tr>
<td>4,5,6-Trichlorosyringol</td>
<td>1.041</td>
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<tr>
<td>2,3-Dimethoxy-4,5,6-trichlorophenol</td>
<td>0.929</td>
</tr>
<tr>
<td>3-Chlorocatechol</td>
<td>0.448</td>
</tr>
<tr>
<td>3,5-Dichlorocatechol</td>
<td>0.573</td>
</tr>
<tr>
<td>3,4,5-Trichlorocatechol</td>
<td>0.936</td>
</tr>
<tr>
<td>3,4,6-Trichlorocatechol</td>
<td>0.783</td>
</tr>
<tr>
<td>Tetrachlorocatechol</td>
<td>1.308</td>
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<tr>
<td>4,5,6-Trichloropyrogallol</td>
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<td>2,4,5-Trichlorophenol</td>
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<td>2,4,6-Trichlorophenol</td>
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<td>3,4,5-Trichlorophenol</td>
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<tr>
<td>2,3,4,5-Tetrachlorophenol</td>
<td>0.684</td>
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</table>

* TMS, Trimethylsilyl.
Although methanogenic consortia utilizing a variety of aromatic compounds have been extensively studied (15, 17, 19, 20, 22, 47, 51), we found only traces of methane in the gas phase of the TMBA consortium, even though substantial concentrations of acetate could be identified in the culture medium (data not shown). Thus methane formation from acetate and carboxylic acids produced by cleavage of aromatic rings appears to be a minor activity in our TMBA consortium.

The anaerobic degradation of CV proceeded by a series of reactions (Fig. 7). The oxidation of the aldehyde group in complex vanillins during methanogenic transformation by rumen bacteria (7, 8) is only formally similar and is certainly mechanistically different.

Results from the kinetic experiments with TMBA, GA, and CV as growth substrates showed that rapid de-O-methylation of the cosubstrates occurred in early growth phases, during which only low cell densities had been attained (Fig. 2). For TMBA and CV, this reaction was involved in their catabolism. We therefore suggest that the metabolism of the growth substrates and that of the cosubstrates were interdependent. De-O-methylation, however, also took place during growth at the expense of GA, which did not require this capability for its degradation.

![Graphs showing kinetics of monodechlorination](image)

**FIG. 5.** Kinetics of monodechlorination of chlorocatechols during growth of a consortium with TMBA. (A) Symbols: ●, 3,4,5-trichlorocatechol; ○, 3,5-dichlorocatechol. (B) Symbols: ▲, tetrachlorocatechol; △, 3,4,6-trichlorocatechol.

![Mass spectra comparison](image)

**FIG. 6.** Comparison of mass spectra of O-trimethylsilyl ethers of dechlorination products from chlorocatechols with those of authentic compounds.
FIG. 7. Postulated metabolic sequence for the degradation of CV by a consortium of anaerobic bacteria.

Polymethoxy compounds were sequentially—and with 3,4,5-
trichloroveratrole, discriminately—de-O-methylated to
catechols (Fig. 4). Microbial de-O-methylation under anaer-
obic conditions has been established for a variety of aro-
matic methoxy compounds lacking chlorine substituents (2,
11, 16, 22, 26, 31), so this activity must be widely distributed.
All of the catechols were apparently stable to further
transformation until catabolism of the growth substrate
was essentially complete (Fig. 2). Only then did dechlorination
begin, and only then was it accomplished effectively by all three
consortia. 3,5-Dichloro- and 3,4,6-trichlorocatechol
appeared as stable end products (Fig. 5). In our experiments
with TMBA as a growth substrate, dechlorination occurred
only with chlorocatechols and was apparently quite specific
in terms of the position of the chlorine atom reduced: none of
the chlorophenols was dechlorinated by the consortium we
studied. The dechlorination appears therefore to differ sig-
nificantly from that examined by other workers (4, 14, 21, 29,
30, 44, 45).

The bacterial transformations described here took place
under anaerobic conditions. Both de-O-methylolation (5, 9, 36,
49) and dechlorination (10, 18, 41) have nonetheless been
observed in strictly aerobic bacteria. The de-O-methylation,
however, is clearly different in the two groups of organisms.
The aerobic reaction is carried out by an oxygenase (3, 6, 46)
with the formation of products at the oxidation level of
formaldehyde, whereas the anaerobic reaction results in the
synthesis of acetate with the methyl group intact (2, 12). On
the other hand, dechlorination is presumably mediated by a
reductase which might be common to both aerobic and
anaerobic microorganisms.

The results of this investigation confirm and supplement
those from a previous study (40), in which natural-sediment
samples and their endogenous microflora were used. The
compounds from all of these experiments may be summa-
ized as follows: de-O-methylation of chloroveratroles and
chloroguaiacols is a relatively rapid reaction which produces
chlorocatechols as primary metabolites. The laboratory
experiments also showed that dechlorination of chlorocate-
chols occurs as a secondary reaction. All of these results are
consistent with the presence of chlorocatechols (40, 50) in
natural-sediment samples. On the other hand, chlorogua-
iacols have also been identified (40, 50), and we therefore
hypthesize that these may be sorbed to the sediment in a
way which shields them from microbial transformation. We
have previously summarized data emphasizing the environ-
mental significance of sediment-substrate interactions (34),
and the recent studies of Garbarini and Lion (13) underscore
the importance of the chemical constitution of sediment
organic material. Even though the structures of the com-
pounds used by these workers differ substantially from the
compounds examined in this study, we feel that this is an
important question, whose magnitude—together with its
effect on the bioavailability to sediment-dwelling biota—
must be taken into consideration in environmental-hazard
assessments.

Two important and hitherto unresolved issues remain to
be investigated: (i) the ultimate fate of chlorocatechols in
anaerobic sediments, and (ii) whether the range of organic
compounds plausibly existing in sediments could support
growth of microorganisms with the catabolic activities
shown in the present study. We suggest that the procedures
developed here provide a suitable methodology for solving
these problems. The successful isolation of pure cultures
would not only make possible a description of the relevant
catabolic pathways, but also reveal the nature of the meta-
abolic interactions between components of the consortia (24).

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