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Transformation of Toluene and Benzene by Mixed Methanogenic Cultures

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The aromatic hydrocarbons toluene and benzene were anaerobically transformed by mixed methanogenic cultures derived from ferulic acid-degrading sewage sludge enrichments. In most experiments, toluene or benzene was the only semicontinuously supplied carbon and energy source in the defined mineral medium. No exogenous electron acceptors other than CO_2 were present. The cultures were fed 1.5 to 30 mM unlabeled or ^{14}C-labeled aromatic substrates (ring-labeled toluene and benzene or methyl-labeled toluene). Gas production from unlabeled substrates and ^{14}C activity distribution in products from the labeled substrates were monitored over a period of 60 days. At least 50% of the substrates were converted to CO_2 and methane (>60%). A high percentage of $^{14}CO_2$ was recovered from the methyl group-labeled toluene, suggesting nearly complete conversion of the methyl group to CO_2 and not to methane. However, a low percentage of $^{14}CO_2$ was produced from ring-labeled toluene or from benzene, indicating incomplete conversion of the ring carbon to CO_2. Anaerobic transformation pathways for unlabeled toluene and benzene were studied with the help of gas chromatography-mass spectrometry. The intermediates detected are consistent with both toluene and benzene degradation via initial oxidation by ring hydroxylation or methyl oxidation (toluene), which would result in the production of phenol, cresols, or aromatic alcohol. Additional reactions, such as demethylation and ring reduction, are also possible. Tentative transformation sequences based upon the intermediates detected are discussed.

The influence of oxygen, either as an electron acceptor or as a substituent, on the degradation of aromatic compounds is well understood. Aerobic (oxygenative) degradation of toluene, benzene, and xylenes has been extensively studied and summarized (9, 11, 17, 18, 28, 29, 40). Under anaerobic conditions, oxygen-substituted aromatic compounds such as aromatic acids (3, 13, 14, 15, 22, 23, 26, 31, 32, 37, 52–54), aldehydes (20, 25), alcohols (19), phenols and catechol (14, 24, 48, 54), trihydroxylated benzenoids (38, 43), and halogenated aromatic acids and phenols (7, 27, 44, 46, 47) are all degraded. The anaerobic transformation reactions include anaerobic oxidation of alcohols to aldehydes and acids, O demethylation, dehydroxylation, addition of water across a double bond in side chains, reduction of the aromatic ring, dehydrogenation, and ring cleavage. The major intermediates are usually benzoic acid, phenol, cyclohexanone, and acetate.

Little is known about the anaerobic fate of aromatic hydrocarbons like toluene, benzene, or xylenes, which contain no oxygen in their molecular structure. These aromatic hydrocarbons are generally believed to be resistant to microbial attack in the absence of oxygen. However, factors other than the lack of oxygen might be important for anaerobic degradation of aromatic compounds to occur (1).

Recently, aromatic hydrocarbons were reported to degrade under anaerobic conditions. Reinhard et al. (39; M. Reinhard, J. W. Graydon, and N. L. Goodman, Proceedings of the Second International Conference on Groundwater Quality Research, Tulsa, Oklahoma, 1984, p. 69–71) noted the selective removal of xylenes from a landfill leachate plume in an anaerobic aquifer. This selective removal was not entirely attributed to adsorption and dispersion. Rees et al. (J. F. Rees, B. H. Wilson, and J. T. Wilson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, Q5, p. 258) observed the conversion of ^{14}C-labeled toluene to $^{14}CO_2$ and labeled nonvolatile intermediates under methanogenic conditions in microcosms containing aquifer material. Battermann and Werner (4) described the anaerobic degradation of toluene, benzene, and smaller amounts of xylenes in a contaminated aquifer after injection of nitrate, as electron acceptor. Kuhn et al. (35) noted the biological degradation of xylenes under denitrifying conditions in the field and in laboratory aquifer columns simulating a river water-groundwater infiltration system. Recent work by Vogel and Grbić-Galić (51) indicated that toluene and benzene are oxidized to the corresponding phenols under methanogenic conditions. The oxygen for ring hydroxylation is apparently derived from water. However, Schink (42) observed no anaerobic transformation of benzene, toluene, xylene, styrene, or naphthalene under methanogenic conditions by enrichment cultures from anaerobic sewage sludge, freshwater sediments, or marine sediments.

In the present paper, the concomitant degradation of ^{14}C-labeled toluene and benzene and the production of $^{14}CO_2$ under methanogenic conditions are described. The results of gas chromatography-mass spectrometry (GC-MS) of intermediates formed from unlabeled toluene and benzene during incubation of mixed methanogenic cultures are also discussed. Possible degradation sequences are proposed.

MATERIALS AND METHODS

Chemicals. ^{14}C-labeled toluene and benzene were obtained from Amersham Corp., Arlington Heights, Ill. The specific activity of the [*methyl*-^{14}C]toluene was 2.3 mCi/mmol (purity, 97%), and that of the ring-labeled toluene was 16.4 mCi/mmol (purity, 95%). The specific activity of benzene was 121.0 mCi/mmol (purity, 99.3%). Unlabeled toluene was Packard Toluene Puresolv (scintillation grade; Packard Instrument Co., Inc., Downers Grove, Ill.), and benzene

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(99.4%) was from J. T. Baker Chemical Co., Phillipsburg, N.J.

Culture procedures. The mixed methanogenic inoculum was derived from stable methanogenic consortia that degrade ferulic acid to CO_2 and methane and that had been originally enriched from sewage sludge (22). These consortia were maintained with ferulic acid as the sole carbon and energy source for 5 years semicontinuously (they were fed 1.5 mM substrate monthly). The inoculum (10%, vol/vol) was taken from ferulic acid cultures at the end of an incubation period, after the substrate had been degraded, to minimize the concentration of organic substances in the inoculum as determined by GC-MS, GC, UV spectrophotometry, and the absence of CO_2 and CH_4 production. No ring condensation products, aromatic compounds, aliphatic alcohols, or acids were found in the inoculum before these experiments were started. The inoculum was injected into 216-ml serum bottles (stoppered with red rubber sleeve serum stoppers [Fisher Scientific Co., Pittsburgh, Pa.]) along with 150 ml of defined mineral salts medium with reducing agents (ferrous chloride plus sodium sulfide), ammonium phosphate as a nitrogen source, vitamins, as described previously (24, 26), and unlabeled toluene and benzene dissolved in 1 ml of methanol in concentrations of 15 to 30 mM. Toluene or benzene and methanol were the only organic carbon sources added and were injected in the serum bottles with a 1-ml sterile glass syringe (series 1000 Hamilton with a Teflon Luer lock). These cultures were maintained on toluene or benzene plus methanol for 9 months before the onset of GC-MS of the intermediates and products of the anaerobic transformation and for 1 year before the onset of ^{14}C labeling experiments. During that time, the cultures were refed every 2 months. They were incubated in a 30% CO_2–70% N_2 gas atmosphere at 35°C in the dark. Repeated measurements with ^{14}C-labeled toluene and benzene showed that 39 ± 8% of the substrates would dissolve into the rubber stoppers (70 stoppers were analyzed) during incubation. To avoid any introduction of oxygen into the cultures, we did not use Teflon-coated rubber stoppers. Previous testing of these stoppers showed fast oxidation (in several hours) of anaerobic media, whereas no oxidation was noticed with plain rubber stoppers.

Experiments with unlabeled substrates. For GC-MS of intermediates and products of the anaerobic transformation of benzene and toluene, the subcultures were fed 15 or 30 mM substrate, respectively, and dissolved in methanol as described above. The high substrate concentrations were used to facilitate the detection of intermediates. Methanol appeared to inhibit toluene and benzene transformation (data not shown). Consequently, addition of methanol was also used to increase the concentrations of intermediates. Two parallel benzene and six parallel toluene cultures were studied. Sterile chemical controls without inocula and autoclaved biological controls (two of each for benzene and for toluene) were also established and monitored. During the incubation period, culture fluid for GC-MS was sampled with disposable syringes seven and eight times for benzene and toluene, respectively. The cultures were incubated until all the initial substrate disappeared, as determined by GC-MS.

Experiments with labeled substrates. The subcultures for labeling experiments were fed mixtures of unlabeled and ^{14}C-labeled toluene or benzene in concentrations of 1.5 or 3 mM. Both methyl-labeled and ring-labeled toluenes were used separately in different sets of serum bottles. Individual series of three cultures were used for each concentration (1.5 and 3 mM) of each labeled substrate. The total initial ^{14}C activities were 38,000 ± 2,000, 450,000 ± 16,000, and 770,000 ± 30,000 dpm/mmol of substrate for [*methyl*-^{14}C]toluene, [*ring*-^{14}C]toluene, and [-^{14}C]benzene, respectively. [*methyl*-^{14}C]toluene dissolved in methanol (0.1 ml/100 ml) was added to the cultures. No [*methyl*-^{14}C]toluene was used without methanol. [*ring*-^{14}C]toluene or benzene was used either as the sole carbon source (without methanol) or in combination with methanol (0.5 ml of methanol/100 ml of culture). Two types of controls (two replicates of each type) were established for each concentration of each labeled substrate with and without methanol, namely, sterile chemical controls without inoculum and sterile controls with autoclaved inoculum (additionally treated with 100 mg of mercuric chloride per liter.) The cultures were incubated for 60 days as described above. Gas production was measured with a glass syringe. Gas composition was determined with a Fisher-Hamilton gas partitioner (model 25 V) on a separate series of duplicate cultures fed only unlabeled toluene or benzene with and without methanol. These cultures were started with the same inoculum as the labeled cultures, run simultaneously, and sampled weekly.

^{14}C-activity measurements. The ^{14}C activity in culture fluid and in the headspace was measured with the Tri-Carb liquid scintillation system (no. 4530; Packard Instrument Co.). Samples were mixed with xylene-based scintillation liquid (ACS; Amersham). Corrections for counting efficiency were made by the channels ratio method (5), resulting in a minimum detectable activity of 3.0 dpm at the 97.5% confidence level (2). The counting efficiency was about 94%. Quenched standards were run weekly to produce a standard curve used to correct for quenching. Deionized water in scintillation liquid (blank) was measured to determine the background activity, which was then subtracted from the results of all other measurements. ^{14}C activity in the liquid phase was determined from the supernatant fluid of undisturbed cultures and of vigorously shaken cultures to measure any of the substrate sorbed to iron sulfide precipitate. Four different measurements were performed on each sample. The first three measurements were done to both the gas phase and the liquid phase separately. First, total ^{14}C activity was measured; 1 ml was added to 10 ml of scintillation liquid. This measurement is the sum of ^{14}C activity of substrate, volatile intermediates, nonvolatile intermediates, CO_2, and CH_4. Second, 1 ml was injected into 2 ml of 1 N NaOH and stripped with nitrogen gas (250 ml/min) for 5 min, after which 10 ml of scintillation liquid was added. This measurement represents the ^{14}C activity of nonvolatile intermediates and carbon dioxide. Third, 1 ml was injected into 2 ml of 1 N hydrochloric acid and stripped with nitrogen gas (250 ml/min) for 5 min, after which 10 ml of scintillation liquid was added. This measurement represents the ^{14}C activity of nonvolatile intermediates only. $^{14}CO_2$ was calculated by the difference between the results of NaOH-treated and HCl-treated samples. Fourth, each rubber stopper from culture and control bottles was shredded at the end of incubation and placed in 10 ml of scintillation liquid. This measurement estimates the amount of substrate dissolved in the rubber stoppers. The background values determined for the controls in all four types of measurements were used to correct the results from the culture measurements.

Analytical procedures. Benzene, toluene, and intermediates of their transformation were identified and quantitated with a Finnigan MAT 4000 GC and 4500 MS with an INCOS data system, by using selective masses. Samples of culture fluid were extracted with diethyl ether under acidic conditions as follows. Samples (5 ml) were acidified with 6 drops

of 1 N HCl and extracted with 1 ml of ether by shaking (by hand) vigorously for 2 min. One microliter of the extract was injected splitless (for 30 s) onto a 60-m DB-5 fused silica capillary column with a 0.32-mm internal diameter and 1.0-μm film thickness. Helium was used as the carrier gas. The initial column temperature was 60°C and was increased to 250°C at a rate of 4°C/min. The injector temperature was 250°C, and the ionizer temperature was 140°C. The forepressure of the column was adjusted to 7.5×10^4 Pa. The scanning rate was approximately 1 scan per s, with an ionization voltage of about 1,500 V. Identifications of the unknowns were based on comparison with a library of known spectra. The external standard procedure was used for quantitation. Only the most abundant intermediates (benzyl alcohol, benzaldehyde, benzoic acid, *o*-cresol, *p*-cresol, 2-hydroxybenzoic acid, benzene, phenol, methylcyclohexane, and aliphatic acids [heptanoic, hexanoic, pentanoic, butanoic, and propanoic]) were run as standards and quantitated.

RESULTS

During the preexperimental period in which the cultures derived from ferulate enrichment were maintained on unlabeled toluene or benzene, an initial lag of 4, 11, and 16 days before the onset of gas production occurred with 1.5 mM toluene and benzene, 15 mM benzene, and 15 mM toluene, respectively. These results are consistent with observations by Schink (41) that toluene and benzene moderately inhibit methanogenesis. After 3 months, our cultures acclimated to the substrates to the extent that the transformations started immediately after the cultures were fed. By the time the experiments were started, no lag was evident. The cultures converted >50% of the available substrate (as determined by GC-MS and GC) to gases, and the percentage of methane in the produced gas was 60.3 ± 2.2% and 65.5 ± 3.4% for benzene- and toluene-fed cultures, respectively, which is close to the theoretically expected values (62.5 and 64.3%, respectively). The theoretical values are based on the following stoichiometric equations for the degradation of benzene and toluene to CO_2 and CH_4 (8): $C_6H_6 + 4.5\ H_2O \rightarrow 2.25\ CO_2 + 3.75\ CH_4$ and $C_7H_8 + 5\ H_2O \rightarrow 2.5\ CO_2 + 4.5\ CH_4$.

Labeling experiments. ^{14}C activity measurements showed no difference between undisturbed and shaken culture fluids, which suggests that very little substrate adsorbed to either the FeS or the cells. Measurement of the activity in shredded rubber stoppers at the end of incubation, however, indicated that 39 ± 8% of the activity was lost into the stoppers. The rest of the activity remained in the gas or liquid phase in the culture bottle, and the $^{14}CO_2$ fraction increased during incubation. Neither the chemical nor the autoclaved biological controls showed any increase of the $^{14}CO_2$ fraction. The data for the total ^{14}C activity in the gas and liquid phases and in the stoppers and for the $^{14}CO_2$ for each substrate concentration are summarized in Table 1. The [*methyl*-^{14}C]toluene cultures produced the highest percentage of $^{14}CO_2$. Methanol-amended ring-labeled cultures produced less $^{14}CO_2$ over the 60-day incubation than did the corresponding cultures without methanol.

On the basis of gas partitioning measurements for the cultures fed unlabeled substrates, up to 12.5% of the nondifferentiated volatile fraction activity (substrates plus volatile intermediates and methane) in the ring-labeled toluene- and benzene-fed cultures could be ascribed to methane. Increased activity in the nonvolatile intermediate fraction was found only with [*methyl*-^{14}C]toluene-fed cultures. With cultures fed ring-labeled toluene and benzene, the ^{14}C activity of the nonvolatile fraction was the same as that of the controls.

Intermediates and products from unlabeled substrates. In 2 days of incubation, the initial toluene level (30 mM) was reduced by 75 ± 3% (to 7.5 ± 0.9 mM), which was greater than the loss due to sorption into rubber stoppers (39 ± 8%). During the same time period, several intermediates, including mainly *p*- and *o*-cresol, benzoic acid, 2-methylcyclohexanol, and hexanoic acid, were detected by GC-MS. Transformation of benzene was slower, so that the original concentration (15 mM) was reduced to only 66 ± 2% (to 5 ± 0.3 mM) in 4 days, with concomitant production of phenol (20 ± 2 μM on day 4). However, complete disappearance of benzene and toluene occurred after 34 and 64 days, respectively. Strictly anaerobic conditions were preserved throughout the incubation, as indicated by resazurin (a redox indicator). No intermediates were detected in sterile chemical or biological controls. Carbon dioxide and methane were the final products of fermentation.

Intermediate products detected in the toluene-fed cultures during a 64-day incubation period are shown in Fig. 1. A total of 8 aromatic, 5 alicyclic, and 10 aliphatic compounds were identified. The appearance and disappearance of three key aromatic intermediates which are likely to be formed through anaerobic oxidation of toluene (*p*- and *o*-cresols and benzoic acid) are shown in Fig. 2. The concentration of *p*-cresol reached 38 ± 4 μM on the day 8 of incubation. The cresols appeared in the culture fluid before benzoic acid did, which might indicate that they were precursors of benzoate. All three key aromatic compounds virtually disappeared by the end of incubation. Other aromatic compounds, like benzene and phenol, were observed to be ≤5 μM throughout the incubation period. All the detected aromatic and alicyclic compounds decreased considerably towards the end of incubation, with the exception of two alicyclic compounds, methylcyclohexane and cyclohexene. These two compounds were seen only after day 57 and possibly represent products of partial substrate reduction. The concentration of methylcyclohexane at the end of incubation was 4 ± 0.2 μM. No dihydroxylated rings or quinones were found. Among the ring cleavage products, propanoic acid reached 50 ± 5 μM on day 26 of incubation in a culture fed 30 mM toluene, dropped to 26 ± 3 μM on day 57, and dropped to 11 ± 2 μM on day 64. Total straight-chain aliphatic acids (propanoic through hexanoic), presumably produced from benzoic acid, reached a maximum concentration of 210 ± 11 μM carbon on day 26 of incubation. Methylated aliphatic acids, presumably produced directly from cresols, reached only 57 ± 3 μM total carbon on day 26. All the aliphatic acids disappeared or decreased considerably by the end of incubation.

In the cultures fed benzene and methanol, phenol was found to be the major aromatic intermediate. Additional compounds detected were cyclohexanone and propanoic acid (data not shown).

DISCUSSION

Methanogenic cultures derived from ferulate enrichment were shown to partially mineralize toluene and benzene to carbon dioxide and methane. The degradation mechanism was biotic and not due to constituents of microbial cells which remained unaffected after sterilization by heat or mercuric chloride. Higher levels of $^{14}CO_2$ were produced

TABLE 1. ^{14}C activity at the beginning and end of 60-day incubation[a]

Substrate[b]	% ^{14}C activity (mean ± SD)			
	Total final activity[c]	Final activity[d]	Initial CO_2	Final CO_2
1.5 mM [*methyl*-^{14}C]T + M				
Culture	96 ± 4.3	51 ± 0.38	0.04 ± 0.02	5.8 ± 2.5
Control	98 ± 2.7	52 ± 3.8	0.03 ± 0.03	0.005 ± 0.005
3 mM [*methyl*-^{14}C]T + M				
Culture	98 ± 0.93	52 ± 0.43	0	15 ± 1.1
Control	102 ± 5.6	65 ± 3.5	0	0
1.5 mM [*ring*-^{14}C]T				
Culture	98 ± 2.0	62 ± 6.0	0	3.6 ± 0.69
Control	99 ± 2.0	53 ± 4.0	0.11 ± 0.11	0
3 mM [*ring*-^{14}C]T				
Culture	98 ± 2.0	59 ± 13	0.01 ± 0.002	4.5 ± 0.74
Control	96 ± 4.6	61 ± 10	0.09 ± 0.09	0
1.5 mM [*ring*-^{14}C]T + M				
Culture	99 ± 1.2	54 ± 12	0	1.3 ± 0.12
Control	100 ± 0.90	70 ± 0.9	0	0
3 mM [*ring*-^{14}C]T + M				
Culture	92 ± 5.9	57 ± 6.8	0.008 ± 0.001	1.0 ± 0.1
Control	96 ± 4.6	61 ± 4.7	0.04 ± 0.004	0
1.5 mM [^{14}C]B				
Culture	103 ± 3.3	68 ± 1.2	0.002 ± 0.0002	5.8 ± 0.36
Control	100 ± 1.6	68 ± 2.3	0.25 ± 0.25	0.1 ± 0.1
3 mM [^{14}C]B				
Culture	95 ± 3.1	64 ± 3.0	0.85 ± 0.044	5.4 ± 0.28
Control	97 ± 2.9	57 ± 5.2	0.39 ± 0.22	0.17 ± 0.17
1.5 mM [^{14}C]B + M				
Culture	94 ± 6.3	53 ± 4.8	0.25 ± 0.019	2.6 ± 0.22
Control	95 ± 4.0	60 ± 1.2	0	0.009 ± 0.009
3 mM [^{14}C]B + M				
Culture	102 ± 2.1	56 ± 3.1	0.05 ± 0.003	2.0 ± 0.2
Control	99 ± 1.3	61 ± 6.3	0.15 ± 0.15	0.03 ± 0.04

[a] Background counts were determined on deionized water in scintillation liquid and ranged from 28.5 to 31.7 dpm; they were subtracted from all the other counts. The values shown were from three parallel cultures and four controls. The values for the controls are the combined results from the two chemical and two biological (sterilized) controls, because there was essentially no difference in behavior of these two types of controls. The carbon dioxide counts were calculated by the difference in the measurements of NaOH-treated and HCl-treated samples.

[b] T, Toluene; M, methanol; B, benzene.

[c] In the liquid and gas phases and in the stopper. Each individual stopper was related to the corresponding culture.

[d] In the liquid and gas phases.

from methyl-labeled toluene than from ring-labeled toluene and benzene, which indicates that the methyl carbon is probably ultimately converted to carbon dioxide and not to methane. The aromatic ring might be the source of methane being produced; however, $^{14}CH_4$ was not measured.

The presence of methanol in the culture fluid seems to have slowed down the transformation of toluene or benzene (Table 1). Consequently, the role of methanol as a possible electron acceptor under these fermentation conditions is unlikely. Since no other exogenous electron acceptors but CO_2 (and possibly also very low concentrations of nondetectable organic compounds introduced with the inoculum) were present, the anaerobic transformation of toluene or benzene might be characterized as a fermentation in which the substrates are partially oxidized and partially reduced. On the basis of the compounds detected in the culture fluid (Fig. 1), the oxidations of the substrates might include both methyl group oxidation (toluene) and ring oxidation (toluene and benzene). Reduction of the substrates resulted in the production of saturated alicyclic rings. Benzyl alcohol might be the primary product of methyl oxidation; it is also the likely source of benzaldehyde and benzoic acid which were found during incubation. A similar sequence of reactions has already been shown for the anaerobic transformation of *p*-cresol under sulfate-reducing (W. J. Smolenski and J. M. Suflita, Abstr. Annu. Meet. Soc. Environ. Toxicol. Chem., 1985, p. 21) and nitrate-reducing conditions (I. Bossert, G. Whited, D. T. Gibson, and L. Y. Young, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K185, p. 224). Benzoic acid is a precursor for ring reduction and cleavage (14, 15, 22, 32, 53). Therefore, the ring-reduction products, as well as the aliphatic compounds which were found in the culture fluid, might be the results of benzoic acid degradation (Fig. 1).

The detection of cresols, probably as the result of ring oxidation, was consistent with the ^{18}O-labeled water experiments previously reported (51). Formation of both *p*- and *o*-cresols might be due to the very complex consortium which contains several different forms of fermentative bacteria, including facultative anaerobes (21, 22). Theoretically,

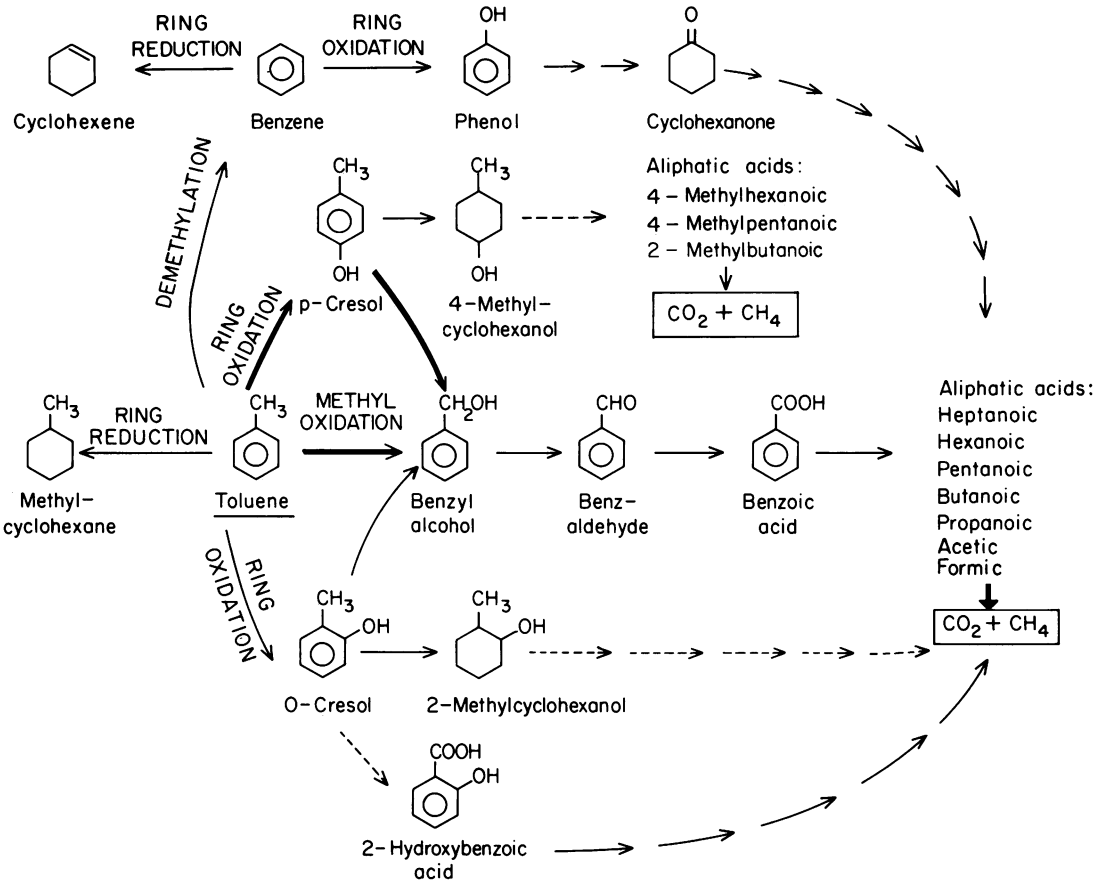

FIG. 1. Tentative sequences of anaerobic toluene degradation by mixed methanogenic cultures. All the compounds shown were detected and identified by GC-MS.

p-cresol would be preferentially formed because of electron-releasing capabilities of the methyl substituent on the toluene ring, as shown for electrophilic aromatic substitution in abiotic systems (16). This preference could explain why higher concentrations of *p*-cresol than of *o*-cresol were found (Fig. 2). Enzymes which introduce hydroxy groups from water into heterocyclic aromatic rings containing nitrogen were previously found in the anaerobic bacteria *Clostridium barkeri* and *Desulfococcus niacini* (29, 30, 45, 50). However, benzene and toluene, with the oxidation potentials of 1.9 and 2.4 V, respectively (49), are more stable than nitrogen heterocyclic compounds and might have to be converted to unstable arene cation radicals to make the nucleophilic attack by water possible. The formation and the hypothesized fate of *p*- and *o*-cresols are shown in Fig. 1. The aliphatic acidic products are assumed to be degraded to methane precursors by hydrogen-producing acetogenic bacteria which are present in mixed methanogenic cultures of this type (6, 36). According to the data presented, a portion of the substrate is completely degraded to CO_2 and CH_4.

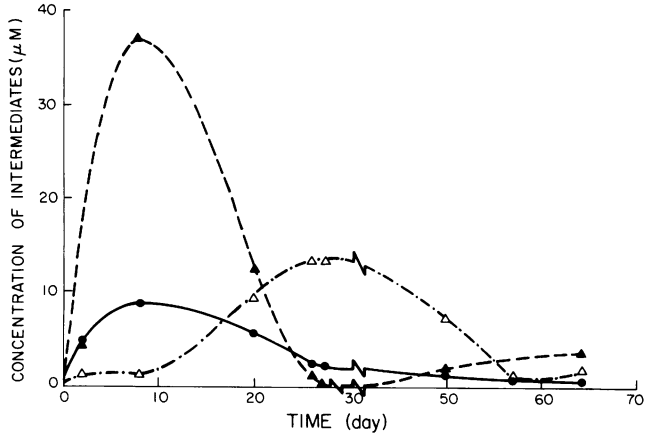

FIG. 2. Transient appearance of the intermediates *p*-cresol (▲), *o*-cresol (●), and benzoic acid (△) (micromolar concentrations) in methanogenic cultures that transformed toluene. The values shown are the means of three replicate cultures.

It must be emphasized that the sequences shown in Fig. 1 are only speculative, that they indicate a complexity of reactions possibly occurring in a mixed culture system, and that they were deduced from the compounds detected in the fluid of the anaerobic cultures transforming toluene. *p*-Cresol transformation under anaerobic conditions was already reported by Bossert et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1986.). Anaerobic transformation of toluene and benzene was documented by Vogel and Grbić-Galić (51). It is important to note that dihydroxylated intermediates typical for the aerobic transformation of toluene (10, 33) were never observed.

The tentative pathway for benzene degradation by meth-

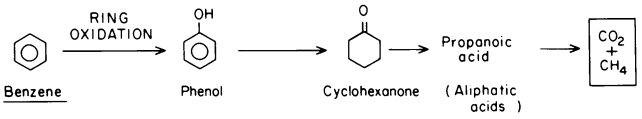

FIG. 3. Tentative sequences of anaerobic benzene degradation in mixed methanogenic cultures. All the compounds shown were detected and identified by GC-MS.

anogenic cultures is presented in Fig. 3 and is based on the finding of phenol in these experiments and the results of the described ^{18}O-labeling experiments (51). Again, a dihydroxylated intermediate (catechol) typical for the aerobic degradation of benzene (12, 17) was not found.

Anaerobic microbial populations which are acclimated to utilization of aromatic derivatives of lignin degradation might have a significant and yet not completely evaluated potential for degradation of aromatic hydrocarbons. Therefore, these organisms could play an important role in determining the fate of petroleum derivatives in anaerobic environments like organic-rich sediments and sewage sludge. Anaerobic microbial transformations of aromatic hydrocarbons might be facilitated in complex natural environments by the presence of other substrates and oxidants, including metals and metal centers (34).

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