Anaerobic Degradation of Alkylated Benzenes in Denitrifying Laboratory Aquifer Columns

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Toluene and m-xylene were rapidly mineralized in an anaerobic laboratory aquifer column operated under continuous-flow conditions with nitrate as an electron acceptor. The oxidation of toluene and m-xylene was coupled with the reduction of nitrate, and mineralization was confirmed by trapping $^{14}$CO$_2$ evolved from $^{14}$C-ring-labeled substrates. Substrate degradation also took place when nitrous oxide replaced nitrate as an electron acceptor, but decomposition was inhibited in the presence of molecular oxygen or after the substitution of nitrate by nitrite. The m-xylene-adapted microorganisms in the aquifer column degraded toluene, benzaldehyde, benzoate, m-tolualdehyde, m-toluate, m-cresol, p-cresol, and p-hydroxybenzoate but were unable to metabolize benzene, naphthalene, methylecyclohexane, and 1,3-dimethylecyclohexane. Isotope-dilution experiments suggested benzoate as an intermediate formed during anaerobic toluene metabolism. The finding that the highly water-soluble nitrous oxide served as electron acceptor for the anaerobic mineralization of some aromatic hydrocarbons may offer attractive options for the in situ restoration of polluted aquifers.

Groundwater aquifers near infiltration zones of polluted rivers or in the proximity of leachate plumes from landfills are usually characterized by high microbial activity and an oxygen deficit. Such aquifers often change from aerobic to denitrifying, sulfate reducing, and eventually methanogenic conditions (4, 10, 19, 23).

The microbial degradation of many pollutants depends largely on the availability of molecular oxygen and on the redox conditions prevailing in the aquifer. Some xenobiotics, such as chlorinated phenols and benzoates, are metabolized under both aerobic and anaerobic conditions, while chlorinated C$_1$ and C$_2$ compounds like chloroform and tetrachloroethylene are preferentially degraded in the absence of molecular oxygen at low redox potentials (4, 5, 8, 12, 34, 39).

The aerobic mineralization of aromatic hydrocarbons such as benzene, toluene, and xylene is well documented, and the catabolic enzymes have been described in detail (13–15, 35). Toluene and xylene, for instance, are initially converted via alcohols and aldehydes to benzoate and toluate, respectively. In contrast, metabolism of such compounds in the absence of molecular oxygen has never been demonstrated under pure culture conditions, and only data from field studies in polluted aquifers suggest that a slow degradation may be possible (27, 38). Only very recently, clear evidence for an anaerobic mineralization of benzene and alkylated benzenes has been presented. Wilson et al. (37) found mineralization of $^{14}$C-toluene to $^{14}$CO$_2$ in methanogenic aquifer material incubated in the laboratory. Grbić-Galić and Vogel (16, 17, 36) reported degradation of $^{14}$Cbenzene and $^{14}$C-toluene to $^{14}$CO$_2$ in a methanogenic microbial culture originally enriched on ferulic acid. This mixed culture was also able to convert toluene to p-cresol slowly, and this compound is known to be degraded anaerobically to p-hydroxybenzoate via the corresponding alcohol and aldehyde (2, 3, 28). Therefore, it was speculated that p-cresol may have been an intermediate of the anaerobic toluene mineralization. In our laboratory, we have observed a rapid anaerobic degradation of m-xylene and toluene in a denitrifying aquifer column (22, 40). On the basis of a carbon and electron balance, we were able to show that m-xylene was quantitatively (80%) oxidized to CO$_2$ with a concomitant reduction of nitrate.

In this paper we report the results of experiments carried out to evaluate the effect of various electron acceptors (NO$_3^-$, NO$_2^-$, N$_2$O) on the anaerobic oxidation of m-xylene and give an overview of the substrate specificity of the m-xylene-adapted microorganisms present in the laboratory aquifer column. We also present results of our efforts to elucidate the pathway of anaerobic toluene degradation.

MATERIALS AND METHODS

Chemicals. Toluene, benzene, benzaldehyde, and sodium benzoate were purchased from Merck (Darmstadt, Federal Republic of Germany), and 3-methylbenzyl alcohol was from Riedel-de Haen (Seelze-Hannover, Federal Republic of Germany). All other aromatic compounds used in this study, as well as methylecyclohexane, 1,3-dimethylecyclohexane, and cyclohexanone, were from Fluka AG (Buchs, Switzerland). All chemicals were of the highest available purity. [ring-$^{14}$C]-m-xylene (specific activity, 93 mCi/mmol) was purchased from Amersham International (Arlington Heights, Ill.), and [ring-$^{14}$C]-toluene (specific activity, 10.9 mCi/mmol) and [methyl-$^{14}$C]-toluene (specific activity, 8.6 mCi/mmol) were from Pathfinder Laboratories Inc. (St. Louis, Mo.). All radiochemicals had purities of greater than 98% according to the supplier.

Laboratory aquifer column. The laboratory aquifer column consisted of a glass cylinder (length, 25 cm; inner diameter, 4 cm) filled with 30% aquifer material from the interface of a river-groundwater infiltration site (22, 30, 40) and 70% expanded slate grain (diameter, 2 mm). The column was maintained at 30°C with a water jacket. Samples for analysis could be withdrawn from the column at the inlet, at the outlet, and at sampling ports located at distances of 1.8, 6.8, and 11.8 cm from the inlet. The experimental setup to

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operate the denitrifying column under continuous-flow conditions in the complete absence of molecular oxygen, as described elsewhere (40). The basal medium (pH 7.5; for composition, see reference 40) contained mineral salts and was supplemented as indicated in Results. It was pumped through the column at a rate of 0.25 ml/min, corresponding to an average flow velocity of 2.6 cm/h. The medium was supplemented with methylated cyclohexanes or aromatic hydrocarbons including benzene, alkylnated benzenes, naphthalene, and methylnaphthalenes in the same way as previously reported for m-xylene (40). Polar substrates like cresols, benzoic acids, and benzyl alcohols as well as radionabeled compounds were added as aqueous solutions to the basal medium with an automatic syringe (Braun, Melsungen, Federal Republic of Germany) directly at the column inlet (22).

Analysis of aromatic compounds. All aromatic compounds were extracted with ethyl acetate from the aqueous samples and qualitatively and quantitatively determined by high-pressure liquid chromatography (HPLC) using a Series 4 liquid chromatograph (Perkin Elmer, Norwalk, Conn.), a Lichrosorb RP 18 column (length, 10 cm; inner diameter, 0.4 cm; particle diameter, 5 µm; Knauer KG, Berlin, Federal Republic of Germany), a Spectroflow 773 photometer with variable wavelength detection (Kratos, Ramsey, N.J.; detection usually between 254 and 280 nm), and an LC-100 integrator (Perkin Elmer). The mobile phase was a mixture of water-methanol (isocratic, 1.5 ml/min, 20 to 60% water depending on the compound). For the analysis of benzoic acid, p-hydroxybenzoic acid, and toluid acid, the pH of the mobile phase was adjusted to 3.

Analysis of nonaromatic organic compounds. After addition of an internal standard (1-chlorohexane or benzoic acid [for the analysis of cyclohexane carboxylic acid]), the acidified water samples (pH 2, 2 ml) were extracted with either 2 ml of hexane (nonpolar compounds) or 2 ml of diethyl ether (polar compounds). The extracts were dried over Na2SO4 and analyzed by gas chromatography (Carlo Erba, model 2101 equipped with a glass capillary column [40 m by 0.32 mm inner diameter], coated with OV-1701, and an FID detector; carrier gas [H2], 0.3 atm; temperature 4 min at ambient and then 6°C/min to 180°C). Before the gas chromatographic analysis, the carboxylic acids present in the samples were converted to their corresponding methyl esters by the following procedure. The diethyl ether was gently evaporated at 10°C, and the sample was incubated with 3% BF3 in methanol for 2 h at 100°C. Some water was added, and the methyl esters were then extracted with diethyl ether.

Analysis of 14C-labeled compounds, nitrate, nitrite, and oxygen. 14C-labeled substrates were supplied to the inflowing medium as described above, and the effluent of the column was collected in an air tight 1-liter glass flask containing 100 ml of concentrated pH 2 buffer to stop metabolic activity and to ensure volatilization of 14CO2. This flask was continuously purged with N2, and the gas was passed through a trap containing 20 ml of isobutanol (to absorb volatile organic compounds) and through three traps, each of which contained 20 ml of 0.1 N NaOH (to absorb 14CO2). The compounds remaining in the acidified solution (pH 2) after purging are referred to as the acidic fraction. This fraction was extracted with ethyl acetate. The content of radioactivity in the acidic fraction and the ethyl acetate extract as well as in the isobutanol and NaOH traps was quantified by supplementing 1-ml samples with 10 ml of Kontrogel (Kontron, Zurich, Switzerland) and counting them in a Tri-Carb liquid scintillation counter (Packard, Downers Grove, Ill.).

Nitrte, nitrite, and oxygen were determined as reported, elsewhere (22); the detection limits were 0.1, 0.03, and 0.001 mM, respectively.

Isotope-dilution experiments: identification of benzoic acid and p-hydroxybenzoic acid. To check whether any 14Clabeled benzoic acid or p-hydroxybenzoic acid had accumulated during the isotope-dilution experiments (see Results), 20 ml of the acidic fraction of the column effluent was extracted at pH 2 with 10 ml of ethyl acetate. The extract was dried with Na2SO4 and split into two 5-ml fractions (A and B). Fraction A was concentrated to about 100 µl. An aliquot (6 µl) was injected into the HPLC, and 1-ml fractions of the HPLC column effluent were collected and counted in a liquid scintillation counter. The radioactivity measured in the various fractions was then compared with the activity determined in another 6-µl sample of the concentrated extract.

The solvent of fraction B was gently evaporated, and the sample was incubated at 100°C for 2 h with either 1 ml of 3% BF3 in methanol (for benzoic acid) or 1 ml of ethanol containing 100 µl of concentrated sulfuric acid (for p-hydroxybenzoic acid). After 1 ml of water was added, the solution containing the corresponding carboxylic esters was extracted with 1 ml of diethyl ether. The extract was then concentrated to about 100 µl and subjected to the same analyses as described above for fraction A.

RESULTS

Electron acceptors of the anaerobic m-xylene oxidation. We have previously demonstrated that the oxidation of 0.3 mM m-xylene in a laboratory aquifer column took place in the absence of molecular oxygen with nitrate as the sole electron acceptor (40). We now show that high concentrations of oxygen inhibit the oxidation of m-xylene under these conditions (Fig. 1). Metabolism of m-xylene was not affected by the presence of 0.03 mM oxygen, but it stopped almost completely upon addition of 0.24 mM oxygen. Similarly, 0.03 mM oxygen had only a slight effect on the denitification process in that the difference between the amounts of nitrate metabolized and nitrite accumulated was reduced. This suggests that oxygen primarily inhibited the formation of gaseous denitrification products. Denitrification, however, stopped completely in the presence of 0.24 mM oxygen (Fig.

FIG. 1. Degradation of m-xylene in a laboratory aquifer column in the presence of molecular oxygen. The inflowing basal medium was supplemented with 5 mM nitrate and 0.17 mM m-xylene. Oxygen was added by flushing the gas exchange chamber of the continuous-flow system (40) with an appropriate nitrogen-oxygen mixture (yielding 0.03 mM O2 in the medium) or with air (yielding 0.24 mM O2 in the medium at equilibrium). The data represent effluent concentrations at a distance of 1.8 cm from the inlet. For nitrate, the difference between influent and effluent concentration is plotted.
1). The degradation of m-xylene and the denitrification were fully resumed upon removal of molecular oxygen (Fig. 1), which demonstrates that the microorganisms metabolizing m-xylene in the absence of oxygen are not irreversibly inactivated by molecular oxygen.

The denitrification of nitrate to molecular nitrogen proceeds through nitrite, nitric oxide, and nitrous oxide (21), and it was of interest to know whether these intermediates can also serve as electron acceptors for the anaerobic degradation of m-xylene.

The degradation of m-xylene continued without any apparent lag period upon the replacement of nitrate with nitrous oxide (Fig. 2). It was confirmed by using 14C-labeled m-xylene that this substrate was completely mineralized to CO2 in the presence of N2O as the sole electron acceptor (data not shown).

The replacement of nitrate by nitrite, however, led to a partial breakthrough of m-xylene (Fig. 3), and the microbial population in the column adapted only poorly to nitrite. Nine days after the replacement, only half (0.08 mM) of the m-xylene was eliminated, and only 0.5 mM nitrite was reduced. An electron balance shows that a reduction of 0.5 mM nitrite to molecular nitrogen does not allow for complete mineralization of 0.08 mM m-xylene. Figure 3 illustrates that m-xylene metabolism stopped completely upon removal of nitrite, but was rapidly resumed upon addition of nitrite.

**Substrate specificity of microorganisms adapted to m-xylene.** Benzene, toluene, ethylbenzene, xylene, naphthalene, and methylated naphthalenes are major pollutants in petroleum-contaminated aquifers (6, 7, 24, 35). We therefore investigated the substrate specificity of the m-xylene-adapted microorganisms towards a number of such aromatic hydrocarbons (Table 1). Toluene and m-xylene were totally eliminated and 3-ethyltoluene was partially eliminated in the column, and the metabolism was coupled with a reduction of nitrate and an accumulation of nitrite. The concentrations of benzene, ethylbenzene, and o-xylene were only slightly reduced in the column, and the reduction of nitrate to nitrite was below the detection limit. A microbial mineralization of these substrates was therefore unlikely. Propylbenzene, p-xylene, and the naphthalenes were not metabolized by the m-xylene-adapted microorganisms. It has to be emphasized, however, that the ability of the m-xylene-adapted microorganisms to degrade the other aromatic hydrocarbons was evaluated only within a 6-day period to avoid prolonged exposure to the new substrate. Whether an extended exposure would have resulted in the evolution of other microbial populations with new metabolic capabilities towards these substrates remains unknown.

To gain insight into the pathway of the anaerobic degradation of toluene and m-xylene in the aquifer column, the activity of the m-xylene adapted microorganisms towards some hypothetical intermediates was determined (Table 2). We found that m- and p-cresol were completely degraded in the column and their metabolism was coupled with a reduction of nitrate and an accumulation of nitrite. o-Cresol was not degraded, which may be due to sterical hindrance. p-Hydroxybenzoic acid was degraded, but very little nitrate was reduced, which may indicate that p-hydroxybenzoic acid was degraded.

**TABLE 1. Anaerobic degradation of selected aromatic hydrocarbons in the laboratory aquifer column adapted to m-xylene**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate influent conc (mM)</th>
<th>Substrate effluent concb (mM)</th>
<th>Nitrate reducedb (mM)</th>
<th>Nitrite effluent concb (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>0.38</td>
<td>0.01</td>
<td>2.8</td>
<td>2.6</td>
</tr>
<tr>
<td>3-Ethyltoluene</td>
<td>0.10</td>
<td>0.06</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Benzene</td>
<td>0.34</td>
<td>0.32</td>
<td>&lt;0.2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>0.22</td>
<td>0.17</td>
<td>&lt;0.2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Propylbenzene</td>
<td>0.11</td>
<td>0.11</td>
<td>&lt;0.2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>o-Xylene</td>
<td>0.21</td>
<td>0.19</td>
<td>&lt;0.2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>m-Xylene</td>
<td>0.19</td>
<td>&lt;0.01</td>
<td>1.6</td>
<td>0.9</td>
</tr>
<tr>
<td>p-Xylene</td>
<td>0.21</td>
<td>0.21</td>
<td>&lt;0.2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>0.17</td>
<td>0.17</td>
<td>&lt;0.2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>1-Methylnaphthalene</td>
<td>0.17</td>
<td>0.16</td>
<td>&lt;0.2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>2-Methylnaphthalene</td>
<td>0.11</td>
<td>0.11</td>
<td>&lt;0.2</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

a The column was adapted to a continuous flow of basal medium supplemented with 5 mM nitrate and 0.2 mM m-xylene for at least 1 week before m-xylene was replaced by another substrate.

b Concentrations were determined 2 to 6 days after the replacement of m-xylene. The data represent effluent concentrations at a distance of 11.8 cm from the inlet. For nitrate, the data represent the difference between the influent (5 mM) and effluent concentrations (at 11.8 cm).
acid was not fully mineralized. Ring-reduced derivatives of toluene and m-xylene (methylcyclohexane and 1,3-dimethylcyclohexane) were not degraded. Alcohol derivatives of toluene and m-xylene were persistent, but the corresponding aldehydes (benzaldehyde, m-toluylaldehyde) and acids (benzoate, m-toluate) were metabolized. It is noteworthy that benzyl alcohol and 3-methylbenzyl alcohol were not metabolized as sole substrates but degradable in the presence of toluene and m-xylene, respectively (data not shown). Of the two common products of the anaerobic benzoate degradation, cyclohexanecarboxylic acid and cyclohexanone, only the acid was rapidly degraded.

Pathway studies based on simultaneous adaptation experiments as presented in Table 2, however, have to be evaluated with great care since they do not directly analyze for the presence of intermediates and products. The experiments may be impeded by uptake and induction effects, or substrates may be metabolized even though they are not intermediates of the catabolic sequence of interest.

Products and intermediates of anaerobic toluene degradation. Toluene was rapidly degraded in the aquifer column (Table 1), and the number of possible intermediates is likely to be smaller for toluene than for m-xylene. Therefore, m-xylene was replaced by toluene as a substrate for the column, and further work on the pathway of anaerobic degradation of alkylated benzenes focused on toluene. The column was operated for months under continuous-flow conditions with 0.25 mM toluene as the only carbon and energy source. Toluene was always degraded completely, and metabolism was coupled with a reduction of nitrate. To establish a carbon mass balance of the degradation and to determine whether the ring carbon or the methylcarbon of toluene would be preferentially mineralized to CO2, the inflowing medium was supplemented with [ring-14C]toluene and [methyl-14C]toluene, respectively, over an 18-h period, and the effluent was collected and analyzed (see Materials and Methods). The addition of [ring-14C]toluene and [methyl-14C]toluene led to a similar pattern of the distribution of 14C-labeled products in the effluent (Fig. 4). The only difference was a slight delay of the maximum 14CO2 evolution from the ring-labeled compound as compared with the methyl-labeled compound. For both cases, however, within 7 days about 82% of the initial radioactivity was evolved as 14CO2, about 1% was extractable at pH 2 from the acidic fraction of the effluent, around 3% remained in the aqueous phase, and less than 0.5% was trapped as undegraded toluene. Around 14% of the injected radioactivity was not eluted within 7 days, which was probably due to the incorporation of carbon into the biomass of the microorganisms attached to the aquifer particles. The acidic fraction and the extractable fraction of the effluent were analyzed by HPLC, but no major metabolite was detected.

To accumulate intermediates of the toluene metabolism in the column, we used an isotope-dilution technique which was successfully applied by Evans and co-workers to elucidate the pathway of the anaerobic benzoate and phenol degradation (9, 12). A continuous flow of 0.25 mM toluene into the column was not only spiked with [ring-14C]toluene (as shown in Fig. 4A), but was also provided with an excess of a suspected intermediate acting as a carrier. The effluent was collected, and each carrier compound was extracted and

<table>
<thead>
<tr>
<th>TABLE 2. Anaerobic degradation of hypothetical intermediates in the laboratory aquifer column adapted to m-xylenea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
</tr>
<tr>
<td>--------------------</td>
</tr>
<tr>
<td>p-Cresol</td>
</tr>
<tr>
<td>m-Cresol</td>
</tr>
<tr>
<td>0-Cresol</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
</tr>
<tr>
<td>Methylcyclohexane</td>
</tr>
<tr>
<td>1,3-Dimethylcyclohexane</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
</tr>
<tr>
<td>Benzoic acid</td>
</tr>
<tr>
<td>3-Methylbenzyl alcohol</td>
</tr>
<tr>
<td>m-Toluylaldehyde</td>
</tr>
<tr>
<td>o-Toluene</td>
</tr>
<tr>
<td>1,3-Dimethylcyclohexane acid</td>
</tr>
<tr>
<td>Cyclohexanone</td>
</tr>
</tbody>
</table>

a See Table 1, footnotes a and b.

FIG. 4. Anaerobic degradation of [ring-14C]toluene (A) and [methyl-14C]toluene (B) in a laboratory aquifer column. A continuous flow of basal medium supplemented with 5 mM nitrate and 0.25 mM toluene was spiked with a total of 6 μCi of [14C]toluene over an 18-h period, and the effluent at a distance of 25 cm from the inlet was daily analyzed as indicated in the text. Symbols: (—) 14CO2; (—) acidic fraction.
tested for incorporation of $^{14}$C from the degradation of [ring-$^{14}$C]toluene (Table 3). The HPLC analysis revealed that none of the carriers was degraded to $>50\%$ over the entire column length.

The addition of benzoic acid as a carrier did not affect toluene degradation (80% $^{14}$CO$_2$), but led to an accumulation (14%) of $^{14}$C-labeled compounds in the acidic fraction of the effluent (Table 3). Over 70% of the radioactivity in this fraction was extractable at pH 2 but not at pH 7, indicating the presence of mostly acidic components. The HPLC analysis of the undervatized (fraction A) and derivatized (fraction B) pH 2 extracts revealed that all radioactivity coeluted with the benzoic acid and the benzoic acid methyl ester, respectively. Thus, the radioactivity extractable from the effluent of the column upon addition of benzoate was $^{14}$C-labeled benzoate.

The addition of $p$-hydroxybenzoate as a carrier also had very little effect on toluene metabolism (79% $^{14}$CO$_2$), but in contrast to the experiment with benzoate, only 2% of the applied radioactivity was extractable at pH 2. More than 90% of this radioactivity, however, again coeluted in the HPLC analysis with the carrier compound, that is, $p$-hydroxybenzoic acid (fraction A) and $p$-hydroxybenzoic acid ethyl ester (fraction B), indicating the formation of some $^{14}$C-labeled $p$-hydroxybenzoate during $^{14}$C]toluene degradation.

Finally, the addition of $p$-cresol led to a weak inhibition (75% $^{14}$CO$_2$) and the addition of benzaldehyde led to a strong inhibition (17% $^{14}$CO$_2$) of toluene degradation. In both cases, again only 1 to 2% of the applied radioactivity was extractable at pH 2. Possible transformation products were not identified.

**DISCUSSION**

In this report and in previous publications (22, 40) we demonstrated that toluene and $m$-xylene are rapidly mineralized under denitrifying conditions in the complete absence of molecular oxygen. The mineralization was confirmed by a carbon mass balance and an electron balance. Additions of molecular oxygen led to an inhibition of the anaerobic toluene degradation. On the basis of these findings and the results of other laboratories (16, 17, 36, 37), we conclude that aromatic hydrocarbons present in anoxic environments such as lake sediments (23), groundwater infiltration zones from landfills (27), and polluted rivers (26) are not necessarily persistent but may be mineralized in the absence of molecular oxygen. The finding that the anaerobic mineralization of aromatic hydrocarbons takes place not only in the presence of nitrate but also with nitrous oxide as an electron acceptor (Fig. 2) may offer attractive options for the in situ restoration of polluted aquifers (20). $N_2O$ is highly water soluble (solubility at room temperature: 26 mM), and its reduction does not yield any toxic products such as nitrite, but instead yields molecular nitrogen (21).

The degradation of 0.17 mM $m$-xylene and 0.38 mM toluene in the presence of 5 mM nitrate yields 0.1 and 2.6 mM (respectively) nitrite which is only slowly reduced (Fig. 1, Table 1). Moreover, the degradation of 0.17 mM $m$-xylene is incomplete in the presence of 3.1 mM nitrite as the sole electron acceptor (Fig. 3). Hence, we conclude that nitrite is a poor electron acceptor at these concentrations. On the basis of these data alone, however, we cannot conclude whether nitrite also has an inhibitory or toxic effect on the microbial population in the aquifer column. In additional experiments under continuous-flow conditions (data not shown), 0.3 mM toluene was completely degraded not only in the presence of 15 mM (inflowing concentration) nitrate but also in the presence of 15 mM nitrate plus 5 mM nitrite. The degradation, however, stopped in the presence of 15 mM nitrate plus 10 mM nitrite. These results suggest that nitrite also exhibits toxic effects at concentrations above 5 mM.

Toluene was selected as a model compound to elucidate the pathway of the degradation of alkylated benzenes in the denitrifying laboratory aquifer column. The isotope-dilution experiments suggested benzoate to be an intermediate of the anaerobic toluene degradation, and we also observed a rapid turnover of benzoate in the aquifer column adapted to $m$-xylene. Several publications describe the metabolism of benzoate and other oxygenated aromatic compounds under anaerobic conditions (5, 8, 12, 34, 38, 39). Therefore, the initial catalytic steps of toluene in the absence of molecular oxygen are of particular interest. Theoretically, toluene degradation can be initiated by three different enzymatic mechanisms, namely, a reduction of the aromatic ring, a hydroxylation of the methyl group, or a hydroxylation of the aromatic ring (Fig. 5).

A reduction of the aromatic ring of toluene to methylcyclohexane would be thermodynamically favorable (16), and reductions of the aromatic rings of phenol and benzoate under anaerobic conditions are frequently observed (12, 38). In contrast to phenol and benzoate, however, toluene has no functional group, and microorganisms which anaerobically reduce the aromatic ring of aromatic hydrocarbons have never been observed. In addition, methylcyclohexane and 1,3-dimethylcyclohexane were not degraded in our column.

### TABLE 3. Anaerobic degradation of [ring-$^{14}$C]toluene in the laboratory aquifer column in the presence of suspected intermediates as carriers

<table>
<thead>
<tr>
<th>Carrier added$^a$</th>
<th>Radioactivity (%) $^{14}$C in effluent$^b$</th>
<th>Radioactivity (%) $^{14}$C in effluent$^b$</th>
<th>Radioactivity (%) $^{14}$C in effluent$^b$</th>
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<th>Radioactivity (%) $^{14}$C in effluent$^b$</th>
<th>Radioactivity (%) $^{14}$C in effluent$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ring-$^{14}$C]toluene$^c$</td>
<td>$^{14}$CO$_2$ evolved</td>
<td>$^{14}$CO$_2$ evolved</td>
<td>$^{14}$CO$_2$ evolved</td>
<td>$^{14}$CO$_2$ evolved</td>
<td>$^{14}$CO$_2$ evolved</td>
<td>$^{14}$CO$_2$ evolved</td>
<td>$^{14}$CO$_2$ evolved</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>$&lt;0.5$</td>
<td>80</td>
<td>44</td>
<td>22</td>
<td>14</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>$p$-Hydroxybenzoate</td>
<td>$&lt;0.5$</td>
<td>79</td>
<td>47</td>
<td>20</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>$p$-Cresol</td>
<td>11</td>
<td>75</td>
<td>45</td>
<td>19</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>59</td>
<td>17</td>
<td>5</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$ A continuous flow of basal medium supplemented with 5 mM nitrate and 0.25 mM toluene was spiked with 6 uCi of [ring-$^{14}$C]toluene (considered to be 100% $^{14}$C) and 2.6 mM (final concentration) carrier compound over an 18-h period.

$^b$ Effluent was collected at a distance of 25 cm from the inlet and analyzed daily as indicated in the text. The data represent total values received over a 3-day incubation period. In addition, for the $^{14}$CO$_2$ evolution data daily values are given.

$^c$ $^{14}$C entrapped in isopropanol was considered to be [ring-$^{14}$C]toluene.

$^d$ Probably exceptionally high due to incomplete trapping of nondegraded [ring-$^{14}$C]toluene in isopropanol.
and are therefore unlikely to be intermediates of the anaerobic metabolism of toluene and m-xylene, respectively.

Under aerobic conditions, microbial metabolism of toluene commonly occurs via the hydroxylation of the methyl group leading to benzyl alcohol. This intermediate is subsequently oxidized to benzaldehyde and benzoate (Fig. 5; 13-15). Since the oxygenase that catalyzes the initial conversion of toluene to benzyl alcohol requires molecular oxygen as a cosubstrate, it does not operate under anaerobic conditions. We are not aware of any publications describing an anaerobic turnover of toluene or benzyl alcohol via benzaldehyde to benzoate. Our own findings are inconclusive. Benzaldehyde was degradable in the m-xylene-adapted column, and it inhibited toluene metabolism. However, the isotope-dilution experiment showed no evidence for this compound being an intermediate of the anaerobic toluene degradation. Benzyl alcohol was not degradable as a sole substrate but only in the presence of toluene. Similarly, Smolenski and Sufita (31) reported that sulfate-reducing aquifer material able to degrade p-cresol was simultaneously adapted to p-hydroxybenzaldehyde and p-hydroxybenzoate but not to p-hydroxybenzyl alcohol.

An alternative pathway for toluene degradation under aerobic conditions includes hydroxylation of the aromatic ring leading to p-cresol or 3-methylcatechol (13, 15; G. M. Whited, L. D. Kwart, and D. T. Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K168, p. 199). These transformations, however, are again catalyzed by oxygenases and do not take place under anaerobic conditions. Only recently, Gribić-Galić and Vogel (16, 17, 36) observed an accumulation of traces of p-cresol in a methanogenic culture growing in the presence of toluene. Using $^{18}$O-labeled water and analyzing the produced p-cresol by mass spectrometry, these authors showed that the oxygen incorporated into toluene came from water. The conversion of toluene to p-cresol was very slow, and it remains to be investigated whether such an enzymatic turnover could account for the rapid metabolism of toluene observed in our denitrifying aquifer column. The aerobic and anaerobic turnover of p-cresol to p-hydroxybenzoate has been described in detail (2, 5, 11, 18, 31), and the anaerobic mineralization of p-hydroxybenzoate (and its analogs protocatechuic [3,4-di-hydroxybenzoate] and gallic acid [3,4,5-trihydroxybenzoate]) via phenols or benzoates has also been well documented (20, 28, 32, 33). We also observed a rapid degradation of p-cresol and p-hydroxybenzoate in our columns. In the isotope-dilution experiments no p-cresol and only very little p-hydroxybenzoate were accumulated during toluene metabolism.

In summary, the experiments conducted so far in our laboratory and elsewhere do not allow us to firmly establish a particular pathway for microbial toluene degradation under denitrifying conditions. There is some evidence that the metabolism may proceed through benzyl alcohol and benzaldehyde or through p-cresol and p-hydroxybenzoate to benzoate. The mechanism of the initial enzymatic attack towards toluene in the absence of molecular oxygen is still obscure. It remains to be investigated whether radical reactions or enzymatic steps similar to those postulated for the anaerobic metabolism of methane (1) or saturated and unsaturated linear hydrocarbons (25, 29) play a role in the anaerobic degradation of toluene.

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