Incorporation of Oxygen from Water into Toluene and Benzene during Anaerobic Fermentative Transformation

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Toluene and benzene were anaerobically transformed and eventually mineralized in mixed methanogenic cultures. However, the source of oxygen for the initial oxidation step had been unknown, owing to the presence of both methanol and water. No exogenous electron acceptors other than carbon dioxide, toluene, and benzene were present in the defined mineral medium. Through the use of ^{18}O-labeled water, the oxygen incorporated into the monoaromatic compounds was shown to come from water. The cresol from the toluene and the phenol from the benzene contained up to 8% ^{18}O label after incubation in 9% ^{18}O-labeled medium. Gas chromatography-mass spectrometry was used to detect the ^{18}O-labeled aromatic metabolites.

Anaerobic degradation of some monoaromatic hydrocarbons has recently been studied. Previously, only monoaromatic compounds containing oxygen in ring substituents were shown to be biodegradable. Reinhard et al. (M. Reinhard, J. W. Graydon, N. L. Goodman, and J. F. Barker, Proceedings of the Second International Conference on Groundwater Quality Research, Tulsa, Oklahoma, 1985, p. 69–71) recently described the selective removal of xylenes from a landfill leachate plume in an anaerobic aquifer. Sorption and dispersion could not account for this selective removal. Rees et al. (J. F. Rees, B. H. Wilson, and J. T. Wilson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, Q5, p. 258) described the disappearance of toluene under methanogenic conditions in microcosms containing aquifer material. They also observed the mineralization of ^{14}C-labeled toluene to ^{14}CO$_2$ in their microcosms. Denitrifying conditions also appear to be favorable for the degradation of toluene, benzene, and xylenes. Battermann and Werner (1) observed the degradation of toluene, benzene, and lesser amounts of xylenes in a contaminated aquifer to which nitrate was added as the main electron acceptor. Kuhn et al. (6) recently described the degradation of xylenes under denitrifying conditions in the field and in laboratory aquifer columns.

In an attempt to clarify the anaerobic oxidation of toluene and benzene, experiments with methanogenic cultures showing the production of ^{14}C-labeled CO$_2$ from ^{14}C-labeled toluene and benzene and the possible metabolites of this anaerobic oxidation process have been described (D. Grbić-Galić and T. M. Vogel, submitted for publication). However, determination of the origin of the first oxygen atom incorporated into toluene and benzene is critical for understanding the apparent anaerobic oxidation which has been reported. In this paper, a description of the anaerobic oxidation of toluene and benzene by oxygen derived from partially ^{18}O-labeled water is presented.

The ^{18}O-labeled H$_2$O (90%) used for the experiments was obtained from Morton Thiokol, Inc. (Alfa Products, Danvers, Mass.). The toluene used was scintillation-grade Packard Toluene Puresolv (Packard Instruments Co., Inc., Downers Grove, Ill.). The benzene used was Baker Ana-

lyzed (99.4%; J. T. Baker Chemical Co., Phillipsburg, N.J.). Mixed methanogenic cultures were derived from stable methanogenic consortia that were degrading ferulic acid to CO$_2$ and methane and that originally had been enriched from sewage sludge (2). The cultures were maintained on toluene or benzene as the sole carbon and energy source for 1 year before the initiation of the experiments with ^{18}O-labeled water. During this year, the cultures were fed toluene or benzene six times, and the intermediates of their degradation were identified and quantified by gas chromatography-mass spectrometry. An abundance of oxidized intermediates was found, whereas none were found in heat-killed controls (Grbić-Galić and Vogel, submitted). These cultures transformed all the added substrate each of the six times within 40 to 60 days of dark incubation at 35°C. The acclimated seed from these cultures was then inoculated into defined mineral salts medium with reducing agents (ferrous chloride plus sodium sulfide), ammonium phosphate as the nitrogen source, and vitamins as described previously (2, 3). The medium also consisted of 9% ^{18}O-labeled water (91% [^{16}O]H$_2$O). Toluene and benzene (3.0 mM each) were added to separate culture tubes containing 10 ml of the described medium, and the tubes were incubated at 35°C in the dark.

Water samples (2 ml) were extracted (after acidification) with ethyl ether in small vials (5 ml) sealed with Teflon-coated silicone septa. The ethyl ether was subsequently subsampled through the septa with a 10-μl glass syringe. The ethyl ether extract was then injected through a Grob-type injector (250°C) with the split closed for 30 s into a DB-5 thick-film, 60-m, fused silica column (Durabond column; J & W, Inc.). The column temperature initially was 60°C and was increased at a rate of 4°C per min to 200°C. The column effluent went directly into a Finnigan mass spectrometer with an INCOS data system. The spectrum of each peak was routinely compared by the data system to the spectra of the 33,000 entries of the National Bureau of Standards. Owing to the potentially low yield of ^{18}O-labeled metabolites, controls without ^{18}O-labeled water were compared. Gas chromatography-mass spectrometry was performed several times during 40 days of incubation.

Significant amounts of ^{18}O-labeled metabolites were detected in the methanogenic incubations of both toluene and benzene. The production of ^{18}O-labeled *p*-cresol from tolu-
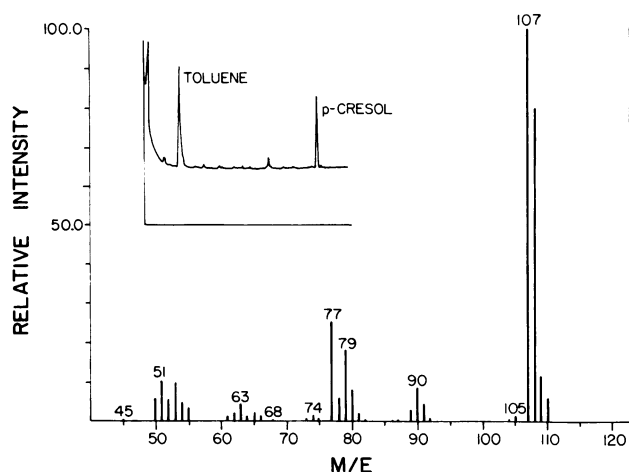
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FIG. 1. Spectrum of p-cresol produced from toluene in partially
[18]O-labeled water medium. The insert is the total ion chromatogram
showing the toluene and p-cresol peaks.

FIG. 3. Spectrum of phenol produced from benzene in partially
[18]O-labeled water medium. The insert is the total ion chromatogram
showing the benzene and phenol peaks.

ene showed the incorporation of [18]O at fairly high levels. The
m/e peak of 110 was 8% of the 108 m/e peak in the p-cresol
spectrum (Fig. 1). On the other hand, no 110 m/e peak
appeared in the spectrum of p-cresol produced in unlabeled
medium (Fig. 2). The 110 m/e peak appeared to be the result
of the 9% ^{18}O-labeled water in the medium.

In the anaerobic oxidation of benzene, phenol was the first
major product. The theoretical value for ^{18}O-labeled phenol
should be similar to the p-cresol value (8%); however, for
some unknown reason, the 96 m/e peak (representing the
labeled phenol) was only 2.5% of the 94 m/e peak (represent-
ing the unlabeled phenol) (Fig. 3). The control spectrum
showed no +2 peak (96 m/e) (Fig. 4).

The results from this experiment show the incorporation
of [18]O from ^{18}O-labeled water as the initial step in the
anaerobic oxidation of toluene and benzene by acclimated
methanogenic cultures. Reduction of toluene and benzene is
the type of transformation expected in anaerobic systems, if
transformation occurs. However, reduction does not explain
the production of [14]CO_2 from[14]C-labeled toluene and ben-
zene, as recently described (Grbić-Galić and Vogel, submit-

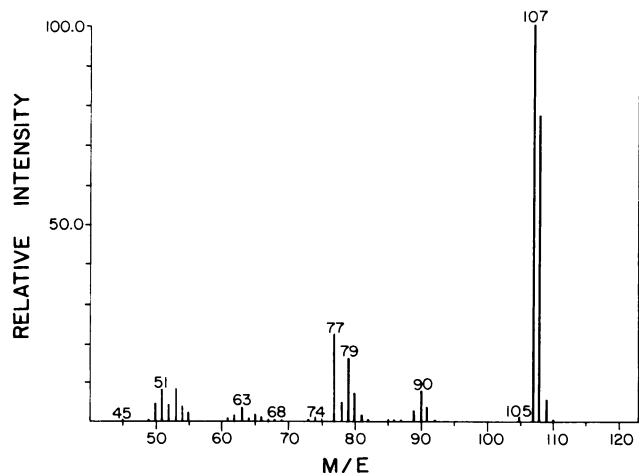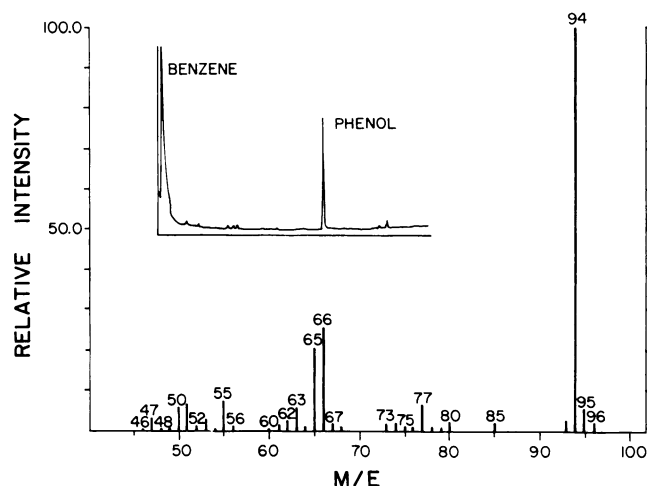ted). Toluene and benzene were, therefore, fermentatively
oxidized by an as-yet-unknown mechanism with water as the
source of oxygen, since no other organic substrates were
involved.

Although this paper is the first to report the introduction of
oxygen from water into toluene and benzene under
anaerobic conditions, enzymes which introduce hydroxy
groups from water into nitrogen-containing heterocyclic
rings have been found in anaerobic bacteria (4, 5, and 7).
Admittedly, toluene and benzene are more stable structures
than heterocyclic rings, with oxidation potentials of 1.9 and
2.4 V, respectively (8), and possibly must be activated by
conversion to unstable arene cation radicals before the
nucleophilic attack by water is possible.

The evidence for the use of water as an oxygen source in
the anaerobic oxidation of toluene and benzene helps toward
understanding the mechanism involved. Certainly, more
work with both labeled water and labeled compounds will
help further elucidate the pathway of anaerobic degradation
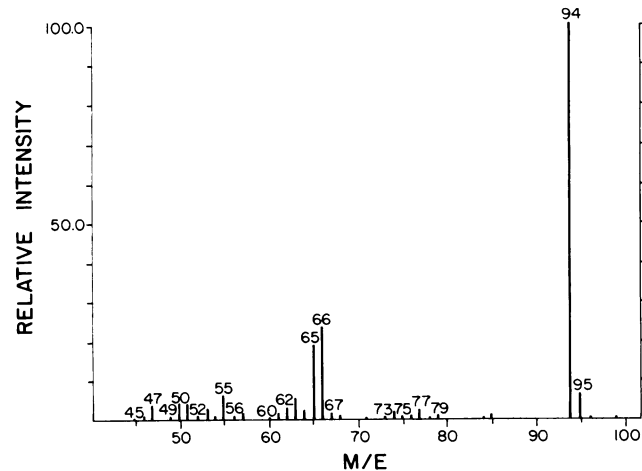of monoaromatic hydrocarbons.

FIG. 2. Spectrum of p-cresol produced from toluene in unlabeled
medium.

FIG. 4. Spectrum of phenol produced from benzene in unlabeled
medium.

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