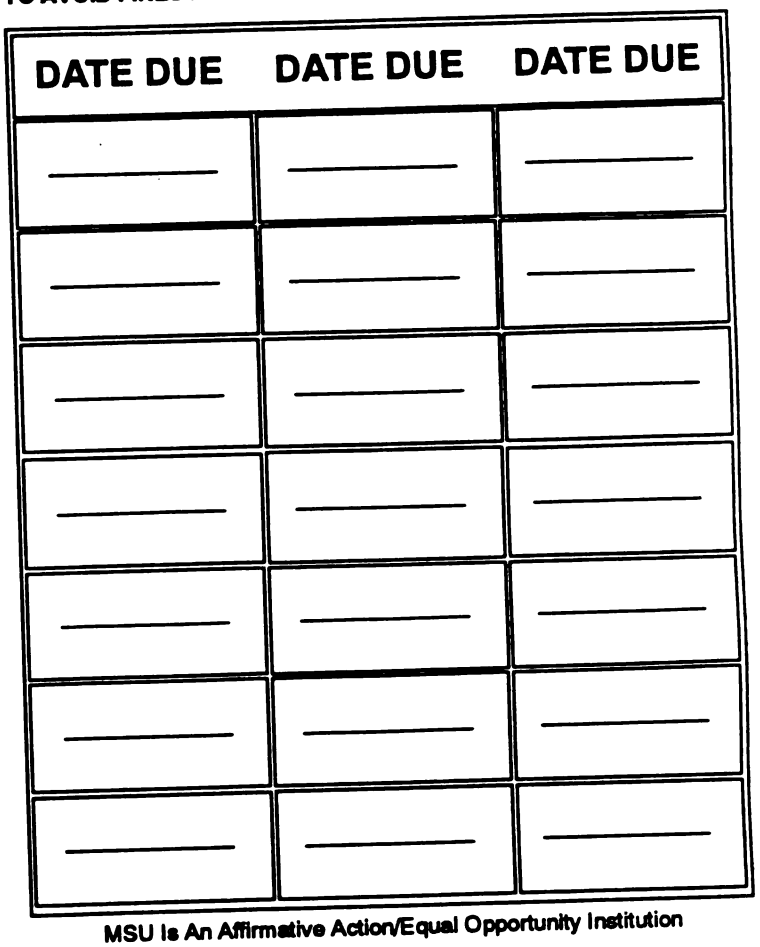

ANAEROBIC BIODEGRADATION OF ETHER COMPOUNDS BY ETHER BOND-CLEAVING BACTERIA AND METHANOGENIC CONSORTIA

by

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1986

ABSTRACT

ANAEROBIC BIODEGRADATION OF ETHER COMPOUNDS BY ETHER BOND-CLEAVING BACTERIA AND METHANOGENIC CONSORTIA

By

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Ether compounds are manufactured for use in nonionic detergents, plastics, pesticides and other products and occur as toxic organic compounds, the most famous being tetrachlorodibenzo-*p*-dioxin. Ether compounds were considered recalcitrant to anaerobic biodegradation due to the lack of an appropriate oxidant for ether bond-cleavage in reducing environments. Many of these compounds reside in anaerobic environments or are exposed to anaerobic waste treatment processes. Thus, it is of interest to identify: (i) whether ether compounds are anaerobically biodegradable, (ii) the anaerobic microorganisms able to degrade these compounds, and (iii) the mechanism(s) of anaerobic ether bond-cleavage.

The ether bonds of polyethylene glycol (PEG; HO-$[CH_2CH_2$-O-$]_n$H), phenyl ether ($[C_6H_5]_2O$), and dibenzo-*p*-dioxin ($[C_6H_4]_2O_2$) were shown to be degraded in methanogenic consortia enriched with these compounds and polyethoxylate (nonionic) surfactants as substrates.

Two anaerobic microorganisms which used PEGs as sole substrates were isolated and characterized. *Desulfovibrio desulfuricans* strain DG2 degraded the monomer ethylene glycol and oligomers up to tetraethylene glycol (HO-$[CH_2CH_2$-O-$]_4$H) in length. *Bacteroides* sp. strain PG1 degraded diethylene glycol and all other polymer lengths of PEG.

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PEGs were degraded by *Bacteroides* sp. strain PG1 via an external depolymerization which was either a hydrolytic or a reductive cleavage of the ether bond. The ether bond of diaryl ethers was apparently cleaved by a reductive mechanism which produced benzene and phenol as products from phenyl ether degradation and benzene and, by indirect analysis, catechol from dibenzo-*p*-dioxin.

A method was devised for immobilizing anaerobic microorganisms in agar and a modified substrate inhibition model was devised to provide estimates for the kinetic parameters V_{max}, K_m, and K_i. By using an immobilized, phenol-degrading methanogenic consortium and phenol as the model inhibitory substrate, it was shown that immobilization appeared to protect the cells from inhibitory concentrations of substrates. This resulted in an increase in the apparent K_i value from 900 to 1725 ug phenol/ml.

ACKNOWLEDGEMENTS

Throughout my stay at MSU there have been many people with whom I have collaborated and also been friends. I would like to acknowledge my committee members whose advice and friendship I will always appreciate: Dr. C. A. Reddy, Dr. F. B. Dazzo, Dr. S. Ferguson-Miller and Dr. M. J. Klug. I would especially like to thank Dr. Klug for the time spent at the Kellogg Biological Station and Dr. Breznak for the time spent in his lab. Dr. Dazzo deserves special thanks for the use of his photography equipment, Dr. Boyd for the use of his equipment and John Weeg for help with computer programming.

My fellow workers and friends in Dr. Tg's lab also deserve thanks for just being there when needed: Dan Shelton, Els Aerssens, Mary Lou Krumme, Chuck Rice, Mark Mikesell, Janet Jansson, Joyce Wildenthal, Joe Robinson, Diane Jordan, Tim Linkfield, Skip Mileski, Angie Frazer, Bill Mohn and just about everyone else, but especially John Golphin and Bob Dietrich. I also want to thank those people who have "worked for me" over the years especially Julie Breitenbach, Kurt Williams, Jeanne Lamont and Teri Aust.

Finally, I would especially like to thank Dr. Jim Tiedje for letting me work in his lab and for letting me try all the different things that I wanted to do. Not many professors would let their students do that and its Jim's patience and guidance to which I owe any success.

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CHAPTER 1

INTRODUCTION

Organic compounds, both naturally occurring and xenobiotic, can be grouped into one of three classifications: biodegradable, persistent, or recalcitrant (18). Often a compound may be placed into more than one category depending on the conditions in which its biodegradability was assessed. In other words, the biodegradability of a compound depends on extrinsic factors such as the presence of microorganisms with appropriate mechanism(s) for degrading the compound, the available concentration of the compound, and whether abiotic conditions are satisfactory for the inititation of biodegradation (1,18,29).

Xenobiotic compounds are manufactured for their own intrinsic properties but also occur as unwanted, and at times toxic, industrial side products. Many of these compounds appear to be recalcitrant to biodegradation, or at least are persistent in the environment. The need to dispose of these compounds created an interest in both identifying and making use of factors which make organic compounds degradable and in creating suitable conditions for degradation to occur. There is also a continuing search for microorganisms able to degrade existing xenobiotic compounds (15,18,29,40).

Until recently, the biodegradative abilities of microorganisms in anaerobic environments received little attention. This is unfortunate since anaerobic environments are often the depositories for large quantities of organic matter from both natural and synthetic sources. Anaerobic biodegradation to dispose of xenobiotic compounds offers an attractive alternative to large-scale aerobic

biodegradation processes (29). Large-scale anaerobic processes are less costly. Also, they (i) do not require the input of energy for the transfer of oxygen that is required by aerobic processes, (ii) produce less quantities of sludge, and (iii) offer the potential for methane recovery.

Anaerobic microorganisms also offer four known biological activities which may be useful in the degradation of organic compounds: reduction, dealkylation, dehalogenation, and hydrolysis (14,40). Because anaerobic microorgaisms have not been extensively studied, the versatility and usefulness of anaerobic biodegradation is perhaps only beginning to become apparent. For example, some reductive dehalogenations occur only under anaerobic conditions (29) and are perhaps the critical factor in initiating the biodegradation of specific halogenated aromatics (14,19).

The focus of this study was to assess the capacity of anaerobic bacteria for the degradation of xenobiotic ether compounds by ether bond-cleavage. Ether bond-containing compounds occur ubiquitously as natural organic compounds and as synthetic compounds released into the environment. For example, ether bonds are the major intermonomeric linkage for lignin and humic substances which account for some 6×10^{12} metric tons of organically bound carbon. It has been suggested from infrared spectral analyses that every second carbon atom of humus participates in an ether bond (4). The most common xenobiotic ether compounds are derived through the polymerization of ethylene oxide to form polyethylene glycol (PEG). These compounds are used primarily as the hydrophilic substituents of nonionic surfactants (11), of which 300,000 metric tons were used in

the U.S. in 1983 with the market increasing at a 2-3% annual rate (20).

Much of the lignin and lignin-derived humic substances reside in anaerobic environments. Nonionic surfactants are discharged primarily to municipal waste streams and may be processed in anaerobic sludge reactors for the removal of organic compounds. Thus, by virtue of the sheer quantity of ether compounds present, there is a selective pressure for ether bond-cleavage mechanism(s) to develop among anaerobic bacteria in those environments.

Ether compounds had been considered recalcitrant to biodegradation by anaerobic microorganisms (29). This was because the known mechanisms for ether bond-cleavage involved either peroxide or peroxide radicals (41) or enzymes associated with aerobic microorganisms: monooxygenases, ether hydrolases, a carbon-oxygen lyase (Enzyme Nomenclature 1984, Academic Press, New York), and an O-decarboxymethylase (24). Thus, ether bond-cleavage by anaerobic microorganisms represents a novel reaction and one which has potential use in the anaerobic transformation of persistent natural and synthetic organic compounds.

Anaerobic Ether Bond-Cleavage

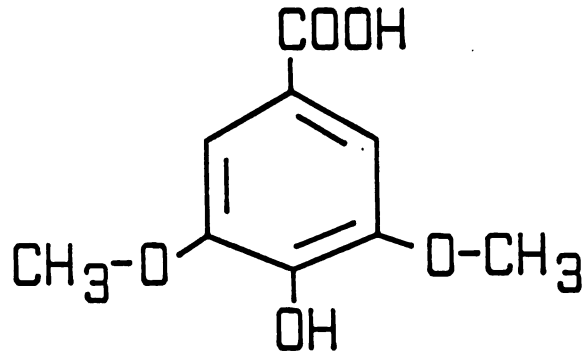
The anaerobic, biological cleavage of ether bonds in a few naturally occurring organic compounds has been demonstrated (Figure 1). The aryl-glycerol-β-aryl ether linkage of lignin model compounds can be cleaved by mixed rumen bacteria (8) and apparently by methanogenic consortia and sediments which degrade those compounds (5,6,9,10,42). The mechanism or point at which ether bond-cleavage occurs is unknown. Aryl methoxy ether bonds apparently are hydrolytically cleaved by monocultures of strictly anaerobic bacteria and the product(s) used as sole carbon and/or energy source (3,16,22,30). The free O-methyl group can be used also as an electron acceptor during carbohydrate oxidation with acetate as product (31).

Some synthetic ether compounds may be anaerobically biodegraded. Anaerobic microorganisms in rumen fluid can degrade organic insecticides by an initial cleavage of the aryl methyl ether bond of disugran (methyl 3,6-dichloro-O-anisate; 21) and 4'-O-methyl genistan and 4'-O-methyldaidzein (34). Mineralization of PEG was first noted to occur in anaerobic sludge reactors (33) and more recently in anaerobic medium inoculated with 10% sludge from municipal anaerobic digesters (37). Recently, Schink and Stieb (35) isolated a strictly anaerobic bacterium which fermented PEG to equimolar quantities of acetate and ethanol apparently via production of an acetaldehyde intermediate.

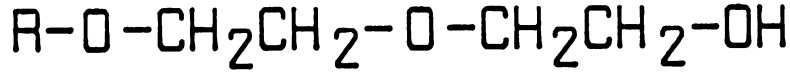
The above compounds represent both alkyl ether and aryl-alkyl ether compounds. The anaerobic cleavage of diaryl ether bonds has not been reported. These ether bonds exist in naturally occurring

Figure 1. Three classes of ether compounds are known to be anaerobically biodegraded. These include: (i) aryl methoxy ethers represented here by syringic acid, (ii) the synthetic alkyl ether, polyethylene glycol (R = (HO-[CH_2CH_2-O-]$_n$), and (iii) aryl-glycerol-β-aryl ethers represented by guaiacylglycerol-β-[O-methoxy-phenyl]ether.

Syringic Acid



Polyethylene Glycol



Lignin Model Compounds
(Guaiacylglycerol-B-
[O-methoxyphenyl] ether)

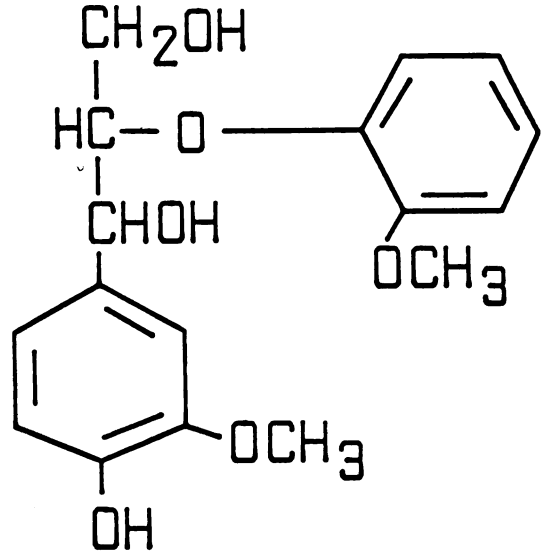


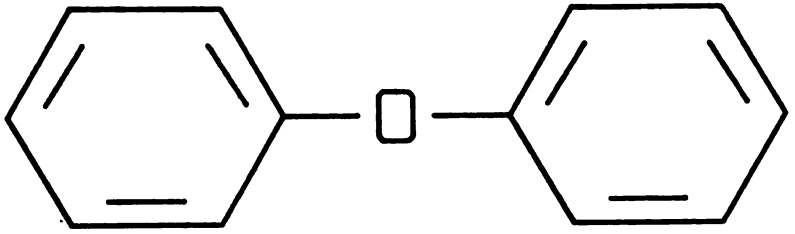
Figure 1

compounds such as lignin and also in the synthetic compounds phenyl ether ($[C_6H_5]_2O$) and dibenzo-*p*-dioxin ($[C_6H_4]_2O_2$) (Figure 2). The dioxin compounds are best known as toxic halogenated congeners. Both 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated diphenyl ethers are on the EPA's list of priority pollutants (26). TCDD is perhaps the most toxic synthetic chemical known with an LD_{50} for mammals of micrograms or less per kilogram (36).

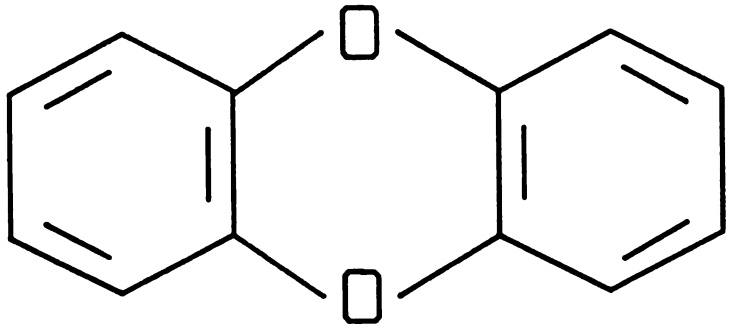
TCDD and 2,7-dichlorodibenzo-*p*-dioxin (DCDD) have been claimed to be degraded in soils (25,32) and model aquatic environments (39). But, due to problems with dioxin extraction from soil, it has been concluded by the Environmental Protection Agency that the biodegradability of dioxins in environmental samples has not been adequately demonstrated (13). One report of partial degradation of the phenyl ether-based microbicide 5-chloro-2-(2,4-dichlorophenoxy)phenol did not identify the mechanism or metabolites (38).

Pure culture studies have been more encouraging with the production and isolation of intermediates from aerobic dioxin biodegradation. Unsubstituted dibenzo-*p*-dioxin is metabolized by a mutant *Pseudomonas* sp. NCIB9816 strain II to 2-hydroxydibenzo[1,4]dioxan via cis-1,2-dihydroxy-1,2-dihydrodibenzo[1,4]dioxan (27). A mutant *Beijerinckia* strain B8/36 oxidized dibenzo-*p*-dioxin and monochlorinated dibenzo-*p*-dioxin to a cis-dihydrodiol compound (28). Recent evidence suggests that TCDD may be degraded by the H_2O_2-dependent extracellular lignin-degrading enzyme system of *Phanerochaete chrysosporium* (7).

Figure 2. Aryl ether compounds examined in this study for potential anaerobic biodegradability were phenyl ether and dibenzo-*p*-dioxin.



Phenyl ether



Dibenzo-p-dioxin

Figure 2

Even though there has been extensive effort expended to detect biodegradation of dioxins (13), no one has apparently approached the problem by attempting to cleave the diaryl ether bonds. Microbial degradation of the ether bonds would destroy the dioxin structure and create potentially degradable products. Thus, aerobic or anaerobic cleavage of the ether bonds of either dioxin or phenyl ether would be a potentially useful method for their detoxification.

Research Objectives

I had four primary objectives in my study of the anaerobic biodegradation of xenobiotic ether compounds: (i) to assess which types of ether compounds can be biologically degraded, ie. alkyl ether, diaryl ether and aryl-alkyl ether compounds, (ii) to isolate and identify the ether bond-cleaving microorganisms, (iii) to identify the mechanism(s) of ether bond-cleavage, and (iv) to develop an whole-cell immobilization technique for anaerobic microorganisms that could be used to degrade inhibitory substrates. This last objective resulted from preliminary studies that showed some ether compounds and their degradative products to be inhibitory to microbial metabolism and growth.

The first goal was to obtain evidence for alkyl ether degradation by anaerobic microorganisms. Previously, only aryl methoxy ether compounds had been shown to be degraded under anaerobic conditions (3). Methanogenic enrichments were established for the degradation of PEGs following the early observations of methane production from PEG made by Daniel Shelton (personal communication). Chapter 1, "Degradation of ethylene glycol and polyethylene glycols

by methanogenic consortia", describes the enrichment procedure. The methods used followed established procedures for assessing anaerobic biodegradability potential (23,37). This Chapter describes the first report of complete alkyl ether degradation by methanogenic consortia in which the sequence and stoichiometry of product formation was identified.

Following the observations of PEG degradation (Chapter 1), we attempted to enrich for methanogenic consortia able to degrade nonionic surfactants having PEG-substituents (polyethoxylate compounds). The biodegradation of several nonionic surfactants was assessed for two reasons: (i) their biodegradation under anaerobic conditions had not been reported and (ii) one type of nonionic surfactant, the alkylphenol polyethoxylates, yields highly toxic alkylphenol compounds after aerobic biodegradation (17). The non-alkylphenol nonionic surfactants were readily degraded, but as in aerobic degradation the alkylphenol moieties were recalcitrant. In an attempt to obtain alkylphenol degradation, several alkylphenols were added as substrates for methanogenic enrichments which metabolized less toxic analogues. This work is described in Chapter 2, "Degradation of several nonionic surfactants and substituted polyethylenes by methanogenic enrichment cultures".

One of our objectives was to isolate ether bond-cleaving microorganisms for the purpose of studying the mechanism of ether bond-cleavage. Two PEG-degrading bacteria were isolated from the methanogenic enrichments discussed in Chapter 1. The isolation procedure, bacterial characterization, and pathways of polyethylene glycol metabolism are described in Chapter 3, "Metabolism of

polyethylene glycol by two anaerobic bacteria, *Desulfovibrio desulfuricans* and a *Bacteroides* sp.". The degradation of PEG and in particular of the ether bonds was studied using cell-free extracts of both microorganisms. A colorimetric assay was devised for measuring PEG-dehydrogenation in the cell-free extracts. One apparent mechanism of ether bond-cleavage was discovered to be extracellular and apparently novel for anaerobic microorganisms.

Chapter 4, "Degradation of dioxin and diphenyl ether by anaerobic ether bond-cleaving methanogenic consortia", describes the enrichment procedure used to obtain the diaryl ether-degrading microorganisms. The mechanism postulated for PEG-depolymerization in Chapter 3 was seen as potentially suitable for the cleavage of diaryl ether bonds as well. We reasoned that PEG could be used as a selective substrate to obtain a microbial population of ether bond-cleaving microorganism(s). Therefore, enrichments were established with PEG and diphenyl ether together as substrates. The cultures were successfully adapted to degrade diphenyl ether and later dibenzo-*p*-dioxin. The degradation pathway was demonstrated to be an initial ether bond-cleavage which yielded phenol (from phenyl ether) and apparently catechol (from dibenzo-*p*-dioxin) plus benzene and was similar to the mechanism proposed for PEG depolymerization.

It is well known that some organic compounds are inhibitory to metabolism when present at relatively high concentrations (2,12). This represents a potential problem when attempting to "clean-up" waste streams containing high levels of pollutants. An immobilization technique useful for anaerobic bacteria was devised and tested with phenol as a model inhibitory substrate and is

described in Chapter 5, "Kinetics of phenol biodegradation by an immobilized methanogenic consortium". In theory, the apparent inhibitory substrate concentration would be less for immobilized cells as a result of diffusion of the substrate into the immobilizing matrix where consumption by the cells would maintain a decreased concentration. A model program based upon the Haldane equation was devised (Appendix B) so that the kinetic constants V_{max}, K_m, and K_i could be obtained for both immobilized and non-immobilized cells. This work was successful in demonstrating that immobilization of bacterial cells protected them from the effects of inhibitory substrates. Diethylene glycol and PEG-1000 were also used as substrates for immobilization experiments using the glycol-degrading isolates in co-culture with *Methanobacterium* strain DG1 (Appendix A).

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CHAPTER 2

DEGRADATION OF ETHYLENE GLYCOL AND POLYETHYLENE GLYCOLS BY METHANOGENIC CONSORTIA

INTRODUCTION

Ethylene glycol (EG; 1,2-ethanediol) and its oligomers and polymers are used in the production of substances such as surfactants, explosives, cosmetics, heat transfer fluids, solvents, lubricants, and plastics (1,3). Little attention has been given to their fate in anoxic habitats, such as those in waste treatment, sediments, and landfills, even though billions of pounds are manufactured and discharged into the environment annually, and the high-molecular-weight polymers (up to 25,000) are relatively resistant to aerobic biodegradation (3). In fact, the biodegradation of most polymers, especially of synthetic polymers, by obligate anaerobes is poorly understood. We felt that the general resistance of polyethylene glycols (PEGs) to rapid aerobic degradation, along with their heavy use, made it important to establish whether or not they can be degraded anaerobically and, if so, to compare the rates and products of degradation with those for aerobic systems.

Aerobic micoorganisms use both EG and PEGs as sources of carbon and energy (8,13,20,21). The aerobic metabolism of EG is relatively common, and the pathways of its metabolism are known (2,7,16,20,22,23). However, the ether bond of the oligomers and polymers is comparatively resistant to microbial attack. This is especially true for the degradation of PEG with a molecular weight of 20,000 [PEG-20,000; $HO\text{-}(CH_2\text{-}CH_2\text{-}O\text{-})_{450}\ H$]. Haines and Alexander (6)

reported the isolation of several monocultures of aerobic bacteria able to grow on PEG-20,000, but did not report carbon balances or demonstrate the extent of polymer degradation. The only other report of PEG-20,000 degradation involves the coculture of a *Flavobacterium* sp. and a *Pseudomonas* sp. (9), in which neither microorganism alone degraded the polymer. In this case, significant degradation of the polymer was not verified.

The anaerobic metabolism of EG has been reported. The fermentation of EG by *Clostridium glycolicum* yields equimolar amounts of acetate and ethanol (5); the metabolism of EG by a *Flavobacterium* sp. under microaerophilic conditions follows the sequence acetyl-CoA, acetylphosphate, and acetate (23). The only apparent example of anaerobic PEG degradation is for PEG-400 in anaerobic sludge reactors, where enhanced methane production was noted (11).

We were able to enrich for glycol-degrading consortia from sludge on EG, diethylene glycol (DEG), PEG-400, PEG-1000, and PEG-20,000. We report here on the degradation rates, intermediate products, specificity of enrichment for polymer length, and extent of substrate conversion to gaseous products for each of the five glycols.

MATERIALS AND METHODS

Cultures. Glycol-degrading bacterial consortia were obtained from sludge of a municipal anaerobic digestor in Mason, Mich. Serum bottles (160 ml) were flushed with a 90% N_2-10% CO_2 gas mixture which had been passed over hot copper filings to remove traces of oxygen. During flushing, 10 ml of sludge was added with 90 ml of reduced medium. The bottles were then sealed with black butyl rubber stoppers (Bellco Glass, Inc.) and crimped with aluminum seals to maintain anaerobic conditions. Enrichments were maintained by the weekly transfer of 10 ml of enrichment to 90 ml of the fresh medium. Incubation was at 37°C and static.

The basic minimal medium (D.R. Shelton, personal communication) was composed of (per liter): 0.30 g of KH_2PO_4, 0.35 g of K_2HPO_4, 0.5 g of NH_4Cl, 0.1 g $MgCl_2$, 70 mg of $CaCl_2 \cdot 2H_2O$, 20 mg of $FeCl_2 \cdot 4H_2O$, 1 ml of trace metals solution (25), 1.2 g of $NaHCO_3$, 120 mg of $Na_2S \cdot 9H_2O$ and 1 ml of vitamin B solution (18). The substrate was added to give a 0.2% final concentration. The enrichment substrates were either EG (Mallinckrodt, Inc.), DEG (J.T. Baker Chemical Co.) PEG-400 (Fisher Scientific Co.), PEG-1000, or PEG-20,000 (both from J.T. Baker Chemical Co.). Substrates at 0.2% concentration are equivalent to 36 mM EG (36 mM ethylene oxide units), 21 mM DEG (42 mM ethylene oxide units), 5 mM PEG-400, 2 mM PEG-1000, and 0.1 mM PEG-20,000 (45 mM ethylene oxide units each). PEG-20,000 manufactured by Union Carbide Corporation, is termed PEG compound 20M and is formed by joining 8,000-molecular weight polymers with a diepoxide. It has an average approximated molecular weight of 20,000, but the molecular weight distribution is from 5,000 to 80,000, with the largest portion

being unreacted 8,000-molecular-weight monomer (L.F. Theiling, Union Carbide Corp., personal communication).

Experimental procedures. Each consortia used in the experiment had undergone 30 weekly transfers after their initial establishment. The substrate range of each enrichment was determined by transferring 10 ml of actively metabolizing culture to 90 ml of medium containing one of the five substrates. Separate cultures were established to determine the rates of product formation and EG and DEG utilization by each bacterial consortium grown on its own substrates. All experiments were done in triplicate and repeated.

Since polymeric substrates cannot be quantified easily, the rates of degradation were assessed as rates of production formation for DEG, PEG-400, PEG-1000, and PEG-20,000. EG was easily and accurately quantified; therefore, the degradation rate of EG was based on a gas chromatographic assay of EG. For the comparison of rates, all values are expressed as millimolar ethylene oxide units degraded, which correspond to the two-carbon product ethanol and acetate. A rate of production formation of 0.5 mM acetate and 0.5 mM ethanol per h, therefore, was presumed to indicate a rate of 1.0 mM ethylene oxide units metabolized per h.

Analytical methods. Aqueous samples (2 ml) were periodically withdrawn by syringe from the incubated bottles, filtered through a 0.45 um filter (Millipore Corp.) into glass vials, and frozen until analyses were done. At time zero and after 126 h, we took 1-ml samples to determine protein concentration by the method of Lowry et al. (24), using bovine serum albumim as the standard. Bacterial protein was made soluble by heating the samples in 0.5 N NaOH at 90°C

for 10 min. Growth yields (Y-substrate) for the percentage of substrate degraded were expressed as micrograms of protein formed per millimole of ethylene oxide units degraded.

EG, DEG, and ethanol were measured with a Perkin-Elmer 900 gas chromatograph equipped with a 2-m Chromosorb 101 packed glass column (Anspec Co., Inc.) and a flame ionization detector. N_2 was the carrier gas at a flow rate of 50 ml/min. For DEG the injector, column, and manifold temperatures were 250°C. For EG and ethanol, the column temperature was 150°C. Acetate was assayed with a 2-m, Carbopack C-0.3% CW 20 M-1% H_3PO_4 packed glass column (Supelco, Inc.). The samples were acidified with formic acid before injection. The injector, column, and manifold temperatures were set at 125°C. Methane was quantified by injecting 0.2 ml of culture headspace gas into a Carle model 8500 gas chromatograph equipped with a Porapak Q column (Anspec Co., Inc.) and a microthermistor detector. When methane derived from acetate oxidation was used in calculations of product accumulation, the methane value was reduced by four-fifths, and the remaining amount was used to infer the amount of acetate oxidized because of the stoichiometry: $4HO\text{-}CH_2\text{-}CH_2\text{-}OH + HCO_3^- + H^+ \rightarrow 5CH_4 + CO_2$.

Microscopy was by phase contrast and fluorescence with a Leitz Ortholux microscope. The fluorescence was used to identify methanogenic bacteria as described by Mink and Dugan (12).

RESULTS

Glycol degradation. Bacterial enrichments were successfully established on each substrate, EG, DEG, PEG-400, PEG-1000, and PEG-20,000, as evidenced by an increase in turbidity and the production of methane. Microscopic examination revealed that the EG, DEG, and PEG-400 consortia were dominated by two morphological types of bacteria. These were isolated from the DEG consortia and tentatively identified as a *Methanobacterium* sp. and a *Desulfovibrio* sp. The loss of the methanogen occurred sporadically in some of the cultures, which resulted in a loss of culture viability. A further characterization of this apparent syntrophic relationship is being conducted. The PEG-1000 and PEG-20,000 consortia exhibited less distinctive, more varied morphologies of bacteria. One week after transfer to fresh medium, both cultures in the high-molecular-weight substrates contained a large number of fluorescent bacteria resembling *Methanosarcina* sp.

Figure 1 depicts the formation of degradation products by the consortia enriched on EG and DEG. The parallel formation of ethanol and acetate suggests that the glycol monomer unit was dismutated. After 70 h, the conversion of ethanol to acetate with concurrent methane formation was readily apparent in the EG consortia; EG degradation appeared complete before ethanol oxidation commenced. In contrast, the DEG consortia (Figure 1) appeared to oxidize ethanol during DEG use, and ethanol was never completely oxidized to acetate. In a 100 ml culture, the dismutation of 40 mM ethylene glycol units would produce 20 mM ethanol and 20 mM acetate; a final methane quantity of 1.0 mmol could theoretically be produced:

Figure 1. Temporal formation of degradation products by the consortia enriched on EG and DEG.

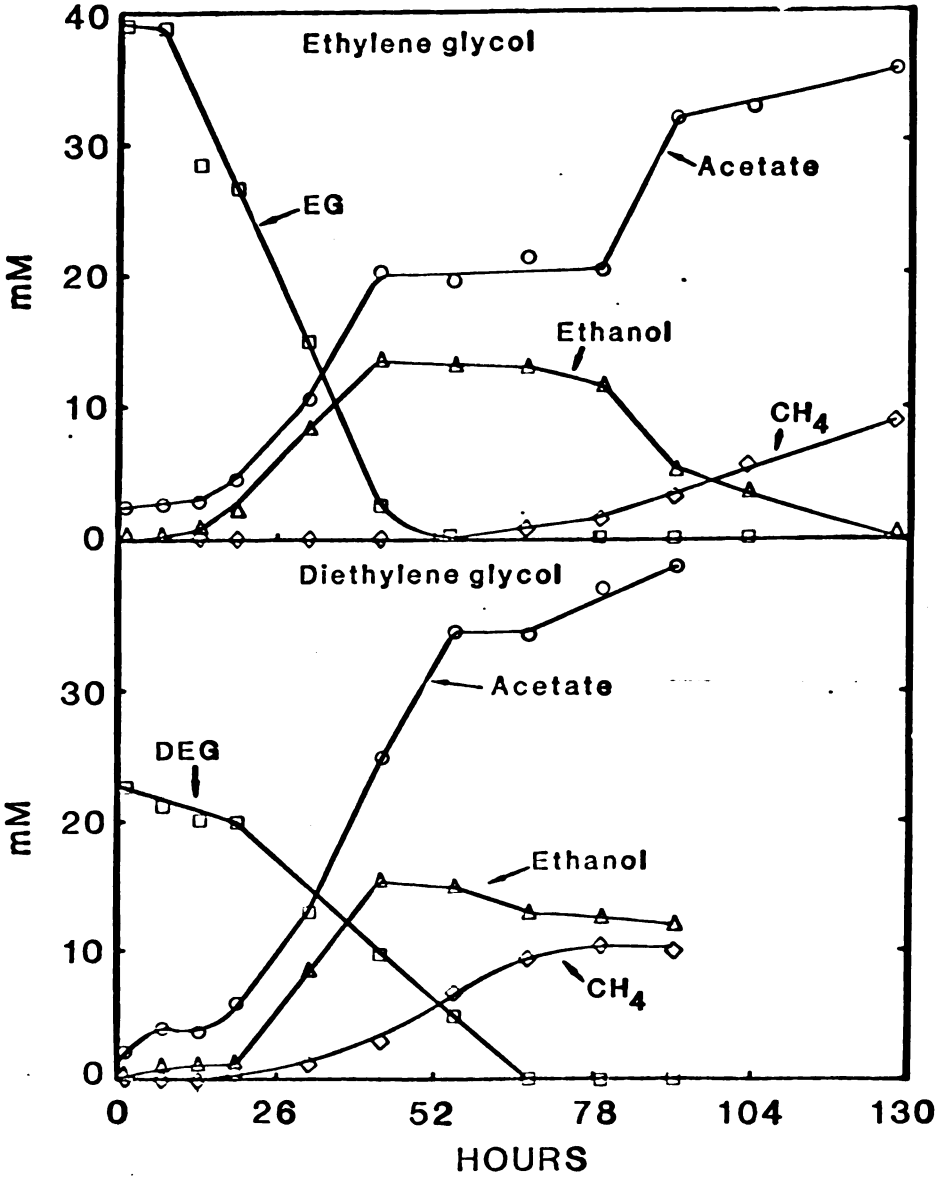
Ethylene glycol
EG
Acetate
Ethanol
CH4
Diethylene glycol
DEG
Acetate
Ethanol
CH4
mM
mM
40
30
20
10
0
30
20
10
0
0
26
52
78
104
130
HOURS

Figure 1

$4HO\text{-}CH_2\text{-}CH_2\text{-}OH \rightarrow 2CH_3CH_2OH + 2CH_3COOH$; $2CH_3CH_2OH + 2H_2O \rightarrow 2CH_3COOH + 4H_2$; and $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$. As expected, the amount of methane in both consortia was one-fourth that of the final acetate concentration and, therefore, is evidence that methane was produced only as a product of ethanol oxidation.

Similar studies with the PEG-400 consortia showed only a transient, low level of ethanol accumulation (18 to 66 h). Methane and ethanol were first detected at the same sampling time; the amount of methane produced subsequently increased until ethanol disappeared (data not shown). This is further evidence that for these consortia, methane was produced only as a product of ethanol oxidation. Methane, ethanol, acetate, and EG (4 to 5 mM) were produced in the PEG-1000 and PEG-20,000 consortia. Ethanol was present only in trace quantities, probably because the rate-limiting step of PEG-1000 and PEG-20,000 degradation was polymer hydrolysis. At 126 h, the amount of methane produced was again one-fourth that of the final concentration of acetate.

Rates of PEG degradation and growth yields. A general decrease in the overall rate of degradation occurred with increases in PEG molecular weight (Table 1). The utilization of glycols as substrates is also shown by the 5-day Y-substrate values (Table 1). The reported DEG consortium growth yield may be too low since ethanol was not totally oxidized to acetate (Figure 1) whereas in the other consortia it was. Three different DEG consortia were subsequently grown until ethanol oxidation was complete; the Y-substrate calculated was 213 ug of protein per mmol of ethylene oxide unit

Table 1. Rates of product formation, carbon recovery, and growth yield of each enrichment on its glycol substrates.

Enrichment and substrate	Rate of product formation (mM C_2 U/h)[a]	% Carbon recovered in products[b]	Y-substrate (ug of protein/ mmol of C_2 U)[c]
EG	0.84 ± 0.04	86-88	144
DEG	0.73 ± 0.05	85-89	148
PEG-400	0.66 ± 0.03	69-75	182
PEG-1000	0.36 ± 0.04	59-63	437
PEG-20,000	0.13 ± 0.01	84-87	512

[a] The rates of product formation (ethanol, acetate) are expressed as the mean ± standard deviation (n = 3) and were calculated for the time period in which a near-constant rate of product formation was observed.

[b] The percent carbon recovered in product is for the period of constant rate and is less than the total carbon recovered in product value found after a long incubation (Table 2).

[c] Y-substrate was calculated at 126 h. The mean is given (n = 3).

degraded. This is still significantly lower than the Y-substrate for the PEG-1000 and PEG-20,000 consortia.

Specificity of consortia for polymer length. Two general observations are evident from the study on substrate specificity (Table 2). First, neither the EG nor DEG consortia were able to significantly attack glycols of higher molecular weight. Second, each PEG enrichment effectively used DEG, perhaps explaining why we were unable to detect DEG as an intermediate. The PEG-20,000 consortia displayed slower degradation of the lower-molecular-weight substrates as compared with the rest of the consortia (e.g. cf. 5-day data on DEG [Table 2]). This may be due to either a lag period in substrate use or to a low relative density of bacteria able to use glycols; microscopic examination of the PEG-20,000 consortia revealed few of the bacterial types which dominated the enrichments metabolizing low-molecular-weight glycols.

The percent degradation for the PEG-1000 and PEG-20,000 consortia was based largely on the accumulation of methane, as the other products were mostly oxidized by day 12 (Table 2). As noted above, both consortia contained *Methanosarcina* sp., which may be responsible for the removal of the acetate. The EG, DEG, and PEG-400 consortia accumulated acetate with no subsequent oxidation. The pH of the consortia fell to 6.0 during their growth period, whereas that of the PEG-1000 and PEG-20,000 consortia remained near 7.0 to 7.2. This may have selected against the *Methanosarcina* sp.

Table 2. Specificity of each consortium for polymer length.

Consortium	% Substrate metabolized after 12 days				
	EG[a]	DEG[a]	PEG-400[b]	PEG-1000[b]	PEG-20,000[b]
EG	100	100(21)[c]	7	7	7
DEG	100	100(100)	9	0	0
PEG-400	86	100(100)	100	62	50(14)
PEG-1000	44	100(83)	60	82	0
PEG-20,000	55	100(60)	90	83	82(33)

[a] Based on substrate disappearance

[b] Based on the accumulation of ethanol, acetate, and methane.

[c] The data given within parentheses are the results after 5 days of incubation and show the slow adaption of the consortia to the given substrates.

DISCUSSION

This study demonstrates significant rates of anaerobic biodegradation of EGs and most importantly, of the recalictrant PEG-20,000 polymer. Heretofore, research has demonstrated the aerobic degradation of PEGs with molecular weights of only 6,000 (16) and less (20) with activated, acclimated sludge. In addition, Cox and Conway (4) found that PEG-1540 was consumed in 2 days, whereas Pitter (17) found that the degradation of 1% PEG-600 and PEG-800 enrichments took 30 days and that of PEG-1000 and PEG-1500 enrichments took 55 and 75 days, respectively. Haines and Alexander (6), using aerobic soil isolates, found that 1% concentrations of PEG-400 were degraded in 5 days and those of PEG-1000 were degraded in 10 days. Our study showed at least an 82% degradation of PEG-20,000 and an 83% degradation of PEG-1000 in 12 days and a 100% degradation of PEG-400 in less than 4 days. Unfortunately, the comparison of our rates of degradation with those obtained aerobically is difficult since the latter data are for biological oxygen demand studies or from the loss of total organic carbon (and thus not specific for substrate conversion), whereas ours rely upon a determination using product recovery. Nonetheless, the substantial rates of anaerobic biodegradation of PEGs by our enrichments obtained from sewage sludge demonstrates a good potential for using anaerobic organisms in PEG removal. Considering the higher cost of aerobic treatment systems the anaerobic process may be advantageous for the removal of this and perhaps other synthetic polymers.

Based on the identity and sequence of the products formed, we are proposing the degradation route for EG in methanogenic consortia

shown in Figure 2. Reactions [1] and [2] have been proposed previously by Weigant and DeBont (22) for *Mycobacterium* E44 which metabolized EG aerobically. For our anaerobic consortia, acetaldehyde is suggested as the electron sink in a dismutation producing acetate and ethanol; reaction [3] thus would account for the early formation of ethanol. The subsequent consumption of ethanol with a concomitant production of acetate and methane is described by reaction [4]. Whereas reactions [1] through [3] are energetically favorable (19), reaction [4], the oxidation of ethanol to acetate, is only favorable if the hydrogen concentration is kept low. This presumably was accomplished by the methanogen(s) and suggests a syntrophic association. The stoichiometry and sequence of the products that we observed (Figure 1) make any other degradative pathway unlikely, although another possibility does exist. EG could be hydrogenated to ethanol ($\Delta G^{o'}$ = -21 kcal [ca. -87.9kJ]) with the hydrogen derived from acetaldehyde oxidation to acetate, but this would require the presence of novel enzyme(s) and, therefore, is less likely.

The ability of our PEG consortia to accumulate EG and to use EG and DEG (Table 2) suggests that glycol units were released from the polymer by hydrolysis before subsequent metabolism to acetate, ethanol, and methane. This is similar to the examples of the aerobic, hydrolytic microorganisms which also use EG and were studied by Haines and Alexander (6). In contrast, other aerobic depolymerizations appear to involve either an initial dehydration of the terminal glycol unit (16) or a dehydrogenation (9,10,15), in which case, the microorganisms appear unable to use EG. Because the

Figure 2. Proprosed pathway for degradation of EG by methanogenic consortium.

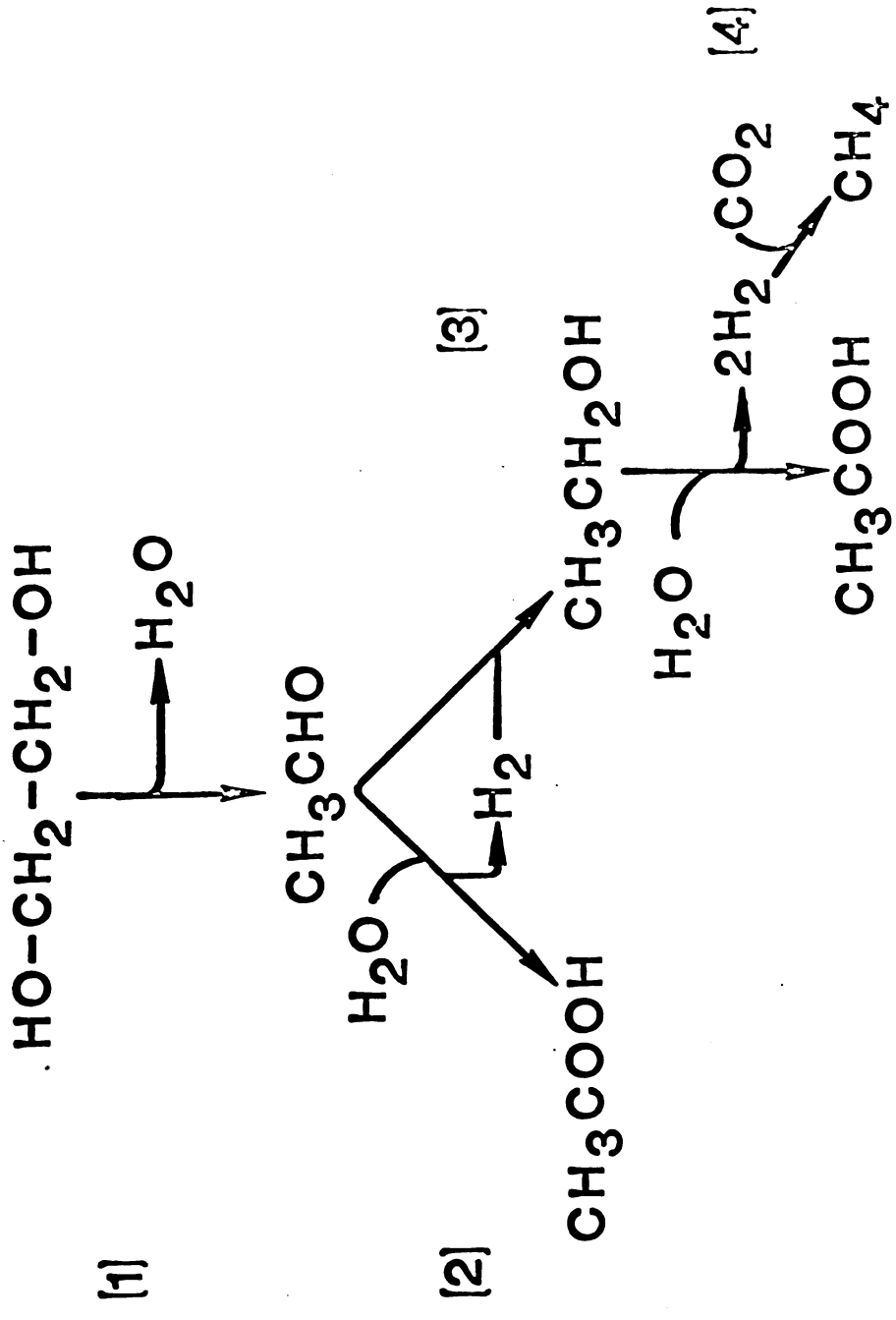
HO-CH2-CH2-OH
H2O
[1]
CH3CHO
H2O
H2
[2]
CH3COOH
[3]
CH3CH2OH
H2O
2H2
CO2
CH4
CH3COOH
[4]

Figure 2

glycol polymers are degraded by a rate one-sixth that of monomer degradation (Table 2), it appears that the polymer first must be hydrolyzed into fragments, from which monomers can then be hydrolytically removed. If enzymatic attack occurred only from the ends, we would expect the degradation rate of the PEG-20,000 polymer [HO-$(CH_2$-CH_2-O-$)_{450}$H] to be less than one-sixth that of the monomer.

Our results demonstrated an inability of the EG and DEG consortia (where selection was for non-depolymerizing bacteria) to metabolize polymers. This is comparable to earlier research with aerobic bacteria (14), which showed that the ability to grow on oligomers, but not polymers, of EG is due to an inability of appropriate enzyme(s) to reach the polymer substrate. We have also demonstrated that each consortium, although adapted to metabolizing glycols with a molecular weight similar to that of its own substrate, also was able to use DEG efficiently. The hydrolytic cleavage of polymers may explain the DEG metabolism, and a difference in the conformational structure of polymers may dictate substrate specificity. Cox (3) noted that PEG biodegradibility may depend on the conformation of the molecule, which appears to be helical in solution (1), whereas low-molecuar weight PEG (< 400) has a zig-zag pattern. Such conformational differences could influence the activity of an enzyme and inhibit, for example, PEG-20,000 utilization by the bacteria of the PEG-400 consortia. In this way, conformational differences could explain the relatively greater ability of the PEG-400 and PEG-1000 consortia to degrade their own respective polymer.

The relatively greater growth yields obtained for high-molecular-weight polymers, as measured by protein concentrations, were unexpected. The results may have been due to either an increase in yield from some acetate metabolism in the consortia or to a difference in metabolism between the dominant bacterial types. Pure culture studies may help clarify this by separating other members of the consortia from the glycol degraders.

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CHAPTER 3

DEGRADATION OF SEVERAL NONIONIC SURFACTANTS AND SUBSTITUTED POLYETHYLENES BY METHANOGENIC ENRICHMENT CULTURES

INTRODUCTION

Nonionic surface-active agents (surfactants) are established alternatives to phosphate and nitrilotriacetic acid (NTA) requiring detergents. In 1983, 300,000 metric tons of nonionic surfactants were used in the U.S. with the market increasing at an annual rate of 2-3% (5). Use of such large quantities has inspired studies to determine biodegradability and toxicity of these compounds.

Alcohol ethoxylates (AE) and alkylphenol ethoxylates (APE) are the principal nonionic surfactants. Both contain a polyoxyethylene $(-O-CH_2-CH_2-)_n$ moiety as the water soluble constituent and an alkyl moiety. A linear alkyl group is preferred (5) because branching interferes with its carboxylation and subsequent degradation (9,10). Both surfactant types are toxic; longer alkyl chain lengths and shorter ethoxylate chain lengths increase the toxicity (1).

AE are generally more degradable than APE (7). Mineralization of trace quantities of AE occurs in estuarine waters (19) and approximately 100% removal occurs in activated sludge (8,9,17,18) with subsequent elimination of AE toxicity (8,18). The APE polyoxyethylene (POE) moiety is partially degraded during aerobic wastewater treatment (12) yielding mono- and di-ethoxylates. These intermediate products can be further degraded in anaerobically stabilized sludge to alkylphenols which are highly toxic (4,15). Since most nonionic detergents end up in municipal wastes, there is

potential for the accumulation of toxic metabolites where such wastes are anaerobically processed.

In contrast to the work done with aerobic systems, little is known concerning the anaerobic degradation of noinionic surfactants. We report here that the entire POE moiety of APE can be degraded with concommitant production of alkyl phenol at concentrations toxic to the methanogenic enrichment cultures. Attempts to obtain alkyl phenol degradation in cultures acclimated to analogous, biodegradable substrates were unsuccessful. Methanogenic enrichments were also established which mineralized AE, sorbitan based nonionic surfactants, and substituted polyethylenes of low solubility.

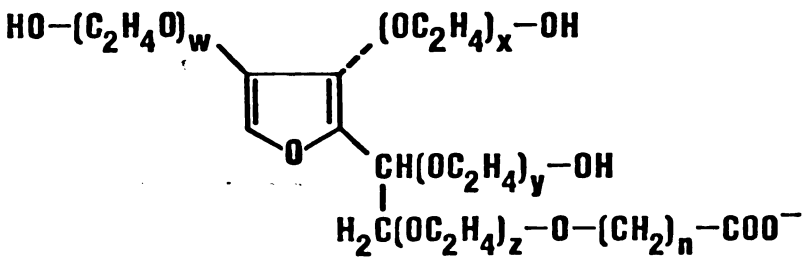
MATERIALS AND METHODS

Chemical abbreviations and sources. Igepal CA-720 (octylphenol polyethoxylate), Igepal CO-720 (nonylphenol polyethoxylate), Igepal CO-210 (nonylphenol diethoxylate) (all three Igepal compounds from GAF Corp.), polyacrilonitril (PACN), polyethylene (PE), polyethylene glycol with a molecular weight of 20,000 g/mol (PEG-20,000) (Sigma Chem. Co.), polyoxyethylene 23-lauryl ether (Brij-35) (Sigma Chem. Co.), polyoxyethylene sorbitan mono-oleate (Tween-80) and mono-laurate (Tween-20) (Sigma Chem. Co.), polystyrene (PSTY), polyvinyl acetate (PVAC), polyvinyl alcohol (PVAL) (PE and all substituted polyethylene compounds from Aldrich Chem. Co.). Other abbrevations: alcohol ethoxylates (AE), alkylphenol (AP), alkylphenol ethoxylates (APE), polyoxyethylene (POE).

Enrichments. Surfactants (Figure 1), substituted polyethylenes (Table 1), ethylphenol, nonylphenol, and PEG-20,000 were screened for degradation in anaerobic medium containing 10% sludge obtained from municipal anaerobic digesters in Michigan. The anaerobic enrichment procedure and medium (RAMM) have been described previously (14). All enrichments (50 ml) were done in 160 ml serum bottles sealed with butyl rubber stoppers. Headspace gas was a 90%/10% mixture of N_2/CO_2. Substrates were at a concentration of 0.2% w/v except for alkylphenols and alkylphenol ethoxylates which, because of their toxicity, were added at concentrations of 0.1, 0.05, and 0.01% v/v. The degree of substrate degradation was measured by quantifying the production of methane. The variation in background methane levels produced by control (unamended) cultures was on the order of 0.8 $\pm$ 0.008 mmol CH_4. This low variability allowed substrate

Figure 1. Chemical structures of surfactants used in the study: (A) Tween-20 (n = 12) and Tween-80 (n = 18): the sum of w, x, y, and z equals 20. (B) Brij-35. (C) 4-alkylphenol polyethyoxylates: Igepal CA-720 (a = 11, b = 7), Igepal CO-720 (a = 11, b = 8), Igepal CO-210 (a = 1, b = 8).

A.



B.

C.

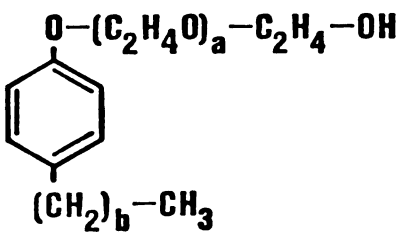


Figure 1

mineralization to be quantitated with good precision. Successful enrichments were maintained by transfer of 10% of the culture into fresh medium. Whole sludge enrichments were established with phenol and p-cresol and fed 2 mM substrate every 2 days.

Analytical methods. Methane was quantified by injecting 0.2 ml of culture headspace gas into a Carle model 8515 gas chromatograph equipped with a Porapak Q packed column (Anspec Co., Inc.) and a microthermistor detector. Phenol, p-cresol, ethylphenol, and nonylphenol were extracted from 1 ml of enrichment cultures with 1 ml acetonitrile. Extracts were filtered through 0.45 um Millipore filters (HVLP) and analyzed by high pressure liquid chromatography (HPLC) using a Waters Liquid Chromatograph, Model 6000A, equipped with a radial compression separation system, Model ACM-100, a Radical-PAK C18 analytical column and a uv absorbance detector, Model 441. The mobile phase was acetonitrile-5% acetic acid (1:1) at a flow rate of 20 ml/min.

Surfactants and PEG were assayed in 5 ml samples of enrichment by both an absorptiometric (16) and a thin-layer chromatographic (TLC) determination (11). Both methods detect POE of five ethoxylate units or more. The absorptiometric determination involved acidification and precipitation of POE containing compounds with two drops each of a 10% w/v solution of barium chloride and phosphomolybdic acid. The precipitate was dried in a stream of air and then dissolved in concentrated sulphuric acid. Absorption was determined at 520 nm and surfactant concentration was obtained from a previously constructed calibration curve. Nonionic material for TLC analysis was extracted from 1 ml of culture with chloroform. The

extract was washed in 1 N HCl followed by an alkaline-wash of 2 N NaOH and was then evaporated to dryness. The resultant residue was dissolved in 0.5 ml chloroform and 10 ul was spotted onto pre-made Kieselguhr G TLC plates (Analtech, Inc.). Chromatograms were developed with solvent mixtures of ethyl acetate-water-acetic acid (40:30:30, Solvent A) or (70:15:15, Solvent B). Solvent A was used to obtain a single spot for quantitative determinations. Solvent B was used to obtain a "polyoxyethylene spectrum" of nonionic substrates resolved by molecular weight such that partial degradation products could be detected. Nonionic material was detected by spraying the developed plates with modified Burger reagent (11).

RESULTS

Degradation of polyethylene compounds. PVAL and PVAC, were degraded to a significant extent in sludge enrichments from two of five sources (Table 1). When background CH_4 was no longer being produced, as evident in unamended sludge cultures, 3-5 umol CH_4/day were produced in the PVAL and PVAC enrichment cultures. The remaining three PVAL and PVAC and other polyethylene enrichment cultures did not produce more CH_4 than unamended sludge. PEG-20,000 was used as a positive control and was readily mineralized to CH_4 and CO_2 in enrichment cultures from all five sources.

Nonionic surfactant degradation. All three non-APE surfactants were readily mineralized to CH_4 and CO_2 (Table 2). Mineralization was complete by day 28 and surpassed the rate of PEG-20,000 degradation. Approximately 75% of the theoretical amount of CH_4 had been produced from Tween-20 and Tween-80 and 5% from Brij-35 by day 11. Absorptiometric and TLC analyses indicated that the POE moiety of Brij-35 was no longer detectable by day 11, but was only partially degraded by day 11 for Tween-20 and Tween-80. CH_4 production values indicated that although the POE moiety was more rapidly degraded in Brij-35 than Tween compounds, mineralization of the degraded POE moiety was not concurrent. PEG-20,000 was initially present as a complete spectrum of POE polymer lengths as shown by TLC analysis using solvent B. Degradation of smaller polymers was more rapid than that of longer polymers which were still present at day 28.

A lag in the onset of mineralization of 4-APEs occurred at a concentration of 0.05% v/v. This was more pronounced for octyl- than nonyl-polyethyoxylate (Table 3). The amount of CH_4 produced

Table 1. Degradation of polyethylenes by methanogenic enrichments.

Substrate mean mw (g/mol)	Structure	Samples showing degradation[a]	CH_4 production[b] (mmol)
PEG (20,000)	$HO-[CH_2CH_2O-]_nH$	5/5	3.3-4.8
PVAL (14,000)	$[-CH_2CH(R)-]_n$ R= -OH	2/5	1.2
PVAC (>1,500)	" R= $-OOCCH_3$	2/5	1.0
c		0/5	0.8

[a] Number of different sludge samples with CH_4 production significantly ($n=3;\alpha=0.05$) above controls after 21 days incubation at 37°C.

[b] Unamended controls produced approximately 0.8 ± 0.008 mmol CH_4.

[c] Substrates not degraded included PE (R = -H), PSTY (R = $-C_6H_5$) and PACN (R = -CN).

Table 2. Degradation of polyethoxylate surfactants by methanogenic enrichments.

Substrate	Theoretical CH_4 production (mmol)[a]	% of Theoretical CH_4 produced[b]	
		Day 11	Day 28
Tween-20	0.7	76	100
Tween-80	0.7	74	100
Brij-35	0.8	5	100
PEG-20,000	0.7	45	60

[a] CH_4 production was calculated for the complete mineralization of substrate carbon.

b Values are the means of two enrichments cultures.

indicated that only the POE moiety was degraded leaving alkylphenols to accumulate. Corresponding HPLC analysis of Igepal CO-720 enrichments showed a peak that appeared, following CH_4 production, with the same retention time as nonylphenol.

Prior acclimation of sludge with either Tween-20, Tween-80, or Brij-35 enhanced the rate of POE degradation of the 4-APEs (Table 3). This was observed for CA-720 in particular. Acclimation also increased the tolerable concentration of 4-APE to 0.1%. Methane was produced from 0.1% 4-APE in non-acclimated enrichment cultures, but as degradation proceeded CH_4 production did not attain its theoretical level unless a 50% transfer of the culture was made to unamended medium. This indicated that a build-up of alkylphenol inhibited further degradation of the original substrate.

Whole sludge and 10% sludge enrichments which mineralized phenol or p-cresol could not degrade either ethylphenol or nonylphenol at concentrations of 0.1, 0.05, and 0.01% v/v. In every case, CH_4 production was depressed below that of unamended controls indicating that these substrate concentrations were toxic. Whole sludge was also given 0.0025% ethylphenol. HPLC analysis indicated that ethylphenol disappeared with time. CH_4 production could not be used as a measure of degradation as ethylphenol at this concentration would not produce CH_4 at levels distinguishable from background CH_4 production.

Table 3. Degradation of 4-alkylphenol polyethoxylates by methanogenic enrichments.

Substrate	Prior acclimation on Brij-35[a]	CH_4 production (mmol) 28 Days	56 Days	% Theoretical CH_4 recovery at 56 days[c]
CA-720	+	0.96	0.96	96
CA-720[b]		0	0.48	96
CO-720	+	0.76	0.85	85
CO-720[b]		0.46	0.46	92
CO-210	+	0.31	0.31	97
CO-210[b]		0.16	0.28	88

[a] Substrate (0.1%) was added to methanogenic enrichments acclimated to Brij-35 (mean of two enrichments). Prior acclimation with Tween-20 and Tween-80 gave similar results.

[b] Substrate (0.05%) was added to 10% fresh sludge enrichments (mean of two enrichments).

[c] Theoretical CH_4 production was calculated for mineralization of POE.

DISCUSSION

The polyethylene compounds in this study (Table 1) are all subject to aerobic degradation by soil bacteria (6). We found that only PVAL and PVAC were degraded anaerobically. Saturated hydrocarbons such as PE appear to be recalcitrant to anaerobic metabolism (13). This is the first indication that PE with oxidizable substituents, in this case hydroxyl and acetyl groups, can be anaerobically degraded. In contrast, PSTY and PACN, which contain non-oxidizable substituents, were recalcitrant to anaerobic degradation.

The hydroxyl substituents of PVAL apparently were sites for oxidation and hydrolysis of the polymer. Prior examples of the anaerobic demethoxylation of aromatic compounds (2) suggest that the acetyl substituents of PVAC might be hydrolytically cleaved to yield an hydroxylated polymer and ethanol. Complete metabolism of both PVAL and PVAC could be expected to occur by such degradative pathways. Both cultures continued to produce additional CH_4 at least through day 56 when compared to controls.

While the presence of oxidizable substituents on PE was sufficient to allow degradation, the low solubilities of the polymers may have contributed to their relatively slow rates of degradation. In contrast, PEG-20,000 which is both soluble and contains sites for polymer cleavage (3) was rapidly degraded.

The nonionic surfactants listed in Table 2 are commonly used for their detergent qualities, but little is known of their anaerobic biodegradability. TLC and absorptiometric analyses on day 11 indicated that the POE moiety of Brij-35 was rapidly degraded. This

should produce an alkyl intermediate in contrast to aerobic degradation of Brij-35 in which the alkyl moiety is degraded first (7). As with the polyethylene compounds, it appears that soluble POEs were anaerobically degraded more rapidly than alkyl moieties. This is corroborated by studies from our laboratory in which 0.2% PEG-1000 (n = 22) was mineralized in 6 days in unacclimated medium containing 10% sludge while degradation of 15 mM (0.3%) lauric acid by a co-culture of fatty acid-degrader and methanogen required 12 weeks.

In contrast to Brij-35, the POE of Tween-20 and Tween-80 was not entirely degraded by day 11. This may be due to the structure of Tween in which one of the POE units is bounded by alkyl structures (Figure 1). It is of interest that the furan moiety of the Tween compounds must also have been ultimately degraded as judged by complete methane recovery.

Although the entire POE moiety of 4-APEs was degraded anaerobically, repeated efforts to degrade ethylphenol and nonylphenol were unsuccessful. Prior acclimation of enrichment cultures with nonionic surfactants increased only the rate of POE degradation, while the apparent concomittant production of toxic 4-alkylphenol from 0.1% 4-APE inhibited POE metabolism. Whole sludge and enrichments which degraded phenol and p-cresol (compounds with structures similar to 4-AP) were unable to degrade alkylphenols at concentrations of 0.01% even after 6 months of incubation.

The toxicity of 4-APs makes them a potentially serious environmental problem especially in light of their accumulation under anaerobic conditions (4,15). The concentration of 4-APs in sludge is

often above that which we found to be inhibitory to our enrichment cultures. Giger *et al.* (4) found 4-nonylphenol in anaerobically stabilized sewage sludge to vary from 0.45 to 2.53 g/kg and noted that the maximum permissible concentrations for substances of similar toxicity was about 0.03g/kg.

Three factors might contribute to the apparent recalcitrance of 4-alkylphenols to anaerobic degradation: (i) The combined aromatic and alkyl structure may be non-oxidizable. Other aromatic structures with hydroxyl groups are anaerobically degraded, but the alkyl chain of AP may inhibit the enyzme(s) which cleave the aromatic ring. (ii) APEs lose their solubility upon losing their POE moiety. Low solubility may be a problem if resultant APs become sequestered in lipophilic flocs and are thus inaccessible to microbial activity (4). (iii) The toxicity of APs to the bacterial community may inhibit their own degradation. At low concentrations of AP, this possibility seems least likely since degradation of 0.05% 4-APE slowly evolved ethylphenol and nonylphenol over a period of many days (Table 3) which were not further degraded.

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CHAPTER 4

METABOLISM OF POLYETHYLENE GLYCOL BY TWO ANAEROBIC BACTERIA, *DESULFOVIBRIO DESULFURICANS* AND A *BACTEROIDES* SP.

INTRODUCTION

Polyethylene glycol (PEG; $HO\text{-}(CH_2\text{-}CH_2\text{-}O\text{-})_nH$) is a nonionic, water soluble polymer of ethylene oxide used in the production of substances such as nonionic surfactants, lubricants, plastics and cosmetics which together consume billions of pounds of ethylene oxide each year (6,12). PEG was once thought to be a recalcitrant xenobiotic with non-degradable ether bonds (6), but both aerobic and anaerobic biodegradation have been reported. Under aerobic conditions, PEG is degraded by dehydrogenation to either carboxylated intermediates (14,15) or glycolaldehyde (24), by acetaldehyde production (18) and by extracellular hydrolytic cleavage with production of ethylene glycol (EG) and diethylene glycol (DEG) (11). Monomeric EG is degraded either by oxidation to glycolic acid with further metabolism through the glycerate pathway (5,10,27) or by dehydration to acetaldehyde with metabolism through the TCA cycle (26).

Recent evidence has suggested that anaerobic depolymerization of PEG results in the production of acetaldehyde which is subsequently dismutated to acetate and ethanol or dehydrogenated to acetate under methanogenic conditions (7,21). Under anaerobic and microaerophilic conditions EG is metabolized by dehydration to acetaldehyde followed by dismutation to acetate and ethanol (1,8,9,27).

Anaerobic cleavage of ether bonds has been reported only for the depolymerization of PEG, the demethoxylation of methoxylated aromatics (2) and in degradation of the aryl-glycerol-β-aryl-ether linkage in lignin (5). The novelty of this reaction has led us to study anaerobic ether bond cleavage of PEGs in more detail. This paper presents evidence that the ether bonds of long chain PEGs are cleaved by either an extracellular hydrolysis or hydrogenation. Two strictly anaerobic bacteria which degrade glycols are identified and compared for their glycol degrading properties.

MATERIALS AND METHODS

Abbreviations used. Ethylene glycol (EG), diethylene glycol (DEG), polyethylene glycols with average molecular weights of 400 (PEG-400), 1000 (PEG-1000) and 20,000 (PEG-20,000), polyoxyethylene 23-lauryl ether (Brij-35), polyoxyethylene sorbitan mono-laurate (Tween-20), polypropylene glycol with average molecular weights of 425 (PPG-425) and 2000 (PPG-2000).

Isolation and characterization of bacterial strains. Methanogenic consortia which used either EG, DEG, PEG-400, PEG-1000, or PEG-20,000 as sole carbon and energy source were obtained from sludge of a municipal anaerobic digester in Mason, MI. The anaerobic enrichment procedure has been described previously (7). Bacterial strains DG2 and PG1 were isolated from the DEG and PEG-1000 degrading consortia, respectively, using anaerobic "roll tubes" (17). Roll tubes were made using an anaerobic mineral medium (RAMM) (22) supplemented with 0.2% substrate, 2% agar and either 20 mM Na_2SO_4, a lawn of *Methanobacterium* strain DG1 as H_2-consumer, or no added electron acceptor. Isolated colonies were transferred from roll tubes with sterile Pasteur pipettes to 50 ml substrate amended RAMM in 160 ml serum bottles which were sealed with butyl rubber stoppers. The procedure was repeated three more times. All transfers were made under a 90%/10% gas mixture of N_2/CO_2. Culture purity was checked both by phase microscopy and by growth on complex media.

The substrate range for both the methanogenic consortia and the isolates was assessed by measuring growth as a change in absorbance at 560 nm of triplicate 10 ml cultures in 20 ml Balch tubes using a Turner Model 350 Spectrophotometer. Various substrates (0.1%) were

tested as the sole carbon and energy source for the following cultures and conditions: (i) the enriched methanogenic consortia; (ii) DG2 under fermentative conditions, with 20 mM Na_2SO_4, and in co-culture with the methanogen *Methanobacterium* DG1; and (iii) PG1 under fermentative conditions and in co-culture with the methanogen. The pH was adjusted to 7.2 where necessary. PPG ($HO-[CH_2-CH_2-CH_2-O-]_nH$) was of particular interest as substrate due to its similarity in structure with PEG. Fermentation balances for PG1 were obtained from 50 ml cultures grown in 160 ml serum bottles and for DG2 in 320 ml cultures grown in 4 L flasks sealed with rubber stoppers. Both growth and fermentation balances were determined after three successive transfers of 10% inoculum grown on the substrate of interest.

Substrate dehydrogenation by cell-free extracts. Cell-free extracts of PG1 and DG2 were obtained by passing washed cell suspensions (10 mM phosphate buffer, pH 7.2) through a French pressure cell (10,000 psi), centrifuging the resultant solution at 15,000 x g for 15 min and collecting the supernatant. All transfers were made under an 80%/20% gas mixture of N_2/CO_2. Substrate oxidation by the cell-free extract was assayed by measuring the initial rate of dichlorophenol indophenol (DCPIP) reduction as a change in absorbance at 600 nm (15) using a Perkin-Elmer Model 85 Spectrophotometer. The reaction mixture contained per ml of cell-free extract: 43 nmol DCPIP, 14 nmol flavin adenine nucleotide (FAD) and 18 umol Tris buffer. The reaction was started by the addition of 1 umol of substrate. All substrates were provided on an equimolar

basis to provide the same number of hydroxyl groups for enzymatic action.

Analytic methods. Aqueous samples (1 ml) were periodically withdrawn by syringe from the incubated cultures and filtered through a 0.45 um filter (Millipore Corp.). EG, DEG, methane, acetate and ethanol were assayed by gas chromatographic methods described previously (7). Acetaldehyde was assayed with a Perkin-Elmer 900 gas chromatograph equipped with a 2 m, 10% SP-1000 on 80/100 Supelcoport packed steel column (Supelco, Inc.). Injector and manifold temperatures were 180°C; column temperature was 130°C. Succinate and lactate were measured after methylation (VPI Anaerobe Laboratory Manual, 1972) using a 2 m Carbopack C-0.3% CW 20 M-1% H_3PO_4 packed glass column. Injector and manifold temperatures were 180°C. The initial and final column temperatures and program rate were 100°C, 160°C, and 16°C/min, respectively. N_2 at 50 ml/min was the carrier gas for both columns. Hydrogen was quantified by injecting 3 ml of culture headspace gas into a Carle Model AGC-111 gas chromatograph equipped with a thermistor detector.

Protein concentrations in the cell-free extracts were determined by the method of Lowry (28) using bovine serum albumin as the standard. Bacterial protein was made soluble by heating samples in 0.5 N NaOH at 90°C for 10 min. Cytochromes were assayed in cell-free extracts of strain PG1 grown on glucose and of strain DG2 grown on pyruvate and Na_2SO_4. The extracts were used to obtain difference spectra (25) using a Perkin-Elmer Model 85 Spectrophotometer. Microscopy was by phase contrast using a Leitz Ortholux microscope. Flagella were observed by transmission electron microscopy using a

Philips 300 electron microscope. Desulfoviridin was assayed by the procedure of Postgate (19).

RESULTS

Characterization of isolates. Two bacteria able to use polyethylene glycol (PEG) as their sole carbon and energy source were recovered as colonies in roll tubes. Strain DG2 was isolated as a DEG-fermenting bacterium from a methanogenic enrichment fed DEG. With Na_2SO_4 provided, colonies with bacteria of morphology similar to strain DG2 were black indicating that SO_4^{2-} reduction had occurred. When a lawn of Methanobacterium DG1 was provided, colonies occurred as mixtures of the proposed DG2 and methanogen. Although colonies of strain DG2 were obtained from every enrichment, DG2 grew in pure culture using as substrate only ethylene oxide oligomers ranging in size from EG through TEEG (Figure 1a). By comparison with the substrate range for the DEG-degrading enrichment (Figure 1a), it was concluded that strain DG2 was the primary oligomeric ethylene glycol-degrading bacterium in the enrichments.

Isolate DG2 was a strictly anaerobic, gram-negative, non-sporeforming, curved rod 1 **x** 3-5 um in size, and motile with a single polar flagellum. The principle cytochrome was c_3; desulfoviridin was present. Growth with sulfate reduction or in co-culture with methanogen occurred with lactate, pyruvate, malate and ethanol as substrate. Growth by fermentation occurred with pyruvate. From these characteristics the bacterium was identified as *Desulfovibrio desulfuricans*. *D. desulfuricans* DG2 fermented 1,2-propanediol and 1,3-propanediol with the production of H_2 and produced only the corresponding acid during growth with sulfate and in co-culture with the methanogen. Substrates not supporting growth included glycolate,

Figure 1a and b. The substrate ranges for (a) the DEG-degrading enrichment and *D. desulfuricans* DG2 at 4 days and (b) the PEG-1000 degrading enrichment and *Bacteroides* PG1 at 10 days were obtained by growth measurements using EG and PEGs (0.2%) as sole substrate.

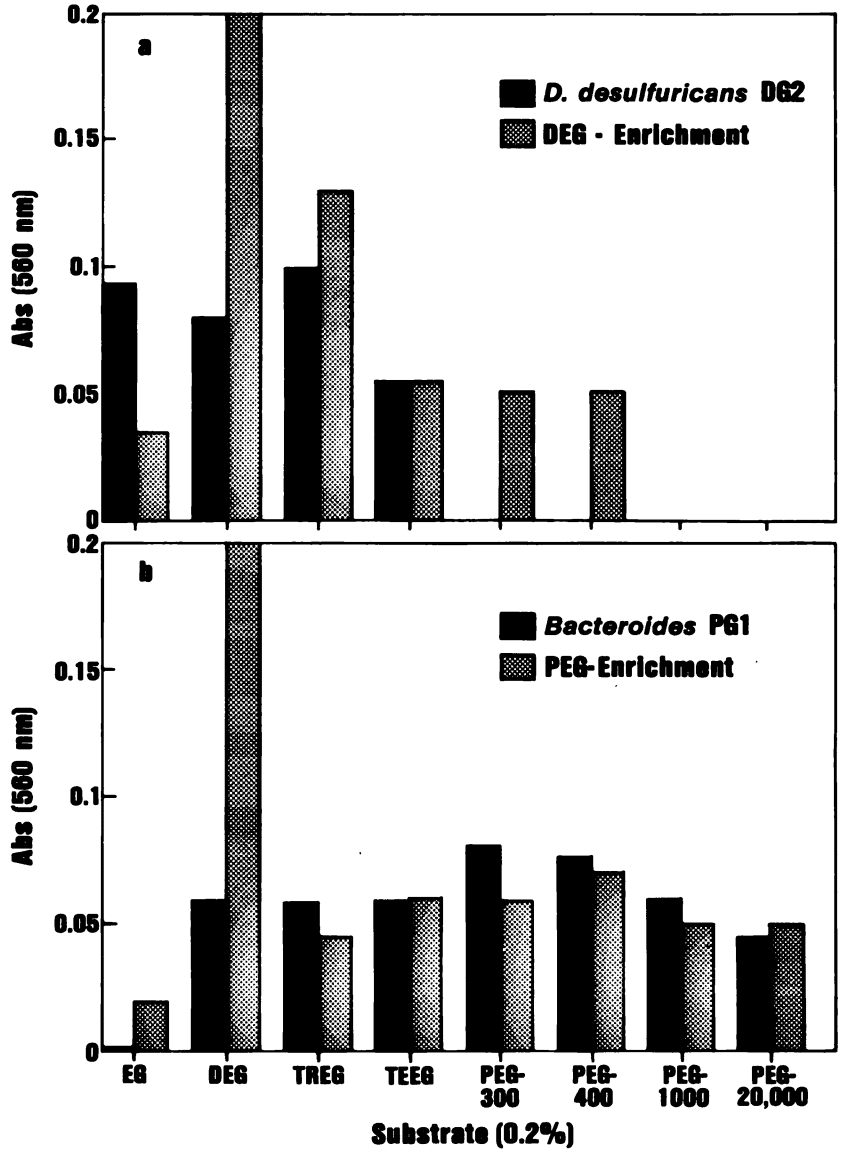

Figure 1a and 1b

glyoxylate, glycerol, ethanolamine, Tween-20, Brij-35, PPG-425 and PPG-2000.

PG1 was isolated as a PEG-fermenting bacterium from a methanogenic enrichment fed PEG-1000. With Na_2SO_4 provided, colonies with bacteria of morphology similar to strain PG1 occurred as mixtures with bacteria resembling *D. desulfuricans* DG2. When a lawn of *Methanobacterium* DG1 was provided, the colonies occurred as mixtures of proposed PG1, DG2 and methanogen. Bacteria with the morphology of strain PG1 occurred only in PEG-degrading enrichments as determined by microscopic observation of enrichments and colonies. A comparison of the substrate range for PG1 and the PEG-1000 degrading enrichment (Figure 1b) indicated that strain PG1 was the primary PEG-degrading bacterium in the enrichments.

Isolate PG1 was a strictly anaerobic, non-motile, gram-negative, pleomorphic rod with rounded ends, occurring singly (1 x 2 um) or in pairs during log phase growth. Long chains of bacteria occurred during late log phase. Growth occurred by fermentation of glucose and of peptone with production of succinate, lactate and acetate. No cytochromes were detectable. Thus, the bacterium was presumed to be a *Bacteroides* sp. *Bacteroides* PG1 grew by fermentation on Tween-20, Brij-35, 1,3-propanediol, 1,2-propanediol, and ethanolamine as substrates. Substrates not supporting growth included glycolate, glyoxylate, glycerol, PPG-425 and PPG-2000. The addition of 0.1% sodium acetate did not improve the growth of either strain with non-growth supporting substrates.

Glycol degradation. Fermentation of DEG by pure cultures of *D. desulfuricans* DG2 resulted in the formation of acetate and H_2 with

Figure 2. Degradation of DEG by *D. desulfuricans* DG2 resulted in the production of acetate, ethanol and H_2. Ethanol was subsequently oxidized to acetate.

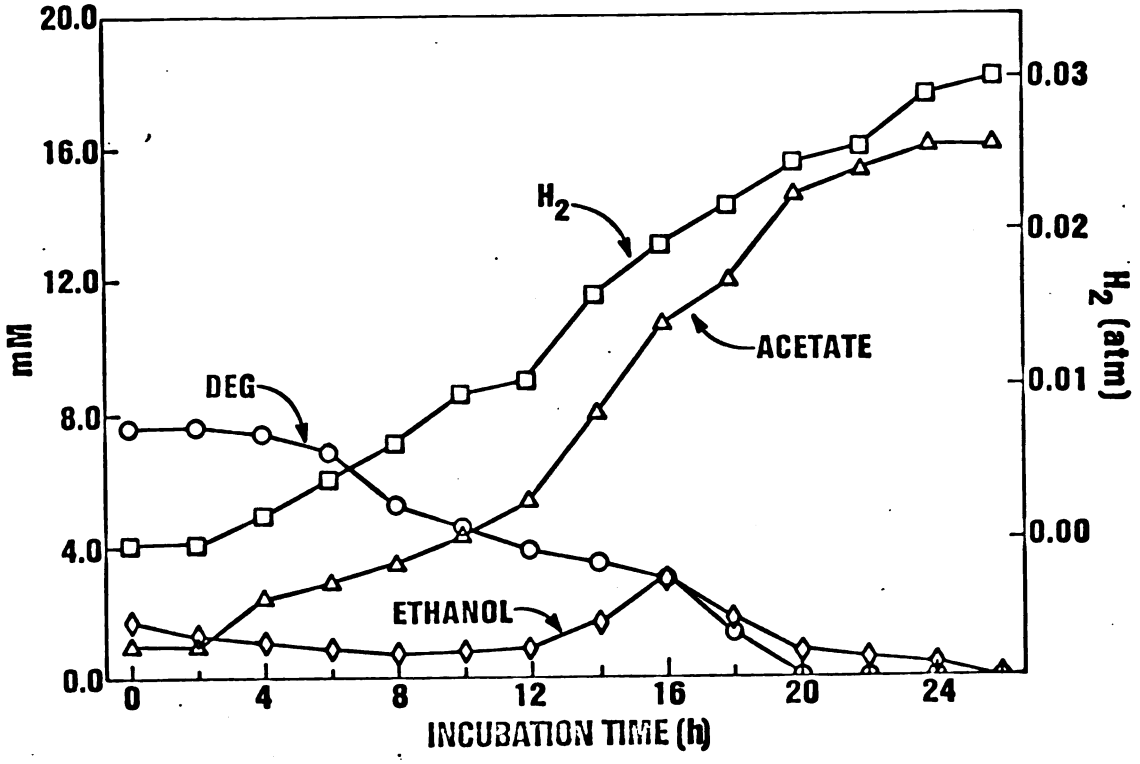
20.0
16.0
12.0
8.0
4.0
0.0
mM
0.03
0.02
0.01
0.00
H_2 (atm)
H_2
ACETATE
DEG
ETHANOL
0
4
8
12
16
20
24
INCUBATION TIME (h)

Figure 2

ethanol as an intermediate (Figure 2). Acetaldehyde was produced at concentrations of 0.1-0.35 mM. The $\Delta G'$ remained favorable for the overall reaction at the maximum H_2 level observed (0.025 atm) ($\Delta G^{o'}$ = -78 kJ/mol, 22). The sequence of reaction products was the same for *Bacteroides* PG1 with PEG-1000 as substrate, although ethanol was not entirely oxidized (Table 1). Both *D. desulfuricans* DG2 and *Bacteroides* PG1 grew well in co-culture with the methanogen as the electron sink and DEG or PEG-1000 as their respective substrate. The stoichiometry for glycol degradation in each case is shown in Table 1.

The cell-free extracts of both *Bacteroides* PG1 and *D. desulfuricans* DG2 dehydrogenated glycols, other hydroxylated substrates and acetaldehyde as determined by the substrate-dependent reduction of DCPIP (Table 2). PG1 extracts were not active on glycerol, a compound with three adjacent hydroxyl groups. Although PG1 was able to use PEG-1000 and PEG-20,000 as its sole carbon and energy source (Figure 1b), the cell-free extract of PG1 dehydrogenated glycols only in the range of EG through PEG-400. After 200 min with no dehydrogenation of either PEG-1000 or PEG-20,000, DEG was added to the reaction mixtures. Reduction of DCPIP commenced after 10 min, demonstrating that the cell-free extract mixture was active.

The cell-free extract of *D. desulfuricans* DG2 dehydrogenated all glycols ranging from EG through PEG-1000; slightly lower rates occurred with PEG-300 through PEG-1000 (Table 2). The ability to dehydrogenate the longer polymers was unexpected for the enzyme(s)

Table 1. Product recovery for degradation of diethylene glycol by D. desulfuricans DG2 and PEG-1000 by Bacteroides PG1.

Culture	Substrate (mmol)	Products formed (mmol)[a] Ethanol	Acetate	H_2	CH_4
DG2	DEG(2.7)	0	5.2	5.0(0.031)[b]	0
DG2 + DG1	DEG(2.5)	0	4.8	0	1.2(1.25)[c]
DG2 + Na_2SO_4	DEG(2.7)	0	5.2	0	0
PG1	PEG-1000(0.10)	1.0	0.73	0.002(0.005)	0
PG1 + DG1	PEG-1000(0.10)	0	1.90	0	0.62(0.58)

[a] Stoichiometry for DEG degradation based on: $HO-CH_2-CH_2-O-CH_2-CH_2-OH + H_2O \longrightarrow 2CH_3COOH + 2H_2$ and for PEG-1000 based on: $PEG\text{-}1000 + 22H_2O \longrightarrow 22CH_3COOH + 22H_2$.

[b] In atmospheres.

[c] Amount of methane expected based on the above stoichiometry of H_2 production.

Table 2. Dehydrogenation of substrates by cell-free extracts of Bacteroides PG1 and D. desulfuricans DG2[a].

Substrate	Bacteroides PG1		D. desulfuricans DG2	
	Rate[b] (pmol/min· ug protein)	Lag time[c] (min)	Rate (pmol/min· ug protein)	Lag time (min)
EG	10.0	15	2.4	15
DEG	9.5	10	3.8	15
TREG	5.0	15	3.6	15
TEEG	N.D.[d]	N.D.	4.5	20
PEG-300	4.2	60	1.4	40
PEG-400	4.3	55	1.5	55
PEG-1000	0.0	>200	1.6	10
PEG-20,000	0.0	>200	N.D.	N.D.
Acetaldehyde	4.4	4	4.5	5
n-Propyl alcohol	5.3	4	N.D.	N.D.
n-Butyl alcohol	4.9	4	2.7	10
Glycerol	0.0	>60	2.9	12

[a] All values represent means of two determinations.
[b] Rates are of dichlorophenol indophenol (DCPIP) reduction.
[c] Time elapsed before start of DCPIP reduction.
[d] Values not determined (N.D.)

since DG2 used only EG through TEEG as carbon and energy sources (Figure 1a).

Polypropylene glycol. Since the PEG-degrading isolates did not degrade PPGs, we attempted to obtain degradation of PPG-425 and PPG-2000 using sludge obtained from digesters in Holt and Mason, MI. Attempts to transfer initial enrichments to PPG-amended medium and to obtain additional CH_4 with PPG additions were unsuccessful.

DISCUSSION

Bacterial strains *D. desulfuricans* DG2 and *Bacteroides* PG1 both fermented PEGs to acetate, ethanol and hydrogen with acetaldehyde as an apparent intermediate. In the presence of methanogen, acetate was the sole oxidized product, the reducing equivalents were consummed in the production of methane (Table 1). This is in agreement with previous studies of anaerobic EG metabolism (1,8,9,27) and of PEG metabolism by *Pelobacter venetianus* (21) and methanogenic consortia (7). Thus, all known anaerobic EG and PEG degradation pathways involve an acetaldehyde intermediate with acetate as the oxidized product.

A dehydrogenase system that was capable of oxidizing PEGs and other hydroxy-compounds was present in cell-free extracts of *both Bacteroides* PG1 and *D. desulfuricans* DG2. A number of factors suggested that substrate dehydrogenation was preceded by a diol dehydratase conversion of EG and PEG to acetaldehyde: (i) DCPIP reduction started 4-5 min after acetaldehyde addition to the enzyme extracts, glycols required at least 10-15 min for DCPIP reduction to start (Table 2), (ii) product recoveries from this (Table 1) and other studies (1,7,9,21,27) account for a single dehydrogenation of individual ethoxy units forming acetate, (iii) acetaldehyde was detected as an intermediate of PEG degradation, reaching quantities representing 15% of the substrate ethoxy units, and (iv) diol dehydratase activity is present in some EG-grown anaerobic bacteria (8).

Extracts of *Bacteroides* PG1 did not dehydrogenate PEG-1000 and PEG-20,000 (Table 2), although whole cells metabolized both polymers

(Figure 1b). This suggests that a prior external depolymerization of PEG was necessary, which contrasts with evidence that *P. venetianus* may take up and internally depolymerize PEG-20,000 (21). Two other factors also suggested that depolymerization was an external event: (i) According to molecular exclusion models, the passage of PEG-20,000 into bacterial cells may not be possible. The cell wall and protoplast of *Bacillus megaterium* have an exclusion threshold for polymers of M_n = 1,200 and an Einstein-Stokes radius (r_{es}) of 1.1 nm. PEG-20,000 appears to be well outside of this limit with M_n = 20,000 and an r_{es} = 4.90 nm (20). *(ii) D. desulfuricans* DG2 grew in spent medium of *Bacteroides* PG1 cultures from which PG1 had been removed by centrifugation and 0.1% w/v PEG-1000 and Na_2SO_4 were then added. This indicated that the depolymerization enzyme(s) of *Bacteroides* PG1 may be external to the cells since DG2 cannot use PEG-1000 as substrate (Figure 1a).

The degradation of polymeric PEG would thus follow the sequence: (i) external depolymerization, (ii) dehydration of the intermediate to acetaldehyde, and (iii) internal dehydrogenation of acetaldehyde to acetate. This final step would alone yield energy for the microorganism.

The product(s) of the proposed depolymerization have not been identified. EG was not detected in cultures of *Bacteroides* PG1 with PEG-1000 as substrate, and since PG1 cannot use EG as substrate, it is unlikely that polymer hydrolysis to monomeric units occurred. A cleavage of ether-bonds to fragment the PEG-chain is more likely, especially considering that cleavage of interior ether-linkages is required for the complete metabolism of PEG-20,000 which consists of

polymers of PEG-6000 linked by aryl epoxides (L.F. Theiling, Union Carbide Corp., personal communication).

The mechanism proposed by Schink and Stieb (21) for anaerobic depolymerization of PEG assumes either a dehydration and rehydration of the terminal EG residue or a co-enzyme B_{12}-dependent shift of the terminal hydroxy function to an acetaldehyde derivative followed in either case by hydrolytic cleavage of the hemiacetal linkage to yield acetaldehyde. In contrast, cleavage of an interior ether bond in PEG requires either hydrolysis with creation of two terminal hydroxy units or hydrogenation to a hydroxy and a methyl unit. Either mechanism also would account for cleavage of the aryl-glycerol-β-aryl-ether linkage in lignin (5); hydrolysis already has been proposed as the mechanism for demethoxylation of methoxylated aromatics (2). In contrast to the lack of a demonstrated mechanism for anaerobic ether bond-cleavage, mono-oxygenases, ether hydrolases, and a carbon-oxygen lyase have been isolated and demonstrated to aerobically cleave ether bonds. Recently, a membrane-bound PEG-dehydrogenase was shown to catalyze the oxidative depolymerization of PEG-6000 (15).

Some aerobic bacteria dehydrogenate oligomers of EG up through PEG-400 in size, but not the higher molecular weight PEGs (13,24); this was also observed with *Bacteroides* PG1 cell-free extracts (Table 2). Oligomeric PEGs ($\leq$ 400 g/mol) have a "zig-zag" shape while longer PEGs have a helical conformation which stablizes the ether bond-linkages of PEG in solution (3). The metabolism of PEG by some bacterial enzymes thus may be dependent on PEG conformation. This

may be the reason long polymers of PEG were not dehydrogenated by the cell-free extracts of *Bacteroides* PG1.

Unlike *Bacteroides* PG1, enzyme(s) in the cell-free extract of *D. desulfuricans* DG2 apparently were not sterically limited, but dehydrogenated EG through PEG-1000 (Table 2). The substrate range of whole cells, though, excluded PEGs greater in size than TEEG. This indicated that *D. desulfuricans* DG2, in contrast to *Bacteroides* PG1, lacked a mechanism for conversion of PEG to a product suitable for cell uptake. TEEG was incompletely utilized by the DEG-enrichment and *D. desulfuricans* DG2 (Figure 1a). TEEG and other such designations denote an average molecular weight (L.F. Theiling, Union Carbide Corp., personal communication) which suggests that not all of the oligomeric size range denoted as TEEG was used as substrate.

EG appeared to be toxic to some component(s) of the methanogenic enrichments which probably accounted for the low productivity observed with that substrate (Figures 1a and 1b). With DEG, metabolism was rapid and conversion of acetate to CH_4 by *Methanosarcina* sp. accounted for the relatively greater productivity observed for enrichments vs. pure cultures.

The substrate range for *Bacteroides* PG1 and *D. desulfuricans* DG2 was limited to a few compounds other than PEGs and the characteristic growth substrates. *Bacteroides* PG1 used ethanolamine and nonionic polyethoxylate surfactants (Tween-20 and Brij-35) as substrates, presumably utilizing only the polyethoxylate moiety of the surfactants. Although EG and n-alcohols were dehydrogenated by cell-free extracts, PG1 whole cells could not use these for growth. The

cells probably lacked a suitable uptake system as had been suggested for *P. venetianus* (21).

Unexpectedly, neither *D. desulfuricans* DG2 nor *Bacteroides* PG1 could metabolize polypropylene glycol (PPG) although propanediol served as substrate for both. The depolymerizing system(s) of both strains thus appear limited to ether-linked ethoxy units. Our inability to obtain PPG degradation in sludge enrichments suggested that PPGs may be recalcitrant to anaerobic degradation.

Bacteria with the morphology of *D. desulfuricans* DG2 and *Bacteroides* PG1 were ubiquitously present in PEG-20,000 degrading enrichments obtained from six different digesters in Michigan. Both microorganisms may thus be of ecological significance in the anaerobic degradation of PEGs and nonionic polyethoxylate surfactants which are present in high concentration in municipal waste (14).

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CHAPTER 5

DEGRADATION OF PHENYL ETHER AND DIBENZO-p-DIOXIN BY ETHER BOND-CLEAVING, METHANOGENIC CONSORTIA

INTRODUCTION

Ether bonds had been considered recalcitrant to anaerobic biodegradative cleavage. Recently, ethoxy ethers (8,22) and aryl methoxy ethers (1,12,19) were shown to be cleaved by monocultures of strictly anaerobic bacteria and the product(s) used as the sole organic carbon source. The O-methyl group of methoxybenzenoids also is used as an electron acceptor with carbohydrates as electron donors and acetate as product (20). Cleavage of the aryl-glycerol-β-aryl-ether linkage in lignin derived compounds and synthetic lignin may occur in methanogenic cultures and sediments which degrade those compounds (2,3,6,27) and by mixed rumen bacteria (5).

Mechanisms for anaerobic ether bond-cleavage have been suggested. A conversion of the terminal ethylene glycol unit of polyethylene glycol (PEG) to a hemiacetal with subsequent hydrolysis of the ether bond was implicated in PEG depolymerization (22). This mechanism requires an adjacent hydroxyl group. Cleavage of interior ether bonds of PEG lacking an adjacent hydroxyl group may occur by hydrolysis or hydrogenation (8). Hydrolysis was suggested as the mechanism for cleavage of methoxyl groups from methoxylated aromatics to produce methanol and hydroxy derivatives of the aromatic compounds (1).

No evidence exists for cleavage of diaryl ether bonds under anaerobic conditions. These bonds exist in naturally occurring

compounds such as lignin and lignin-derived peat and humus. Phenyl ether ($[C_6H_5]_2O$) is manufactured for use as a perfuming agent, as a heat transfer agent, and in pesticides. A double diaryl ether bond links the benzene rings of dioxin (dibenzo-*p*-dioxin, $[C_6H_4]_2O_2$) compounds.

Dioxin compounds are of considerable interest due to the toxicity of the halogenated congeners and their persistence in the environment. Tetrachloro-dibenzo-*p*-dioxin (2,3,7,8-TCDD, TCDD) is the most toxic man-made chemical with an LD_{50} for mammals of micrograms per kilogram (23). TCDD appears to be recalcitrant to both aerobic and anaerobic microbial degradation (10) or anaerobically degraded only to a minor extent (25). Recent evidence suggests that TCDD may be degraded by the H_2O_2-dependent extracellular lignin-degrading enzyme system of *Phanerochaete chrysosporium* (4). Pure cultures of *Pseudomonas* sp. NCIB9816 strain II and *Beijerinckia* strain B8/36 aerobically degrade unsubstituted dioxin when an alternate source of carbon and energy is present (16,17). Anaerobic degradation of unsubstituted dioxin has not been reported.

The ability of anaerobic bacteria to cleave alkyl ethers and aryl methoxy ethers suggested that a mechanism for cleavage of diaryl ethers may be obtained by initially enriching for alkyl ether-cleaving methanogenic consortia and then switching the substrate to a diaryl ether compound. We report here on the success of this approach leading to anaerobic cleavage of the ether bonds of both diphenyl ether and dibenzo-*p*-dioxin. The metabolic intermediates and end-products of subsequent substrate metabolism are identified.

MATERIALS AND METHODS

Chemical abbreviations and sources. Dibenzo-*p*-dioxin (Dioxin, $[C_6H_4]_2O_2$), 1-Cl-dibenzo-*p*-dioxin (1-Cl-dioxin), and 2-Cl-dibenzo-*p*-dioxin (2-Cl-dioxin) were kindly supplied by Gary M. Klecka. The purity of all dioxin compounds was reported to be 99+%. Phenyl ether ($[C_6H_5]_2O$) and polyethylene glycol with an average molecular weight of 1000 (PEG-1000, $HO\text{-}[CH_2\text{-}CH_2\text{-}O\text{-}]_nH$) were obtained from Aldrich Chem. Co. and J. T. Baker Chem. Co., respectively.

Enrichment cultures and experimental procedures. Enrichment cultures were established in anaerobic medium containing 10% sludge obtained from a municipal anaerobic digester in Holt, Michigan. The anaerobic enrichment procedure and medium (RAMM) have been described previously (21). The enriching substrates were 0.1% w/v Bacto-peptone (Difco), 0.1% w/v PEG-1000 (1.0 mM), and 0.05% v/v (3.2 mM) phenyl ether. Cultures (110 ml) were grown in 160 ml serum bottles sealed with butyl rubber stoppers. The headspace gas was a 90%/10% mixture of N_2/CO_2. Cultures were maintained at 37°C either stationary or on a rotary shaker at 120 rpm. The degree of substrate degradation was assessed by measuring the production of methane. When CH_4 production ended, 25 ml of enrichment culture was transferred by syringe to 75 ml fresh medium containing 0.4 mM phenyl ether, 0.01 % yeast extract (Difco) and 0.05% sodium acetate. These enrichments were monitored for the production of methane and fed ethanol and phenyl ether when previous additions of substrate were depleted as described below.

The degradation of phenyl ether was assessed both by measuring the stoichiometric production of CH_4 from phenyl ether and by an

absorptiometric determination of phenyl ether concentration in the enrichment cultures. Both phenol and benzene were also monitored as products of phenyl ether degradation.

When phenyl ether had been successfully degraded in the enrichment cultures after three successive additions, the cultures (100 ml) were given 50 ug/ml dioxin (0.3 mM) and 2.5 ul ethanol (0.43 mM). Dioxin degradation was assessed by measuring the accumulation of the presumed degradation products CH_4, phenol, catechol and benzene. After dioxin had been successfully degraded upon three successive additions, the cultures were given 100 ug 1-Cl-dioxin/ml (0.45 mM) plus 5.0 ul ethanol (0.86 mM). The appearance of degradation products was assessed, as well as disappearance of 1-Cl-dioxin by an absorptiometric determination.

Analytical methods. Samples of cultures were periodically withdrawn by syringe. Phenyl ether was extracted by addition of 10 ml of ethyl ether to 5 ml of culture. The ethyl ether fraction was collected and evaporated in a stream of air. The residue was dissolved in 3 ml ethanol, passed through a 0.45 um Millipore filter (HA) and the absorption obtained with a Perkin-Elmer Model 320 Spectrophotometer at the $_{max}$ for phenyl ether (265, 272, and 279 nm). The concentration of phenyl ether was assessed by comparison with the absorption values of standard solutions of phenyl ether in ethanol. The 1-Cl-dioxin was extracted from 1 ml of enrichment culture with 2 ml of methylene chloride (CH_2Cl_2). The organic layer of the extract was filtered through a 0.45 um Millipore filter (HA) into as glass flask. The methylene chloride was evaporated and the

residue redissolved in 2 ml of hexane. An absorption spectrum of the solution was obtained.

Methane was quantified by injecting 0.2 ml of culture headspace gas into a Carle model 8515 gas chromatograph equipped with a Porapak Q column and a microthermistor detector. Phenol and catechol were solubilized from 1 ml of enrichment culture with 1 ml of acetonitrile. Extracts were filtered through 0.45 um Millipore filters (HVLP) and analyzed by high pressure liquid chromatography (HPLC) using a Waters Liquid Chromatograph, Model 6000A, equipped with a radial compression separation system, Model ACM-100, a Radical-PAK C18 analytical column and a uv absorbance detector, Model 441. The mobile phase was acetonitrile-5% acetic acid (1:1) at a flow rate of 20 ml/min. Benzene was detected by injecting 0.5 ml of culture headspace gas into a Perkin-Elmer 900 gas chromatograph equipped with a 2 m 10% SP-1000 on 80/100 Supelcoport packed glass column (Supelco) and a flame ionization detector. N_2 was the carrier gas at a flow rate of 50 ml/min. Column temperature was 160°C; injector and manifold temperatures were 200°C.

RESULTS

Establishment of enrichment cultures. Enrichment cultures containing 10% sludge, 0.1% PEG-1000, 0.05% phenyl ether and 0.1% Bacto-peptone produced CH_4 for 30 days. When CH_4 production ended, 25 ml of enrichment culture was transferred to serum bottles containing 75 ml of medium with 0.05% sodium acetate, 0.01% yeast extract and 4 mM phenyl ether (two replicate bottles) and 0.4 mM phenyl ether (two replicate bottles). CH_4 was produced for two weeks in these four new cultures. Twenty-five ml of enrichment culture was then transferred from these new enrichments to 50 ml of fresh medium. These transfers and all subsequent attempts to transfer cultures failed. The four transferred culture bottles were fed 0.4 mM phenyl ether and 7 mM ethanol on two subsequent occassions. Both substrates were degraded within 30 days as indicated by measurement of methane production.

Several attempts were made to establish phenyl ether-degrading methanogenic cultures with 10% sludge inocula and using only 3.2 mM phenyl ether as the enriching substrate. Phenyl ether was not degraded in any attempt as indicated by no methane production in excess of the unamended control cultures.

Phenyl ether degradation. A third addition of 3.4 mM ethanol and 0.2 mM phenyl ether to the four enrichment cultures was degraded rapidly (Table 1). The degradative pathway and stoichiometry for phenyl ether conversion to CH_4 was assumed to be: 1 mol phenyl ether $\rightarrow$ 1 mol phenol + 1 mol benzene, 1 mol phenol $\rightarrow$ 3.5 mol CH_4. An amount of methane stoichiometrically equal to that which could be derived from the added ethanol (1 mol ethanol $\rightarrow$ 1.5 mol CH_4) was

Table 1. Degradation of Phenyl Ether by Methanogenic Consortia

Culture	Degradation Products (days)				
A	0	2	3	4	8
Phenyl ether (umol)	200	N.D.	78	0	0
Phenol (umol)	0	N.D.	16	33	0
CH_4 (umol)	0	200	400	430	440
B					
Phenyl ether (umol)	200	N.D.	→	→	200
Phenol (umol)	N.D.	→	→	→	0
CH_4 (umol)	0	→	→	→	0

[a] Total CH_4 recovery should equal 510 umol (70 umol from phenyl ether plus 440 umol from ethanol).
[b] Not determined (N.D.)
[c] Culture B was a non-active control.

subtracted as necessary. CH_4 production was monitored in all four cultures, while aqueous samples were taken from one for measurement of phenyl ether and degradation product concentrations (Table 1). Eight days after addition of substrates, the amount of CH_4 produced was equal to 86% of the theoretical amount produced from ethanol plus one ring of the phenyl ether. The absorption spectrum for detection of phenyl ether upon initial addition to the cultures showed three peaks corresponding to the max for phenyl ether (Figure 1). After five days, the phenyl ether spectrum had disappeared. Ether extractions of 10% sludge medium without added phenyl ether did not have the corresponding absorption peaks. Phenol was detected as an intermediate of phenyl ether degradation on days four and five.

Three culture bottles were subsequently given 0.20 mM phenyl ether plus 0.43 mM ethanol. Cultures were kept stationary and serum bottles were turned upside down so that benzene derived from phenyl ether degradation would accumulate in the headspace gas and not be as readily absorbed into the butyl rubber stoppers. Again, CH_4 production equaled the theoretical amount derived from ethanol plus one aryl ring of phenyl ether after 50 days incubation. This is in contrast to eight days required for degradation previously observed when the cultures were shaken. Benzene was detected at concentrations ranging between 13% and 19% of the expected value (Table 2). The remainder was probably lost into the rubber stoppers.

Dioxin degradation. One of the four phenyl ether-degrading cultures was given 0.3 mM unsubstituted dioxin and 0.43 mM ethanol. The remaining three cultures were maintained with additions of ethanol and phenyl ether. Substrates were degraded in the apparent

Figure 1. Absorption spectrum for (A) phenyl ether extracted from the enrichment culture at time of addition and (B) after five days of incubation.

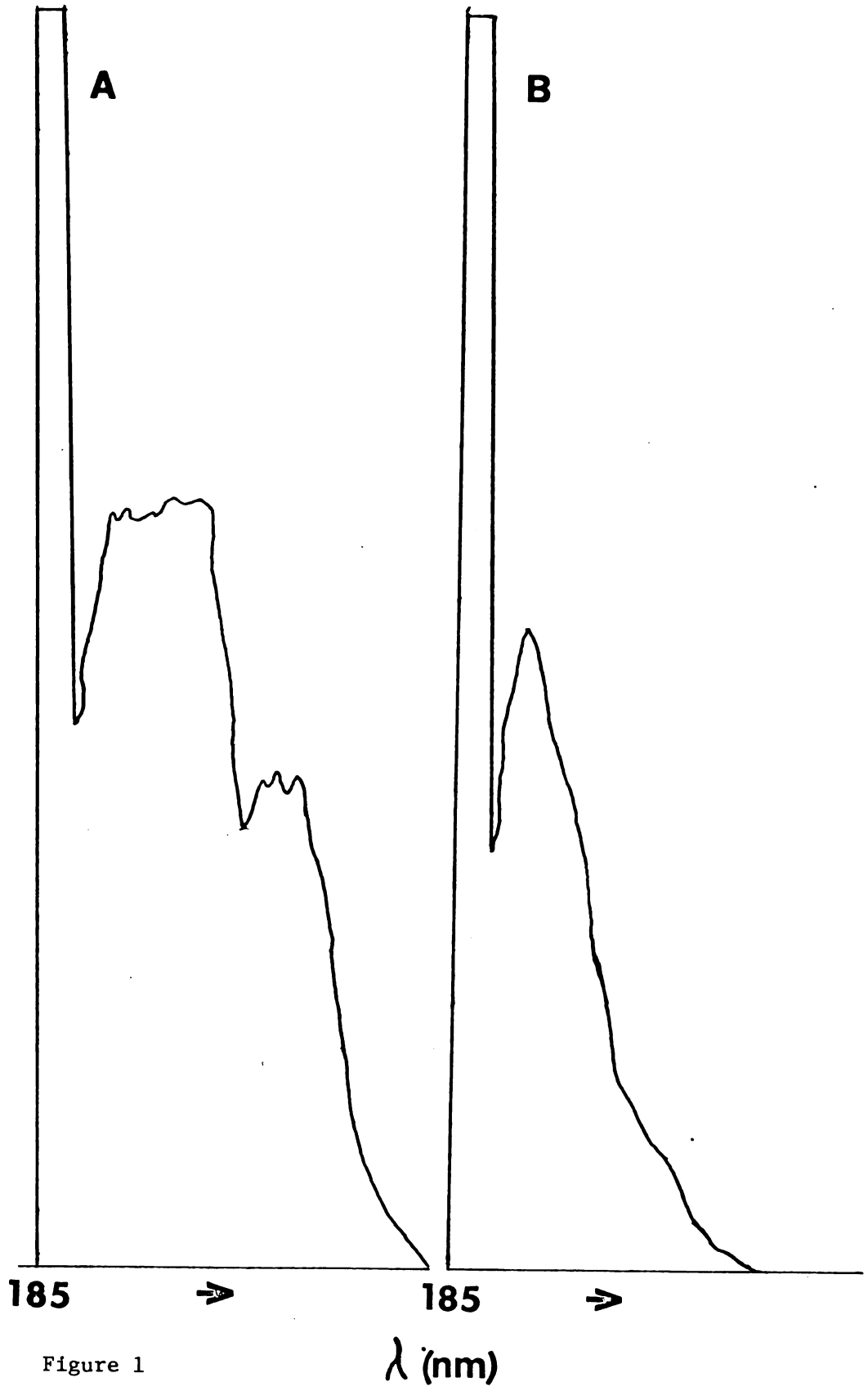


Figure 1

Table 2. Recovery of Degradation Products from Phenyl Ether Degradation.

Culture	Products Recovered (umol)[a,b]	
	CH_4	Benzene
A	125	2.6
B	130	3.8
C	110	3.0

[a] Products measured after 50 days incubation.
[b] Predicted recovery of products: 130 umol CH_4 (65 umol CH_4 from ethanol plus 65 umol from phenyl ether) and 20 umol benzene.

order such that ethanol was converted to CH_4 and CO_2 by day six and dioxin was more slowly degraded from day 10 through day 28 (Figure 2). The amount of CH_4 recovered was 90% of the theoretical.

The culture was given dioxin on two more subsequent occasions (Figure 3). At time (A), 0.3 mM dioxin and 0.43 mM ethanol was added to the culture, the culture bottle was incubated without shaking and upside down. By day 24, 1.1×10^{-4} mol CH_4 had been produced which was 73% of the total theoretical production from one ring of dioxin plus ethanol. The amount expected from ethanol alone was 0.64×10^{-4} mol indicating that some dioxin had been degraded. Corresponding analysis for benzene showed that 0.85 umol of benzene had been produced. This was 50% of the expected benzene production at this point in the degradation of dioxin.

At time (B), the headspace of the culture bottle was flushed with N_2/CO_2 and the culture was given 0.6 mM dioxin and 0.86 mM ethanol. By day 37 dioxin had not been degraded as determined by CH_4 production and visual observation of dioxin crystals present in the culture. The culture was again given 0.86 mM ethanol and 0.4 mM phenyl ether at time (C). The culture was put on a rotary shaker at 120 rpm. After 45 more days incubation (day 77), the amount of CH_4 produced (6.4×10^{-4} mol) was equal to the total predicted from 1.7 mM ethanol (2.5×10^{-4} mol), 0.4 mM phenyl ether (1.3×10^{-4} mol) plus 0.6 mM dioxin (1.7×10^{-4} mol) and the remaining amount of dioxin from the previous experiment (0.5×10^{-4} mol). Because we had both added phenyl ether and placed the culture on a rotary shaker, it wasn't known whether the substrate or the shaking had the positive effect on dioxin degradation.

Figure 2. Temporal production of CH_4 from ethanol (0.43 mM) and dibenzo-p-dioxin (0.30 mM). Theoretical yield is 64 umol CH_4 from ethanol (▲) and 84 umol CH_4 from one aryl ring of dioxin (total of 148 umol CH_4). Production (125 umol CH_4) was 85% of theoretical.

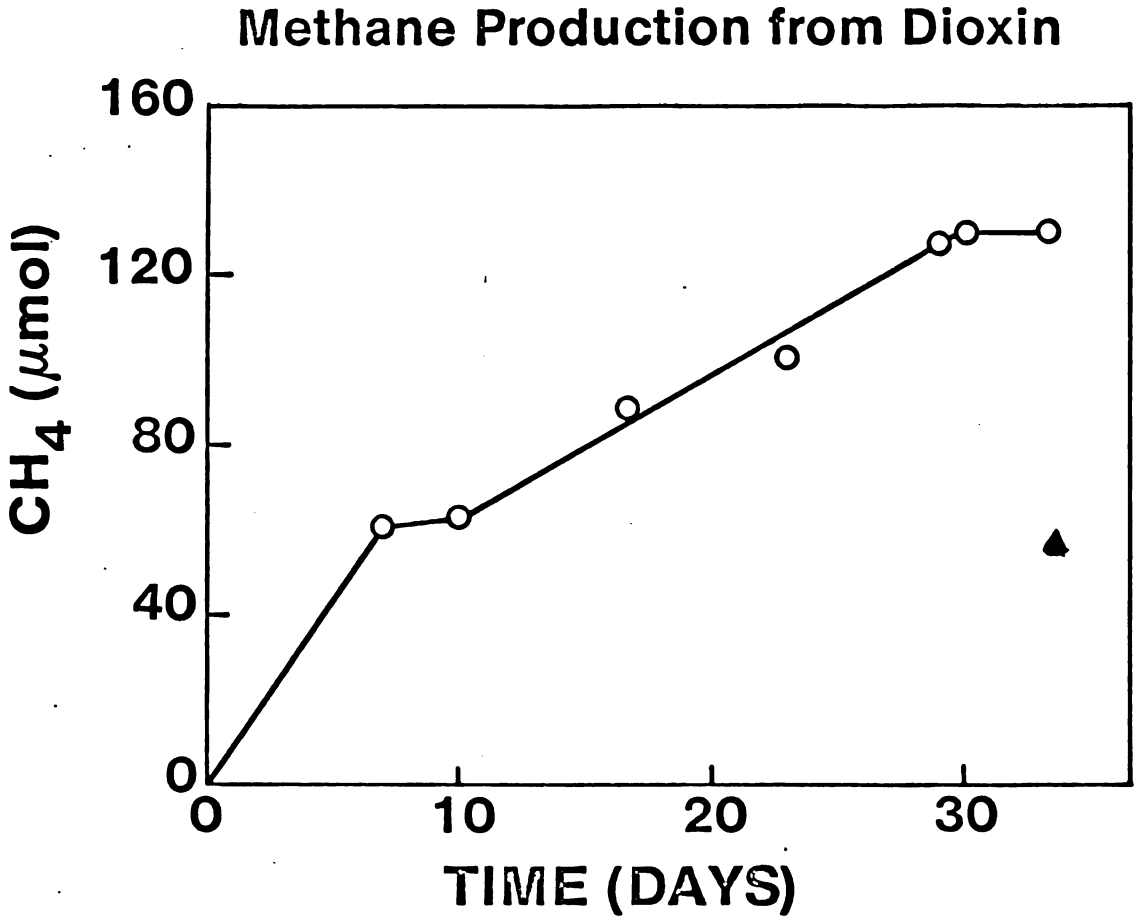

Methane Production from Dioxin
160
120
80
40
0
CH_4 (μmol)
0
10
20
30
TIME (DAYS)

Figure 2

Figure 3. Temporal production of CH_4 from dibenzo-p-dioxin, ethanol, and phenyl ether added to the ether bond-cleaving methanogenic consortium: (A) Addition of 0.30 mM dioxin and 0.43 mM ethanol; the total theoretical amount of CH_4 expected is denoted by (▲), (B) Headspace of the bottle was flushed with N_2/CO_2, addition of 0.60 mM dioxin and 0.86 mM ethanol, (C) Culture bottle placed on rotary shaker, addition of 0.86 mM ethanol and 0.40 mM phenyl ether; the amount of CH_4 expected from ethanol alone is denoted by (▲).

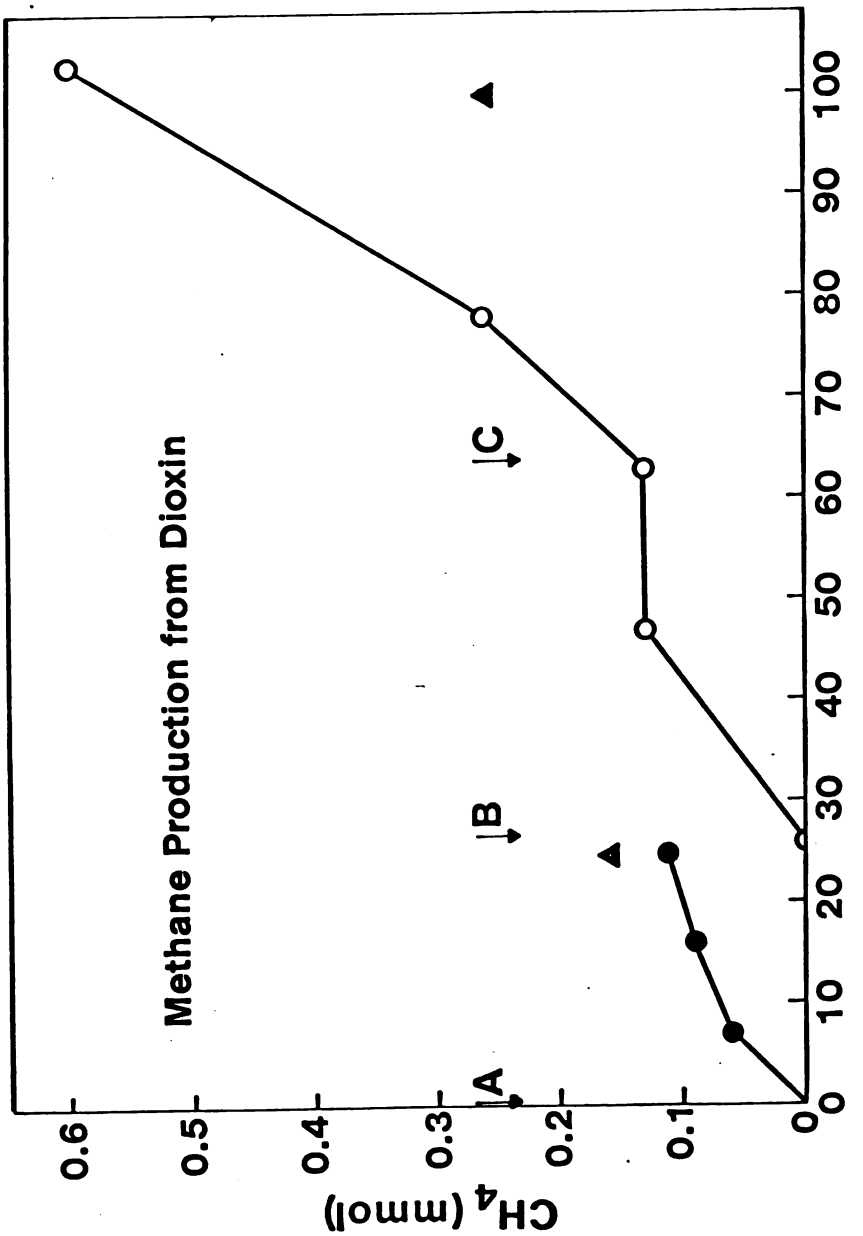

Methane Production from Dioxin
CH_4 (mmol)
TIME (DAYS)
A
B
C
0
0.1
0.2
0.3
0.4
0.5
0.6
10
20
30
40
50
60
70
80
90
100

Figure 3

Transformation of 1-Cl-dioxin. Following the successful degradation of dioxin on three successive occasions, the culture was given 0.45 mM 1-Cl-dioxin plus 0.86 mM ethanol. The culture produced CH_4 in the amount equal only to that expected from the degradation of ethanol (1.3×10^{-4} mol). The dioxin compound was extracted from 1 ml culture with 3 ml methylene chloride, the methylene chloride was evaporated and the glassware washed with 3 ml hexane. A uv absorbance analysis of the hexane-wash identified an extracted compound that had a maximum absorbance at $\lambda =$ 324 nm (Figure 4); 1-Cl-dioxin (maximum absorbance at $\lambda =$ 287 nm) was no longer detectable.

Figure 4. Absorption spectrum for (A) 1-Cl-dibenzo-p-dioxin (0.45 mM) extracted from the enrichment culture at time of addition and (B) absorption spectrum for compound extracted after two weeks of incubation.

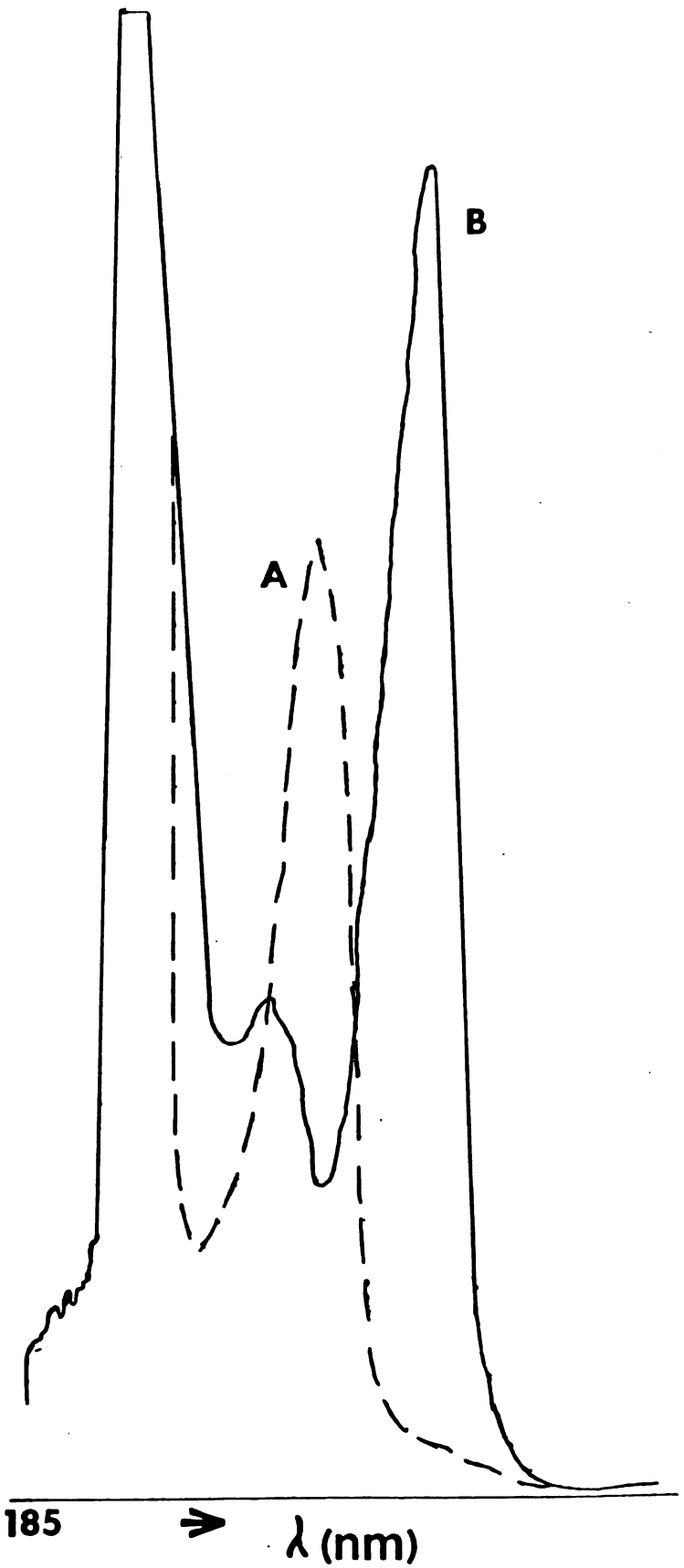


Figure 4

DISCUSSION

The microbial conversion of phenyl ether to phenol and benzene (Tables 1 and 2) was the first evidence of diaryl ether biodegradation under anaerobic conditions. The production of phenol as an intermediate and the stoichiometric production of CH_4 equal to the mineralization of one aryl group indicated that ether bond-cleavage was a reductive mechanism. Only reductive cleavage accounts for the amounts of observed degradation products (Figure 5). This assumes that benzene was recalcitrant to further biodegradation and that the hydroxylated compounds were completely mineralized. We have recently proposed that long polymers of PEG are cleaved externally to the microorganisms by a similar reductive mechanism (8). The known mechanisms for anaerobic ether bond-cleavage (Figure 6) involve hydrolysis of methoxy aryl groups (1,20) or hydrolysis of a proposed hemiacetal formed during the degradation of PEG (22).

The degradation of dibenzo-*p*-dioxin also appeared to be by a reductive cleavage of the diaryl ether bonds as determined by the quantity of CH_4 produced. In this case catechol is the proposed degradative intermediate (Figure 5). Its presence was not detected by HPLC analysis, probably because ether bond-cleavage was the rate limiting step in the degradative sequence. The stoichiometric production of CH_4 from one aryl group of dioxin was unexpected as it appearred that two phenols could as easily be formed as catechol and benzene.

Ether linkages are known to make compounds resistant to biodegradation (18). In the case of lignin, this was presumed due to the lack of a chemical oxidant in reducing environments (27). The

Figure 5. Two possible methods of ether bond-cleavage of phenyl ether and dioxin are compared. Hydrolysis (H_2O) yields two hydroxylated aryl rings while reduction ($NADH_2$) yeilds only one hydroxylated ring. $NADH_2$ was assumed to be involved in the reductive cleavage and has not yet been identified.

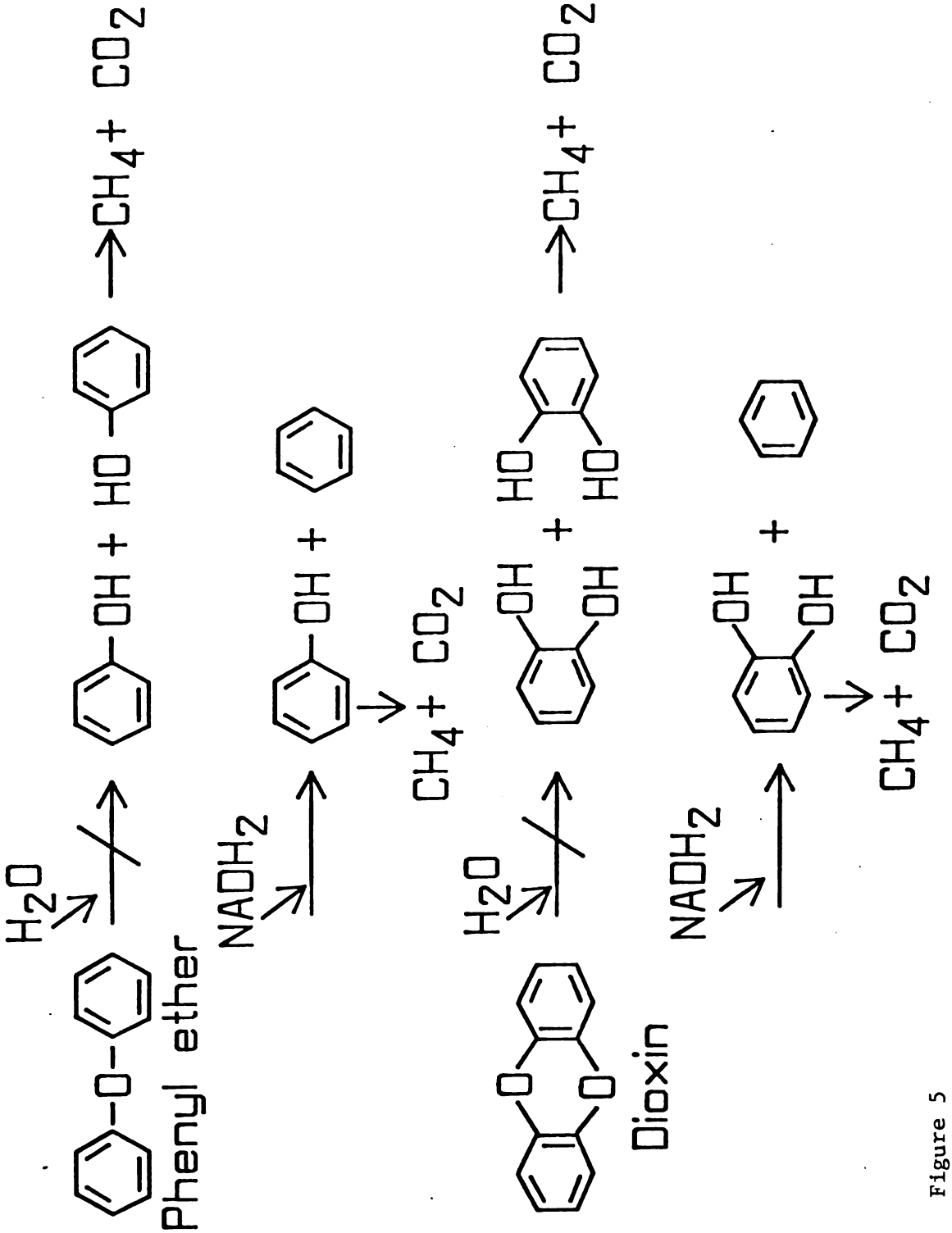
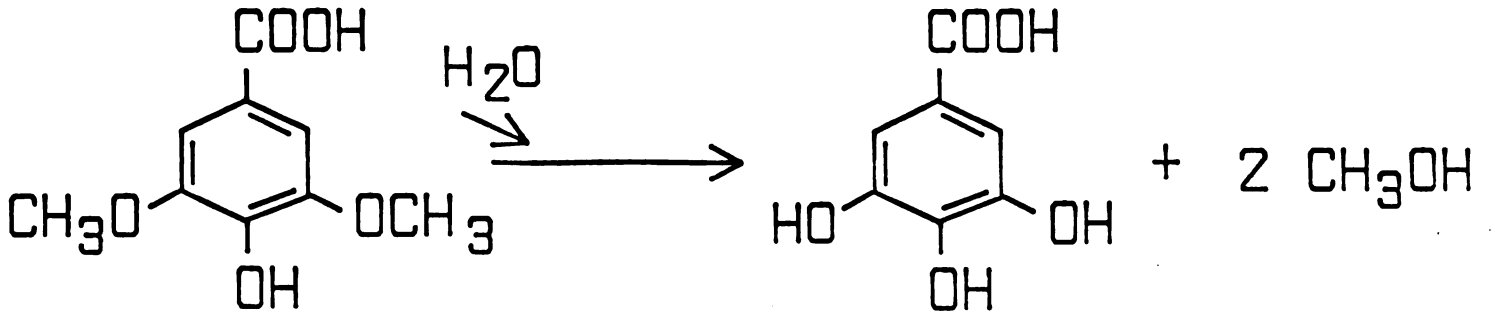

Phenyl ether
H_2O
OH + HO
CH_4+ CO_2
$NADH_2$
OH +
CH_4+ CO_2
Dioxin
H_2O
OH OH
+
HO HO
CH_4+ CO_2
$NADH_2$
OH OH
+
CH_4+ CO_2

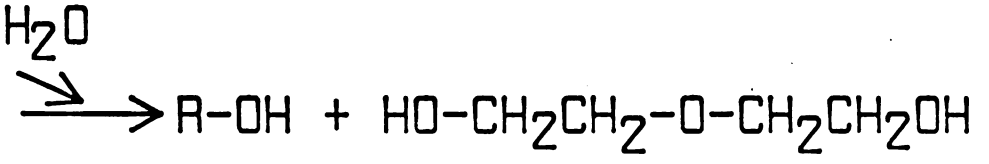
Figure 5

Figure 6. Previously proposed mechanisms for anaerobic ether bond-cleavage involve hydrolysis of methoxy aryl groups eg., by *Syntrophococcus sucromutans* and *Acetobacterium woodii* and of PEG by *Pelobacter venetianus*. Either hydrolysis or reductive cleavage of PEG by a *Bacteroides* sp. strain also occurs.



Syntrophococcus sucromutans (Krumholz and Bryant, 1986)
Acetobacterium woodii (Bache and Pfennig, 1981)

Pelobacter venetianus (Schink and Stieb, 1983)



Bacteroides sp. (Dwyer and Tiedje, 1986)

Figure 6

identification of anaerobic cleavage of aryl methoxy ether, alkyl ether, and now diaryl ether bonds demonstrates the versatility shown by anaerobic microorganisms in degrading persistent organic compounds.

The enrichment procedure used to obtain diaryl ether bond-cleavage was successful and perhaps necessary since without an initial enrichment of PEG, phenyl ether was not degraded under otherwise identical conditions. The initial biodegradation of phenyl ether may have been either gratuitous ie., making use of enzymes that degrade analogs of compounds such as PEG, or cometabolic ie., degradation of a compound that requires an additive source of energy or reducing power. The apparent recalcitrance of dioxin to biodegradation without phenyl ether present as a substrate (Figure 4) was not studied further nor confirmed, but may indicate that its degradation was also cometabolic.

Due to the apparent recalcitrant nature of xenobiotic compounds such as dioxin and phenyl ether it may be best to strive for cometabolic biodegradation. Thus, the concentration of substrates added to the enrichment cultures at any one time was kept low. This was done to maintain a substrate-limited condition, which for mixed substrate systems, is important when either gratuitous or cometabolic metabolism of xenobiotic compounds occurs (13). The advantages of substrate limitation are that: (i) the environment is maintained for the synthesis and functioning of xenobiotic-degrading, inducible enzymes, (ii) the concentration of possible toxic intermediates from xenobiotic degradation is kept low, and (3) a continuous pressure is maintained for anaerobic microorganisms to adapt to xenobiotic

compound degradation. The use of analog compounds (such as PEG) may be especially useful in obtaining a microbial population with specific enzymatic functions.

We attempted to obtain degradation of 1-Cl-dioxin since the halogenated congeners of dioxin are of primary environmental concern (10). It appearred that 1-Cl-dioxin was altered in structure by the bacterial consortium (Figure 4). The altered compound is postulated to arise from one ether bond-cleavage with the other ether bond protected from degradation by the proximity of the chlorine substituent. The presence of a hydroxyl substituent is consistent with the upward shift in the maximum wavelength of absorption by the compound (9). Several attempts were made to analyze the extracted compound by mass-spectrometry. These attempts were unsuccessful due to an apparent instability of the compound once extracted.

The most toxic halogenated dioxin congener is 2,3,7,8-TCDD (23). Because the chlorine substituents are on the aryl rings in positions opposite to the ether bonds, it may be possible to obtain anaerobic biodegradation of both ether linkages for the toxic congeners. These results also raise the possibility of adapting the microbial populations of large TCDD contaminated areas to ether bond-cleavage in an attempt to destroy the pollutant. Contaminated sediments may already be anaerobic, soils can be flooded to achieve anaerobiosis, and anaerobic waste treatment facilities may be the most easily adapted for ether bond-cleaving microorganism(s).

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CHAPTER 6

KINETICS OF PHENOL BIODEGRADATION BY AN IMMOBILIZED METHANOGENIC CONSORTIUM

INTRODUCTION

Immobilized cells embedded in a matrix offer several potential advantages for the treatment of processing and waste streams (21). These include the retention of catalytic activity, protection of cells from the effects of inhibitory substrates, and more efficient substrate mineralization through retention of intermediary products. This last advantage may offer both thermodynamic and kinetic advantages when mineralization involves syntrophic bacteria of methanogenic consortia.

We chose to study the effect of immobilization on the activity of a phenol-degrading methanogenic consortium. Phenol and phenolic compounds are toxic pollutants (9) and inhibitors of biodegradation (6,7,13) and have been used as model compounds to test the effect of inhibitory substrates on cellular metabolism and growth kinetics (14,15). The phenol-degrading methanogenic consortium is postulated to contain three interacting physiological groups of bacteria: a phenol-metabolizer, an H_2-utilizing methanogen, and an acetotrophic methanogen, all of which are required to complete the mineralization of phenol to CH_4 and CO_2 (2,4). Therefore, with this consortium it should be possible to determine the effect of immobilization on both the degradation of an inhibitory substrate and on the kinetic parameters of a catabolic pathway involving more than one species.

Our main goals were to: (1) develop a mild immobilization technique convenient to use under anaerobic conditions which would maintain the activity of a variety of syntrophs, (2) maintain long term survivability under toxic concentrations of phenol, and (3) formulate a kinetic model which describes the rate of biodegradation of toxic substrates at both inhibitory and non-inhibitory concentrations.

Kinetic models are of value in investigating both the capacity and stability of biological processes which utilize inhibitory substrates. We have modified a model based the Haldane equation which describes the kinetics of inhibitory substrate utilization (12) to incorporate a substrate diffusion-limitation parameter for immobilized cells. Experimental data were used to test the model's success in fitting measured values of kinetic parameters to both stimulatory and inhibitory substrate concentrations.

MATERIALS AND METHODS

Enrichment. The phenol-degrading consortium was enriched from anaerobic digestor sludge obtained from a municipal plant in Jackson, MI. The enrichment was maintained for 2 years with bimonthly transfers of 25% inocula to fresh revised anaerobic mineral medium (RAMM) (17). The enrichment was grown at 37°C under stationary conditions. Approximately 2 mM phenol was added to the enrichments every 2 days. Phenol was stoichiometrically converted to methane and CO_2.

Immobilization of bacterial cells. All media and transfers were made using anaerobic gases and gassing probes. Cells from 1200 ml of phenol enrichment were collected as pellets by centrifugation at 15,000 x g for 15 min. Centrifuge tubes were preflushed with an oxygen-free 80% N_2/20% CO_2 gas mixture. The pelleted cells were suspended and washed in an equal volume of growth medium, recentrifuged and then suspended in 20 ml of medium. This cell suspension contained approximately 10 mg protein/ml. A 3 ml aliquot was then added by syringe to each of three serum bottles (160 ml) containing 50 ml of anaerobic medium. These were used as native cell cultures; the remaining suspension was used for immobilization.

The immobilization matrix was 2% agar. To make the anaerobic matrix, three 2 m sections of Tygon tubing (id of 1 mm) were connected to gas lines and flushed with 80% N_2/20% CO_2. The bulk of the tubing was placed in a double-valved anaerobic jar which was also flushed with the oxygen-free gas. The distal ends of tubing were inserted into three 25 mm test tubes in a 50°C water bath on a hot plate/magnetic stirrer. The tubes were filled with 3 ml of anaerobic

growth medium with 4% agar at 50°C. Three milliliters of cell suspension was then slowly added to the medium while mixing with a magnetic stir bar. The gas line was disconnected from each section of tubing and the cell-agar mixture was drawn rapidly into the oxygen-free tubing by suction with a 50 ml syringe. After solidifying, the matrix was slowly ejected by syringe into a pre-flushed serum bottle (160 ml) containing 50 ml of growth medium. This resulted in a 1 mm diameter agar gel matrix which resembled a long strand of spaghetti. The matrix was washed twice with 50 ml of medium to remove loose cells and fine colloidal particles before addition of 50 ml of incubation medium. Bottles were then sealed with butyl rubber stoppers. Both native and immobilized cultures contained the same amount of cells.

Experimental procedure. Triplicate serum bottles containing either native or immobilized cells were incubated at 37°C while shaking at 120 rpm. The rate of phenol degradation was assayed by monitoring the production of methane. The range of initial concentrations of phenol was from 10 to 2000 ug/ml. Methane was quantified by injecting 0.2 ml of culture headspace gas into a Carle model 8515 gas chromatograph equipped with a Porapak Q packed column and a microthermistor detector. Protein concentrations were determined by the method of Lowry et al. (24). Microscopy was by phase contrast and fluorescence with a Leitz Ortholux microscope. Fluorescence was used to identify methanogenic bacteria. Samples of "spaghetti agar" for scanning electron microscopy (SEM) were dehydrated in ethanol and freeze-fractured. Dried specimens were mounted on stubs and coated with gold before viewing with a JEOL JSM-

35-C scanning electron microscope. Samples for thin-section transmission electron microscopy (TEM) were fixed in 2% glutaraldehyde and 0.1 M phosphate buffer (pH 7.2), dehydrated in ethanol followed by propylene oxide and embedded in epoxy resin. Electron micrographs were taken on a Philips 300 electron microscope.

Kinetic Analysis. Andrews (1) and Edwards (3) proposed mathematical growth models based on Monod biokinetics for both batch and continuous cultures of native cells. These kinetic models were based on the Haldane equation and made use of an inhibition function, K_i, to relate substrate concentration to specific growth rate. The model was successfully used to predict biomass production during treatment of phenolic wastes by activated sludge (14,15). A modification of the basic model was proposed by Neufield et al. (12):

$$V = V_o/[1 + K_s/S + (S/K_i)^n] \qquad [1]$$

where S is the available substrate concentration, V is the specific substrate utilization rate, V_o is the maximum substrate utilization rate, K_s is the substrate concentration at 0.5 V_o, K_i is the inhibition constant, and n is an empirical value describing the order of inhibition.

For immobilized cells, the available substrate concentration, S', is decreased from S because of diffusion-limited mass transport. We have, therefore, altered model [1] to describe the utilization of an inhibitory substrate by either native or immobilized microorganisms:

$$v = V_{max}/[1 + K_m/(S \cdot D) + (S/K_i)^n] \quad [2]$$

where D is an empirical parameter ranging from 0 to 1 that changes S to S' to reflect the diffusion limited substrate concentration available to immobilized cells. For native cells D = 1. For

immobilized cells, D will equal a value that reflects lowered mass transport such that $S' = S \cdot D$.

Estimates of V_{max}, K_m, K_i, and D were obtained from initial velocity experiments with substrate concentrations of 10, 20, and 30 ug/ml phenol. Values for V_{max} and K_m were estimated directly from Lineweaver-Burk plots of the resultant data. An initial estimation of K_i was then made with Andrews' (1) batch culture model:

$$v = V_{max} \cdot S/[S^2/K_i) + S + K_m] \qquad [3]$$

using the estimated values for V_{max} and K_m and the observed initial velocity, v, at an inhibitory substrate concentration of 1000 ug/ml phenol for native cells and 2000 ug/ml phenol for immobilized cells. Thus, by use of the Lineweaver-Burk plot and equation [3] we obtained estimates for V_{max}, K_m, and K_i. Because rates are dependent on substrate concentration, the difference in the rate of methane production between native and immobilized cells was used to obtain a value for D to convert S to S'. Since v'/v is a constant S'/S must also be a constant equal to v'/v and V'_{max}/V_{max}. Given that $D = S'/S$ (equation 2), the ratio of V'_{max}/V_{max} was used to calculate a value for D. Values for n were estimated by fitting the model to experimental data.

A range of values around these estimates was used in a program, implemented in BASIC, that simulated data points for a plot of v vs. S as per equation [2]. The simulated data were compared to experimental data by a nonlinear least squares regression analysis. Kinetic parameters were then selected for which the sum of errors between simulated and experimental data was minimal. Because equation [2] contains four constants, an almost infinite number of

numerical combinations would suffice to fit the data. By first obtaining independent estimates for three of the constants we were able to set a range for each value such that the parameters selected by the program were as accurate as possible.

RESULTS

Phenol-degrading consortium. The "spaghetti agar" containing the cells broke into 2-50 cm lengths when initially placed on the rotary shaker, but then maintained both integrity and phenol degrading activity for over one month. Observation of the agar matrix by SEM showed the cells to be in an undisrupted state (eg., Figure 1). Stoichiometric conversion of phenol to CH_4 and CO_2 was evidence that the consortium remained active after immobilization.

Three distinct bacterial morphotypes which dominated the liquid enrichments were also evident within the agar matrix (Figure 1). These are proposed to be the following three physiological types: an acetoclastic methanogen, recognized by its fluorescence and Methanothrix-like morphology (a); an H_2-utilizing methanogen which also fluoresced (b); and a phenol-oxidizing bacterium (c). The proposed phenol-oxidizer was oval-shaped 0.8 um x 1.2-3.3 um in size (Figure 2a) and stained gram-negative. These cells had the same undulating membrane (Figure 2b) as two strains of anaerobic benzoate-oxidizing bacteria (11, 18). Approximately 80% of the bacteria in the enrichment had the morphology of the proposed phenol-oxidizing bacterium as shown by random counts under phase microscopy. Both benzoate and p-cresol were rapidly metabolized to CH_4 and CO_2 in the enrichment (data not shown) indicating that the phenol-oxidizing bacterium might use these substrates as well.

The rate of phenol degradation was affected by immobilization of the cells; the type of effect depended on the concentration of phenol initially available. At concentrations of 200 ug/ml phenol (Figure 3), or less (data not shown), a lag in methane production occurred

Figure 1. Scanning electron micrograph of a freeze fractured surface of the agar matrix containing cells of the phenol-degrading consortium. Cells present are the proposed *Methanothrix*-like organism (a), H_2-utilizing methanogen (b), and the proposed phenol-oxidizing bacterium (c). Bar = 10 um.

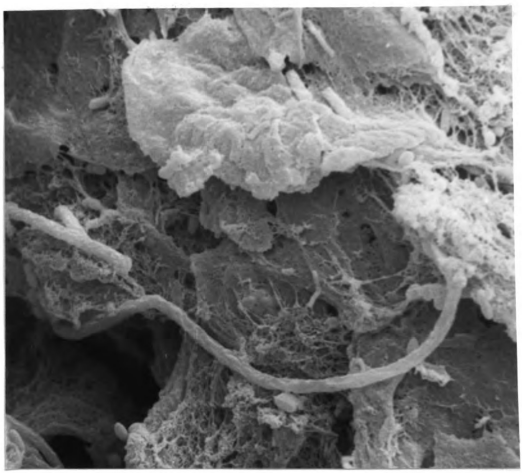

Figure 1

Figure 2a. Phase-contrast photomicrograph of the consortium showing the shape and numerical dominance of the organism proposed to be the phenol-oxidizing bacterium. Both single cells and cells in division are shown. Bar = 10 um.

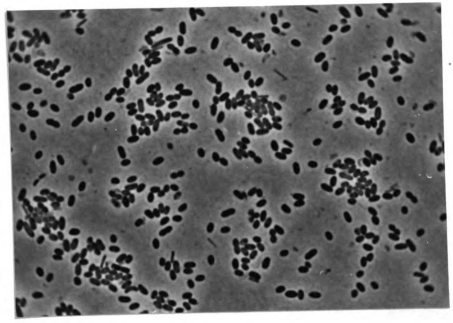

Figure 2a

Figure 2b. Transmission electron micrograph of a thin section of the proposed phenol-oxidizing bacterium.

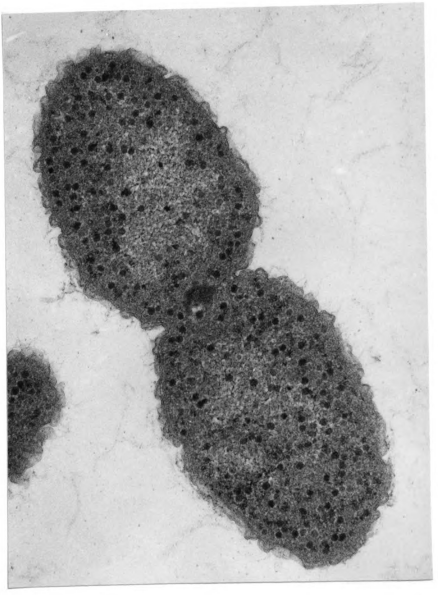

Figure 2b

Figure 3. Temporal production of methane from 200 ug/ml phenol by native (o) and immobilized (Δ) cells. Complete conversion of phenol should yield 0.35 mmol CH_4.

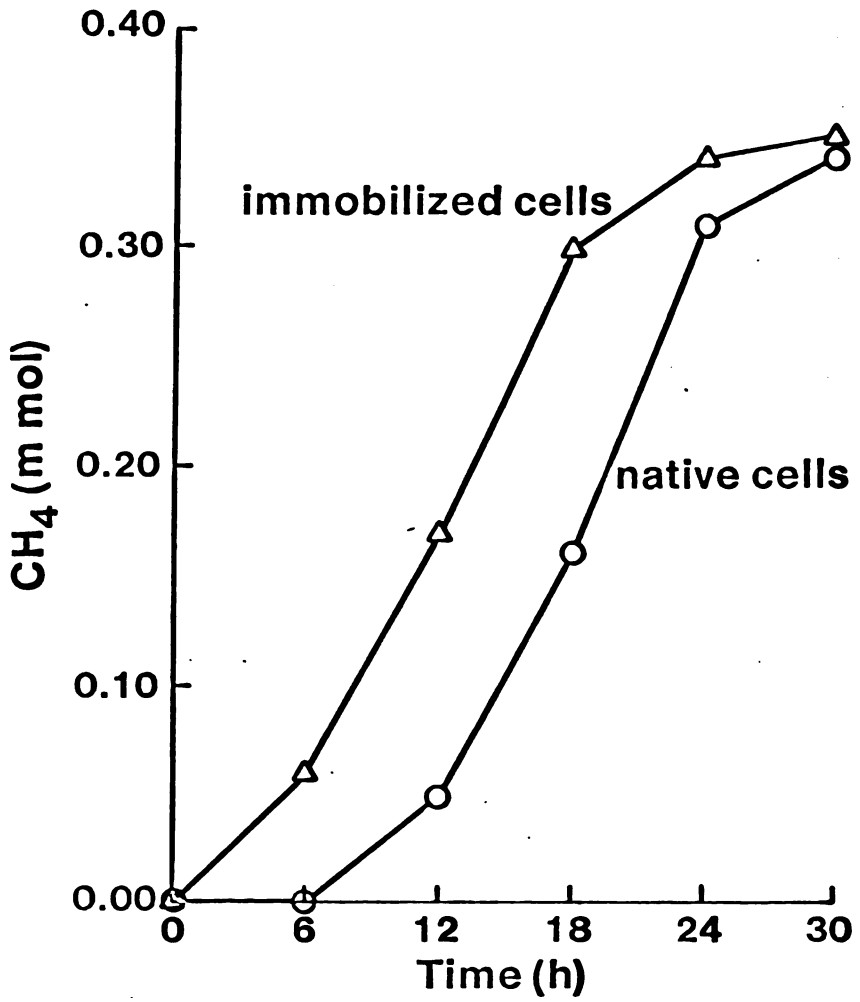
0.40
0.30
0.20
0.10
0.00
CH_4 (m mol)
immobilized cells
native cells
0
6
12
18
24
30
Time (h)

Figure 3

for native cells, but not for the immobilized consortium. At 500 ug/ml phenol, or more, neither system exhibited an apparent lag (Figure 4). The rate of phenol degradation for native cells decreased at concentrations above 500 ug/ml, and for immobilized cells at concentrations above 1000 ug/ml. At 2000 ug/ml phenol, native cells were completely inhibited, while the immobilized cells maintained some activity.

Kinetics of phenol degradation. The kinetic parameters V_{max}, K_m, K_i and D were compared between native and immobilized consortia. Rates of methane production were obtained during the initial 45 min of linear productivity with 10, 20, and 30 ug/ml phenol. These rates were used to construct Lineweaver-Burk plots (Figure 5). Low phenol concentrations were used to avoid substrate inhibition. The fit of linear plots in double-reciprocal co-ordinates was taken as support for the Michaelis-Menten model of substrate dependence and as evidence that these phenol concentrations were not inhibitory. Inhibition would have been expressed as higher reciprocal-rate values due to a decrease in the degradation rate with higher phenol concentration.

The linearity between rate and phenol concentration (Figure 5) indicated that S (equation 2) was the rate limiting factor. The rates for immobilized cells, v', were proportionately less than for native cells, v, probably due to mass transport limitation of substrate which reduced S to S', the apparent initial substrate concentration for immobilized cells. The estimated kinetic parameters from these plots are listed in Table 1.

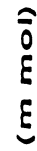

Figure 4. Temporal production of methane from phenol biodegradation by native (o) and immobilized (Δ) cells. Initial phenol concentrations are in parentheses.

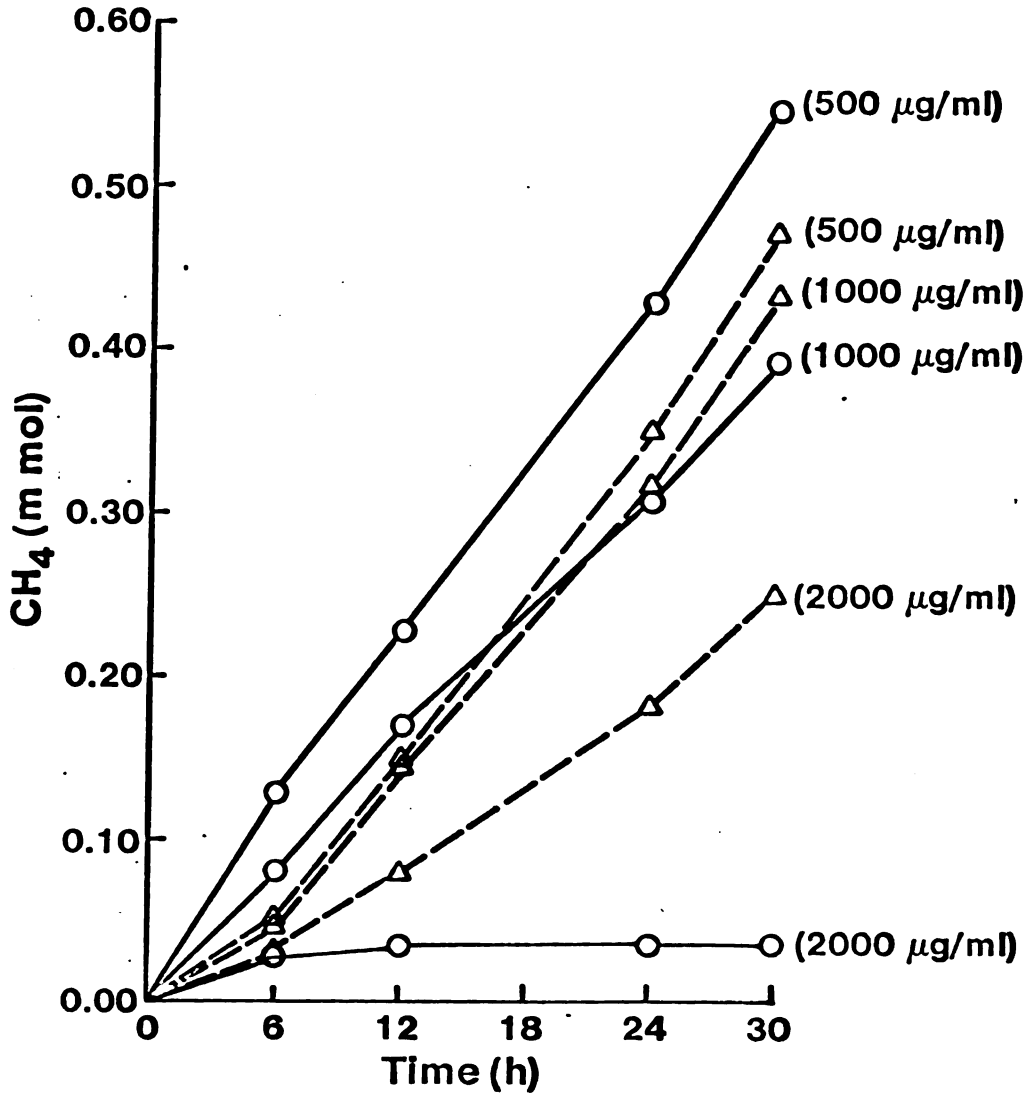
0.60
0.50
0.40
0.30
0.20
0.10
0.00
CH_4 (m mol)
0
6
12
18
24
30
Time (h)
(500 μg/ml)
(500 μg/ml)
(1000 μg/ml)
(1000 μg/ml)
(2000 μg/ml)
(2000 μg/ml)

Figure 4

Figure 5. Lineweaver-Burk plots of initial methane production rates by native (o) and immobilized cells (Δ). Rates are mean values of duplicate cultures. Initial phenol concentrations were 10, 20, and 30 ug/ml. V_{max} and K_m estimates were obtained from these plots. Regression coefficients were $r^2 = 0.999$ for native cells and $r^2 = 0.986$ for immobilized cells.

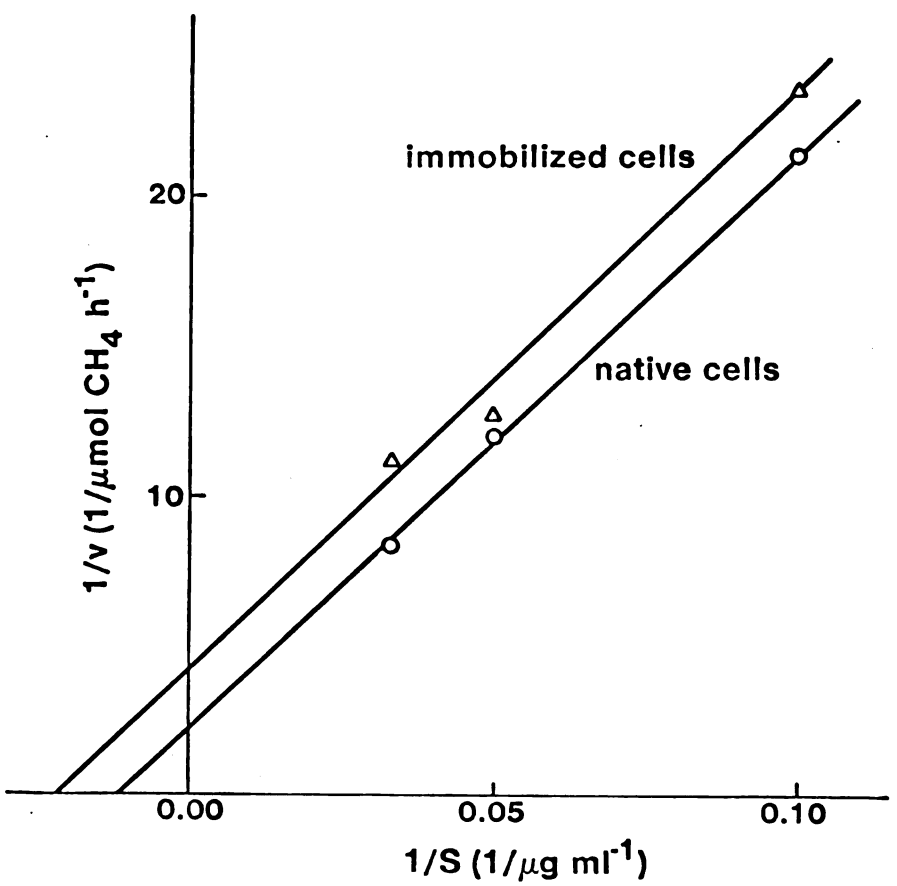
immobilized cells
native cells
20
10
1/v (1/μmol CH4 h-1)
0.00
0.05
0.10
1/S (1/μg ml-1)

Figure 5

Model. Neufield's modified model [2] was used to obtain kinetic parameters (Table 1) which best fit a series of experimental rate determinations. The values obtained from the model were used to construct simulated curves for rate vs. concentration of phenol for both native and immobilized cells. The curves shown in Figure 6 are superimposed with the experimental data. Both plots show an area of substrate stimulation (a), saturation (b), and inhibition (c). Immobilized cells did not attain rates as high as native cells but were protected from the inhibitory effect of high phenol concentrations. This protection was also shown by the K_i values (Table 1) of 900 ug/ml (native) vs. 1725 ug/ml (immobilized). The accuracy of this model for predicting phenol oxidation rates was further proven since the peak of activity occurred for both native and immobilized cells at the predicted ($S = (K_m K_i)^{0.5}$) phenol concentrations of 285 and 282 ug/ml, respectively.

The experimental rate for native cells at 1500 ug/ml appeared to cause the simulated curve to overestimate rates for region (c). If the curve was computed without this value it approached a rate of zero at 2000 ug/ml.

Table 1. Kinetic parameters for phenol utilization by native and immobilized cells.

Culture	Estimation Method	V_{max} (ug/ml)	K_m (ug/ml)	K_i (ug/ml)	D[a]	n[b]
Native cells	Lineweaver-Burk	14.0	78	1400	-	-
	Model	20.0	90	900	-	2.5
Immobilized cells	Lineweaver-Burk	7.8	47	1783	0.6	-
	Model	12.0	46	1725	0.6	3.6

[a] D is an empirical parameter used to change the real substrate concentration of the culture, S, to the apparent substrate concentration in the immobilizing matrix, S'.

[b] n is an empirical value which describes the order of substrate inhibition and is obtained by fitting the kinetic model to rate data.

Figure 6. Simulated curves of phenol degradation vs. phenol concentration are shown for both native and immobilized cells. Experimental data for native (o) and immobilized (Δ) cells are presented in the same plot to show their fit with the model derived curves. Each data point is a mean of triplicate determinations.

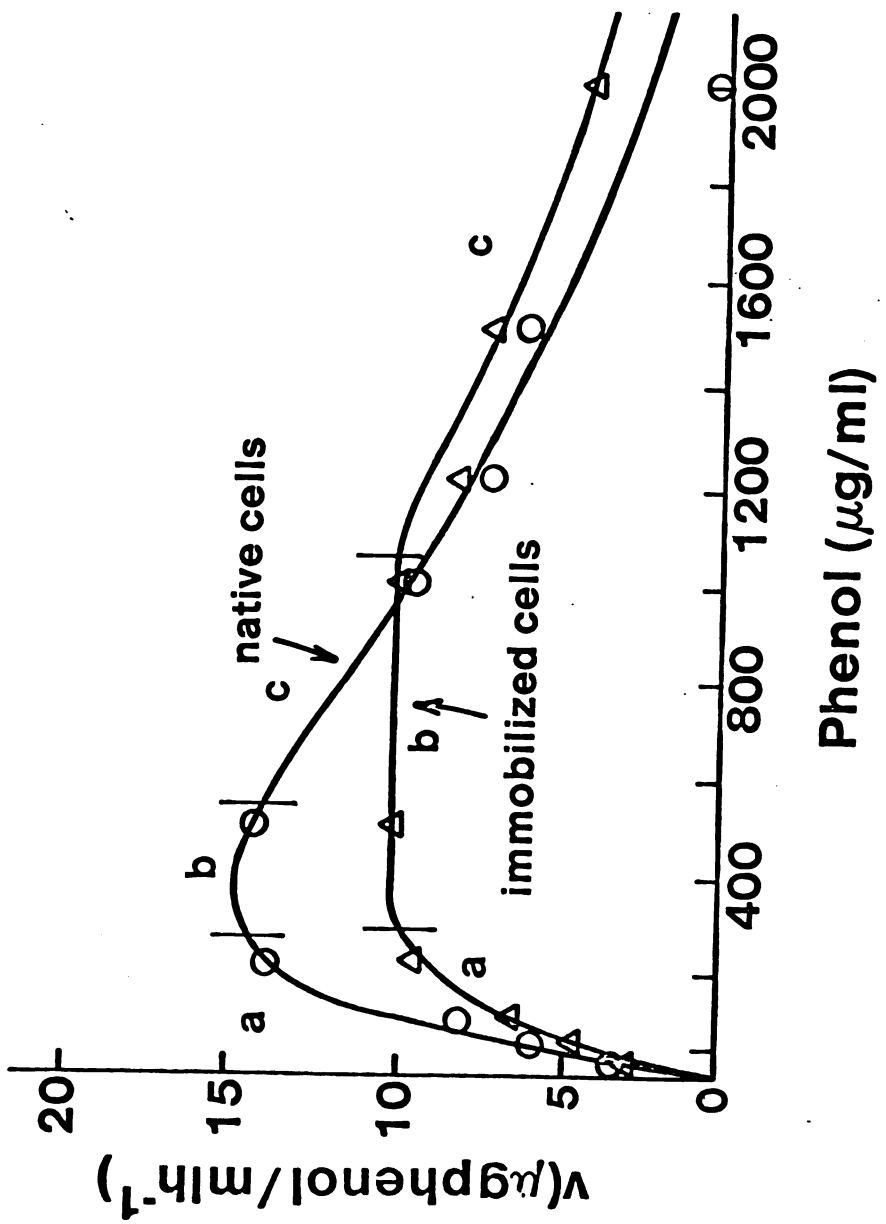
v(µg phenol/min⁻¹)
20
15
10
5
0
400
800
1200
1600
2000
Phenol (µg/ml)
native cells
immobilized cells
a
b
c
a
b
c

Figure 6

DISCUSSION

We were successful in devising a form of immobilization mild enough to maintain the syntrophic activity necessary for phenol biodegradation with minimal exposure of cells to oxygen. Many immobilizing agents, e.g. polyacrylamides, either may be toxic (10), use oxidative polymerizing agents (8) or cause cell disruption (23). We, therefore, used cell entrapment in agar since both agar and alginate gel immobilization methods are relatively mild and maintain anaerobic cellular activity (8,16). Observation of many samples by SEM (eg., Figure 1) demonstrated the good physical condition of the cells of the phenol-degrading enrichment. Stoichiometric production of methane from phenol demonstrated activity by all three physiological groups.

The "spaghetti" agar was of a consistent size (1 mm in diameter), which is important for comparative kinetic studies. The immobilized cells also maintained long term phenol-degrading activity (>1 month). Thus, our immobilization method is useful both for laboratory screening of anaerobic cellular activity and for determining the kinetic changes associated with immobilization.

The effect of immobilization on the methanogenic consortium was twofold: it protected the cells from inhibitory concentrations of phenol, and altered the kinetic characteristics of the food chain. The latter is shown by a decrease in the apparent Km (Table 1), the lag period (Figure 3), and the rate of phenol mineralization (Figure 5). Since activity was measured as methane production, we feel these effects were due to retention of methanogenic substrates during the mineralization of phenol coupled with a closer proximity of

interacting cells within the agar matrix. This is in contrast to results from studies involving single enzymatic reactions. A kinetic analysis of -glucosidase activity by a pure culture of immobilized *Alcaligenes faecalis* showed that the apparent K_m increased above that for the free enzyme (22). This occurred while the apparent V_{max} decreased and reflected restriction of substrate transport across the immobilizing matrix. In our study this same restriction likely caused the retention of methanogenic substrates and is reflected in the decrease in the apparent K_m.

The response of methane production to phenol concentration (Figures 4 and 6) was similar to that for previous batch culture studies (6). Three distinct regions of methane producing activity were evident in the range of 10 to 2000 ug phenol/ml (Figure 6). Region (a) demonstrated first-order kinetics and initial rates increased with phenol concentration. Immobilized cells had lower rates probably due to mass transport limitations of phenol into and CH_4 out of the agar matrix. In region (b) both native and immobilized cells reached a maximum rate of activity. For native cells, this equaled the rate predicted by the Lineweaver-Burk estimation of kinetic parameters (14 ug/h phenol). In contrast, the maximum rate of activity for immobilized cells (10 ug/h phenol) was greater than predicted because the Lineweaver-Burk analysis was affected by diffusion-limited mass transport at low phenol concentrations. Higher concentrations of phenol may have increased diffusion into the agar thereby increasing S' and causing the faster rates. Compared to native cells region (b) included a wider range of substrate concentrations for immobilized cells. This is a result of

the protection against substrate inhibition afforded by immobilization. While the phenol concentrations of region (c) inhibited activity, immobilized cells were somewhat protected. At 2000 ug/ml phenol, native cells were completely inhibited while immobilized cells maintained about one-half their phenol-degrading activity. This protection from phenol inhibition also is evident from comparison of the K_i values (Table 1).

Our estimated K_i for immobilized cells was 1725 ug/ml phenol whereas for native cells it was 900 ug/ml which is comparable to the K_i value of 500-700 ppm and 966 ppm found by Pearson et al. (13) and Neufield et al. (12), respectively. Our results indicated that immobilization might be an alternative to the dilution treatment now used by many facilities for lessening the effect of toxic substrates. This is important considering that wastewater from coal conversion techniques may contain phenolic compounds up to 7600 ppm (5). Other microbial processes should be amenable for immobilization since agar gels will allow diffusion of substrates with molecular weights of less than 2×10^4 g/mol (20). As an example, we have successfully immobilized polyethylene glycol degrading methanogenic co-cultures (Dwyer and Tiedje, unpublished results).

Our diffusion-limited rate data were successfully used in a kinetic model designed to describe substrate inhibited activity. Kinetic growth models for inhibitory substrates have been tested (12,19), but an empirical model which incorporates mass transport limitation has not. Our modification of Neufield's (12) model [2] was made to compensate for the limiting effect that immobilization should have upon apparent substrate concentrations and substrate

dependent kinetics (22). An analysis of the model shows why this is true. At low substrate concentrations $K_m/(S \cdot D) > (S/K_i)^n$ and the equation reduces to its simple Monod function. In this form, V'_{max} is overestimated for immobilized cells without the term D to effectively change S to S'. With D, V_{max} is reduced to V'_{max} by the percentage underestimated by the Lineweaver-Burk analysis. Simulated values for v' would then reflect activity at S' which was the case as the close agreement between our experimental data and simulated data shows (sum of errors = 1.9). At high inhibitory substrate concentrations $(S/K_i)^n > K_m/(S \cdot D)$ and the inhibition term decreases V_{max}. Thus, the importance of obtaining estimates for both K_m and K_i is readily evident. Where $K_i > K_m$ the system can approach a maximum rate at $S = (K_m K_i)^{0.5}$, but where $K_i < K_m$ substrate inhibition would occur at low concentrations thus preventing the system from reaching its apparent V_{max}.

This model can serve as a framework for understanding the effects of immobilization on consortia which utilize inhibitory substrates. Process stability for continuous culture systems should be increased both by virtue of the higher K_i obtained by immobilization and by prevention of cell washout at dilution rates higher than maximum growth rates. While our modified inhibition model may not be directly applicable to all types of substrate inhibited systems (1), the diffusion-limitation parameter is both easily understood and quantifiable which makes it a useful concept for other models as well.

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APPENDICES

APPENDIX A

The "spaghetti agar" technique for immobilizing anaerobic bacteria (1) was developed with two glycol-degrading, methanogenic co-cultures. One co-culture contained bacterial strain DG2, which was isolated from a methanogenic enrichment fed DEG (diethylene glycol; 2 ethyoxylate units) and was identified as a *Desulfovibrio desulfuricans* strain. The other co-culture contained bacterial strain PG1, which was isolated from a methanogenic enrichment fed PEG-1000 (polyethylene glycol with a molecular weight of 1000; 22 ethoxylate units) and was identified as a *Bacteroides* sp. PG1 and DG2 were grown in co-culture with methanogen, *Methanobacterium* strain DG1 and fed PEG-1000 and DEG, respectively. Both substrates were stoichiometrically metabolized to acetate and methane. The isolation, identification and growth conditions have been described (3).

Three factors were assessed during development of the anaerobic immobilization technique:

1. We determined if the procedure maintained anaerobic conditions by observing glycol degradation by the obligately anaerobic bacteria.
2. We determined the effect that immobilization has on production, accumulation and transfer of intermediate products (in this case H_2) during the syntrophic metabolism of a substrate.
3. Because of their size differences, DEG and PEG-1000 may have different mass transport limitations in the immobilizing agar gel. We determined the effect of substrate size on lag time and rate of substrate degradation by immobilized cells.

Both DEG and PEG-1000 were stoichiometrically metabolized to CH_4 and acetate by native (non-immobilized) and immobilized co-cultures. A lag of approximately 3 h in the degradation of DEG and of 8 h in the degradation of PEG-1000 occurred for immobilized cells (Figures 1 and 2), due to the time necessary for transport of substrate across the agar matrix. The larger size of PEG-1000 probably created the relatively longer lag time observed for that co-culture. The immobilizing agar matrix had little effect on activity as indicated by similar rates of methane production for immobilized and native cells once the lag period ended.

These results showed that the "spaghetti agar" immobilization technique was adequate for maintaining anaerobic conditions and mild enough to maintain appropriate conditions for syntrophic metabolism. Methane production by co-cultures requires whole cell metabolism with cofactor regeneration that enzymatic preparations or destructive immobilization techniques do not allow. Thus, this procedure may be useful for studying the metabolism of substrates by anaerobic cells that are immobilized under more satisfactory conditions than previously available (4).

The usefulness of the immobilization technique was further studied by a kinetic analysis of phenol degradation by an immobilized, methanogenic phenol-degrading enrichment (1). Mass transport limitations by the agar matrix were shown to lower the apparent K_i (inhibitory substrate concentration constant) of phenol for immobilized cells relative to that for native cells. By retention of the methanogenic substrates, acetate and H_2, in the vicinity of methanogens the apparent K_m was also lowered. Such

Figure 1. Temporal production of methane from DEG by native (x) and immobilized (□) co-cultures. Values are the means of triplicate cultures. Total methane production equaled the theoretical yield of 0.52 mmol from 0.2% substrate.

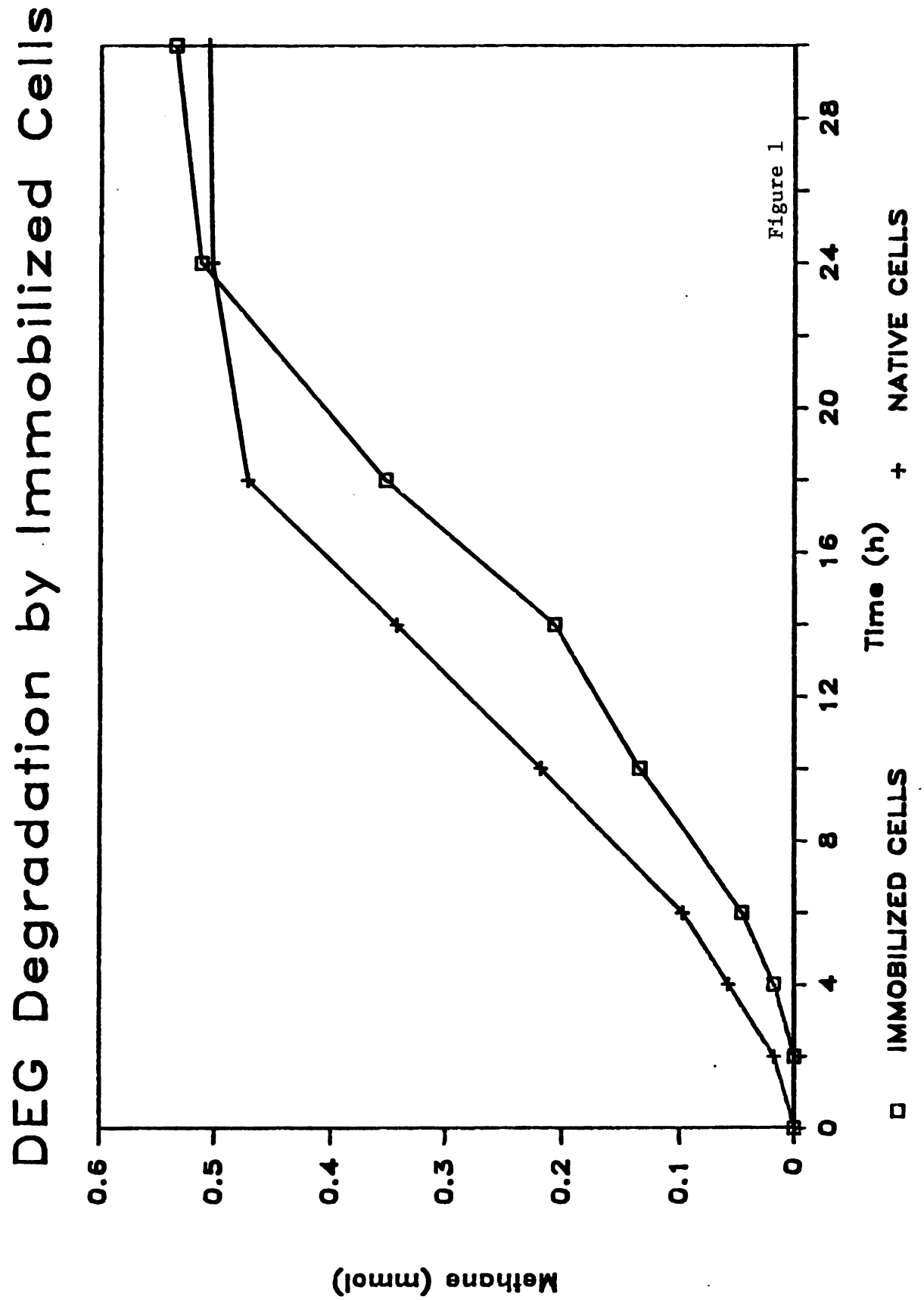
DEG Degradation by Immobilized Cells
Methane (mmol)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
8
12
16
20
24
28
Time (h)
□ IMMOBILIZED CELLS
+ NATIVE CELLS

Figure 1

Figure 2. Temporal production of methane from PEG-1000 by native (x) and immobilized (□) co-cultures. Values are the means of triplicate cultures. Total methane production equaled the theoretical yield of 0.58 mmol from 0.2% substrate.

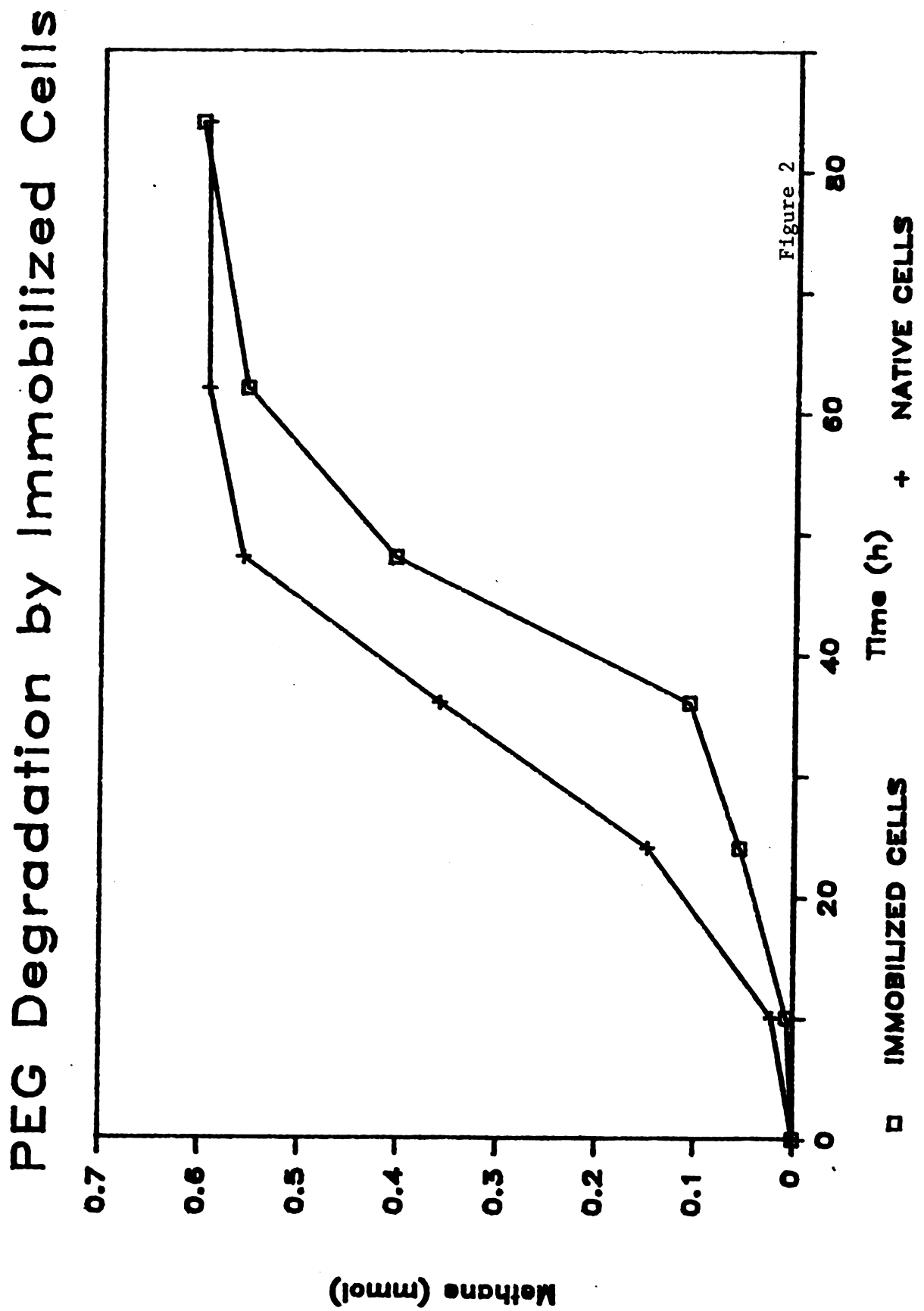
PEG Degradation by Immobilized Cells
Methane (mmol)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
Time (h)
□ IMMOBILIZED CELLS
+ NATIVE CELLS

Figure 2

useful qualities are in addition to other positive qualities gained by using immobilized cells for bioconversions such as catalyst retention and relatively clean effluents.

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APPENDIX B

PROGRAM FOR ESTIMATING KINETIC PARAMETERS

The program requires two sets of variables. First, enter the data points for substrate (S) and the corresponding rate (V). Second, give it the best estimates for K_s, V_{max}, K_i, n and D. This is done by setting a range for each of these parameters. The program will vary each parameter over this range by increments which are also specified. For each permutation of the parameters the program simulates data points for S and V and calculates the sum of the square differences between each data point and the simulated data. It then selects the kinetic parameters for which the sum of the squared differences is minimal.

Variables within the program: S and V are the data points, Num is the number of S-V pairs, and Sumer is the sum of the squared deviations for all data points.

List

```
 10  CLS :  PRINT "WELCOME TO THE FIND A CURVE FOR DARYL'S";
 20 PRINT " POINTS PROGRAM"
 30 PRINT "HI User!"
 40 PRINT
 50 DIM S(20), V(20), OUTER$(20,60)
 60 S(1) = 30   : V(1) = 5.6
 70 S(2) = 50   : V(2) = 6.8
 80 S(3) = 100  : V(3) = 7.8
 90 S(4) = 200  : V(4) = 13.9
100 S(5) = 500  : V(5) = 13.9
110 S(6) = 1000 : V(6) = 9.4
120 S(7) = 1200 : V(7) = 7.7
130 S(8) = 2000 : V(8) = 0.0
140 MIN = 9999
150 NUM = 8
160 FOR I = 1 TO 20
170   FOR J = 1 TO 60
180     OUTER$(I,J) = "."
```

```
190   NEXT J
200 NEXT I
210 FOR N           = 2.2 TO 2.2 STEP .1
220   PRINT "N = ';N
230   FOR KS        = 97 TO 97 STEP 1
240     FOR VMAX    = 20 TO 20 STEP 1
250       FOR KI    = 950 TO 950 STEP 5
260         FOR D   = 1 TO 1 STEP 1
270           FOR I = 1 TO NUM
280             TEMPV = VMAX / (1 + KS / (D*S(I)) + ((S(I) / KI) ^
N))
290             DIFF  = TEMPV - V(I)
300             SUMER = SUMER + (DIFF * DIFF)
310           NEXT I
320           IF SUMER > MIN THEN GOTO 390
330           KEEP = KI
340           MIN = SUMER
350           KEEPN = N
360           KEEPS = KS
370           KEEPV = VMAX
380           KEEPD = D
390         NEXT D
400       NEXT KI
410     NEXT VMAX
420   NEXT KS
430 NEXT N
440 BEEP : BEEP
450 PRINT "VALUES WITH MINIMUM ERROR"
460 PRINT "D =";KEEPD
470 PRINT "KI =";KEEP
480 PRINT "N =";KEEPN
490 PRINT "KS =";KEEPS
500 PRINT "VMAX =";KEEPV
510 PRINT "mean SSE = "; MIN/B
520 PRINT
530 PRINT "PRESS RETURN TO SEE THE PLOT"
540 INPUT GOON
550 PRINT
560 FOR I = 1 TO NUM
570   OUTER$(INT(V(I)),INT(S(I) / 40)) = "*"
580 NEXT I
590 FOR S = 40 TO 2400 STEP 40
600   V = KEEPV/ (1 + KEEPS / (KEEPD*S) + ((S / KEEP) ^ KEEPN))
610   OUTER$(V, S/40) = "+"
620 NEXT S
630 PRINT "THE ORIGINAL POINTS ARE * AND THE APPROXIMATIONS ARE +"
640 FOR I = 20 TO 1 STEP -1
650   PRINT USING "## "; I;
660   FOR J = 1 TO 60
670     PRINT OUTER$(I,J);
680   NEXT J
690   PRINT
700 NEXT I
710 PRINT "   :     :     :     :     :     :     :     :     :     :     :     :
```

```
720 PRINT "  40   240  440  640  840  1040 1240 1440 1640 1840 2040
2240
730 END
```