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Microbial Metabolism of Homocyclic and Heterocyclic Aromatic Compounds under Anaerobic Conditions

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INTRODUCTION

The biodegradation of both naturally occurring and xenobiotic chemicals has been investigated extensively in the last few decades. The metabolic fate of xenobiotics under aerobic conditions has been widely studied, and the predominant persistence patterns and degradation pathways are well elaborated. In contrast, the metabolism of naturally occurring organic and xenobiotic compounds by anaerobic microbial populations has largely been ignored; this is especially true of the metabolism of heteroaromatic compounds. However, recent studies on the anaerobic biodegradation of haloaromatic and aromatic compounds, as reviewed by Young (114) and Sleat and Robinson (86), clearly demonstrate the importance of anaerobic microbial transformations of organic compounds in anoxic environments.

Anaerobiosis usually occurs in any habitat in which oxygen consumption exceeds its supply and is a common phenomenon in many natural environments. Examples include flooded soils, sediments, landfills, lagoons, anaerobic fresh and ocean waters, and some groundwaters.

One of the most important, yet least understood, of the anaerobic habitats is the saturated subsurface which is known to harbor biologically active anaerobic microorganisms (35, 67, 103, 104). A variety of organic compounds, e.g., heteroaromatics often associated with energy production activities, are disposed of in these subsurface environments. The contamination of groundwater by leaching of these organic chemicals through soil and subsoils is a major environmental concern, since even in small quantities many of these organic compounds are considered toxic or even carcinogenic. Although very little is known about the abilities of anaerobic microorganisms to influence the subsurface transport of organic contaminants, their ability to transform or mineralize a variety of organic compounds is now recognized as a significant factor in the fate of contaminants in the anoxic subsurface environment. Investigators have thus begun to probe not only substrate utilization, metabolic pathways, and microbial taxonomy but also habitat range of anaerobic microorganisms. The purpose of this paper is to present a critical review of the available scientific information on the anaerobic microbial metabolism of homo- and heterocyclic aromatic compounds, with emphasis on various anaerobic microbial processes and metabolic pathways.

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ANAEROBIC MICROBIAL PROCESSES

Microbial Diversity

Within anoxic ecosystems, the availability of electron donors and acceptors plays a crucial role in influencing microbial activity and diversity. Presumably as a result of the ubiquitous nature of organic matter, organic carbon predominates as the electron donor in many anoxic environments and is required by many anaerobic microorganisms

TABLE 1. Relationship between representative microbial processes and redox potential

Process	Reaction (electron donor + electron acceptor)[a]	Physiological type	Redox potential (mV)	Reference
Respiration	OM + $O_2 \rightarrow CO_2$	Aerobes	700–500	13
Denitrification	OM + $NO_3^- \rightarrow N_2 + CO_2$	Faculative anaerobes	300	29
Fermentaion	OM → organic acids (most notably acetate, propionate, and butyrate)	Faculative or obligate anaerobes		
Dissimilatory sulfate reduction	OM (or H_2) + $SO_4^{2-} \rightarrow H_2S + CO_2$	Obligate anaerobes	−200	91
Proton reduction	OM (C_4–C_8 FA) + $H^+ \rightarrow H_2$ + acetate (propionate) + CO_2	Obligate anaerobes		
Methanogenesis	$CO_2 + H_2 \rightarrow CH_4$	Obligate anaerobes	<−200	87
	Acetate → $CO_2 + CH_4$	Obligate anaerobes	<−200	87

[a] OM, Organic matter; FA, fatty acid.

for their energy-yielding, oxidation-reduction reactions. Table 1 summarizes several microbial processes, such as respiration, denitrification, fermentation, dissimilatory sulfate reduction, proton reduction, and methanogenesis. In addition, an approximate relationship between the microbial process and the environmental redox potential is indicated.

In many anoxic ecosystems, food chains or syntrophic associations are necessary for anaerobic microorganisms to completely mineralize an organic compound. For instance, it is now believed that three major groups of microorganisms are essential for complete mineralization of organic carbon to CO_2 and CH_4 in anoxic sites that are without light and are low in electron acceptors other than CO_2. These three groups are the fermenters, the proton reducers, and the methanogens (14, 65, 66).

Representative Degradative Pathways of Aromatic Compounds under Anaerobic Conditions

Aromatic compounds consisting of either a homocyclic (e.g., benzoate, Fig. 1a) or a heterocyclic (e.g., nicotinate, Fig. 1b) aromatic nucleus can be metabolized by microorganisms under anaerobic conditions. A general degradative pathway for anaerobic metabolism of benzenoid compounds is illustrated by the anaerobic photocatabolism of benzoate. Figure 2 illustrates the well-elucidated benzoate reductive pathway first described by Dutton and Evans (25). The initial step in this degradative pathway is ring hydrogenation. Following the ring reductive phase is a hydration ring cleavage sequence. Evans (30) theorizes that the anaerobic metabolism of benzenoid compounds can proceed only through the reductive pathway. Until now, Evans's postulation has remained largely unchallenged. Recently, however, Grbić-Galić and co-workers (105) have obtained experimental evidence suggesting that benzenoid compounds may be metabolized through an oxidative pathway (i.e., the initial step is ring oxidation rather than reduction). Investigations revealed that the anaerobic degradation of benzene and toluene to CO_2 and CH_4 proceeded by way of a hydroxylation reaction. Thus, the intermediate phenol and cresol were formed from benzene and toluene, respectively (105).

The degradative pathway for fermentation of nicotinate by a *Clostridium* species is illustrated in Fig. 3. The initial step in nicotinate fermentation is ring hydroxylation, which is followed by a ring reduction hydrolytic ring cleavage sequence. Although the degradative pathways of benzoate and nicotinate share some similarities, it should be pointed out that the basic sequence of events is reversed. It is interesting to note that the benzoate pathway features an initial ring reduction step, whereas the initial step in the nicotinate pathway is a ring hydroxylation reaction.

The mechanism of enzymatic catalysis of aromatic compounds is dependent upon energy relationships that are determined, at least in part, by the physicochemical nature of the aromatic substrate. Replacement of an annular

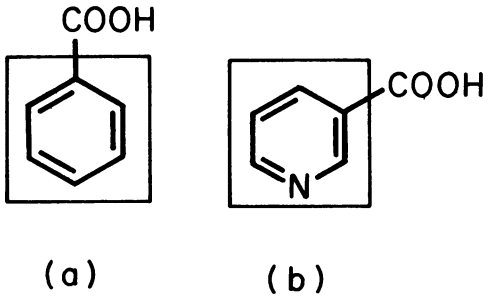

FIG. 1. Homocyclic aromatic "benzenoid" nucleus (enclosed) of benzoate (a) and heterocyclic aromatic "pyridine" nucleus (enclosed) of nicotinate (b).

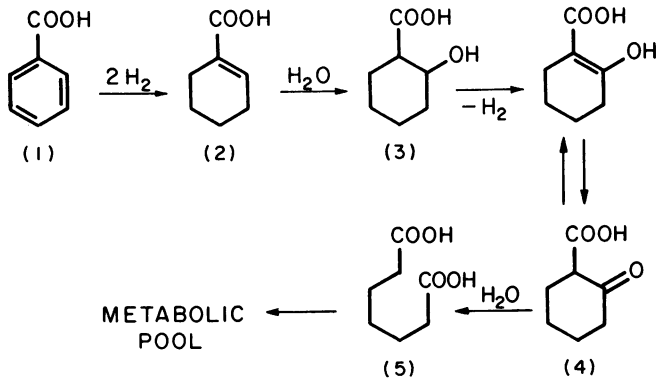

FIG. 2. Proposed reductive pathway for photocatabolism of benzoate by *R. palustris*. (1) Benzoate; (2) cyclohex-1-ene-1-carboxylate; (3) 2-hydroxycyclohexane carboxylate; (4) 2-oxocyclohexane carboxylate; (5) pimelate (25).

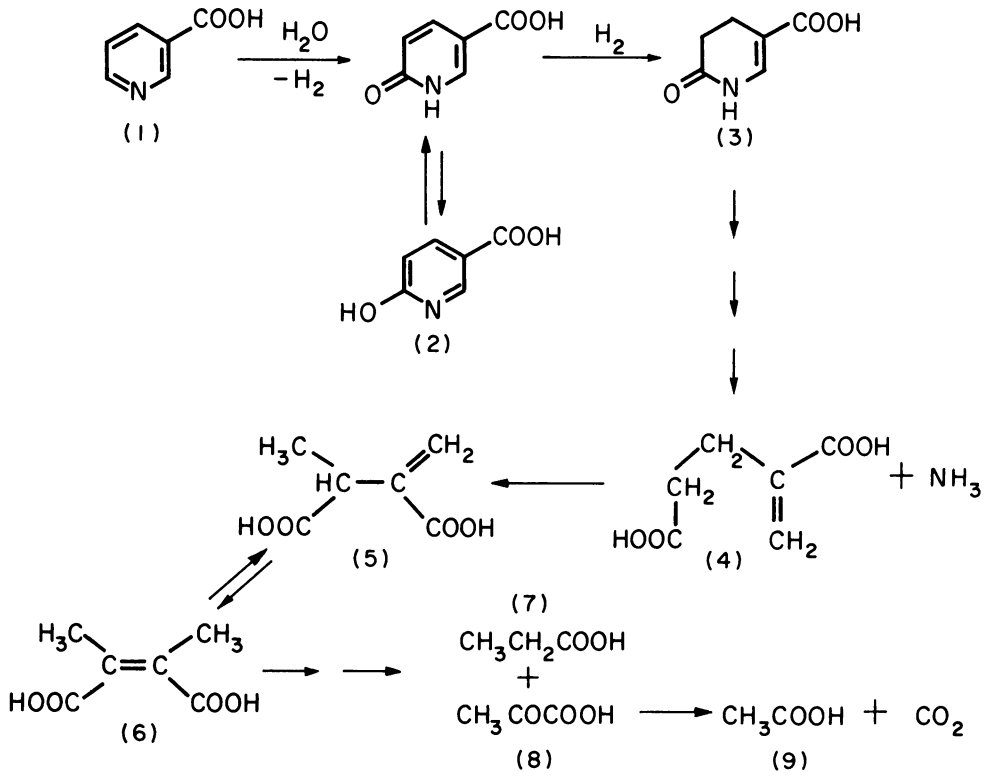

FIG. 3. Pathway of nicotinic acid fermentation by *C. barkeri*. (1) Nicotinate; (2) 6-hydroxynicotinate; (3) 1,4,5,6-tetrahydro-6-oxonicotinate; (4) α-methylene-glutarate; (5) methylitaconate; (6) dimethylmaleate; (7) propionate; (8) pyruvate; (9) acetate (89).

—CH= group of the benzene nucleus with an —N= heteroatom group, forming pyridine, results in an aromatic nucleus with different physical and chemical characteristics. An important measure of this difference between the benzenoid (or the cyclopentadienyl carbanion) and the heteroaromatic nucleus is aromatic stability (Table 2). Equally important are the differences in chemical behavior between these classes of aromatic species. To a large extent, the chemical behavior or reactivity of an aromatic ring is dependent upon the distribution of the electron density. Substitution of a heteroatom for a carbon atom in an aromatic system results in a distortion of the π-electron density; i.e., the π-electron density of the carbon sites in pyridine is lower than that in benzene, causing these sites to be "π deficient." This distortion is responsible for the change in chemical behavior (50, 70). For example, protonation of the heterocyclic ring constituents, either π deficient or π excessive, generally renders heteroaromatics more susceptible to nucleophilic attack than benzenoid compounds. Under biologically suitable conditions, protonation of the benzene nucleus as a means of ring activation is unlikely.

TABLE 2. Stability ratings of several aromatic compounds

Compound	Aromaticity (kcal/mol)[a]	Reference
Benzene	36–39	100
Pyridine	32	100
Pyrimidine	26	100
Cyclopentadienyl anion	42	64
Thiopene	29	64
Pyrrole	21	64
Furan	16	64

[a] 1 cal = 4.184 J.

The benzoate and nicotinate metabolic pathways (Fig. 2 and 3, respectively) share two common features: (i) destabilization of the aromatic ring as the initial step, and (ii) attachment of an oxygen atom to the ring prior to ring cleavage. Apparently, attachment of an oxygen atom to the ring structure facilitates ring catabolism under anoxic conditions. The dearomatization of benzoate through addition of hydrogen is apparent. However, the aromatic destabilization of nicotinate by hydroxylation is less evident. In aqueous solutions the hydroxypyridine is in thermodynamic equilibrium with pyridone, which is the favored "less aromatic" tautomer (9). The nitrogen heteroatom of pyridone is in the preferred sp^3 hybridized molecular orbital configuration, as opposed to the hydroxypyridine tautomer, where the nitrogen atom is in the sp^2 hybridized configuration. The sp^3 hybrid configuration allows for a greater degree of π-electron localization and a decrease in aromatic stability. Although, there is a loss of aromatic conjugation energy, additional stabilization arises from the resonance energy of the —N—C(=O)— group (12).

METABOLISM OF HOMOCYCLIC AROMATIC COMPOUNDS UNDER ANAEROBIC CONDITIONS

Anaerobic Photometabolism

For the phototrophic purple nonsulfur bacteria (i.e., *Rhodospiriaceae*), organic compounds serve as the major source of electrons and carbon for cellular components.

Organic substrates used by the purple nonsulfur bacteria vary from species to species and include fatty acids, alcohols, carbohydrates, and aromatic compounds (90). Some species of purple nonsulfur bacteria are able to grow aerobically in the dark by respiratory metabolism of organic compounds. A few species, such as *Rhodopseudomonas palustris* (36) or *Rhodocyclus purpureus* (76), can use thiosulfate or sulfide as an electron donor in addition to organic compounds. In general, however, purple nonsulfur bacteria are sensitive to sulfide and tend to grow only in those environments in which sulfide concentration is low (90).

R. palustris can use benzoate as the sole substrate under aerobic conditions via respiration or anaerobically by photometabolism (M. H. Proctor and S. Scher, Biochem. J. **76**:33p, 1960). Dutton and Evans (25) investigated aromatic catabolism by *R. palustris* under aerobic and anaerobic conditions. For substrate utilization experiments, *R. palustris* cells were grown either photosynthetically under O_2-free N_2 in a mineral salts-vitamin medium containing the various test substrates or aerobically in the dark with the same supplemented mineral salts medium (25). Under anaerobic conditions, benzoate and *m*- and *p*-hydroxybenzoate supported growth of the organism. Growth was not observed with *o*-hydroxybenzoate, protocatechuate, or nicotinate as substrate. When aerobic conditions prevailed, *R. palustris* was able to use *p*-hydroxybenzoate or protocatechuate as sole source of carbon. Benzoate, *o*-, and *p*-hydroxybenzoate, and catechol did not support aerobic growth of *R. palustris*. Using an isotope dilution technique with buffered cell suspensions, these investigators proceeded to determine the intermediates involved in the degradation of [^{14}C]-benzoate by *R. palustris*. Thin-layer chromatographic analysis allowed tentative identification of the labeled intermediates as follows: (1) cyclohex-1-ene-1-carboxylate, (2) 2-hydroxycyclohexane carboxylate, (3) 2-oxocyclohexane carboxylate, and (4) pimelate. Identification of these intermediates provided evidence to support a novel reductive pathway for the anaerobic catabolism of the benzenoid ring (Fig. 2). In addition to the metabolites mentioned above, significant amounts of labeled cyclohexane carboxylate and cyclohex-2-ene-1-carboxylate were also detected in the isotope dilution study. Just exactly where these two intermediates fit in the reductive pathway scheme for benzoate catabolism remains unclear. Dutton and Evans (25) further showed that the enzyme system used by *R. palustris* to photometabolize aromatic substrates is inducible and lacks substrate specificity. Guyer and Hegeman (39) provided further evidence to support the reductive pathway of benzoate degradation by using mutant strains of *R. palustris*.

Dutton and Evans (25) proposed that the reduction of aromatic (benzenoid) compounds by *R. palustris* involves a reductase that is coupled to some low-redox-potential component (e.g., ferredoxin) of the light-induced electron transport system. Dutton and Evans (25) also demonstrated that aerobic catabolism of benzenoid compounds by *R. palustris* proceeded via the protocatechuate 4,5-oxygenase pathway. Furthermore, they concluded from their experiments that protocatechuate and oxygen were obligatory for the induction of protocatechuate 4,5-oxygenase in *R. palustris*. Further efforts to elucidate the photocatabolic pathway of benzoate degradation by cell-free extracts of *R. palustris* were hampered by the presence of inhibitory components in the extracts. Fatty acids were implicated as the inhibitory components of photoassimilation in cell-free extracts of *R. palustris* (26).

Catabolism by Denitrifying Microorganisms

Microorganisms that carry out nitrate respiratory metabolism (e.g., the denitrifiers) are facultative in character and appear to prefer oxygen as their electron acceptor (36). Under aerobic conditions, this group of microorganisms uses a wide range of organic compounds as carbon and energy sources. In many instances the same range of organic carbon is used under denitrifying conditions. Active nitrate-respiring microorganisms are found in a variety of anoxic environments, including soils, lakes, rivers, and oceans (29, 72, 82, 101).

Microbial catabolism of aromatic (benzenoid) compounds under anoxic conditions and in the presence of nitrate has been reported by several authors (2, 18, 73, 99). Taylor et al. (98) isolated a pseudomonad, designated PN-1, from soil by enrichment culture techniques on a mineral salts-trace metal medium with *p*-hydroxybenzoate as the carbon source and nitrate as the sole electron acceptor. *Pseudomonas* sp. strain PN-1 was able to use *p*-hydroxybenzoate, benzoate, and *m*-hydroxybenzoate to grow under both aerobic and nitrate-reducing conditions. Protocatechuate was able to support growth only under anaerobic conditions. However, *o*-hydroxybenzoate would support anaerobic growth of *Pseudomonas* sp. strain PN-1 only after a 9- to 11-day lag period. The following organic compounds were not degraded by *Pseudomonas* sp. strain PN-1 under aerobic and anaerobic conditions: phenol, catechol, benzylalcohol, cyclohexane carboxylate, cyclohex-3-ene carboxylate, *cis*- or *trans*-1:2-cyclohexane diol, and 6-hydroxynicotinate. Substrate utilization experiments were conducted by using respirometric methods with tris(hydroxymethyl)aminomethane-buffered cell suspensions of *Pseudomonas* sp. strain PN-1 grown anaerobically on one of several aromatic substrates and incubated anaerobically in the presence of various other aromatic compounds. The pathway shown in Fig. 4 proposed by Taylor et al. (98) for the anaerobic degradation of aromatic compounds by *Pseudomonas* sp. strain PN-1 is partly based on their interpretation of the substrate utilization experiments (i.e., as determined by production of N_2).

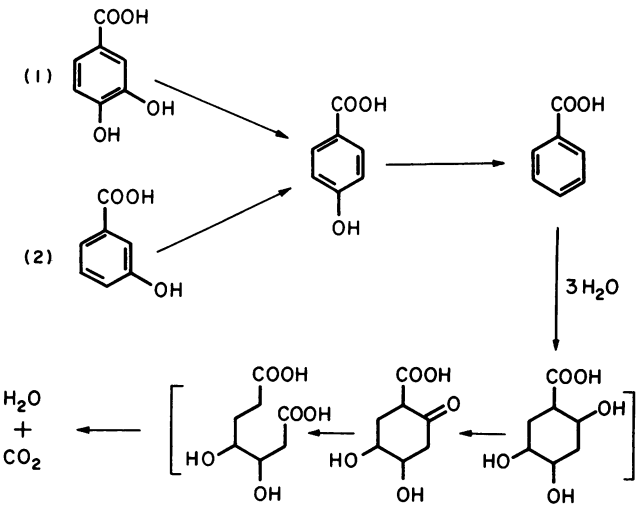

FIG. 4. Anaerobic catabolism of aromatic compounds by *Pseudomonas* sp. strain PN-1. The part of the pathway enclosed in brackets is hypothetical. (1) Protocatechuate; (2) *m*-hydroxybenzoate (98).

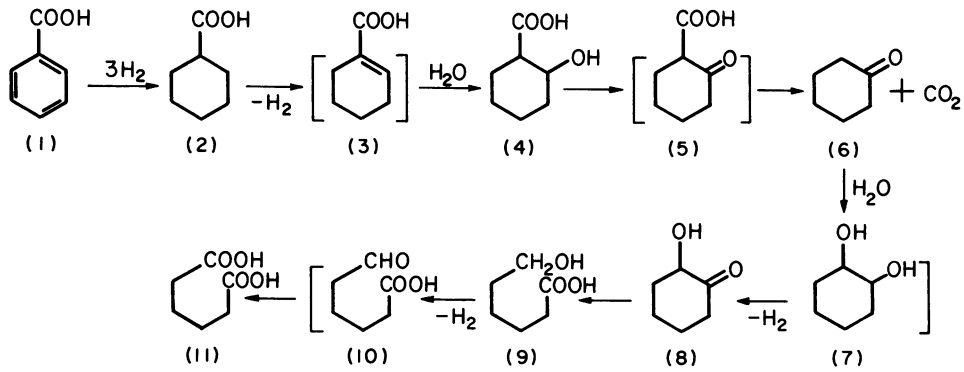

FIG. 5. Proposed pathway for degradation of benzoic acid by *Moraxella* sp. Compounds enclosed in brackets are hypothetical; all others were tentatively identified with gas or thin-layer chromatography. (1) Benzoate; (2) cyclohexane carboxylate; (3) cyclohex-1-ene-carboxylate; (4) 2-hydroxycyclohexane carboxylate; (5) 2-oxocyclohexane carboxylate; (6) cyclohexanone; (7) 1,2-dihydroxycyclohexane; (8) 2-hydroxycyclohexanone; (9) 6-hydroxyhexonate; (10) adipate, semialdehyde; (11) adipate (adapted from Williams and Evans [112]).

The portion of the pathway starting from benzoate was proposed on the basis of indirect evidence, stemming from the observation that *Pseudomonas* sp. strain PN-1 could not use cyclohexane carboxylate, a metabolite generated during benzoate degradation by *R. palustris*, as a substrate under nitrate-reducing conditions. Furthermore, this compound was not even oxidized in the presence of nitrate by tris(hydroxymethyl)aminomethane-bufferedcell suspensions grown anaerobically on *p*-hydroxybenzoate (98). Thus, a nonreductive pathway (Fig. 4, bracketed) was proposed as an alternative to the reductive pathway suggested earlier by Dutton and Evans (25) for benzoate catabolism by *R. palustris*. In other experiments, Taylor et al. (98) demonstrated that *Pseudomonas* sp. strain PN-1 tris(hydroxymethyl)aminomethane-buffered cell suspensions, grown aerobically on *p*-hydroxybenzoate, oxidized protocatechuate to a yellow intermediate subsequently identified as α-hydroxy-γ-carboxymuconic semialdehyde. Solubilized extracts of these cells contained significant levels of protocatechuate 4,5-oxygenase. Taylor et al. (98) concluded from this evidence that aerobic catabolism of aromatic (benzenzoid) compounds by *Pseudomonas* sp. strain PN-1 proceeded via the protocatechuate 4,5-oxygenase *meta*-cleavage pathway. Additional experiments, involving cell-free extracts of this organism, demonstrated that cells grown aerobically produced 52-fold more oxygenase than cells grown anaerobically. Apparently, *Pseudomonas* sp. strain PN-1 possesses an oxygenase enzyme system that does not require oxygen as an inducer.

In a similar investigation, Williams and Evans (111) isolated in pure culture a bacterium from soil that was able to grow anaerobically (nitrate respiration) or aerobically on benzoate-mineral salts medium. They classified their bacterial strain as *Pseudomonas stutzeri*. From their observation that aerobically grown cell-free extracts contained catechol 1,2-oxygenase, they concluded that the aerobic degradation of benzoate proceeded via the *ortho*-cleavage pathway. In contrast to *Pseudomonas* sp. strain PN-1, anaerobically grown buffered cell suspensions of *P. stutzeri* exhibited no oxygenase activity (111). Using anaerobically grown phosphate-buffered cell suspensions, Williams and Evans (111) discovered that *P. stutzeri* transformed ^{14}C-labeled benzoate to *trans*-2-hydroxycyclohexane carboxylate. These results suggested to the investigators that degradation of aromatic (benzenoid) compounds under nitrate-respiring conditions proceeded via the novel reductive pathway.

Support for the reductive pathway as a universal pathway common to all physiological groups was substantially strengthened in a subsequent investigation by the same two authors. Williams and Evans (112) isolated a *Moraxella* sp. from garden soil by anaerobic enrichment culture techniques on a mineral salts-trace element medium with benzoate as the carbon source and nitrate as the sole electron acceptor. Benzoate supported both anaerobic and aerobic growth of the organism. The presence of oxygenase in *Moraxella* sp. was established only for cells grown under aerobic conditions and not for cells grown under anaerobic conditions. The reductive pathway for benzoate degradation by *Moraxella* sp. was proposed (112) on the basis of isolation and identification of intermediates (Fig. 5). By gas-liquid and thin-layer chromatographies, cyclohexane carboxylate, 2-hydroxycyclohexane carboxylate, and cyclohexanone were first identified from extracts of anaerobically grown benzoate cultures. These same two intermediates were also identified, along with adipate, in phosphate-buffered cell suspensions incubated in the presence of ^{14}C-labeled benzoate. Several organic compounds were tested and found to be incapable of supporting growth of *Moraxella* sp.; some of these included *p*-hydroxybenzoate, protocatechuate, cinnamate, cyclohexa-2,5-diene carboxylate, cyclohexane carboxylate, adipate, pimelate, and phloroglucinol. Substrates used by the organism grown anaerobically on benzoate that could not support anaerobic growth alone were cyclohex-1-ene carboxylate, *cis*- or *trans*-hydroxycyclohexane carboxylate, cyclohexanol, cyclohexanone, and 2-hydroxycyclohexanone, to mention a few. Several organic compounds such as phenol, catechol, and phthalate were not metabolized.

Aftring and Taylor (2) have recently isolated a *Bacillus* sp. from Miami River mud by anaerobic enrichment culture techniques on a mineral salts-trace metal medium with phthalic acid as the carbon source and nitrate as the sole electron acceptor. The organism grew either aerobically or anaerobically (nitrate respiration) on phthalate. Further experiments with fluorobenzoate compounds provided evidence that benzoate was a common intermediate in the degradation of phthalate by this *Bacillus* species under aerobic and anaerobic conditions. No other intermediates were determined for the anaerobic catabolism of phthalate by this organism. In a closely related investigation, Aftring et al. (1) reported decomposition of phthalic acid by a microbial consortium from freshwater and marine sedi-

ments. This consortium was necessary for complete mineralization of phthalate under nitrate-respiring conditions.

Taylor (97) investigated the anaerobic and aerobic catabolism of vanillic acid and other aromatic compounds by *Pseudomonas* sp. strain PN-1. A mineral salts medium containing vanillate supported anaerobic (nitrate respiration) but not aerobic growth of the organism. In addition, phosphate-buffered cell suspensions of organisms grown anaerobically on vanillate immediately oxidized protocatechuate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, and benzoate in the presence of nitrate. This observation supported the hypothesis that the anaerobic degradative pathway of substituted aromatic compounds (e.g., vanillic acid) proceeded via the sequential elimination of substituent groups, resulting in the formation of benzoate, which subsequently enters the reductive pathway of aromatic catabolism. Taylor (97) also observed that cells grown anaerobically on vanillate oxidized protocatechuate in the presence of oxygen. Presumably, protocatechuate 4,5-oxygenase was responsible for the oxidation reaction; this finding substantiates an earlier observation by Taylor et al. (98) that cells grown anaerobically contain oxygenase activity. It thus appears that some facultative microorganisms retain low levels of oxygenase activity when grown in the presence of aromatic compounds even under anaerobic conditions (e.g., *Pseudomonas* sp. strain PN-1), while others do not (e.g., *R. palustris*, *P. stutzeri*, and *Moraxella* sp.).

Fermentation

Many microorganisms that inhabit anoxic environments obtain their energy for growth through fermentation of organic carbon. Fermentation is a process that can be carried out in the absence of light by facultative or obligatory anaerobes. Growth-sustaining energy results from substrate-level phosphorylation reactions. In fermentation, organic compounds serve not only as electron donors but also as electron acceptors.

Schink and Pfennig (80) isolated five strains of strictly anaerobic bacteria from limnic and marine mud samples on a medium of mineral salts, trace elements, and vitamins with gallic acid or phloroglucinol as the sole carbon source. All five isolates were rod shaped, gram negative, and nonsporing and were assigned to the genus *Pelobacter* gen. nov. The five isolates fermented only gallic acid, pyrogallol, 2,4,6-trihydroxybenzoate, or phloroglucinol. Several organic compounds were not transformed by these organisms including among others, phenol, glucose, fructose, catechol, resorcinol, syringic acid, protocatechuate, cyclohexane carboxylate, benzoate, and nicotinate. The fermentation products of aromatic catabolism were acetate (3 mol) and CO_2 (1 mol per mol of substrate). Using cell-free extracts, Schink and Pfennig (80) also obtained experimental evidence which suggested that the initial step in phloroglucinol fermentation by the new bacterium *Pelobacter* sp. was a reduced nicotinamide adenine dinucleotide phosphate-dependent reduction to dihydrophloroglucinol. This same reaction has also been proposed as the initial step in the anaerobic degradative pathway of phloroglucinol by a *Coprococcus* species (75). Further syntrophic studies with the newly isolated organism gave some very interesting results. Schink and Pfennig (80) incubated in coculture *Pelobacter acidigallici* nov. sp. (the marine isolate designated Ma Gal2) and *Acetobacterium woodii*, a demethylating microorganism (3). Although neither of these two bacteria was able to degrade the aromatic ring when cultured separately, the coculture completely metabolized syringic acid to acetate and CO_2. In addition, if the isolates were cocultured with *Methanosarcina barkeri*, any metabolizable aromatic substrate could be completely mineralized to CO_2 and CH_4.

In a related study, Kreikenbohm and Pfennig (57) used a mixed culture of *Pelobacter acidigallici* and *Desulfobacter postgatei* to degrade 3,4,5-trimethoxybenzoate. The rate of degradation varied greatly depending on the composition of the consortium and also on culture (batch versus continuous) conditions. Kreikenbohm and Pfennig (57) found that under continuous-culture conditions *A. woodii* had a greater ability to metabolize 3,4,5-trimethoxybenzoate when cocultured with *Pelobacter acidigallici* than when incubated alone. In addition, they discovered that mixed populations of *A. woodii*, *Pelobacter acidigallici*, and *D. postgatei* completely mineralized 3,4,5-trimethoxybenzoate to CO_2 under batch culture conditions, whereas under continuous-culture conditions the same consortium formed >3 mM acetate. The reason for the differential rates of acetate metabolism is unknown. The results of these mixed culture investigations are very important insofar as they serve to elucidate the interrelationships among various members of the anaerobic community.

In a recent investigation, Krumholz and Bryant (58) isolated a strictly anaerobic chemoorganotroph from the rumen which, like *A. woodii*, has the ability to cleave methyl-ether linkages of monobenzenoids. The newly isolated fermenter, *Syntrophococcus sucromutans* sp. nov. gen. nov., utilizes carbohydrates such as fructose, glucose, galactose, ribose, and xylose as electron donors. Compounds serving as electron acceptors include formate and methoxyl groups of methoxymonobenzenoids, i.e., syringate, caffeate, and vanillin. *S. sucromutans* is also able to grow well in coculture with *Methanobrevibacter smithii*. In this case *Methanobrevibacter smithii* functions as the electron acceptor system. Krumholz and Bryant (59) have also isolated a strictly anaerobic chemoorganotroph from the rumen which degrades gallate, pyrogallol, and phloroglucinol to acetate, butyrate, and on occasion CO_2. This newly isolated fermenter, *Eubacterium oxidoreducens* sp. nov., requires either formate or hydrogen as an electron donor to catabolize the above-mentioned monobenzenoids.

Catabolism by Sulfate-Reducing Microorganisms

Those microorganisms that carry out dissimilatory sulfate reduction to obtain energy for growth are strict anaerobes. For these bacteria, organic carbon serves as a source of both carbon and energy. Reducible sulfur compounds (e.g., sulfate, thiosulfate) serve as terminal electron acceptors, and adenosine triphosphate is generated as a result of electron transport or substrate-level phosphorylation reactions (36). Dissimilatory sulfate-reducing bacteria (sulfidogens) are most commonly associated with aquatic environments (i.e., marine and freshwater sediments) (21, 53, 61, 62, 88, 113), although sulfate reducers can also be found in soil. However, soil is not usually considered a favorable environment for growth (101).

Widdel et al. (110) recently isolated a new sulfate-reducing organism (*Desulfonema magnum*) from marine sediment capable of mineralization of various fatty acids and benzoate to CO_2 in the presence of reducible sulfur compounds. The organism was isolated by enrichment culture techniques on a mineral salts-trace element-vitamin medium containing acetate as the electron donor and carbon source. Several organic compounds supported the growth of *Desulfonema*

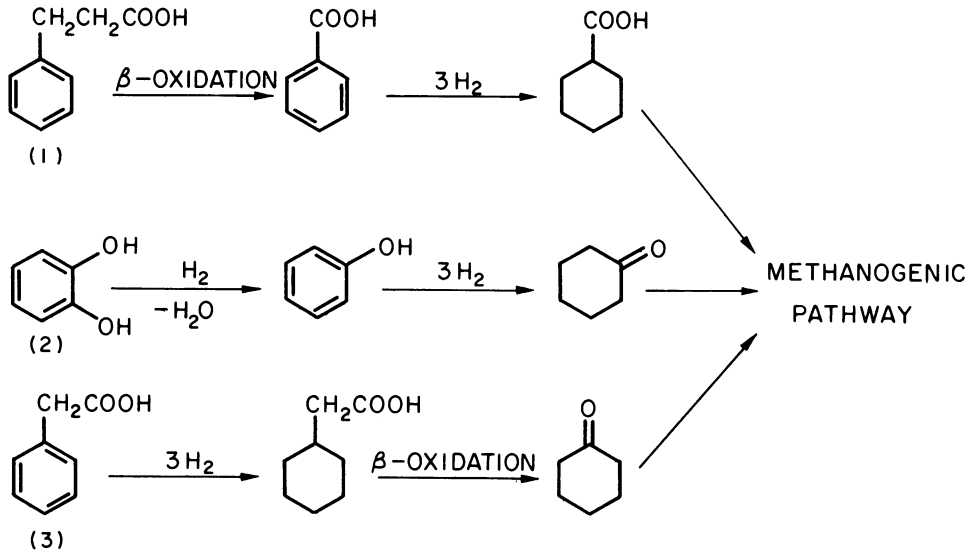

FIG. 6. Proposed pathway for the degradation of (1) phenylpropionate, (2) catechol, and (3) phenylacetate (adapted from Balba and Evans [5–7]).

magnum. These included acetate, propionate, butyrate, valerate, caproate, benzoate, 4-hydroxy-benzoate, and phenylacetate. Compounds tested which were not able to support growth included ethanol, cyclohexane carboxylate, adipate, pimelate, glucose, 2-hydroxybenzoate, 3-hydroxybenzoate, urate, and nicotinate. Organic substrates were oxidized at the expense of sulfate, which was reduced stoichiometrically to H_2S. Widdel et al. (110) detected no intermediates from the degradation of benzoate.

Metabolism under Methanogenic Conditions

Bacterial methanogenesis is a process common to many anoxic environments. This strictly anaerobic process is frequently associated with the decomposition of organic matter in ecosystems such as anoxic muds and sediments, the rumen and intestinal tract of animals, and anaerobic sewage sludge digesters (63, 90, 115).

As illustrated below, methane bacteria are able to use only a few simple compounds to support growth:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

$$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$$

$$CH_3COOH \rightarrow CH_4 + CO_2$$

Acetate and CO_2 plus H_2 are probably the most important substrates for methane bacteria in natural ecosystems (36). Since these organisms can use only simple compounds to support growth, they must rely on syntrophic associations with fermenters which degrade complex organic compounds (i.e., aromatic compounds) into usable substrates. For the most part, these syntrophic associations between methane bacteria and fermenters are obligatory. Thus, fermenters play a crucial role in mineralizing organic matter in methanogenic habitats. The syntrophic association between methane bacteria and fermenters may be similar in nature from one methanogenic habitat to another as determined by the susceptibility of a variety of chemicals to anaerobic biodegradation (47, 94).

An early investigation by Tarvin and Buswell (96) demonstrated that the methanogenic consortium present in anoxic sludge could degrade aromatic (benzenoid) compounds to CO_2 and CH_4. They observed a quantitative decomposition, including the aromatic ring, of phenylacetic acid, hydrocinnamate, cinnamate, tyrosine, and benzoate. The consortium could not degrade bromobenzene, toluene, aniline, or benzene. Several investigators have since confirmed benzoate degradation by the methanogenic consortium present in sludge (22, 31, 33, 71). Further investigations of this type of habitat have established that the major intermediates formed by these benzoate-degrading methanogenic consortia are butyrate, propionate, and acetate along with CO_2 and H_2 (32, 56, 84). Using methanogenic enrichment cultures, Shlomi et al. (84) demonstrated, in benzoate-amended cultures, increased levels of 2-hydroxycyclohexane carboxylate, 2-oxocyclohexane carboxylate, and pimelate compared with unamended control cultures. As a result of these observations, the investigators proposed that benzoate degradation by a methanogenic consortium proceeded via the reductive pathway (Fig. 2).

Subsequent investigations involving aromatic degradation by methanogenic consortia have been primarily directed toward one of three areas: (i) elucidation of the range and pathways of degradable aromatics, (ii) isolation and characterization of microbial components, or (iii) investigation of new methanogenic habitats.

Healy and Young (42) demonstrated that acclimated methanogenic enrichment cultures (derived from sewage sludge) could degrade catechol and phenol in 32 and 18 days, respectively. Basing their study on carbon balance measurements, they showed the aromatic ring to be completely mineralized to CO_2 and CH_4. In a subsequent study, these same investigators examined anaerobic degradation of lignin-derived aromatic compounds by a methanogenic consortium obtained from sludge. Subsequently, they were able to demonstrate the anaerobic degradation of the following compounds (numbers in parentheses are acclimation lag days): vanillin (12), vanillate (9), ferulate (10), cinnamate (13), benzoate (8), catechol (21), protocatechuate (13), phenol (14), *p*-hydroxybenzoate (12), syringate (2), and

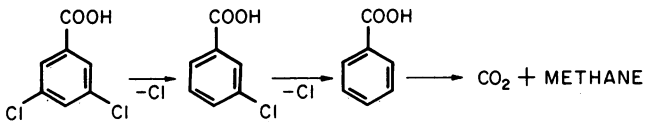

FIG. 7. Reductive dehalogenation of 3,5-dichlorobenzoate by a methanogenic consortium (93).

syringaldehyde (5) (43). Substrate conversion was again determined by carbon balance measurements.

The degradation of catechol, phenylacetate, and phenylpropionate by methanogenic consortia was investigated by Balba and Evans (5–7). On the basis of their observations, they proposed a ring reduction β-oxidation degradative sequence for phenylacetate (and vice versa for phenylpropionate), while for catechol they proposed a ring dehydroxylation reaction prior to the ring reduction step (Fig. 6). In other investigations, Healy et al. (44) established the degradative pathway of ferulate by a methanogenic consortium obtained from sludge enrichments. Intermediates were detected by gas chromatography and gas chromatography/mass spectrometry analysis of culture fluid and revealed the presence of phenylacetate, phenylpropionate, benzoate, cyclohexane carboxylate, pimelate, and adipate. Further investigation of ferulate metabolism resulted in the identification of caffeate, *p*-hydroxycinnamate, cinnamate, cyclohexanone, 2-oxocyclohexane carboxylate, methylcyclohexanone, caproate, heptanoate, and isocaproate (38). Presumably, then, ferulate undergoes β-oxidation, demethylation, and dehydroxylation reactions (forming benzoate) before entering the reductive pathway (44). Similar results were obtained by Grbić-Galić (37) when she investigated the pathway of coniferyl alcohol degradation by methanogenic sludge enrichment cultures.

In another investigation involving lignin-derived aromatic compounds, Kaiser and Hanselmann (54) observed complete mineralization of 3,4,5-trisubstituted aromatic compounds to CO_2 and CH_4 by a methanogenic consortium obtained from lake sediment enriched with syringic acid. This same consortium was unable to mineralize 3,4-disubstituted aromatic compounds. For example, aromatic compounds such as 3,4,5-trimethoxybenzoate and 2,3,4-trihydroxybenzoate were mineralized to CO_2 and CH_4 via 3,4,5-trihydroxybenzoate and pyrogallol, respectively. The 3,4-disubstituted aromatic compounds such as vanillin, protocatechuate, and veratrate were converted only to catechol. Detection of aromatic substrates and intermediates was carried out by spectrophotometric (54) and high-performance liquid chromatography (55) analyses, and the results show that basically this methanogenic consortium was responsible for not only demethylation but also decarboxylation reactions.

Another important class of aromatic compounds degraded by methanogenic consortia is the halogenated benzoates and phenols. Suflita et al. (93) observed the dehalogenation of several mono-, di-, and trihalogenated (e.g., —I, —Cl, and —Br) benzoates in a stable methanogenic consortium enriched (on 3-chlorobenzoate) from sludge. The reductive dehalogenation process did not take place in sterilized control cultures or anaerobic cultures that were exposed to air and thus was presumed to be a biologically mediated event. Horowitz et al. (48) demonstrated that complete removal of the halogens to yield benzoate was required before mineralization to CO_2 and CH_4 could take place (Fig. 7). Presumably, benzoate degradation proceeded via the reductive pathway (30). The sequence of events leading to anaerobic degradation of halogenated aromatic compounds (i.e., dehalogenation ultimately followed by ring cleavage) is quite different from the aerobic degradation pathway of the same compounds. Under aerobic conditions, the metabolism of halogenated benzenoids generally proceeds through one of two pathways: (i) replacement of a halide by a hydroxyl group after ring cleavage or (ii) ring cleavage followed by dehalogenation (78, 79, 116). Other investigations have shown that reductive dehalogenation carried out by methanogenic consortia is not limited to benzoates but also includes halogenated phenols (15, 16).

Boyd et al. (16) observed that, in general, the presence of —Cl or —NO_2 groups on phenol inhibited methane production. Elimination or transformation of these substituent groups was accompanied by increased methane production. The nitro substituent group was presumed to undergo reduction to form an amino group. The resulting intermediate (aminophenol) subsequently underwent mineralization.

In other investigations, Shelton and Tiedje (83) isolated seven bacteria from a stable methanogenic consortium able to use 3-chlorobenzoate as the sole source of carbon and energy. The original source of the inoculum was anaerobic sewage sludge. The organisms isolated in mono- or coculture were as follows: one dechlorinating bacterium (strain DCB-1), one benzoate-degrading bacterium (strain BZ-2), two butyrate-oxidizing bacteria (strains SF-1 and USF-2), two H_2-consuming methanogens (*Methanospirillum hungatei* PM-1 and *Methanobacterium* sp. strain PM-2), and a sulfate reducer (*Desulfovibrio* sp. strain PS-1). The dechlorinating organism (DCB-1) was described as a gram-negative, obligate anaerobe with a unique "collar" surrounding the cell. Sleat and Robinson (85) were able to isolate a methanogen from a stable benzoate enrichment culture established from lake sediment. They identified their methanogenic organism as *Methanobacterium soehngenii*.

Mountfort and Bryant (69) isolated and characterized an organism, *Syntrophus buswellii*, from anaerobic digester sludge that was also found in aquatic sediments (68). *Syntrophus buswellii* is a motile, gram-negative strict anaerobe which ferments benzoate and hydrocinnamate to acetate, CO_2, and H_2 (or formate) only when cocultured with an appropriate hydrogenotroph. The organism can grow when in coculture with *Desulfovibrio* sp. (strain DM-3) plus sulfate or *Methanospirillum hungatei* (strain SF-1). It could not utilize organic compounds such as acetate, adipate, butyrate, *n*-heptanoate, succinate, phenylacetate, pimelate, propionate, cyclohexanone, ferulate, phenol, and vanillate.

Anaerobic biodegradation of aromatic compounds by methanogenic consortia from anaerobic sludge, sediments, and rumen has been well documented (refer to preceding discussion). Recently, Ehrlich et al. (27, 28) and Godsy et al. (35) demonstrated the presence of a creosote-degrading methanogenic consortium in a near-surface aquifer located

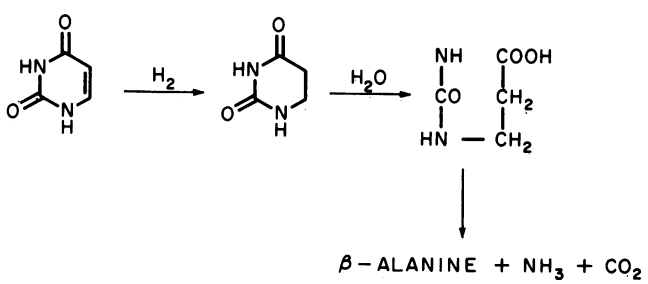

FIG. 8. Degradation of uracil by *C. uracilicum* (20).

TABLE 3. Metabolism of homocyclic aromatic compounds under anaerobic conditions

Energy-yielding process	Compound	Organism(s)	Enrivonmental source	Reference(s)
Photoreduction	Benzoate *m*,*p*-Hydroxybenzoate	*Rhodopseudomonas palustris*	Not mentioned	25
Denitrification	2-Aminobenzoate	*Pseudomonas* sp.	Soil, compost	18
	Benzoate	*Pseudomonas stutzeri*	Soil	111
	Benzoate *o*,*m*,*p*-Hydroxybenzoate Protocatechuate Vanillate	*Pseudomonas* sp.	Soil	97, 98
	Benzaldehyde Benzyl alcohol Benzoate Caffeate Cinnamate Phloroglucinol	*Moraxella* sp.	Soil	112
	Phenol *o*,*m*,*p*-Cresol	Nonspecific	Soil	4
	Phthalate	*Bacillus* sp.	River mud	2
Fermentation	Gallate Pyrogallol 2,4,6-Trihydroxybenzoate Phloroglucinol	*Pelobacter* sp.	Limnic and marine mud	80
Sulfate reduction	Benzoate *p*-Hydroxybenzoate	*Desulfonema magnum*	Marine sediment	110
	Benzoate Phenol	Nonspecific	Subsurface (groundwater)	92
Methanogenic fermentation	Phenylacetate Benzoate Cinnamate Hydrocinnamate Tyrosine	Nonspecific	Sludge	96
	Benzoate Cinnamate Catechol Ferulate *p*-Hydroxybenzoate Phenol Protocatechuate Syringaldehyde Syringate Vanillate Vanillin	Nonspecific	Sludge	42, 43
	Benzoate Ferulate	Stabilized consortia	Sewage	38
	Phenylacetate Phenylpropionate Catechol	Nonspecific	Not mentioned	5–7
	3,4,5-Trimethoxybenzoate Sinapate 2,3,4-Trihydroxybenzoate 2,6-Dimethoxyphenol	Nonspecific	Lake sediment	54
	o- or *m*-Chlorophenol *o*- or *p*-Cresol *o*-, *m*-, or *p*-Methoxyphenol *o*-, *m*-, or *p*-Nitrophenol	Nonspecific	Sludge	16

Continued on following page

TABLE 3—*Continued*

Energy-yielding process	Compound	Organism(s)	Enrivonmental source	Reference(s)
	Coniferyl alcohol	Nonspecific	Sludge	37
	Benzoate	Nonspecific	Sludge	85
	2-,2-, or 4-Bromobenzoate 3-Chlorobenzoate 2-, 3-, or 4-Iodobenzoate 3,5-Dichlorobenzoate	Nonspecific	Sludge	93
	Phenol	Nonspecific	Subsurface (groundwater)	28, 34
	Benzoate	Nonspecific	Sludge	22, 31, 33, 71, 84
	Benzoate *o*-, *m*-, or *p*-Chlorobenzoate *o*-, *m*-, or *p*-Bromobenzoate *o*-, *m*-, or *p*-Iodobenzoate 3,5-Dichlorobenzoate Phenol *o*-, *m*-, or *p*-Chlorophenol 2,4-Dichlorophenol 2,5-Dichlorophenol 2,4,5-Trichlorophenol Phenoxyacetate 2,4-Dichlorophenoxyacetate 2,4,5-Trichlorophenoxyacetate	Nonspecific	Subsurface	92

adjacent to a coal-tar distillation and wood-treating plant in St. Louis Park, Minn. Creosote is a complex mixture of organic compounds composed primarily of polynuclear aromatic hydrocarbons, phenolics, and various nitrogen and sulfur-containing heterocyclic compounds. Apparently, coal-tar and runoff wastes from the plant (in operation from 1918 to 1972) were discharged in a nearby wetlands, and the waste material contaminated a near-surface aquifer (28). Analysis of water samples obtained from several sampling wells located downfield from the wetlands site revealed the presence of polynuclear aromatic hydrocarbons and phenolic compounds. Furthermore, methane was evolved from laboratory anoxic cultures when the contaminated groundwater was inoculated with bacteria from the contaminated zone (27). In addition, methanogenic bacteria were isolated from a laboratory fermentor charged with contaminated aquifer water from one of the wells located within the contaminated zone (27). Other bacterial species were isolated from the fermentor and included a nitrate-respiring bacterium (*P. stutzeri*) capable of using phenol as substrate.

Summary

It is evident from the above discussion that microorganisms are able to catabolize a wide variety of aromatic homocyclic compounds under anaerobic conditions. The types of compounds metabolized by anaerobic microorganisms are summarized in Table 3.

METABOLISM OF HETEROCYCLIC AROMATIC COMPOUNDS UNDER ANAEROBIC CONDITIONS

Anaerobic Photoreduction

Heterocyclic aromatic compounds transformed under photosynthetic conditions include α-picolinate, furan-2-carboxylate, and thiophene-2-carboxylate. Tanaka et al. (95) used enrichment techniques to isolate from sewage mud a benzoate-degrading photosynthetic bacterium (strain H45-2) similar to *R. palustris*. Although the organisms could not use thiophene-2-carboxylate as a growth-supporting substrate, under anaerobic conditions, washed cell suspensions of this organism were apparently able to transform thiophene-2-carboxylate to (+)-3-hydroxytetrahydrothiophene-2-carboxylate and tetrahydrothiophene-2-carboxylate.

Fermentation

Heterocyclic aromatic compounds serve as substrates for a variety of microorganisms. For example, pyridine is a compound that can be metabolized by microorganisms under either aerobic or anaerobic conditions (8, 10, 40, 41, 45, 74, 77, 109). Under anaerobic conditions, the initial step in pyridine metabolism can be either ring reduction or ring hydroxylation (46, 49, 109). The source of atomic oxygen for the ring hydroxylation reaction can be molecular oxygen or water (46, 49); under anoxic conditions the source is water (46).

To date, investigations dealing with fermentation of heterocyclic aromatic compounds have centered on the N-heterocyclic bases pyridine, pyrimidine, and purine. The fermentative pathways for N-heterocyclic aromatic compounds, observed thus far, seem to involve an initial ring hydroxylation step prior to the ring reduction ring cleavage sequence. An exception to this statement seems to be, for example, the reductive degradation of uracil by *Clostridium uracilicum* (soil isolates of Campbell [20]) (Fig. 8). However, in the context of this review the highly oxidized pyrimidine uracil is considered to be beyond the initial step of ring hydroxylation and well into the reductive phase of the degradation sequence mentioned above.

Pastan et al. (74) isolated a nicotinate fermenter from Potomac River mud by anaerobic enrichment culture techniques, using a phosphate-buffered medium consisting of mineral salts and nicotinate. After two successive transfers, growth was no longer supported by this medium unless supplemented with 0.2% yeast extract or 0.2% peptone. This

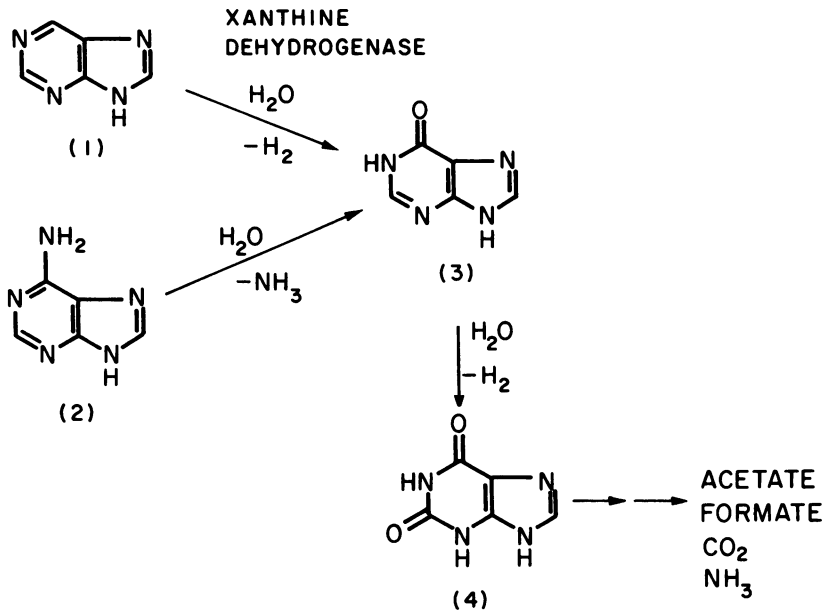

FIG. 9. Transformation of adenine and purine to xanthine by cell-free extracts of *C. purinolyticum*. (1) Purine; (2) adenine; (3) hypoxanthene; (4) xanthine (24).

isolate was later identified as *C. barkeri* sp. nov. by Stadtman et al. (89). Fermentation balance studies with this organism were conducted in Warburg flasks containing a nicotinate carbonate phosphate-buffered solution and washed bacterial cells (74). The products of fermentation were determined to be 1 mol each of propionate, acetate, CO_2, and ammonia per mol of nicotinate.

Additional experiments with *C. barkeri* phosphate-pyruvate-buffered cell suspensions and [^{14}C]nicotinate revealed the presence of several intermediates (102), three of which were identified as 6-hydroxynicotinate, 1,4,5,6-tetrahydro-γ-oxonicotinate, and α-methyleneglutanate. Previous work by Tsai et al. (102) formed the foundation for explaining the degradative pathway of nicotinate by *C. barkeri* (Fig. 3). Studies with cell-free extracts established that the first step in the catabolism of nicotinate (i.e., formation of 6-hydroxynicotinate) was catalyzed by a nicotinamide adenine dinucleotide phosphate-dependent nicotinate hydroxylase (46). Imhoff and Andreesen (51) have since demonstrated that the formation of active nicotinate hydroxylase is selenium dependent.

Two strains of anaerobic sporeforming bacteria with the ability to use adenine as a carbon and energy source have recently been isolated from soil (strain WA-1) and sewage sludge (PD-1) (23). Isolated by enrichment culture techniques by using a mineral salts-yeast extract medium with adenine as substrate, the two isolates were identified as *C. purinolyticum* sp. nov. Growth of the isolates was supported by several organic compounds, including purine (isolate WA-1 only), glycine, xanthine, 2-hydroxypurine (isolate WA-1 only), guanine, uric acid, and formiminoglycine. The isolates could not use the following organic compounds: benzoate, pyrimidine, imidazole, phenylacetate, acetate, and nicotinate, to name a few.

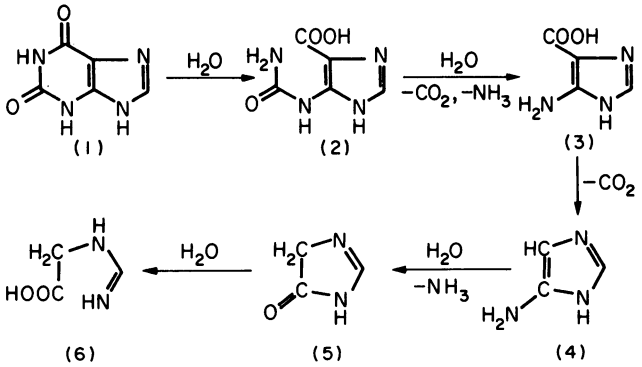

FIG. 10. Conversion of xanthine to formiminoglycine by *C. purinolyticum*. (1) Xanthine; (2) 4-ureido-5-imidazole carboxylate; (3) 4-amino-5-imidazole carboxylate; (4) 4-aminoimidazole; (5) 4-imidazolone (unstable); (6) formiminoglycine (24).

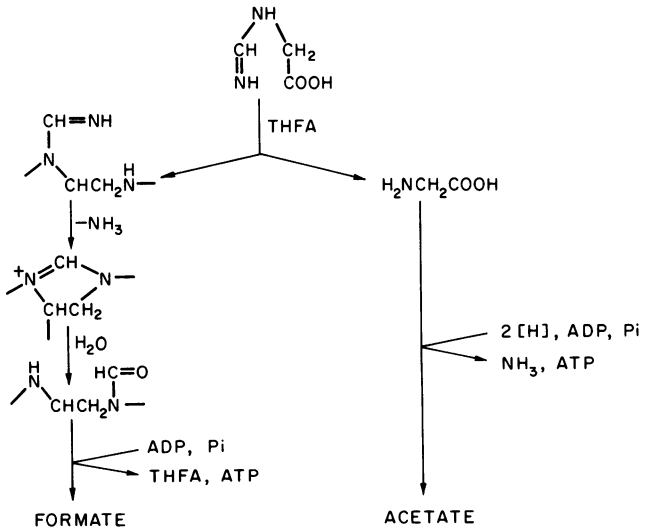

FIG. 11. Degradation of formiminoglycine by *C. purinolyticum*. THFA, Tetrahydrofolic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; 2[H], reducing equivalents (24).

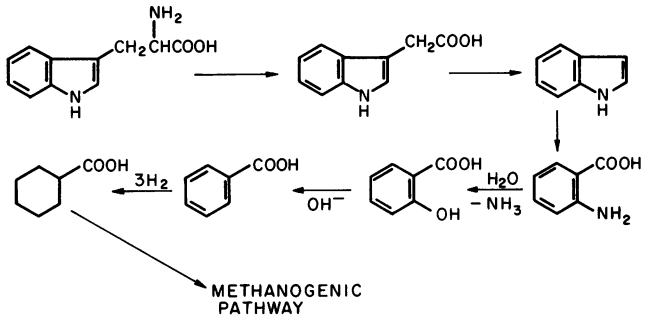

FIG. 12. Proposed pathway for degradation of tryptophane by a methanogenic consortium (adapted from Balba and Evans [7]).

The substrate utilization pattern of *C. purinolyticum* (23) was determined to be similar to that of the purine-fermenting soil isolates *C. cylindrosporum* and *C. acidiurici* (i.e., organisms capable of fermenting a purine derivative such as guanine, uric acid, or xanthine). Although growing cultures of *C. purinolyticum* strain WA-1 catabolyzed adenine to acetate, formate, ammonia, and CO_2 (23), neither *C. acidiurici* nor *C. cylindrosporum* was able to use purine or adenine to support growth. Growth of *C. purinolyticum* on either adenine or purine required the presence of the trace element selenium, presumably because selenium is necessary to promote the formation of xanthine dehydrogenase (23, 107). Purine or purine derivatives are converted by cell-free extracts to a central intermediate (xanthine) by all three organisms, *C. purinolyticum*, *C. acidiurici*, and *C. cylindrosporum* (17, 24). Figure 9 illustrates the xanthine dehydrogenase-mediated transformation of purine and adenine to xanthine by *C. purinolyticum*. As determined by high-performance liquid chromatography, this organism then degrades the highly oxidized purine derivative xanthine to formiminoglycine via 4-ureido-5-imidazole carboxylate, 4-amino-5-imidazole carboxylate, and finally 4-aminoimidazole (Fig. 10) (24). (It is interesting to note that the aromatic ring was cleaved without a preceding ring reduction step.) The entire complement of enzymes required to degrade formiminoglycine to formate, acetate, and ammonia was present in cell-free extracts of *C. purinolyticum*. The pathway of formiminoglycine degradation and the responsible enzymes were first worked out for *C. acidiurici* and *C. cylindrosporum* (see Vogels and Van Der Drift [106] for a complete review of this pathway). Partly on the basis of this information, the pathway of formiminoglycine degradation by *C. purinolyticum* was determined (Fig. 11) by Dürre and Andreesen (24), who demonstrated that the trace element selenium is important in influencing substrate specificity and carbon flow in these purine-degrading microorganisms.

Metabolism by Sulfate-Reducing Microorganisms

In a recent investigation, Imhoff-Stuckle and Pfennig (52) isolated and characterized a nicotinate-degrading, sulfate-reducing bacterium, *Desulfococcus niacini*. Three strains of this organism were enriched for and isolated from marine mud samples, using a mineral salts-trace element-vitamin medium with nicotinate as the electron donor and carbon source. Nicotinate was completely mineralized according to the equation $4C_5H_4N \cdot COO^- + 11SO_4^{2-} + 20H_2O \rightarrow 24HCO_3^- + 11HS^- + 4NH_4^+ + 5H^+$; its use was dependent on selenite supplementation of the growth media. *Desulfococcus niacini* was able to use several organic compounds to support growth; some of these compounds included acetate, propionate, butyrate, caproate, pimelate, and pyruvate. Some organic compounds that did not support growth were adipate, cyclohexane carboxylate, 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, pyridine, urate, and benzoate. Additional experiments with cell-free extracts established the presence of a selenium-dependent nicotinate dehydrogenase. Presumably, then, the first step in nicotinate degradation by this sulfate reducer is the formation of 6-hydroxynicotinate. However, no attempts have been made to isolate intermediates.

Apparently sulfate reducers can also degrade oxygen-containing aromatic heterocyclic compounds, as demonstrated by Brune et al. (19). From a digestor containing sulfite evaporator condensate, they were able to isolate *Desulfovibrio* sp. strain F-1 on a mineral salts-trace metal medium with small amounts of furfural as substrate. Furfural was degraded to acetate in the presence of sulfate, which was reduced to sulfide. Brune et al. (19) did not attempt to isolate or identify intermediates of furfural metabolism.

Metabolism under Methanogenic Conditions

Balba and Evans (7) have demonstrated that a methanogenic consortium from sewage sludges, soils, and anoxic sediments can completely mineralize tryptophan to CO_2 and CH_4. The primary inocula, obtained from the sites mentioned above, were incubated in a mineral salts-vitamin-supplemented medium under a gas phase of CO_2/H_2 (1:4). Enrichment procedures yielded a methanogenic fermentation consortium capable of tryptophan mineralization. The pathway for tryptophan degradation is illustrated in Fig. 12. The intermediates were tentatively identified by thin-layer and gas-liquid chromatographic analyses (of neutral or acid ether extracts of the culture medium). In a more recent investigation, Wang et al. (108) showed that methanogenic enrichment cultures were able to degrade indole to CH_4 and CO_2. Experiments were conducted with bacteria-laden granules of activated carbon acquired from an anaerobic filter that had been previously exposed to a synthetically prepared mixture of indole, quinoline, and methyl quinoline. Although these experiments provided evidence for methanogenic fermentation of indole, the results are in no way conclusive due to sorption phenomena.

Strong evidence for the methanogenic fermentation of indole has been provided by Berry et al. (11), who clearly established the mineralization of indole to methane by a

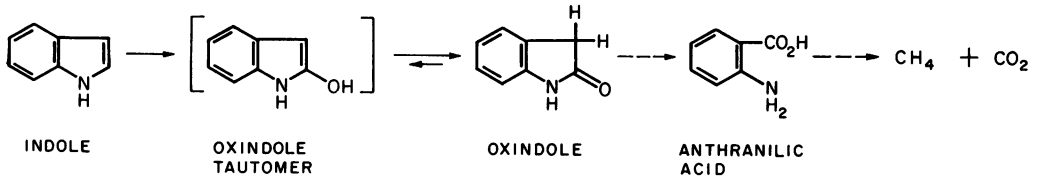

FIG. 13. Degradative pathway of indole by a methanogenic consortium (11).

TABLE 4. Metabolism of heterocyclic aromatic compounds under anaerobic conditions

Energy-yielding process	Compound	Organism	Enrivonmental source	Reference(s)
Fermentation	Uracil	*Clostridium uracilicum*	Soil	20
	Nicotinate	*Clostridium barkeri*	River mud	74
	Adenine Purine	*Clostridium purinolyticum*	Marine mud	23
Sulfate reduction	Nicotinate	*Desulfococcus niacini*	Marine mud	52
	Furfural	*Desulfovibria* sp.	Sulfate evaporator, digestor	19
Methanogenic fermentation	Tryptophan	Nonspecific	Sewage sludge, soil, sediments	7, 6
	Indole	Nonspecific	Activated carbon filter	11, 108

microbial consortium present in 10% anaerobic digested sludge. The degradative pathway was at least partially revealed, as they were able to isolate and identify the initial intermediate product (Fig. 13). High-performance liquid chromatography was used to simultaneously follow indole disappearance and the appearance of the intermediate oxindole (1,3-dihydro-2H-indol-2-one). The identification of oxindole was substantiated by mass and ultraviolet spectra. Methane was determined by gas chromatography. This research is interesting because it establishes the fact that both π-deficient (pyridine, pyrimidine, and the purine nucleus) and π-excessive (indole nucleus) aromatics undergo similar hydroxylation reactions as an initial step in their respective degradation pathways. Activation of indole towards hydroxylation is probably achieved through protonation (forming the 3H-indolium ion).

Summary

Although there are large numbers of heterocyclic compounds in nature as well as in waste materials, to date we have little information on their biodegradation pathways and persistence in nature. Table 4 summarizes the existing information on the anaerobic metabolism of heterocyclic compounds.

IN SITU BIODEGRADATION OF AROMATIC COMPOUNDS IN ANAEROBIC SUBSURFACE ENVIRONMENTS

Most work involving the anaerobic biodegradation of aromatic compounds is conducted in the laboratory under carefully controlled experimental conditions. The results obtained from such controlled experiments (i.e., those involving enrichment cultures, pure cultures, or cell-free extracts of microorganisms) are important insofar as they serve to elucidate and verify the anaerobic metabolic pathway. However, because of the complexity of natural ecosystems, extrapolations of laboratory results to in situ environments are often difficult.

In situ monitoring of anaerobic microbial transformations of organic pollutants in subsurface environments is important to assess their fate and transport, but is technically difficult. Ehrlich et al. (28) were able to measure methane production at specific saturated subsurface sites that had been contaminated with creosote. Methane production was found only at those sites which had been contaminated with creosote. These results implied the anaerobic biodegradation of the waste material. Further investigations of the contaminated site revealed the presence of several physiological types of bacteria including denitrifiers, iron reducers, sulfate reducers, and methanogens (27). The presence of these microorganisms at the contaminated site was highly correlated with the biodegradation of creosote. In a similar investigation, Godsy and Goerlitz (34) found that methanogenesis occurred in contaminated aquifers near a creosote plant located in Pensacola, Fla. Analysis of contaminated water from various wells at the site established the presence of acetate and formate, which are known to be important intermediate products in methanogenic fermentation cultures (65, 66, 115).

Recently, Suflita and Miller (94) investigated the fate of chlorophenols and phenol in a contaminated shallow aquifer located next to a municipal landfill in Norman, Okla. Inocula for the anaerobic degradation investigations were obtained from either one of two sites bordering the landfill area. One site was designated methanogenic, on the basis of the chemical and physical characteristics of the aquifer at that locality, including an "obvious" volatile fatty acid odor, an undetectable level of dissolved oxygen, and a sandy-brown coloration of water. In addition, active methane production was monitored by a gas detector placed down a bore hole leading to the top of the water table. The other site was designated as a sulfate-reducing site (92). Aquifer material from this site was colored grey to black (iron-sulfide precipitates) and had a slight hydrocarbon and sulfide odor. The dissolved oxygen concentration for the groundwater at this site was <2 ppm (<2 μg/ml). Furthermore, no gas evolution could be detected with an electronic gas detector sensitive to methane.

Anaerobic incubations of aquifer material from the methanogenic site readily transformed 2,5-dichlorophenol and 4-chlorophenol to 3-chlorophenol and phenol, respectively. Mineralization of the chlorophenols proceeded only after complete dehalogenation. Subsurface inocula from the sulfate-reducing site presumably contained microorganisms that readily degraded phenol. Inoculum from this site was unable to transform (dehalogenate) or mineralize the chlorophenols. When sulfate-reducing metabolism served as the terminal process, only phenol and benzoate were mineralized; most of the halogenated benzoates were not metabolized.

Schwarzenbach et al.(81), in a 2-year field study, investigated the fate of organic pollutants, including alkylated benzenes and chlorinated phenols, in contaminated river water during the natural infiltration of river water to ground-

water. The field study of primary interest was conducted in the Lower Glatt Valley near Zurich, Switzerland. An aquifer was located just beneath the river at a 60° to 90° angle to the river flow. In the near field of the Glatt River, observation wells were installed in the direction of groundwater flow, and pollutant movement was determined by analyzing water samples collected from this network of wells. Toluene, naphthalene, 1,3-dimethylbenzene, and 1,4-dichlorobenzene were always present in the Glatt River but were almost never detected in any of the observation wells. The disappearance of these organic pollutants during infiltration is difficult to explain since these chemicals are both unreactive and weakly sorbed to subsurface materials. Elimination of these compounds from infiltrated, polluted river water of the saturated subsurface suggested the occurrence of biological transformations or mineralization or both under aerobic conditions. Interestingly, 1,4-dichlorobenzene (which was normally "eliminated" from infiltrated river water much more slowly than the other aromatic hydrocarbons) was detected in one well (G2) at a concentration nearly equal to that found in the river water. This phenomenon occurred only during the summer months when conditions for denitrification prevailed. Subsequently, Kuhn et al. (60) attempted to explain the fate of the aromatic hydrocarbons by using laboratory column studies. The flowthrough columns used in these studies were packed with subsurface material collected from the river-groundwater infiltration site. Under denitrifying conditions, evidence was obtained for the degradation of *o*-, *m*-, and *p*-xylene. *m*-[U-^{14}C]xylene was degraded to $^{14}CO_2$ in the columns with concomitant production of nitrite. Under those same denitrifying conditions, dichlorobenzenes were not transformed.

Information obtained from field studies by in situ monitoring of the organic contaminants provide relevant insights into the behavior of these compounds in anaerobic habitats as well as validate the conclusions drawn from laboratory investigations.

CONCLUSIONS

The ability of microorganisms to degrade aromatic compounds in a given habitat depends on a variety of physical, chemical, and environmental factors. From the standpoint of the compound itself, properties such as water solubility, volatility, molecular size, number and type of functional groups, stability, and presence of a substituent group(s) play a crucial role with respect to the biodegradability of a compound. Equally important in determining the biodegradability of a compound are the environmental factors that influence microbial activity and diversity. Some of these factors include temperature, moisture, pH, oxygen concentration, hydrostatic pressure, salinity, and concentration of chemicals.

With regard to biodegradation of heterocyclic and homocyclic aromatic compounds under anaerobic conditions, the availability of electron donors and electron acceptors plays a crucial role in influencing microbial activity and diversity. If organic carbon is readily available, the diversity of heterotrophic populations is largely dependent upon the presence of electron acceptors, i.e., NO_3^-, SO_4^{2-}, and CO_2.

Homocyclic aromatic compounds such as benzoate can be mineralized by microorganisms under anoxic conditions. At the present time, only the reductive pathway for the anaerobic metabolism of benzenoid compounds has been elucidated. The reductive pathway consists of an initial ring hydrogenation step (ring reduction) followed by a ring hydration ring cleavage reaction sequence. This pathway is believed to be common to all microorganisms involved in benzenoid metabolism, including the denitrifiers, the sulfate reducers, and the fermenters.

Nicotinate fermentation by *C. barkeri* serves as an example of heteroaromatic metabolism. In contrast to the reductive metabolic pathway, the initial step in nicotinate fermentation is ring hydroxylation, rather than ring reduction. The ring hydroxylation step is followed by a ring reduction ring cleavage reaction sequence. Although the degradative pathways of nicotinate and benzenoid compounds exhibit some differences, they also share some interesting similarities. For instance, the initial step in the metabolism of homocyclic and heterocyclic aromatic compounds involves an aromatic destabilization reaction. The reductive and hydroxylation pathways have in common a hydration reaction. Apparently, the attachment of oxygen to the ring structure facilitates ring cleavage.

Laboratory studies have established the ability of microorganisms to degrade homocyclic and heterocyclic aromatic compounds under anaerobic conditions. The results obtained from laboratory investigations (i.e., those involving enrichment cultures, pure cultures, or cell-free extracts of microorganisms) are important because they allow the investigator to define an anaerobic pathway. However, caution must be exercised when attempting to predict the environmental fate of any organic compound based on laboratory results alone. Whenever possible, results from laboratory investigations should be critically correlated with results obtained from in situ investigations. The information obtained from such field studies, together with information obtained through laboratory studies, should provide a framework for predicting the fate of organic compounds in natural environments.

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