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Microbial Reductive Dehalogenation

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INTRODUCTION 483
Definition of Reductive Dehalogenation 483
Scope of This Review 484
SUBSTRATES AND HISTORICAL PERSPECTIVE 484
Organochlorine Pesticides 484
Alkyl Solvents 484
Aryl Halide Compounds 485
Polychlorinated biphenyls 486
Chlorobenzenes 487
Other xenobiotic compounds 487
Natural haloaromatic compounds 487
UNDEFINED CULTURES 488
Biological Activity 488
Acclimation 488
Enrichment 490
Specificity 490
Electron Acceptors 491
Other Nutrients 492
Temperature 492
Substrate Availability 493
Conclusion 493
PURE CULTURES 493
Alkyl Dehalogenators 493
Substrate specificity 494
Products of alkyl solvents 494
An Anaerobic Aryl Dehalogenator, *D. tiedjei* 494
Dehalogenation activity 495
Induction 495
Inhibition 495
Morphology 496
Syntrophy 496
Physiology 497
Taxonomy 498
Attempts To Find Other Aryl Dehalogenators 498
Sulfate-reducing bacteria 498
New isolates 498
Highly enriched cultures 498
Aerobic Aryl Dehalogenators 499
Pentachlorophenol 499
Dichlorobenzoate 499
Conclusion 499
CELL-FREE ACTIVITY 499
Cell Extracts 500
D. tiedjei extract 500
A Purified Enzyme, P-450 cam 501
Transition Metal Complexes 501
Iron porphyrins 501
Co- and Ni-containing porphyrins 501
Conclusion 502
GENERAL CONCLUSIONS 502
ACKNOWLEDGMENTS 502
REFERENCES 503

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INTRODUCTION

Reductive dehalogenation is an important means of biodegradation of numerous compounds, including organochlorine pesticides, alkyl solvents, and aryl halides. These compounds include many of our most toxic and environmentally persistent pollutants. Reductive dehalogenation is the only known biodegradation mechanism for certain significant pollutants including highly chlorinated polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), tetrachloroethene (PCE), and pentachlorophenol (PCP) (Table 1 lists common names and chemical names of compounds). Dehalogenation generally makes xenobiotic compounds less toxic and more readily degradable. Reductive dehalogenation is mainly known to occur under anaerobic conditions and is the initial step in anaerobic biodegradation of most aryl halides. In addition, reductive dehalogenation is involved in aerobic degradation of certain highly halogenated compounds. Hence, reductive dehalogenation is of particular interest because of its involvement in the environmental fate of pesticides and industrial chemicals and its potential application to bioremediation of pollutants and hazardous wastes.

The study of reductively dehalogenating microorganisms is essential for their exploitation by humans but is also intriguing from a more fundamental scientific perspective. Studies of reductive dehalogenation have contributed significantly to basic microbiology in the areas of ecology, physiology, and phylogeny. Reductive dehalogenation of many compounds is known to occur only within mutualistic anaerobic microbial communities. Using undefined cultures, researchers are beginning to understand the ecology of these communities which occur in soils, sediments, intestinal tracts, and bioreactors. Many pure cultures are reported to catalyze reductive dehalogenation of alkyl halides (alkyl reductive dehalogenation). On the other hand, only a few organisms capable of reductive dehalogenation of aryl halides (aryl reductive dehalogenation) are currently available in pure culture. The study of aryl reductive dehalogenation has led to the discovery of one novel and unusual organism, *Desulfomonile tiedjei* DCB-1, and continued study of this process may lead to other novel organisms. Studies of cell-free systems, including cell extracts, one purified enzyme, and several transition metal complexes, are beginning to lend insight into how and why microorganisms catalyze reductive dehalogenation.

Definition of Reductive Dehalogenation

Reductive dehalogenation involves the removal of a halogen substituent from a molecule with concurrent addition of electrons to the molecule. Essentially, two processes have been identified. The first process, hydrogenolysis, is the replacement of a halogen substituent of a molecule with a hydrogen atom (Fig. 1A and B). The second process, vicinal reduction (dihaloelimination), is the removal of two halogen substituents from adjacent carbon atoms with the formation of an additional bond between the carbon atoms (Fig. 1C). Hydrogenolysis can transform alkyl or aryl halides, whereas vicinal reduction can transform only alkyl halides. Both processes require an electron donor (reductant). In all reported examples of biologically catalyzed reductive dehalogenation, the halogen atoms are released as halide anions. Possible mechanisms for these reactions are discussed below under Transition Metal Complexes.

TABLE 1. Common and chemical names of compounds

Common name	Chemical name
Alachlor	2-Chloro-2′,6′-diethyl-*N*-(methoxymethyl)-acetanilide
Aldrin	1,2,3,4,10,10-Hexachloro-1,4-endo,exo-5,8-dimethanonaphthalene
Benthiocarb	*S*-4-Chlorobenzyl-*N*,*N*-diethyl thiocarbamate
Bromacil	5-Bromo-3-*sec*-butyl-6-methyl uracil
Carbon tetrachloride	Tetrachloromethane
Chloroform	Trichloromethane
Chloronitrofen	4-Nitrophenyl-2,4,6-trichlorophenyl ether
Chloropicrin	Trichloronitromethane
DDD	1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethane
DDT	1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane
Dieldrin	1,2,3,4,10,10-Hexachloro-6,6-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo,exo-5,8-dimethanonaphthalene
Diuron	3-(3,4-Dichlorophenyl)-1,1-dimethyl urea
Ethylene dibromide	1,2-Dibromoethane
HCB	Hexachlorobenzene
Heptachlor	1,4,5,6,7,8,8-Heptachloro-3a,5,7,7a-tetrahydro-4,7-methanoindene
Lindane	γ-1,2,3,4,5,6-Hexachlorocyclohexane
Methoxychlor	1,1,1-Trichloro-2,2-bis(*p*-methoxyphenyl)ethane
Methyl chloride	Monochloromethane
Methylene chloride	Dichloromethane
Mirex	Dodecachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta(cd) pentalene
PCB	Polychlorinated biphenyl
PCE (perchloroethene)	Tetrachloroethene
PCP	Pentachlorophenol
Propanil	*N*-(3,4-Dichlorophenyl) propanamide
TCE	Trichloroethene
Techloftham	*N*-(2,3-Dichlorophenyl)-3,4,5,6-chloro-phthalamic acid
TPN	2,4,5,6-Tetrachloroisophthalonitrile
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
Vinyl chloride	Monochloroethene

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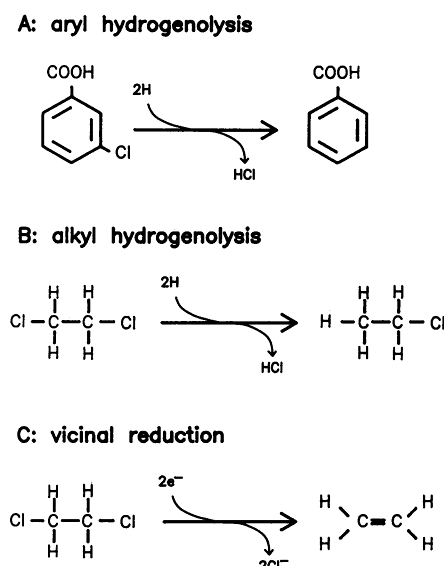

FIG. 1. Examples of reductive dehalogenation. (A) Aryl hydrogenolysis of 3-chlorobenzoate to benzoate; (B) alkyl hydrogenolysis of 1,2-dichloroethane to chloroethane; (C) vicinal reduction of 1,2-dichloroethane to ethene.

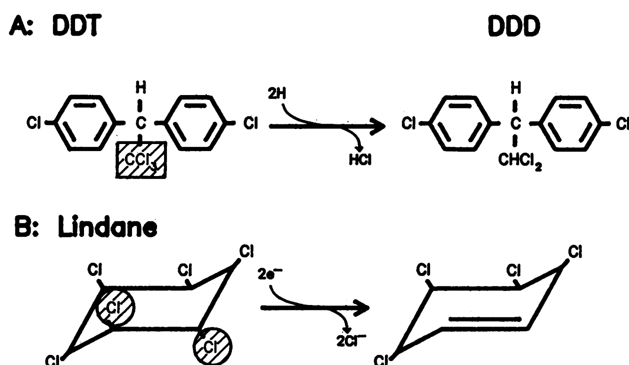

FIG. 2. Alkyl reductive dehalogenation of organochlorine pesticides. (A) Hydrogenolysis of DDT to DDD; (B) Vicinal reduction of lindane to γ-3,4,5,6-tetrachloro-1-cyclohexene. (Based on reference 98.)

Scope of This Review

Certain aspects of reductive dehalogenation have been previously reviewed by Essac and Matsumura (65), Kuhn and Suflita (115), Tiedje et al. (199), and Vogel et al. (206). Reductive dehalogenation has been less extensively covered in a number of other reviews, most of which are on more general aspects of biodegradation (1, 20, 28, 41, 66, 118, 127, 153, 154, 171, 173, 178, 196). The above reviews have thoroughly cataloged the reported range of substrates for reductive dehalogenation and the various biological systems that catalyze this activity. We will therefore not describe individual reports of reductive dehalogenation, except for important new examples of this activity. Our focus is instead on the ecological and physiological principles which are beginning to emerge from a broad perspective of reductive dehalogenation. We note both trends and differences in the many known biological systems capable of reductive dehalogenation and in the different reductive dehalogenation activities described. When possible, we relate studies with undefined cultures, pure cultures, and cell-free systems. In many cases available information on reductive dehalogenation activities is limited; for example, particular organisms which are capable of catalyzing certain activities have not yet been identified. Thus, in many ways, the ecological and physiological understanding of reductive dehalogenation is in a relatively early stage.

SUBSTRATES AND HISTORICAL PERSPECTIVE

During the last half of the 20th century, various xenobiotic compounds have been widely applied or discharged to the environment. The environmental persistence and toxicity of many compounds has raised public concern, and research reviewed here follows a chronology of response to such concerns. The relevant compounds can be roughly categorized as organochlorine pesticides, alkyl solvents, and aryl halides. Often these compounds are persistent in aerobic environments but degraded in anaerobic environments (reviewed in references 65 and 115). This realization has led researchers to study relatively unknown anaerobic organisms and communities and to find that reductive dehalogenation is often an important fate of halogenated compounds in anaerobic environments.

Organochlorine Pesticides

As concern for the environmental fate of widely used pesticides grew in the 1960s, reductive dehalogenation, particularly in anaerobic environments, was found to be an important fate of several organochlorine pesticides, including DDT (Fig. 2A), lindane (Fig. 2B), mirex, and toxaphene (Table 2). With the exception of a single report of PCP degradation (97), these were reports of alkyl reductive dehalogenations. Alkyl reductive dehalogenation of dieldrin (Fig. 3) (134) and alachlor (Fig. 4) (22) were reported more recently. Pure cultures of a wide variety of microorganisms were found to catalyze the reductive dehalogenation of organochlorine pesticides (see Table 4), as were cell-free systems (see Table 5). In many cases further degradation of the dehalogenation products was not observed, and those products often persist in the environment. Such partial degradation has several implications. In monitoring the environmental fate of a chemical, one must also monitor the products formed, not simply the disappearance of the chemical. In assessing the effects of degradation of a chemical, one must consider possible decreases or increases in toxicity and mobility resulting from partial degradation.

Alkyl Solvents

Halogenated C_1 and C_2 solvents became a major concern in the 1970s. Included in this class of compounds are chloromethane, chloroform, tetrachloroethene (PCE), and trichloroethene. These compounds have been widely used for dry cleaning, degreasing machinery, and manufacturing electronic components and as pesticides. Such compounds can also form as a result of chlorination of water. Alkyl solvents are relatively water soluble. Consequently, alkyl solvents have become major contaminants of soil and groundwater. In the 1980s, degradation of these solvents was studied and intricate degradation pathways were observed, including presumably both biological and abiological (chemical) steps and involving several dehalogenation mechanisms (reviewed in reference 206). Among the several dehalogena-

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TABLE 2. Alkyl reductive dehalogenation in undefined anaerobic communities

Substrate	Inoculum	Reference(s)
Alachlor	Stream sediments	22
DDT	Anchovy intestine	130
	Bovine rumen	76, 142
	Estuarine sediment	216
	Lake sediments	133, 142, 164
	Mammalian gut	29
	Ovine rumen	186
	Sewage sludge	91, 164, 216
	Silage	89
	Soil	34, 82, 107, 159
	Stream sediments	164
1,2-Dibromo-3-chloro-propane	Soil	36
2,3-Dibromobutane	Soil	36
Dieldrin (and other cyclodienes)	Soil	134
	Sheep rumen	134
	Chicken feces	134
Halomethanes	Soil	25, 121
	Sewage sludge	193
Haloethanes	Bioreactor	208
	Subsoil	47
	Marl, muck	160
Haloethenes	Bioreactor	73, 176, 207, 208
	Enrichment culture	12, 215
	Marl, muck	14, 160
	Soil	36, 106
	Subsoil	106, 196, 211
Heptachlor	Soil	140, 141
Toxaphene	Bovine rumen	174
	Sewage	174
Lindane	Flooded soil	128, 129, 170, 202
	Soil	134
	Sheep rumen	134
	Chicken feces	134
Methoxychlor	Soil	39
Mirex	Sewage sludge	6

tion mechanisms is reductive dehalogenation, which occurs especially (but not exclusively) in anaerobic environments. Reductive dehalogenation of alkyl solvents has since been reported in a wide range of undefined communities (Table 2), pure cultures (see Table 4), and cell-free systems (see Table 5). Reductive dehalogenation degrades alkyl solvents to

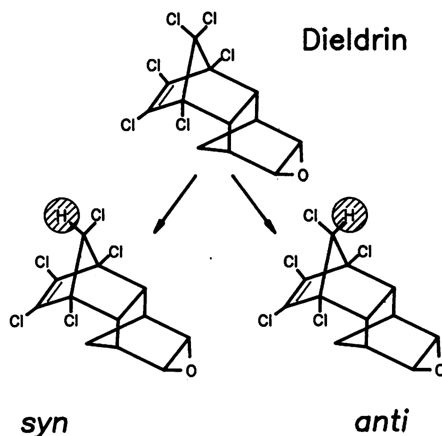

FIG. 3. Alkyl dehydrogenolysis of dieldrin to both *syn*- and *anti*-monodechlorodieldrin. (Based on reference 134.)

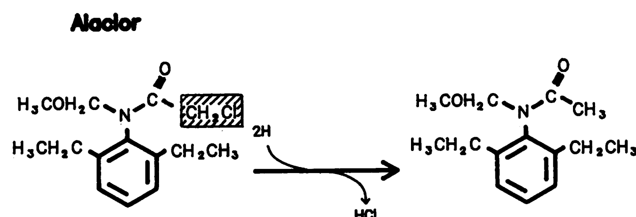

FIG. 4. Alkyl hydrogenolysis of alaclor. (Based on reference 22.)

various extents, depending on the solvent, the physicochemical environment, and the microorganisms present. In most studies of microbial dehalogenation of methanes, ethanes, and ethenes listed in Tables 2 and 4, only partial dehalogenation was reported. The less-halogenated products may not be degraded under the same conditions. Accordingly, in contaminated anaerobic natural environments, partial dehalogenation products of alkyl solvents are often found. In fact, *cis*-dichloroethene is frequently the major environmental contaminant when PCE or trichloroethene has entered anaerobic aquifers. Complete dehalogenation is possible with PCE (Fig. 5) (73) and dihaloethanes (19). CO_2 is frequently also a product of anaerobic degradation of alkyl solvents (24, 25, 45, 46, 63, 64, 139, 207, 208). The relative amount of the CO_2 product appears to be variable, and it is not clear whether reductive dehalogenation or a degradation pathway distinct from reductive dehalogenation leads to CO_2 formation. A proposed mechanism leading to both reductive dehalogenation and CO_2 formation is discussed in Transition Metal Complexes, below.

Aryl Halide Compounds

Concern about numerous aryl halide pollutants also grew during the 1970s. Aryl halides include pesticides, solvents,

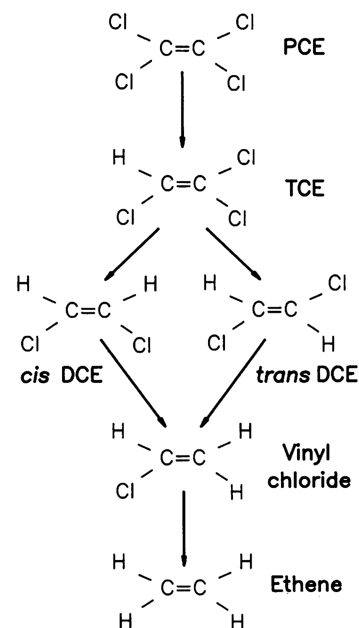

FIG. 5. Sequential hydrogenolysis of PCE. Abbreviations: DCE, dichloroethene; TCE, trichloroethene. (Based on references 73 and 207.)

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TABLE 3. Aryl reductive dehalogenation in undefined anaerobic communities

Substrate	Inoculum	References
Benthiocarb	Flooded soil	148
Bromacil	Subsoil	2
Chloroanilines	Aquifer	116
	Pond sediment	194
Chlorobenzenes	Rat gut	201
	River sediment	23
	Sewage sludge	72
	Subsoil	122
Chlorocatechols	Marine sediment	5, 155
	Reactor column	85
Chloroguaiacols	Reactor column	85
Chloronitrofen	Flooded soil	213
Chlorophenoxy-acetates	Sewage sludge	137
	Subsoil	79, 80
	Pond sediment	79
Chlororesorcinol	Sewage sludge	71
Diuron	Pond sediment	11
Halobenzoates	Lake sediment	95, 123, 197, 198
	Subsoil	79
	Estuary sediment	180, 181
	River sediment	180, 181
	Sewage sludge	197, 198
Halophenols	Estuary sediment	84, 180, 181
	Flooded soil	97
	Lake sediment	86, 108, 109, 214
	Marine sediment	103
	River sediment	180, 181
	Sewage sludge	26, 27, 54, 96, 138
	Subsoil	79, 195
Polyhalogenated biphenyls	River sediment	156, 166, 169, 205
Propanil	Pond sediment	190
Techloftham	Flooded soil	104
2,4,5,6-Tetrachloro-isophthalonitrile	Flooded soil	175

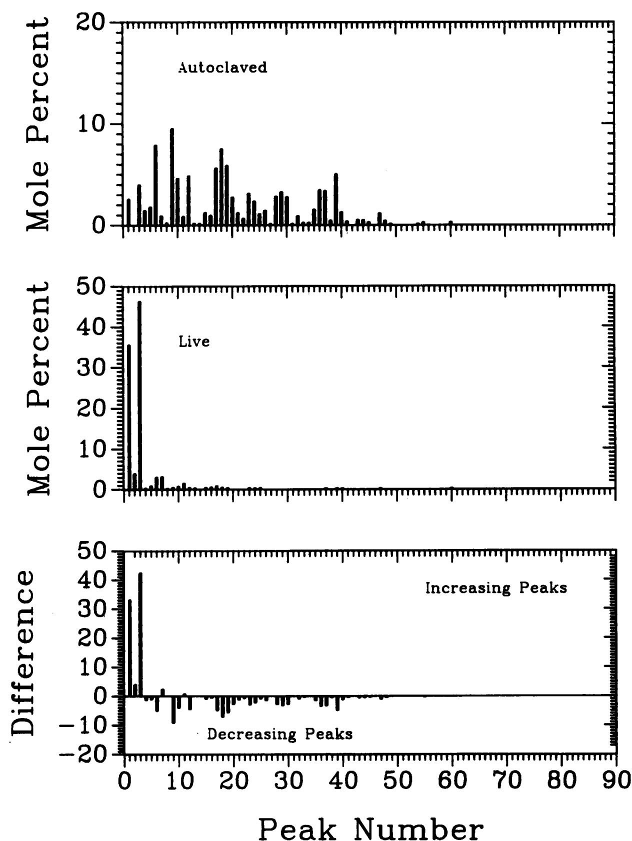

FIG. 6. Transformation of a mixture of PCBs to less chlorinated congeners by live anaerobic sediment organisms (courtesy of J. F. Quensen III). Histograms represent PCB mixtures analyzed by gas chromatography, with increasing peak numbers corresponding to congeners with increasing chlorine contents.

heat transfer agents, and waste products from numerous industrial processes. These compounds include halogenated anilines, benzenes, biphenyls, phenoxyacetates, and phenols, and they have become widespread environmental contaminants. Except for the above-mentioned report of PCP degradation (97), reductive dehalogenation of aryl halides, like that of alkyl solvents, was not recognized until the 1980s. Aryl reductive dehalogenation has been found and studied in a variety of undefined anaerobic communities (Table 3) but, unlike alkyl reductive dehalogenation, has not been found in many pure cultures or cell-free systems. Pure cultures of only a single anaerobe (185) and three aerobes (9, 188, 203) are reported to catalyze aryl reductive dehalogenation. Initial studies of aryl reductive dehalogenation focused on relatively simple compounds (e.g., mono- or dihalogenated benzoates and phenols). For such compounds reductive dehalogenation is the primary step in anaerobic mineralization (reviewed in references 115 and 199). More recent studies have found that the same activity can transform compounds which are considered much more significant as pollutants. These compounds tend to be more complex, less water soluble, and more toxic. These recent findings greatly increase the significance of aryl reductive dehalogenation from an applied perspective and are briefly summarized below.

Polychlorinated biphenyls. PCBs are currently of great concern owing to their recalcitrance and toxicity. They were formerly used widely as heat transfer agents in electrical transformers and capacitors, as hydraulic fluids, and in carbonless copy paper. Commercial PCBs are very complex mixtures of congeners (analogs which vary in numbers and arrangements of chlorine substituents). There are 209 theoretically possible PCB congeners, and commercial mixtures contain up to 70 PCB congeners. Laboratory studies have now demonstrated that the more highly chlorinated PCB congeners in commercial mixtures, such as Aroclors 1242, 1248, 1254, and 1260, can be reductively dechlorinated by anaerobic microorganisms from PCB-contaminated sediments (4, 156, 166, 169, 205). These studies support the previous interpretation that dechlorinated PCBs found in anaerobic sediments resulted from in situ biological activity (30–32). Highly chlorinated PCBs, as in Aroclor 1260, are dechlorinated relatively slowly, but this activity is still significant because it is the only biodegradation of these compounds observed so far. The transformations observed generally reduce the chlorine content of PCB mixtures (Fig. 6), and the major products are mono- and dichlorobiphenyls.

Various patterns of PCB dehalogenation are observed depending on the source of anaerobic sediments (Fig. 7). Reductive dehalogenation activity is most commonly observed at the *meta* and *para* positions, but recent reports indicate that dehalogenation of *ortho* chlorines is also possible (205). The different patterns of PCB dehalogenation

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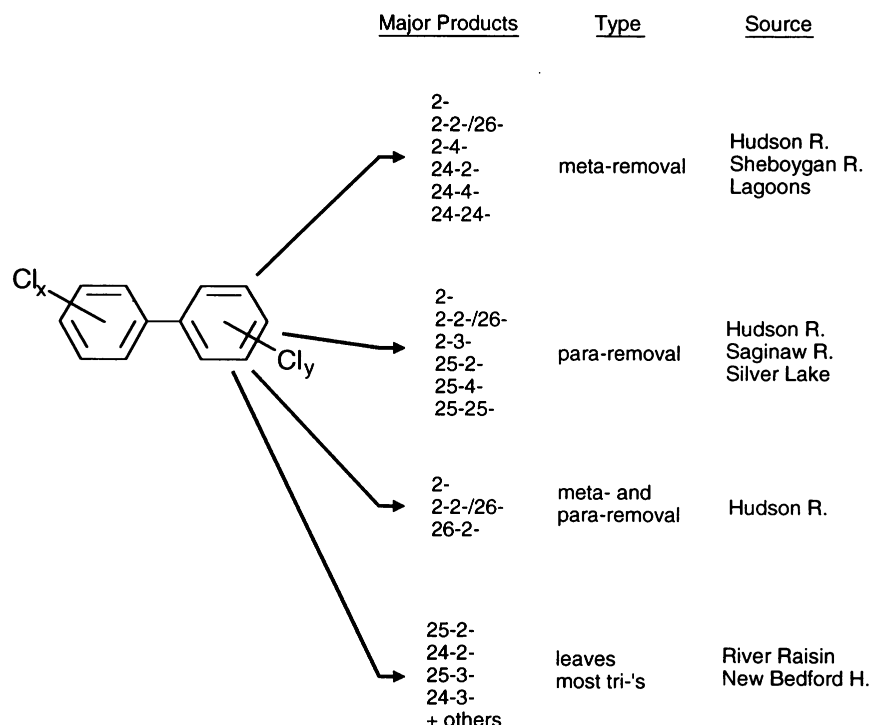

FIG. 7. Different patterns of PCB dehalogenation depending on the inoculum source. (Based on reference 200. Courtesy of J. F. Quensen III.)

may be due to distinct dehalogenating microbial populations or to different environmental conditions. Significant detoxification of Aroclors probably results from dehalogenation, since the reported transformations would degrade the most toxic PCB congeners, 3,4,3′,4′-tetra-, 3,4,5,3′,4′-penta-, and 3,4,5,3′,4′,5′-hexachlorobiphenyl (167, 172). The less-chlorinated, *ortho*-substituted biphenyls which result from anaerobic PCB degradation can be mineralized aerobically (reviewed in references 1 and 16), suggesting that with a sequence of anaerobic and aerobic conditions, microbial communities might mineralize all PCB congeners. Biological reductive dehalogenation of polybrominated biphenyls has also been demonstrated recently (168).

Chlorobenzenes. HCB and other chlorobenzene congeners are also widespread pollutants with very low water solubility. Undefined cultures from a number of sources appear to reductively dehalogenate HCB (Table 3). Patterns of dehalogenation vary somewhat (Fig. 8), but several generalizations are possible. HCB is most readily dehalogenated to 1,3,5-trichlorobenzene (72, 122, 151). Further dehalogenation to monochlorobenzene is possible but apparently less readily catalyzed (23, 122, 151). Monochlorobenzene appears to be stable under anaerobic conditions. Some variation in dehalogenation pathways can occur, and in some cases, as for PCB dehalogenation, variation is due to different inoculum sources (151). Together, these studies indicate that anaerobes are capable of the necessary steps for degradation of HCB to monochlorobenzene; however, this process has not been observed in a single system. Di- and monochlorobenzenes can be mineralized aerobically (49, 177, 187, 204). Thus, as with PCBs, the proper sequence of environmental conditions may allow biological mineralization of all chlorobenzene congeners.

Other xenobiotic compounds. Chloroanilines are used in industrial syntheses and are degradation products of pesticides. Recent studies indicate that chloroanilines can be reductively dechlorinated by organisms from aquifer material (116, 117) and pond sediment (194). In those studies, more highly chlorinated anilines were dechlorinated but monochloroanilines persisted. Chlororesorcinols are possible by-products of industrial syntheses, and 4-chlororesorcinol has been shown to be reductively dechlorinated in anaerobic sewage sludge (71). The resorcinol product subsequently disappeared from enrichment cultures from the sludge, suggesting that anaerobic communities may be capable of mineralizing 4-chlororesorcinol. Chlorocatechols apparently result from the process of paper bleaching and other industrial processes and have recently been reported to be dehalogenated by enrichment cultures from marine sediments (5). Only certain chlorocatechol congeners were dehalogenated, and some variation in pathways was observed (Fig. 9). In the first report of biological reductive dehalogenation of a heterocyclic compound, Adrian and Suflita (2) clearly demonstrated removal of bromine from the herbicide bromacil by aquifer slurries (Fig. 10). No further evidence concerning the fate of the debrominated product was given. Chlorinated pyridines have now also been reported to be reductively dehalogenated by aquatic sediments (126).

Natural haloaromatic compounds. Not all haloaromatic compounds are xenobiotic. The burrows of a hemichordate inhabiting a marine sediment were found to contain 2,4-dibromophenol (102). The worm apparently synthesizes the compound, which inhibits the growth of aerobic bacteria in the burrow. Subsequent investigation showed that an anaerobic microbial community from the sediment could first reductively debrominate and then mineralize this compound (103). Red algae (Rhodophyta) also produce aryl halides. Marine sediments containing a red alga more rapidly deha-

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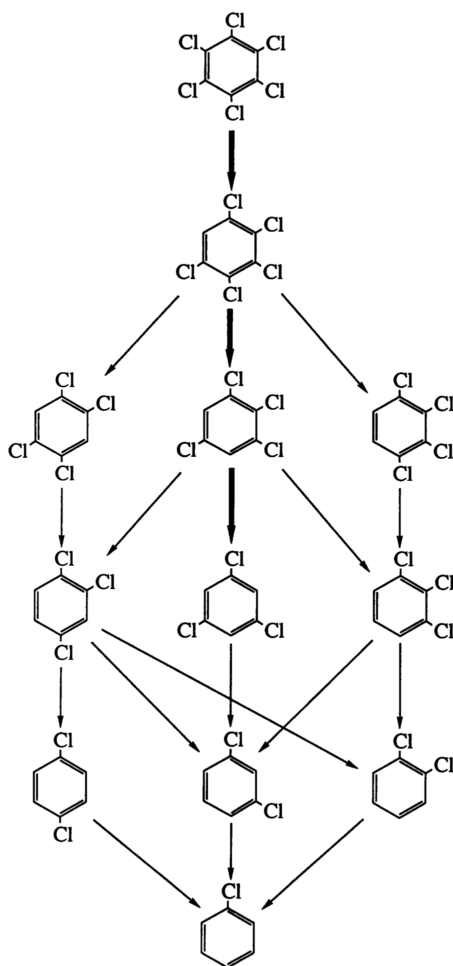

FIG. 8. Inferred pathway of anaerobic dehalogenation of chlorobenzenes (based on references 23, 72, 93, 122, and 151). Bold arrows indicate a common pathway which results in accumulation of 1,3,5-trichlorobenzene. The complete sequence has not been observed in a single system.

logenated various aryl halides than did sediments that apparently did not contain the alga (161). This observation would be consistent with selection for a dehalogenating population during decomposition of the alga. Organochlorine compounds can also result from combustion of organic matter, as during forest fires (3), and from natural transformations of humic matter in soil (10). The existence of such naturally occurring compounds suggests that selective pressure for reductive dehalogenation may have existed during the evolution of bacteria. Further investigation of the metabolism of such natural compounds will probably contribute greatly to our understanding of the metabolism of xenobiotic compounds.

UNDEFINED CULTURES

Most, but not all, reported examples of reductive dehalogenation are in anaerobic systems. Like other anaerobic processes, reductive dehalogenation has typically been found to occur in complex, mutualistic communities. Ecological understanding of such communities will probably be important in using reductive dehalogenation in bioremediation processes. Aryl reductive dehalogenation has been observed almost exclusively in undefined communities, and so ecological understanding of those communities is especially important. As a consequence, laboratory investigations with undefined anaerobic communities have been performed in the study of aryl reductive dehalogenation out of necessity. Far fewer investigations with undefined anaerobic communities have been performed in the study of alkyl reductive dehalogenation, perhaps because of the availability of pure cultures that catalyze this activity. The study of undefined communities can identify ecological factors that affect reductive dehalogenation but is of limited value in determining the mechanisms of these effects. However, this approach does have predictive value, since undefined cultures are probably more likely than pure cultures to behave like populations in natural habitats or habitats engineered for bioremediation. We next examine several aspects of reductive dehalogenation in undefined communities.

Biological Activity

In undefined cultures reductive dehalogenation is typically biologically dependent, but it is not clear that the activity is biologically catalyzed. Reductive dehalogenation can usually be inhibited by sterilization. Autoclaving is the main method of sterilization, and it was found to inhibit dehalogenation in many of the undefined cultures listed in Tables 2 and 3. Chemical sterilization with propylene oxide (22) or formaldehyde (95), as well as sterilization by gamma irradiation (95), has also been shown to inhibit dehalogenation. The inhibition of reductive dehalogenation by sterilization indicates either biological or biologically dependent catalysis. Reductive dehalogenation requires a reductant which, in natural systems, may be a product of biological activity. Thus, sterilization could abolish the source of reductant instead of the catalyst of reductive dehalogenation. Zoro et al. (216) demonstrated that sterilized sewage sludge and sterilized yeast cultures catalyze the hydrogenolysis of DDT if a reductant is provided. Abiological mechanisms have been implicated in reductive dehalogenation of alkyl solvents (206, 208). There is little direct evidence for the relative significance of living and nonliving catalysts in natural environments or undefined cultures.

Perhaps a more useful distinction is between two types of biological catalysts, namely enzymes and subenzymatic catalysts (e.g., prosthetic groups or transition metal complexes). Both are normally of biological origin, although the former is encoded by a structural gene and can be produced by molecular biology techniques and the latter can often be chemically synthesized. Living cells are normally required to stabilize enzymes, whereas subenzymatic catalysts are stable in many environments. Subenzymatic catalysts typically have less substrate specificity than enzymes do. At the mechanistic level, reductive dehalogenation is always a chemical reaction, regardless of the catalyst. However, the catalyst is often rate limiting for a reaction, and so it is important to understand the nature of the catalyst, especially if one wants to optimize the reaction. Examples of abiological, subenzymatic, and enzymatic catalysts of reductive dehalogenation are discussed below under Pure Cultures and Cell-Free Activity.

Acclimation

The term "acclimation" is used here to describe the initial period of an incubation during which reductive dehalogena-

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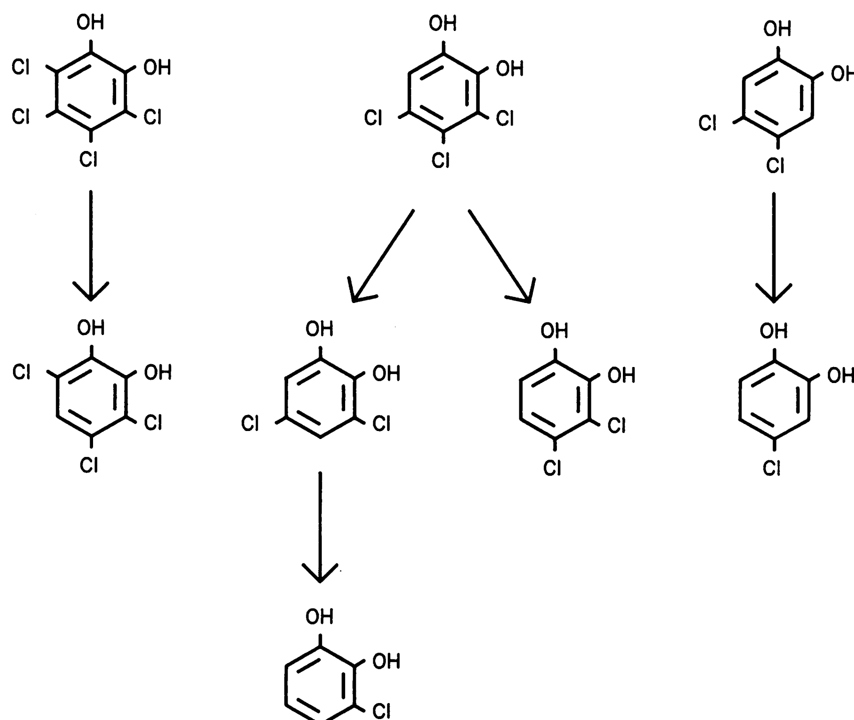

FIG. 9. Chlorocatechol hydrogenolysis reactions catalyzed by marine sediment enrichment cultures. (Based on reference 5.)

tion is not detectable. Such periods are also sometimes referred to as lag times. Acclimation periods appear to be more commonly associated with aryl than alkyl reductive dehalogenation. Alkyl reductive dehalogenation of many organochlorine pesticides and alkyl solvents readily occurs in a variety of anaerobic communities without acclimation (14, 69) or with relatively short acclimation times (less than 1 month) (22, 74, 82, 134, 216). In some cases, longer acclimation periods were necessary before alkyl reductive dehalogenation (211). Cases in which acclimation was not required are probably due to the continual presence of the dehalogenation catalyst in the natural community, suggesting that a non-specific catalyst is operative. In contrast, aryl reductive dehalogenation in natural samples usually requires long acclimation periods, often several months (23, 33, 86, 87, 95, 108, 109, 123, 194, 197, 214). Cultures inoculated with natural samples have required up to 6 months to exhibit reductive dehalogenation of halobenzenes (23) or halobenzoates (95, 123, 197). One must therefore allow for the possibility of such a requirement in studies of aryl reductive dehalogenation. Aryl halides added after acclimation of a culture to those compounds are usually dehalogenated immediately.

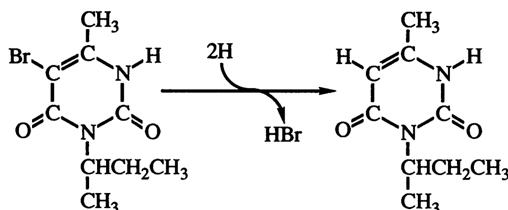

FIG. 10. Hydrogenolysis of the heterocyclic pesticide bromacil. (Based on reference 2.)

Most studies of aryl reductive dehalogenation have focused on the activity following acclimation, leaving the acclimation process itself poorly understood. Acclimation to halobenzoates is addressed in one study by Linkfield et al. (123). The periods of acclimation varied for different substrates but were reproducible for the same substrate over time and with different inoculum sources (all from sediment of the same lake). Acclimation periods were found to be inversely correlated to subsequent dehalogenation rates. These authors identify the following possible explanations for the periods of acclimation: (i) genetic change, (ii) induction, (iii) exhaustion of a preferred substrate (diauxy), or (iv) growth of the active population from a very low initial density. The first three explanations involve an acclimation period when no dehalogenation activity occurs, whereas the last explanation involves activity which is initially present at undetectable rates and is not true acclimation. The authors suggest that induction best explains the observed patterns. Kinetic analyses of the dehalogenation rates indicated that there probably was not activity in the cultures initially. Genetic change, which might involve mutation or genetic exchange, was considered unlikely because of the reproducibility of the acclimation periods. A diauxy response was considered unlikely because sediment refrigerated for 2 years exhibited the same acclimation period as fresh sediment; however, this conclusion is based on the questionable assumption that refrigerated sediment would significantly deplete available substrates.

Recently dehalogenation of benzoates was found to be inducible in a pure culture, *D. tiedjei* (see below). In a study of halophenol dehalogenation, Kohring et al. (108) found that storage of lake sediment for 2 months at 12°C increased the acclimation time. This finding would also not be consistent with a diauxy response, since storage should then decrease the acclimation time, but would be consistent with acclima-

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tion as a result of growth of a dehalogenating population which decreased in viability during storage. The inverse relation of acclimation time and dehalogenation rate found in the former study is consistent with the notion that the acclimation time is due to dehalogenation-dependent growth. It seems possible that growth of the dehalogenating population was at least partly responsible for the acclimation times in both studies. If such an explanation is true, the requirement for an acclimation period for aryl, but not always for alkyl, reductive dehalogenation suggests that populations capable of the former activity are more rare in nature than those capable of the latter. This is consistent with the greater difficulty in identifying pure cultures capable of the former activity (see Pure Cultures, below).

It is possible that there are different explanations for acclimation periods in different systems or that combinations of the above-mentioned explanations apply. One possibility which combines several explanations is that a community succession is required for establishment of the dehalogenating population(s). In this case, climax communities presumably catalyze dehalogenation, since the activity persists after acclimation. Such a succession might be required to create favorable environmental conditions for dehalogenation, including, perhaps, exhaustion of substrates inhibitory to dehalogenation (see Electron Acceptors, below) and reduction of redox potential. This possibility would be consistent with the apparent lack of any initial activity found above. Different haloaromatic substrates might affect the acclimation time by having differential toxicity toward one or more of the organisms involved in the succession. Finally, the rate of dehalogenation would affect the growth of the dehalogenating community if that growth was dependent on dehalogenation, either directly (below) or through use of the dehalogenation product. Such a community succession, although occurring in laboratory cultures, would not necessarily occur in natural systems, and so the resulting reductive dehalogenation must be regarded as a potential activity. All of the above explanations for acclimation periods are consistent with reductive dehalogenation catalyzed by living organisms and, in most cases, by enzymes of those organisms. Clearly our present understanding of the acclimation periods preceding reductive dehalogenation by natural communities is highly speculative and points to gaps in our understanding of factors affecting the activity.

Enrichment

Enrichment of reductive dehalogenation activity is possible and indicates that selection for that activity can occur. Certain communities, often in polluted habitats, are adapted to certain xenobiotic compounds. Thus, inocula from PCB-contaminated river sediments dechlorinated PCBs during prolonged incubations whereas inocula from uncontaminated sediments did not (169); also, inocula from PCB-contaminated river sediments required a shorter acclimation time to dechlorinate PCBs than did inocula from uncontaminated sediments (150). Polluted river and estuary sediments had greater potential than unpolluted ones for dehalogenation of monochlorophenols and monochlorobenzoates (180). Sewage sludges from different sources varied in their potentials to dehalogenate various substrates (184). Additionally, by serial transfer of laboratory cultures, it has been possible to enrich for aryl reductive dehalogenation of a number of substrates, including halobenzoates (180, 197), halophenols (114, 180, 214), 4-chlororesorcinol (71), dichloroanilines (194), and PCBs (149). Evidence for enrichment includes increases in dehalogenation rates and decreases in acclimation periods in transfers from primary cultures. Adaptation and enrichment are common observations in microbial cultures, but they are not trivial observations for xenobiotic substrates. One would not necessarily expect such substrates to support growth, especially in cases, such as PCBs, HCB, and chloroanilines, when only some of the chlorine substituents are removed and degradation of the remaining hydrocarbon compound does not occur. In these cases, adaptation and enrichment clearly indicate selective pressure for reductive dehalogenation per se. Such selective pressure could be positive (e.g., for use as a substrate) or negative (e.g., for detoxification).

The above examples of enrichment of reductive dehalogenation activity all involve aryl halide substrates. There are far fewer reports of serial transfers of enrichment cultures with alkyl halide substrates. The lack of enrichment studies is probably due to identification of numerous pure cultures able to dehalogenate alkyl halides (see below). Undefined cultures that dehalogenate PCE (73, 215) and dieldrin (134) have been maintained through serial transfers, and adaptation of continuous cultures (bioreactors) to alkyl halides does appear to have occurred in some cases (207). However, it is not clear that there was selection for a discrete dehalogenating population in those systems. From the available evidence, it is impossible to determine whether alkyl dehalogenation activity provides any selective advantage to organisms in undefined cultures.

Specificity

The above-mentioned acclimation and enrichment of cultures for aryl reductive dehalogenation usually involve some substrate specificity. Such specificity is for the type of aromatic compound (e.g., benzoate or phenol), for the ring position of the halogen (regiospecificity; i.e., *ortho*, *meta*, or *para*), and for the halogen atom removed (e.g., Cl, Br, or I). Cultures enriched for 3-chlorobenzoate (3CB) dehalogenation were able to dehalogenate bromine and iodine from the *ortho*, *meta*, and *para* positions of benzoate but were unable to dehalogenate a variety of other haloaromatic compounds (53, 95). Boyd and Shelton (26) acclimated different cultures to each of the three monochlorophenol isomers and found two distinct activities, one specific for the *ortho* and *para* positions and the other specific for the *meta* and *para* positions. Subsequent studies have found similar regiospecificity for phenol dehalogenation (33, 86, 144). Various marine sediment enrichment cultures had distinct specificities for chlorocatechols which they dehalogenated (5). These marine sediment cultures differed in the substrates used for enrichment, which were not chlorocatechols but instead were structural analogs of chlorocatechols. In other cases, lesser degrees of specificity have been found, as when Struijs and Rogers (194) found that dichlorophenols and dichloroanilines each caused acclimation for the other (cross-acclimation). Interestingly, in the last study, the cultures did not cross-acclimate for the monochlorinated products.

Various specificities in pathways of reductive dehalogenation have been observed for less polar aryl halides. Different patterns of PCB dechlorination yielding different product mixtures were observed in various cultures (Fig. 7) (17, 150, 166). These patterns resembled those previously elucidated for PCB dechlorination in natural sediments (30–32). The variation in dechlorination patterns of HCB (Fig. 8) resembles that found for PCBs. Various dehalogenation patterns were also observed for 3,4,5-trichlorocatechol (Fig. 9) (5).

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The above specificities have led to the hypothesis that the activities are catalyzed by distinct organisms with different dehalogenating enzymes. Specificities could reside at the level of enzymes, organisms, or broad physiological groups. However, until the individual organisms or communities are tested under identical conditions, the possibility remains that environmental factors also have a direct effect on patterns of product formation.

The substrate specificity of undefined anaerobic communities for aryl reductive dehalogenation appears to be in contrast to an apparent lack of specificity for alkyl reductive dehalogenation. This lack of specificity is suggested by several observations, including (i) the ubiquitous nature of alkyl reductive dehalogenation (Table 2), (ii) the broad range of alkyl halides dehalogenated by certain pure cultures (see Table 4), and (iii) the broad range of alkyl halides dehalogenated by cell-free systems (see Table 5). On the other hand, certain alkyl reductive dehalogenation reactions do exhibit substrate specificity. It appears that vicinal reduction of lindane and hydrogenolysis of dieldrin are catalyzed by different organisms since amino acids that support Stickland fermentations stimulated only the former activity (134). Other examples of alkyl dehalogenation specificity in pure cultures and cell-free systems are discussed below.

When polyhalogenated alkyl and aryl compounds are dehalogenated in undefined communities, a series of less chlorinated intermediates frequently accumulate transiently (14, 72, 73, 79, 82, 138, 194, 195, 198, 207, 214). Such sequential dehalogenation has the benefit for researchers of revealing degradation pathways and may have several explanations, including the following: (i) dehalogenation of the more halogenated congeners may be thermodynamically more favorable; (ii) the more-halogenated congeners may inhibit dehalogenation of the less-halogenated ones; and (iii) different organisms may catalyze the different dehalogenation steps. The first possibility has frequently been cited and is probably important, but it is difficult to prove in undefined systems. The other two possibilities imply specificity. Kinetic analyses support the second possibility in one system in which dichlorobenzoate apparently inhibited dechlorination of its monochlorobenzoate product (198). The third possibility seems likely in other cases, in which different populations appear to remove the different halogen substituents of phenols (26, 214).

Electron Acceptors

Electron acceptors are frequently the limiting resource for anaerobic communities and a major determinant of the species composition of anaerobic communities. Therefore, the presence of reductively dehalogenating organisms in a community may be affected by electron acceptors. Furthermore, it might be expected that the availability of electron acceptors affects the flow of electrons (i.e., availability of reductant) required for reductive dehalogenation. This effect might occur via intracellular channeling of electrons or via interspecific competition for electron donors. Accordingly, evidence which indicates that electron acceptors do affect dehalogenation activity in anaerobic communities is accumulating. However, this relationship appears to be complex.

Sulfate, an electron acceptor used by sulfate-reducing bacteria, can inhibit reductive dehalogenation. The laboratory of J. M. Suflita has examined two closely located sites within an aquifer contaminated by landfill leachate (18). The sites differed in being dominated by either methanogenesis or sulfate reduction. Only samples from the methanogenic site demonstrated the ability to dechlorinate and mineralize chloroanilines (116), chlorobenzoates (79), chlorophenols (79, 195), and chlorophenoxyacetates (79, 80). The potential for 2,4,5-trichlorophenoxyacetate dehalogenation existed at both sites, with sulfate apparently inhibiting dehalogenation in samples of the sulfate-reducing site, since addition of sulfate inhibited dehalogenation by samples from the methanogenic site and since depletion of sulfate by addition of acetate (as an electron donor) allowed dehalogenation by samples from the sulfate-reducing site. Additionally, amendment of aquifer slurries with nitrate or sulfate was found to inhibit debromination of the heterocyclic ring of bromacil (Fig. 10) (2). Amendment of aquifer slurries with sulfate did not prevent dechlorination of 2,3,4,5-tetrachloroaniline but did prevent further dechlorination of the trichloroaniline product (117). These data clearly indicate inhibition of dehalogenation by sulfate in this aquifer; however, caution should be used when extrapolating this conclusion to other habitats or other xenobiotic compounds.

In another case, it appears that sulfate does not inhibit reductive dehalogenation directly; rather, sulfate inhibits enrichment of dehalogenation activity. Kohring et al. (109) examined dechlorination of phenols by samples from freshwater sediments. Addition of nitrate to samples completely inhibited dechlorination of 2,4-dichlorophenol. Sulfate increased the adaptation time, decreased the rate of the initial dechlorination at the *ortho* position, and prevented dechlorination of the 4-chlorophenol product. However, when samples were first acclimated to 4-chlorophenol without sulfate, subsequently added sulfate was reduced to sulfide and had little effect on dechlorination of 4-chlorophenol.

Sulfate and nitrate do not always inhibit reductive dehalogenation. In a survey of a variety of sediments taken from river and estuary locations, Sharak Genthner et al. (180, 181) tested the effects of sulfate and nitrate on the degradation of monochlorobenzoates and monochlorophenols. Added sulfate and nitrate generally inhibited degradation by the samples, but there were several exceptions. An inhibitor of methanogenesis, bromoethane sulfonate (BES), also generally inhibited degradation by the samples. The effect of BES may imply that methanogenesis is required for degradation for various possible reasons; e.g., (i) methanogens simultaneously metabolize natural substrates and the xenobiotic compounds and (ii) the dehalogenating organisms are dependent on methanogens. However, the effect of BES on methanogenesis was not verified, and a direct effect on dehalogenation was not excluded. Sulfate and nitrate more consistently inhibited degradation in transfers of the original samples, but there were still notable exceptions. Transfers of one sample required nitrate for degradation of 3CB and 4-chlorophenol; another transfer was stimulated by sulfate in the degradation of 4-chlorophenol. All three monochlorophenols and 2,4-dichlorophenol could be degraded in river sediments incubated under sulfate-reducing conditions (84). 4-Chlorophenol was a transient intermediate of the dichlorophenol, suggesting that at least that compound was degraded via reductive dehalogenation. In these cultures an inhibitor of sulfate reduction, molybdate, inhibited chlorophenol degradation, implicating the direct or indirect involvement of sulfate-reducing bacteria in chlorophenol degradation.

Dehalogenation of 2,4-dibromophenol in marine sediment also presumably occurred concurrently with sulfate reduction (103). In this case molybdate did not affect debromination of 2,4-dibromophenol but did prevent degradation of the phenol product. The effect of the inhibitor on sulfate reduction was not verified, but the effect on phenol degradation

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probably indicates that sulfate reduction was blocked. It is not clear that, as the author suggests, sulfate reducers were not responsible for debromination, since the specific blockage of ATP sulfurylase by molybdate would not necessarily stop all other activities of these organisms.

Alkyl reductive dehalogenation also appears to have variable responses to electron acceptors and inhibitors of electron-accepting metabolic reactions. PCE was reductively dehalogenated in cultures that were either methanogenic (73) or sulfate reducing (12). Dehalogenation was complete (Fig. 5) in the methanogenic cultures but only partial in the sulfate-reducing ones. Activity was inhibited by BES in the methanogenic but not the sulfate-reducing cultures, suggesting that different organisms are responsible for dehalogenation of PCE under the two conditions. Pure cultures of methanogens are known to dehalogenate PCE (see Table 4), and so methanogens could reasonably be catalyzing dehalogenation in the methanogenic cultures. Dehalogenation of PCE by presumably methanogenic cultures inoculated with soil and subsoil was inhibited by nitrate, molybdate, and BES (179), yet in another study PCE was reductively dehalogenated in cultures inoculated with subsoil and provided with nitrate (25).

It is widely assumed, although not always true, that oxygen inhibits reductive dehalogenation. This assumption is based on the observation that reductive dehalogenation typically occurs in natural environments only if they are anaerobic and usually requires low redox potentials (reviewed in references 65, 115, and 178). However, this assumption has only rarely been confirmed in laboratory cultures (46, 95). Exceptions to inhibition by oxygen do exist, including reduction of hexachloroethane to PCE (apparent vicinal reduction) under presumably aerobic conditions (47) and aryl hydrogenolysis by pure cultures which are aerobic (below).

One caveat for studying the effects of electron acceptors on reductive dehalogenation is that an apparent lack of inhibition by an electron acceptor might be due to exhaustion of the electron acceptor followed by dehalogenation. This would be an example of an acclimation period due to diauxy (see above). This is possible because of the long-term incubations often used in such studies. One must therefore verify the presence of the electron acceptor before concluding that it does not inhibit dehalogenation.

The evidence available suggests that oxygen, nitrate, and sulfate most often inhibit dehalogenation by anaerobic communities but that the nature of such inhibition varies under different biological and chemical conditions. A critical factor in the response of communities to electron acceptors would be the presence or absence of populations capable of using the electron acceptors, since such populations might compete with dehalogenating populations for available electron donors. Additionally, direct inhibition of the dehalogenation process by electron acceptors appears to occur in some communities, and in other communities inhibition by electron acceptors is apparently growth dependent via selection for a nondehalogenating population. A nondehalogenating population might outcompete dechlorinators by virtue of a higher growth rate, but once established, certain dechlorinating populations apparently can compete successfully for electron donors. In a minority of cases tested, sulfate and nitrate did not inhibit dehalogenation or were even required for activity. The use of CO_2 as an electron acceptor by methanogens does not appear to inhibit reductive dehalogenation. Methanogenesis may even be required for dehalogenation in certain systems, possibly because of its involvement in syntrophic associations which sustain most anaerobic communities and thereby sustain certain physicochemical conditions. Although fewer undefined cultures that catalyze alkyl reductive dehalogenation have been studied, the effects of certain electron acceptors on both alkyl and aryl dehalogenation appear similar. The effects on dehalogenation of other catabolic electron acceptors such as iron and manganese have yet to be examined.

Other Nutrients

In contrast to the effects of electron acceptors, the effects of other nutrients on reductive dehalogenation have not been examined extensively. Such nutrients include electron donors; carbon, nitrogen, and phosphate sources; and micronutrients. In most studies of aryl reductive dehalogenation in which undefined communities were used, such nutrients were not added but were presumably supplied by the source material (e.g., sediment, sludge, or aquifer solids). Electron donors may have been rather recalcitrant and slowly degraded materials (e.g., humic matter). When halogenated compounds were completely degraded, they also provided electrons and carbon. In some cases, enrichment cultures were serially transferred, mineralizing haloaromatic compounds and using them as sole organic carbon and energy sources (71, 114, 149, 180, 194, 197, 214). Studies of alkyl reductive dehalogenation in undefined cultures have more frequently included addition of an organic substrate as a potential electron donor and carbon source.

Addition of various nutrients to undefined cultures can stimulate reductive dehalogenation activity or enrichment of that activity. Sugars, organic acids, amino acids, methanol, formate, and H_2 have been variously found to stimulate dehalogenation of chlorophenols (54), dieldrin (134), lindane (134), 4-chlororesorcinol (71), 2,4,5-trichlorophenoxyacetic acid (80), PCBs (149, 156), and PCE (12, 73). Undefined substrates such as yeast extract, trypticase, rumen fluid, sludge supernatant, and alfalfa have been variously found to stimulate dehalogenation of 4-chlororesorcinol (71) and DDT (107). In some cases added nutrients probably serve as electron donors for reductive dehalogenation. In other cases the nutrients may support the growth of the dehalogenating organisms. Undefined substrates may provide essential amino acids, vitamins, and trace elements. Additionally, if dehalogenation activity requires a population succession (e.g., to establish a low redox potential) or syntrophy (e.g., to provide vitamins or to prevent inhibitory product accumulation), nutrients might stimulate dehalogenation by supporting the growth of nondehalogenating organisms. Knowledge of the nutritional requirements of undefined, reductively dehalogenating communities will be essential for their use in bioremediation, although the complexity of undefined communities makes elucidation of these requirements difficult.

Temperature

Temperature is likely to affect reductive dehalogenation by having a direct effect on reaction rates and by exerting selective pressure on populations. Both effects were indicated in a study of lake sediment samples to which 2,4-dichlorophenol was added (108). Temperature affected both the acclimation period and the rate of dechlorination activity. Activity was found between 5 and 50°C, with distinct rate maxima at 30 and 43°C, suggesting selection for two distinct dehalogenating populations. A direct correlation of

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temperature and dehalogenation rate was found only from 15 to 30°C. Thus, it may be difficult to extrapolate laboratory rate measurements at higher temperatures to natural habitats which are below 15°C. In another study, PCB dechlorination was found to occur at 25°C and, more slowly, at 12°C but not at 37°C or above (200). The upper limits of temperature are consistent with the conclusion that reductive dehalogenation is catalyzed by living organisms. Clearly, low temperatures would severely limit dehalogenation rates in many habitats, but activity is possible at sediment temperatures commonly found in situ in temperate regions.

Substrate Availability

The hydrophobicity of many haloaromatic compounds affects their biological dehalogenation through its effect on their availability to microorganisms. The availability of substrates will have a direct effect on dehalogenation rates. In addition, because of the toxicity of many dehalogenation substrates, their availability may affect such toxicity, which, in turn, may affect dehalogenation rates. A large body of literature describes the various nonbiological fates of haloaromatic compounds in natural habitats (for general discussions, see references 42 and 88). Here we simply point out that fates such as sorption, volatilization, leaching, chemical hydrolysis, and photolysis will affect the aqueous concentrations and hence the first-order degradation rates of these compounds. Additionally, physical surfaces may juxtapose or isolate sorbed hydrophobic compounds and microorganisms attached to those surfaces.

Studies of the dehalogenation of very hydrophobic aromatic compounds in liquid cultures have involved the use of various carriers for these compounds, including a liquid organic phase (93), sediments (169), bark chips (8), and granular activated carbon. In the above studies, the carriers may have enhanced the dehalogenation of chemicals by microorganisms via the following means: (i) dispersal of insoluble chemicals (with a subsequent increase in the chemical surface area), (ii) reduction of dissolved concentrations below toxic levels, and (iii) concentration of dilute chemicals (with a subsequent increase in the chemical-microorganism contact). Dispersants have been developed for delivery of hydrophobic antibiotics. These are molecules designed to be water soluble but to have a hydrophobic interior region which may contain a hydrophobic compound. These and other compounds may have potential for use in studies of aerobic biodegradation of hydrophobic compounds. Studies of reductive dehalogenation have not yet addressed optimization of the availability of haloaromatic compounds.

Conclusion

Most of the studies of undefined cultures described above involved sealing natural samples in serum bottles and incubating them in the laboratory. Such experiments have logistical advantages and adequately address certain questions, but these cultures differ from in situ conditions in input of soluble nutrients, removal of soluble products, temperature, and other aspects. Such experiments indicate potential activities of organisms in the natural samples but do not necessarily indicate the fate of haloaromatic compounds in situ, as some researchers have suggested. Because of the complexity of the degradation pathways of alkyl solvents, it is especially difficult to predict their environmental fate. Empirical knowledge of the predictive values for natural and derived habitats requires experiments which more closely approximate those habitats or experiments performed in situ. The latter experiments have yet to be undertaken.

Studies with undefined cultures clearly indicate the potential of anaerobes to catalyze reductive dehalogenation. Such studies suggest that aryl, but not necessarily alkyl, reductive dehalogenation activity can confer a selective advantage to populations, allowing for their enrichment. Enriched populations typically demonstrate substrate specificity for aryl dehalogenation. Ecological factors, such as the availability of electron acceptors, the availability of other nutrients (particularly electron donors), and temperature, affect dehalogenation by undefined communities. Often results do not distinguish between direct effects of such factors on activity and effects on population selection. The observed effects of ecological factors are not always consistent among different inocula and different substrates; therefore, in these cases it is not yet reasonable to generalize conclusions. It seems likely that reductive dechlorination is catalyzed by physiologically diverse organisms in different anaerobic communities. In many cases a better understanding of ecological factors will require studies with pure cultures. Paradoxically, isolation of more anaerobes capable of aryl reductive dehalogenation probably depends on better understanding of their ecology.

Certain aspects of reductive dehalogenation will be understood only when the responsible organisms are studied in pure culture. Pure-culture studies may answer the following essential questions. (i) Which organisms have dehalogenation activity? (ii) Which enzymes and cofactors are responsible for the activity? (iii) What are the chemical mechanisms involved in activity? (iv) How do various factors directly affect activity? (v) How does the activity benefit (or harm) the responsible organisms? In addition to satisfying basic scientific interest, answers to such questions are of obvious importance in applications of reductive dehalogenation.

PURE CULTURES

The vast majority of pure cultures reported to catalyze reductive dehalogenation have activity on alkyl halides (Table 4). This suggests a fundamental difference between alkyl and aryl reductive dehalogenation. Only one strict anaerobe in pure culture capable of aryl reductive dehalogenation has been reported. This organism, *D. tiedjei* DCB-1, provides a singular opportunity to examine certain aspects of aryl reductive dehalogenation. Several efforts to isolate other anaerobes capable of aryl reductive dehalogenation have been reported, but so far these organisms remain elusive. Aryl reductive dehalogenation is also used by some aerobic bacteria for degrading certain substrates in which halogen substituents probably prevent oxidative degradation. Thus, at least some aryl reductive dehalogenation reactions are compatible with aerobic conditions.

Alkyl Dehalogenators

Organisms capable of alkyl reductive dehalogenation are numerous and phylogenetically diverse, encompassing eukaryotes and prokaryotes, including strict anaerobes, facultative anaerobes, and aerobes (Table 4). These organisms were not isolated by selection for reductive dehalogenation activity; rather, most were previously isolated cultures which were tested and found competent for dehalogenation. In some cases the organisms were isolated from undefined cultures with dehalogenation activity, but selection for dehalogenation per se was not necessarily involved. The possibility that alkyl dehalogenation confers a

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TABLE 4. Reductive dehalogenation in pure microbial cultures

Substrate	Culture	References
Alkyl halides		
3-Chloropropionate	*Clostridium* spp.	152
DDT	*Aerobacter aerogenes*	135
	Baker's yeast	100, 216
	Bacteria	99, 216
	Proteus vulgaris	13
	Serratia marcescens	136, 162, 189
	E. coli	120
	Actinomycetes	40
	Trichoderma viride	132
	Sewage sludge isolates	165
	Lake sediment isolates	133
	Algae	163
Dieldrin	*Clostridium* spp.	134
Haloethanes	*Acetobacterium woodii*	64
	M. thermoautotrophicum	19, 62, 64
	Methanococcus spp.	19
	Desulfobacterium autotrophicum	62
Haloethenes	*A. woodii*	64
	M. thermoautotrophicum	19, 62, 63
	Methanococcus spp.	19
	Methanosarcina spp.	68, 70
	D. tiedjei	70
Halomethanes	*A. woodii*	64
	Clostridium spp.	77
	D. autotrophicum	62, 64
	E. coli	46, 64
	M. thermoautotrophicum	62, 64
	Methanosarcina spp.	139
	Pseudomonas putida	37, 38, 119
Heptachlor	Bacteria	140
	Actinomycetes	140
Lindane	*Clostridium* spp.	128, 157
	Facultative anaerobes	98
	P. putida	131
Methoxychlor	*Aerobacter aerogenes*	136
Trichloroethane	*Clostridium* sp.	77
Aryl halides		
Chlorohydroquinones	*Flavobacterium* sp.	188
(PCP metabolites)	*Rhodococcus* spp.	9, 83
Chlorophenols	*D. tiedjei*	144
Halobenzoates	*Alcaligenes denitrificans*	203
	D. tiedjei	43, 124, 158

selective advantage to an organism apparently has not been tested. Organisms that catalyze the reduction of DDT to DDD are particularly diverse, and the activity appears common. When large numbers of bacteria were screened for DDT dechlorination, most tested positive (133, 165). Entire physiological groups may be capable of alkyl solvent dehalogenation. Using a limited sample size, Egli et al. (64) found a correlation between the use of the acetyl coenzyme A (acetyl-CoA) pathway by organisms and their ability to reductively dehalogenate chloromethanes. Methanogenic, homoacetogenic, and certain sulfate-reducing bacteria all use the acetyl-CoA pathway, and members of each group catalyze reductive dehalogenation. The acetyl-CoA pathway functions in CO_2 fixation or acetate oxidation and involves a methyl group transfer catalyzed by a corrinoid-containing enzyme system (further discussed under Transition Metal Complexes, below). Pure cultures reported to catalyze reductive dehalogenation of alkyl solvents include only anaerobes, but it is not clear whether this reflects a physiological limitation of aerobes or simply the failure to examine aerobes. Comprehensive studies relating alkyl reductive dehalogenation activity to phylogenetic groups or physiological features are lacking.

Substrate specificity. There appears to be limited substrate specificity for alkyl reductive dehalogenation, since organisms capable of this activity frequently have a wide substrate range (Table 4). Examples are *Methanobacterium thermoautotrophicum*, which dehalogenates methanes, ethanes, and ethenes (19, 62), and *Escherichia coli*, which dehalogenates DDT (120) and methanes (46). The variety of microorganisms capable of alkyl reductive dehalogenation and the broad substrate range of some of those organisms suggest that this activity is general and widespread. This would account for the observations that many alkyl halides are degraded in a broad range of reduced environments (Table 2) and that acclimation periods are not typically required for alkyl reductive dehalogenation activity (see above). Possible explanations for the frequency of this activity are discussed under Cell-Free Systems, below.

There are limits to the generalization that alkyl reductive dehalogenation is nonspecific. One exception appears to be the vicinal reduction of lindane (Fig. 2B). Jagnow et al. (98) have demonstrated that in several anaerobes this activity is bona fide vicinal reduction, rather than hydrogenolysis plus dehydrohalogenation. In the same study, this activity was specific to organisms capable of H_2 evolution by a ferredoxin-dependent system. In another study, 20 organisms that catalyze the reductive dehalogenation of DDT were unable to degrade lindane (162). Some specificity is also apparent in organisms capable of reductive dehalogenation of alkyl solvents, as in the ability of *M. thermoautotrophicum* but not *Desulfobacterium autotrophicum* to dehalogenate PCE (62). Unfortunately, there are few reports of substrate ranges for alkyl dehalogenation by particular organisms.

Products of alkyl solvents. Anaerobic bacteria form products from alkyl solvents which are a variable mixture of reductive dehalogenation products (from single or multiple dehalogenations), CO_2, and other (primarily unidentified) compounds. Interspecific differences may account for some of the observed differences in product mixtures. For example, $CHCl_3$ was a major product of CCl_4 in *D. autotrophicum* but only a minor product in *Acetobacterium woodii* (64). However, intraspecific differences in product mixtures, resulting from variable environmental conditions, can also occur. For example, the products of carbon tetrachloride degradation by *E. coli* varied depending on whether culture conditions favored metabolism which was aerobic, microaerophilic, fumarate respiring, or fermentative (46). The above differences in product formation have applied significance, since the less-toxic CO_2 would be preferred over partially dehalogenated compounds (e.g., vinyl chloride) as products in a bioremediation system. Recent progress toward understanding the formation of the product mixtures is described under Transition Metal Complexes, below.

An Anaerobic Aryl Dehalogenator, *D. tiedjei*

D. tiedjei DCB-1 has offered the only opportunity to study anaerobic reductive dehalogenation of aromatic compounds in pure culture. Results of studies of *D. tiedjei* in pure culture or in defined mixed cultures have implications for several unresolved questions arising from studies of reductive dehalogenation in undefined cultures (see above). The ability of *D. tiedjei* to gain energy for growth from reductive dehalogenation may have broad implications. This unusual organ-

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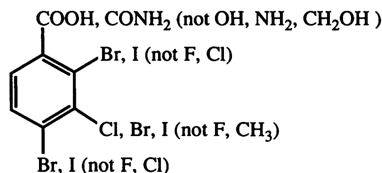

FIG. 11. Monohalogenated aromatic substrates for reductive dehalogenation by *D. tiedjei*. One of the substituents shown in the C-1 position is required, and the various halogen substituents shown can be removed. (Based on references 43, 53, and 185.)

ism also furthers our appreciation of microbial diversity. Recent studies have greatly improved our understanding of this organism, and because of the broad relevance of these findings we will discuss them in detail.

Dehalogenation activity. Aryl reductive dehalogenation by *D. tiedjei* exhibits substrate specificity. Dehalogenation activity is preferentially, but not exclusively, directed to the *meta* position of aromatic substrates (43, 144, 185). Monohalogenated benzoates and benzamides can be dehalogenated (Fig. 11). Monochlorinated phenols are not dehalogenated, but more highly chlorinated phenols are (144). The latter finding indicates the complexity of determining substrate ranges for aromatic dehalogenation. By simply testing monohalogenated compounds, one may overlook substrates, but there is a very large number of haloaromatic compounds (e.g., there are 19 chlorophenol congeners alone). *D. tiedjei* is also capable of the alkyl reductive dehalogenation of PCE (70). It is not yet clear whether the different dehalogenation activities of *D. tiedjei* share the same catalyst.

Using *D. tiedjei*, Dolfing and Tiedje (59) have examined the effects of substituents on dehalogenation rates of 3CB analogs in an attempt to establish structure-activity relationships which, if representative of other systems, could be of broad predictive value. Although substituents such as amino, chloro, hydroxyl, and methyl groups had clear effects on dehalogenation (generally inhibitory), these effects did not fit a general structure-activity model, based on the Hammett equation. It was concluded that the substituents probably affect not only the chemical reactivity of the substrates but also their uptake into the cells and their fit into enzyme active sites. This and other biological systems that catalyze reductive dehalogenation are probably too complex to understand and predict by consideration of the chemical reaction alone. Studies with purified enzymes, when available, would be free from some of this complexity.

The hydrogen atom which replaces the chlorine substituent during hydrogenolysis of 3CB by *D. tiedjei* appears to originate from water (81). When the reaction occurred in the presence of deuterated water, a single deuterium atom was incorporated at the C-3 position of the benzoate product and no deuterium was found at other positions. This observation is not consistent with a dechlorination mechanism involving proton exchange at other positions. Specifically, this observation would appear to rule out partial ring hydrogenation at ring positions *ortho* or *para* to the chlorine which is removed. The authors suggest that likely mechanisms would include, but not be limited to, direct electron addition and nucleophilic attack at the position of the chlorine substituent (Fig. 12). The former mechanism might involve transition metal-containing coenzymes (e.g., iron porphyrins, corrinoids, F_{430}), and the latter might involve a hydride ion or its equivalent (e.g., NADH).

Induction. Aryl reductive dehalogenation activity in *D. tiedjei* is inducible. Initially it was reported that dehalogenation activity in *D. tiedjei* was dependent on growth in the presence of 3CB (52). More recently *meta*-halobenzoates or analogs were found to specifically induce dehalogenation activity (Fig. 13) (44). There are a number of inducers which are not substrates (gratuitous), as well as a number of substrates which are not inducers (compare Fig. 11 and 13). Inducers must be *meta* substituted, but dehalogenation activity can also act at the *ortho* and *para* positions. Inducers can have certain *meta* substituents which are not transformed (e.g., F, CH_3, CF_3). The dehalogenation of chlorophenols appears to be coinduced with dehalogenation of benzoates and not to be induced by chlorophenols (144). Similarly, the alkyl dehalogenation of PCE appears to be coinduced with dehalogenation of benzoates and not to be induced by PCE (67). Thus, although *D. tiedjei* can dehalogenate 2-bromobenzoate, PCP, and PCE, these activities would have been undetected in tests for dehalogenation which did not include an inducer. Likewise, other dehalogenators may have gone undetected in studies which tested for dehalogenation of substrates that do not induce dehalogenation in those organisms.

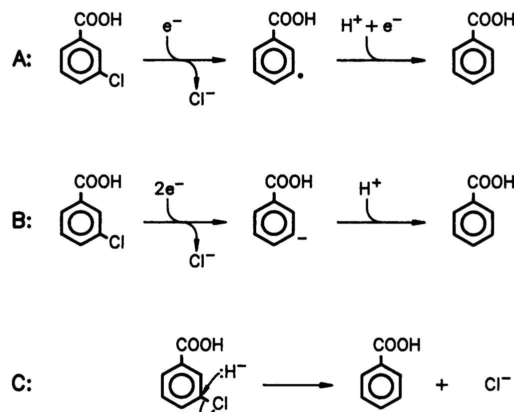

FIG. 12. Proposed reaction mechanisms for hydrogenolysis of 3CB. (A) Stepwise one-electron transfers with alkyl radical intermediate; (B) two-electron transfer with carbanion intermediate; (C) nucleophilic attack.

Inhibition. The relationship between dehalogenation and potential electron acceptors in the pure culture of *D. tiedjei* is complex, just as in the undefined cultures described above. Thiosulfate and sulfite inhibit dechlorination of 3CB by growing cells (53, 124) and by resuspended cells (50). Sulfate inhibits dechlorination of 3CB by cells growing on one medium (124) but not by cells growing on a different medium (43) or by resuspended cells (50). The latter study found that both 3CB and sulfoxy anions could support H_2 consumption by resuspended cells, the H_2 consumption rate

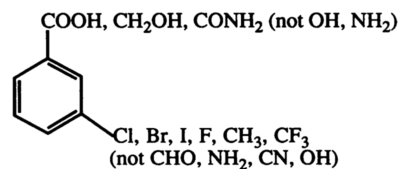

FIG. 13. Inducers of reductive dehalogenation activity in *D. tiedjei*. One of the substituents shown in both the C-1 and C-3 positions is required. (Based on reference 43.)

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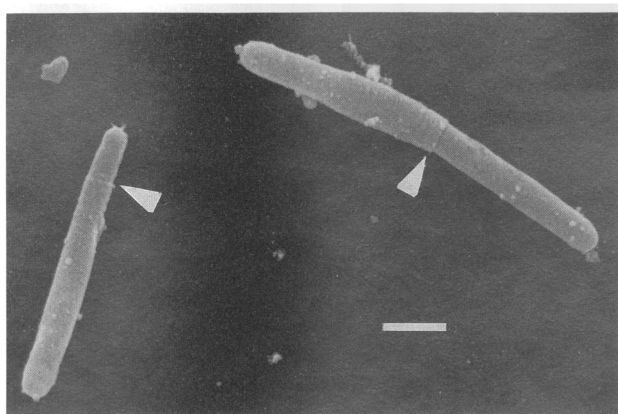

FIG. 14. Scanning electron micrograph of *D. tiedjei* showing a collar structure (arrows). Bar, 1 μm.

being higher with sulfoxy anions. With both electron acceptors present, an intermediate rate of H_2 consumption was observed, suggesting to these authors that dechlorination and sulfoxy anion reduction are enzymatically distinct pathways which compete for limited electron donors. The above results suggest that inhibition of dehalogenation by thiosulfate and sulfite observed in undefined cultures may occur via intraspecific channeling of electrons to the different electron acceptors. However, if dehalogenating organisms in such undefined cultures resemble *D. tiedjei*, inhibition by sulfate must involve interspecific competition for electron donors.

Morphology. The morphology of *D. tiedjei* is unlike that of any previously described organism. The organism is a large, nonmotile rod with a unique feature, a collar which girdles each cell (Fig. 14). The large size of this organism distinguished it in the enrichment culture from which it was isolated and was the key to its isolation (183, 185). The involvement of the large rod in dehalogenation was implicated because that organism was more abundant in the enrichment culture if it was fed 3CB than if it was fed benzoate. Colonies in roll tubes were therefore microscopically examined for the large rod. Eventually the large rod was found in very slow-growing pinpoint colonies and transferred to liquid medium. It was later realized that the rod was isolated on a substrate(s) provided by undefined medium components, not on the substrate for dehalogenation, 3CB. After some optimization of the medium, the organism dehalogenated 3CB. This experience reveals two related points which may be critical to isolation of organisms with functions of interest. First, it may be possible to track the organism by using a feature other than the function of interest, and second, it may be necessary to separately verify the success of the isolation step since conditions required for the function of interest may not be understood. Although the collar was not used to track *D. tiedjei* during its isolation (it had not yet been observed), we have since used this feature to identify *D. tiedjei* or possibly related organisms in other dehalogenating enrichment cultures (43, 143).

Syntrophy. Studies of *D. tiedjei* suggest possible reasons why aryl reductive dehalogenation is found most readily in complex communities and why other anaerobes capable of aryl reductive dehalogenation are difficult to isolate. Initially *D. tiedjei* could be cultured only on an undefined medium including rumen fluid, and pyruvate was the only substrate found to stimulate growth (185). However, Dolfing and Tiedje (56) were able to recombine three organisms from the

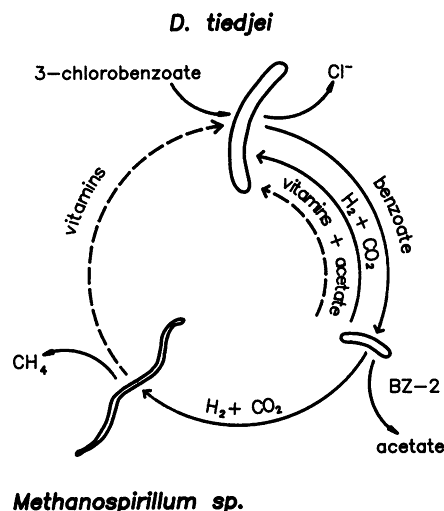

FIG. 15. Syntrophic relationships in a defined consortium growing on 3CB. Solid arrows show demonstrated nutrient flow, and dashed arrows show hypothesized nutrient flow. (Based on references 7, 50, 56, 58, 185, and 197.)

original enrichment source of *D. tiedjei* in a stable, defined consortium which dechlorinated 3CB and used it as the sole organic substrate in defined medium. In addition to *D. tiedjei*, the consortium contained a benzoate-fermenting rod (BZ-2) and an H_2-consuming methanogen (*Methanospirillum* sp.). The three organisms participated in a syntrophic relationship (Fig. 15), with *D. tiedjei* dehalogenating 3CB, BZ-2 fermenting the benzoate intermediate, and both *D. tiedjei* and the *Methanospirillum* sp. using the H_2 product of benzoate fermentation. Benzoate fermentation was dependent on a low H_2 partial pressure, which was maintained by H_2 consumption. The H_2 was partitioned between *D. tiedjei* and the *Methanospirillum* sp., the former using H_2 as an electron donor for dehalogenation and the latter using it for methanogenesis. There was apparently no significant competition for H_2 between the two organisms; rather, in combination they stimulated the rate of benzoate fermentation and consequent H_2 production (60). Such neutralism is consistent with the occurrence of reductive dehalogenation in numerous methanogenic undefined cultures (see above).

Dechlorination by *D. tiedjei* in pure culture on defined medium was possible only after the vitamin requirements were determined. DeWeerd et al. (51) found the vitamins hematin, lipoic acid, 1,4-naphthoquinone, nicotinamide, and thiamine to be stimulatory or, possibly, required by *D. tiedjei*. It was later found by Apajalahti et al. (7) that growth on pyruvate could occur in a defined medium but that dehalogenation activity required a factor which could be provided by rumen fluid, a *Propionibacterium* sp. in coculture, or the culture fluid of the *Propionibacterium* sp. The factor in the culture fluid was extractable, and its chemical properties suggested that it was a quinoid compound. The factor could be replaced by 1,4-naphthoquinone or menadione (vitamin K_3). Thus, naphthoquinone or an analog appears to be required for dehalogenation activity and was presumably provided in the above-mentioned culture systems by (i) rumen fluid, (ii) the *Propionibacterium* sp., and (iii) either the benzoate fermentor or the H_2-consuming methanogen. Whether the other vitamins mentioned are required for growth or dehalogenation activity remains to be determined.

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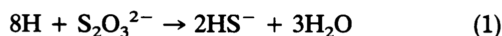
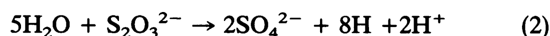
D. tiedjei living in natural habitats probably depends on other organisms to furnish a number of other factors in addition to vitamins. First, as an obligate anaerobe, *D. tiedjei* requires a reduced, oxygen-free environment created by other organisms. As described below, *D. tiedjei* uses electron donors which probably originate as end products of other anaerobes. Finally, products of *D. tiedjei* which at high concentrations are toxic to the organism, such as sulfide (see below) and benzoate (185), may be removed by other organisms. Thus, *D. tiedjei* conforms to the general rule of syntrophy in anaerobic ecosystems. The niche of this organism might be as a scavenger which relies on electron donors and vitamins from other organisms and exploits a broad range of electron acceptors (below). Such a niche would exist in most anaerobic communities but probably would support only relatively low populations. Low populations of aryl-dehalogenating organisms would be consistent with the requirement of long acclimation periods before aryl dehalogenation is detectable.

Physiology. In general, *D. tiedjei* appears to be relatively versatile in the use of electron acceptors but not so in the use of electron donors. Growth of the organism is stimulated by sulfoxy anions (191). *D. tiedjei* is a bona fide sulfate-reducing bacterium (145), with the usual ability to reduce sulfate or thiosulfate to sulfide as well as the ability, in the absence of a suitable electron donor, to gain energy from the disproportionation of thiosulfate to sulfide plus sulfate (equations 1 to 3):



$$8H + S_2O_3^{2-} \rightarrow 2HS^- + 3H_2O \quad (1)$$



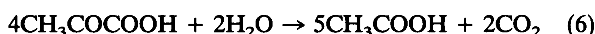
$$5H_2O + S_2O_3^{2-} \rightarrow 2SO_4^{2-} + 8H + 2H^+ \quad (2)$$

$$2H_2O + 2S_2O_3^{2-} \rightarrow 2HS^- + 2SO_4^{2-} + SH^+ \quad (3)$$

Growth by the latter lithotrophic fermentation is apparently possible for only a few of the sulfate reducers presently in pure culture (110). *D. tiedjei* grows mixotrophically on pyruvate plus CO_2 (192) by an unusual fermentation (145). The fermentation involves the oxidation of pyruvate to acetate plus CO_2 (equation 4) and the reduction of CO_2 to acetate (equation 5), with the oxidative and reductive processes balancing one another (equation 6):

$$4CH_3COCOOH + 4H_2O \rightarrow 4CH_3COOH + 4CO_2 + 8H \quad (4)$$

$$8H + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (5)$$



$$4CH_3COCOOH + 2H_2O \rightarrow 5CH_3COOH + 2CO_2 \quad (6)$$

Thus, CO_2 serves as an electron acceptor in this process. In many respects this resembles the terminal steps in homoacetogenic fermentation of sugars; however, *D. tiedjei* is unable to use sugars. Like homoacetogens, *D. tiedjei* has carbon monoxide dehydrogenase activity and is believed to employ the acetyl-CoA pathway for CO_2 reduction. Unlike many homoacetogens, *D. tiedjei* cannot grow on H_2 plus CO_2 or formate plus CO_2.

Reductive dehalogenation is yet another reaction exploited by *D. tiedjei* for energy metabolism. As mentioned above, Dolfing and Tiedje (56) constructed a defined consortium, including *D. tiedjei*, which used 3CB as the sole organic substrate. Growth of the consortium was stimulated by dechlorination per se (57). Subsequently, dechlorination was found to support the growth of *D. tiedjei* in pure culture (55, 146) and to support ATP synthesis in stationary-phase cultures which were limited by 3CB (55). If *D. tiedjei* is representative of other dehalogenating anaerobes and they can also gain energy from dehalogenation, this novel form of chemotrophy may account for the above-mentioned enrichment of cultures on substrates which are reductively dehalogenated but are not further metabolized (i.e., dehalogenation itself supported growth).

The possibility that dehalogenation of other substrates can support energy metabolism is supported by the calculations of Dolfing and Harrison (61). Free energies of formation were calculated for benzenes, phenols, and benzoates. These data indicate that, of all those compounds, 3CB has among the lowest energy yields from reductive dehalogenation. Thus, dehalogenation of most of these compounds can theoretically support growth.

The use of dehalogenation for energy metabolism bears on the fundamental question of the evolutionary origin of reductive dehalogenation activity. This activity may have been independently selected or may be coincidentally catalyzed by an enzyme(s) evolved for a different activity (fortuitous dehalogenation). Natural selection for this activity is possible because of the existence of naturally occurring haloaromatic compounds (see above). The ability of *D. tiedjei* to use dehalogenation for energy metabolism indicates one selective advantage of this activity; detoxification might be another. The specific induction of dehalogenation by *D. tiedjei* suggests that at least the regulation of this activity has been selected. The apparent distinction between the enzymatic pathways of dehalogenation and sulfoxy anion metabolism by *D. tiedjei* indicates that the former activity is not simply a fortuitous consequence of the latter. The evidence is not conclusive, but it suggests that, for *D. tiedjei*, dehalogenation per se may have been selected.

In *D. tiedjei* energy conservation from reductive dehalogenation of 3CB (Fig. 1A) appears to be via a respiratory process. Dechlorination can be coupled to formate oxidation (146) and H_2 oxidation (50, 60). Since formate and H_2 are not known to support substrate-level phosphorylation, energy conservation from metabolism of formate or H_2 plus 3CB would presumably have to be respiratory. Further evidence now indicates such a respiratory mechanism (147). Dehalogenation directly supports ATP synthesis in suspended cells. Dehalogenation supports proton translocation by the cells and hence formation of a proton motive force. Negative effects of respiratory inhibitors on the efficiency of dechlorination-dependent ATP synthesis suggest that a proton motive force couples dechlorination and ATP synthesis via a proton-driven ATPase. This evidence indicates the presence of chemiosmotic coupling of dechlorination and ATP synthesis according to the principles illustrated in Fig. 16. In this model the arrangement of formate dehydrogenase and the dechlorinating enzyme results in vectorial proton extrusion from the cell. The resulting proton motive force supports ATP synthesis via a proton-driven ATPase. The model is consistent with the data currently available; however, the arrangement of the enzymes is entirely speculative, and other arrangements could serve the same function equally well. Localization within the cell of the required respiratory components or development of an in vitro system capable of dechlorination-dependent ATP synthesis would greatly further our understanding of this novel respiratory process.

Like other sulfate reducers, *D. tiedjei* appears to have several respiratory electron carriers. Desulfoviridin and cytochrome c_3 were isolated from *D. tiedjei* (51). In addition to their probable role in sulfate reduction, these electron car-

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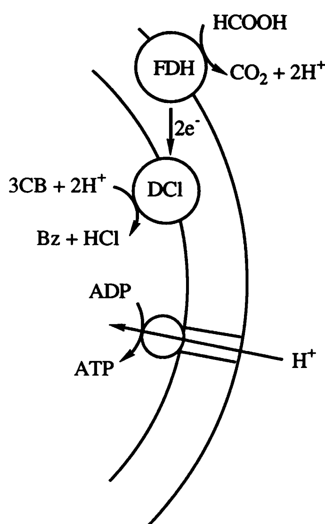

FIG. 16. Respiratory model illustrating how a proton motive force might couple reductive dehalogenation and ATP synthesis by a proton-driven ATPase. Abbreviations: Bz, benzoate; DCl, dechlorinating enzyme; FDH, formate dehydrogenase.

riers may participate directly in dehalogenation or in respiratory energy conservation from dehalogenation. The abovementioned requirement for 1,4-naphthoquinone or menadione for the dehalogenation activity of *D. tiedjei* suggests the possible involvement of a menaquinone in that process. It has not been reported whether *D. tiedjei* has the same requirement for sulfoxy anion metabolism; however, the vitamin stimulates growth on pyruvate plus thiosulfate (51).

D. tiedjei appears to have a relatively limited range of electron donors. In addition to pyruvate, formate, and H_2 (mentioned above), this organism oxidizes CO, lactate, butyrate (145), 3- and 4-methoxybenzoates and their derivatives, and benzoate (51). Acetate can also be used as an electron donor, with haloaromatic compounds (58) and possibly sulfoxy anions (145) as electron acceptors; however, it is not clear that such oxidation of acetate supports growth. Oxidation of methoxybenzoates is via O-demethylation to the corresponding hydroxybenzoates (53), which are not further degraded (145). The latter activity is characteristic of organisms that use the acetyl-CoA pathway. The electron donor range of *D. tiedjei* is typical of sulfate-reducing bacteria and, as mentioned above, could allow *D. tiedjei* a terminal position in anaerobic food chains, oxidizing products of fermentative organisms.

Taxonomy. DeWeerd et al. (51) determined the 16S rRNA sequence of *D. tiedjei*, which clearly indicates that the organism is a member of the delta subdivision of the class *Proteobacteria* (purple bacteria). The degree of distance between *D. tiedjei* and *Desulfovibrio desulfuricans* was greater than between *D. tiedjei* and *Desulfuromonas acetoxidans* (an elemental sulfur-reducing bacterium) or *Desulfobacter* spp. It was concluded from the 16S rRNA sequence that *D. tiedjei* represents a new genus among the sulfate-reducing bacteria. However, comparisons were made with only three other sulfate reducers, and the phylogenetic position of *D. tiedjei* within that group remains unclear. The unique physiological characteristics of *D. tiedjei* (see above) and the sequence analysis led DeWeerd et al. (51) to assign the name *Desulfomonile tiedjei* gen. nov. and sp. nov. to the organism, previously called strain DCB-1.

Attempts To Find Other Aryl Dehalogenators

Although studies of *D. tiedjei* have greatly aided our understanding of aryl reductive dehalogenation, it is risky to base generalizations on a single model. Hence, efforts to isolate additional aryl dehalogenators are very important. One strategy for finding such organisms is to examine existing pure cultures with physiological similarities to *D. tiedjei*. Another strategy is to isolate organisms from dehalogenating enrichment cultures by selecting for physiological similarities to *D. tiedjei*. So far, these strategies have been unsuccessful. Other strategies have yielded highly enriched cultures capable of aryl reductive dehalogenation, but the dehalogenating organisms have proven difficult to isolate.

Sulfate-reducing bacteria. After *D. tiedjei* was identified as a sulfate-reducing bacterium, a variety of other sulfate reducers were tested for the ability to reductively dehalogenate haloaromatic compounds. Linkfield (125) tested three *Desulfovibrio* spp. for dechlorination of 3CB under sulfate-reducing conditions. Later, 10 *Desulfovibrio*, *Desulfobacter*, *Desulfobacterium*, and *Desulfococcus* species were tested (143). In the latter test, conditions used were based on optimal conditions for *D. tiedjei* (i.e., in the presence of required electron donors and vitamins and in the absence of competitive electron acceptors); the substrates tested included halobenzoates and halophenols. No dehalogenation activity was detected. With the caveat that not all possible culture conditions could be tested, it appears that dehalogenation activity is not a general property of sulfate reducers. It should also be noted that a major group, gram-positive sulfate reducers, has not been tested.

New isolates. Numerous isolation strategies based on physiological characteristics of *D. tiedjei* were used in an attempt to obtain dehalogenators from four different enrichment cultures growing on halobenzoates and a chlorophenol (143). More than 114 isolates were obtained and tested for dehalogenation activity. The only dehalogenating organism thus obtained came from the same enrichment culture as *D. tiedjei* and was indistinguishable from *D. tiedjei* on the basis of morphology and limited physiological characteristics. The new isolate was obtained on medium selecting for the ability to grow diazotrophically on pyruvate plus thiosulfate. Other isolates were selected for growth on pyruvate, pyruvate plus rumen fluid, rumen fluid, thiosulfate (by disproportionation), formate plus halobenzoates, or methoxybenzoate plus thiosulfate. These negative results suggest that, in the enrichment cultures from which *D. tiedjei* was not isolated, the dehalogenating organisms are physiologically distinct from *D. tiedjei*. Isolation of dehalogenating organisms from PCB-dehalogenating enrichment cultures was also attempted (150). Six isolates capable of growth on H_2 (five with and one without sulfate) were tested for PCB dechlorination activity, but no dechlorination was observed. The above strategies failed to select novel dehalogenating organisms. Direct selection for dehalogenation-dependent growth appears to be a more promising strategy; however, low growth rates will probably make this difficult.

Highly enriched cultures. Other workers report being very close to obtaining anaerobic aryl dehalogenators in pure culture. Zhang and Wiegel (214) have recently reported the establishment of a stable culture which reductively dechlorinates 2,4-dichlorophenol to 4-chlorophenol without further degrading this product. The dechlorinating organism is believed to be a spore-forming rod (because it resists pasteurization) but has not yet been obtained in pure culture (48). Sharak Genthner et al. (182) report a gram-positive curved

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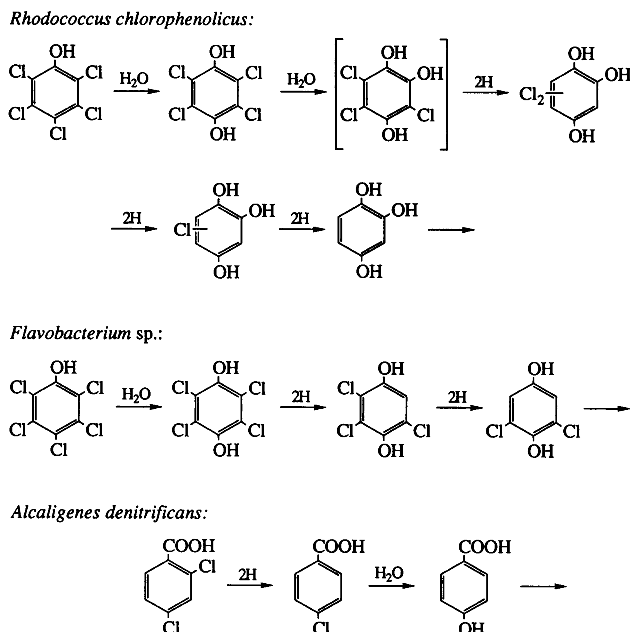

FIG. 17. Initial steps in aerobic degradation pathways which include reductive dehalogenation reactions. (Based on references 9, 188, and 203.)

rod that is presumably able to dehalogenate 3CB. The organism was obtained by serial dilution of an enrichment culture but has yet to be isolated as a single colony. The curved rod is dependent on a second organism, or spent medium of the second organism, for growth and dehalogenation activity on pyruvate-containing medium. Dietrich and Winter (54) report a spirochete which appears to catalyze *ortho* dehalogenation of phenols, including 2-bromo-, 2-chloro-, 2,6-dichloro- and 2,6-dichlorophenols. This spirochete appeared to confer dehalogenation activity to an enrichment culture. The dehalogenation activity required undefined medium components, yeast extract, and peptone. The culture used *n*-butyrate, or other organic acids, as a cosubstrate and electron donor for dehalogenation. The spirochete has not been isolated.

Aerobic Aryl Dehalogenators

Reductive dehalogenation also appears to be used by aerobic bacteria in the degradation of highly chlorinated aromatic rings which are invulnerable to ring-cleaving oxygenases. Although such organisms live in oxidized environments, their cytoplasm probably has a low redox potential and would be favorable for reductive reactions. Aryl reductive dehalogenation occurs in aerobic degradation of at least two compounds, but few further details have been reported.

Pentachlorophenol. The pathways of aerobic mineralization of PCP by a *Flavobacterium* sp. (188), *Rhodococcus chlorophenolicus* (9), and another *Rhodococcus* sp. (83) involve reductive dehalogenation steps. The degradation pathways differ between the first and the last two organisms, but they both begin with hydrolytic dechlorination(s) followed by reductive dechlorinations (Fig. 17). Chlorophenols are required to induce their own degradation by either organism, but for the *Flavobacterium* sp., only the initial hydroxylation activity is induced and the reductive dechlorination activities appear to be constitutive (212). This may also be the case for *R. chlorophenolicus*. The dechlorination activities of cell extracts of both organisms have been studied (see below).

Dichlorobenzoate. A facultative anaerobe, *Alcaligenes denitrificans*, also uses both hydrolytic and reductive dechlorination reactions for the aerobic mineralization of 2,4-dichlorobenzoate (203). It was proposed that reductive dechlorination preceded hydrolytic dechlorination (Fig. 17), although the evidence admits the possibility that the order is reversed. The consumption of 2,4-dichlorobenzoate, presumably including the reductive dechlorination reaction, was catalyzed by cells grown on 4-iodo-, 4-bromo-, and 4-chlorobenzoates. Thus, the reductive dechlorination activity was not specifically induced by its substrate. Whole cells of *A. denitrificans* required O_2 for the dechlorination reactions. This requirement may indicate that energy is required directly or indirectly for dehalogenation activity, but other explanations cannot be ruled out until the activity in cell extracts is examined. Regardless, *A. denitrificans* and the other aerobes described above would experience a net gain of energy from complete degradation of aromatic compounds.

Conclusion

An examination of the pure cultures reported to catalyze reductive dehalogenation suggests a fundamental difference between alkyl and aryl dehalogenation, the latter activity being more widespread and less substrate specific. We lack comprehensive and systematic studies of the occurrence of reductive dehalogenation activity in various microorganisms and of the substrate range for that activity in particular microorganisms. Such studies are difficult since negative results may only indicate oversights in the experimental method resulting from insufficient information about the activity being tested.

Many aspects of *D. tiedjei* are consistent with undefined cultures which catalyze aryl reductive dehalogenation. If this organism is representative of other aryl dehalogenators, several inferences about aryl reductive dehalogenation are possible: (i) aryl reductive dehalogenation is probably catalyzed by inducible proteins with distinct substrate specificity; (ii) aryl dehalogenators are adapted to living in syntrophic communities and may therefore be difficult to isolate; and (iii) aryl dehalogenators can be enriched because they use reductive dehalogenation for energy metabolism. The selective advantage conferred by dehalogenation may aid in exploitation of the activity by humans for bioremediation. Aryl reductive dehalogenation can also occur under aerobic conditions and is apparently used by aerobic bacteria in degradation of compounds resistant to oxidative attack as a result of the presence of halogen substituents. The frequency of this aerobic activity and its substrate range are not known.

CELL-FREE ACTIVITY

The study of reductive dehalogenation in cell-free systems gives insight into the cell components that might catalyze the activity and the reaction mechanism by which they do so. Studies of cell-free systems suggest that reductive dehalogenation may represent both enzymatic and nonenzymatic activities and both selected and fortuitous activities. Reductive dehalogenation has been found in a number of cell-free systems including cell extracts, a purified enzyme, and transition metal complexes (Table 5).

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TABLE 5. Reductive dehalogenation in cell-free biological systems

Substrate	Cell-free system	References
Alkyl halides		
DDT	*Aerobacter aerogenes* extract	209, 210
	E. coli extract	75
	Hydrogenomonas sp. extract	164
	Ferroheme	35, 142, 216
	Flavin	65
	Vitamin B_{12}	21
	P-450 cam enzyme of *P. putida*	38
Halomethanes	Ferroheme	37, 38, 78, 105
	Corrinoids	78, 112, 113
	F_{430}	78, 111
	P-450 cam enzyme of *P. putida*	38
Haloethanes	Ferroheme	105
	Methanosarcina barkeri extract	92
Haloethenes	Hematin	78
	Vitamin B_{12}	78
	F_{430}	78
Lindane	*Clostridium rectum* extract	158
	Clostridium sphenoides extract	90
Mirex	Hematin	94
Toxaphene	Hematin	101, 174
Aryl halides		
Chlorohydro-quinones	*Flavobacterium* sp. extract	212
(PCP metabolites)	*Rhodococcus* spp. extract	9, 83
Halobenzenes	Hematin	78
	Vitamin B_{12}	78
	F_{430}	78
Halobenzoates	Cell extract of *D. tiedjei*	52
PCP	Vitamin B_{12}	78

Cell Extracts

Cell extracts from bacteria have been found to catalyze reductive dehalogenation of DDT (75, 164, 209, 210), lindane (90, 158), chloroethanes (92), halobenzoates (52), and chlorohydroquinone intermediates of aerobic PCP mineralization (9, 83, 212). Most of these systems have not been extensively characterized, nor have the activities been further purified. Four distinct dechlorination reactions of DDT appear to be catalyzed by discrete enzymes with different responses to temperature, pH, and inhibitors (210). Usually activity was found to be heat labile, suggesting the possible involvement of an intact protein structure. However, Holliger et al. (92) found that heat did not destroy the reductive dechlorination of dichloroethane by *Methanosarcina barkeri* extract. In this case, a strong reductant, Ti(III), was provided. In most of the above cases in which activity was found to be heat labile, reductants were not provided, and it is possible that heat damaged an enzymatic system providing reductant and did not damage the dehalogenation catalyst per se. Catalysis of reductive dehalogenation by nonprotein transition metal complexes is discussed below. Activity in some cell extracts is heat labile even when reductants are provided, as with dechlorination of hydroquinones (9, 83, 212) and benzoates (52). These observations suggest that dehalogenation of these aromatic substrates is catalyzed by proteins.

Despite general similarity in pathways of aerobic PCP degradation by the *Flavobacterium* sp. and *R. chlorophenolicus* (see above), it is not yet clear how similar are the reductive dechlorination activities of each organism. Reductive dechlorination in cell extracts from both organisms has been studied (9, 212). Extracts from the *Flavobacterium* sp. catalyze the removal of two chlorine substituents from tetrachloro-*p*-hydroquinone via reductive dechlorination. Extracts from *R. chlorophenolicus* catalyze the removal of all four chlorine substituents from tetrachloro-*p*-hydroquinone, one via hydroxylation and three via reductive dechlorination. However, like extracts from the *Flavobacterium* sp., extracts from *R. chlorophenolicus* catalyze the removal of only one chlorine substituent from trichloro-*p*-hydroquinone. From the available evidence, it is difficult to establish whether these reductive dechlorination activities of the two organisms are distinct or whether other activities (e.g., hydroxylation) account for the different PCP degradation pathways observed. Both extracts catalyzed reductive dehalogenation under anaerobic conditions. Extracts of *R. chlorophenolicus* also did so in the presence of oxygen. Chlorophenols specifically induced their own degradation by both organisms. Reductive dechlorination of trichloro-*p*-hydroquinone is inducible in *R. chlorophenolicus*, but reductive dechlorination of *p*-hydroquinones appears to be constitutive in the *Flavobacterium* sp. Thus, only the initial hydroxylation step of the PCP degradation pathway appears to be inducible in the *Flavobacterium* sp. Ascorbic acid was required for tetrachloro-*p*-hydroquinone degradation by the *R. chlorophenolicus* extract and so may have served as the electron donor for dechlorination by this extract. Glutathione, and not ascorbic acid, was specifically used as the electron donor for dechlorination by the *Flavobacterium* extract. Characteristics of the above two cell extracts differ significantly, but variations in preparation of these extracts may account for some of the differences.

***D. tiedjei* extract.** DeWeerd and Suflita (52) have obtained a *D. tiedjei* cell extract with reductive dehalogenation activity. Thus far, the dehalogenation activity of this extract is consistent with that of whole cells. This extract has the same substrate specificity for benzoates as do whole cells, removing I and Br from all positions but removing Cl from only the *meta* position (Fig. 11). The dehalogenation rate of the extract increases with the size of the halogen; however, this was not the case for whole cells (124). It was not reported whether benzamides or ethenes are also substrates for cell-free activity, as they are for whole cells. As in whole cells (above), cell-free dehalogenation activity appears to require induction. Purification of dehalogenation activity in the crude extract would be of interest, among other reasons, to determine whether the dehalogenation activities observed in *D. tiedjei* are due to a single enzyme.

Dehalogenation by the above cell extract was dependent almost entirely on methyl viologen, which could not be replaced by a number of biological electron carriers tested, including reduced NAD, NADP, flavin adenine dinucleotide, flavin mononucleotide, desulfoviridin, and cytochrome c_3. Methyl viologen has the lowest redox potential of all the cofactors tested, suggesting that the cofactor in vivo may have a similarly low redox potential. H_2, formate, and CO were found to support the reduction of methyl viologen by the cell extract and to stimulate dehalogenation by the extract in the presence of methyl viologen. These findings are consistent with the above-mentioned abilities of whole cells of *D. tiedjei* to use these electron donors for growth and to couple formate or H_2 oxidation to dechlorination. Thiosulfate and sulfite inhibit cell-free dehalogenation activity, whereas sulfate does not, as observed with whole cells (50).

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Kinetic experiments determining the nature of this inhibition might be very useful in resolving the relationship between sulfoxy anion metabolism and dehalogenation, especially if the dehalogenase can be purified. The cell-free dehalogenation activity appears to be membrane associated. This location may be significant to the respiratory system apparently involved in dehalogenation-dependent ATP synthesis (see above).

A Purified Enzyme, P-450 cam

P-450 cam, a previously purified enzyme containing an iron porphyrin, was found to dehalogenate halomethanes (38). It should be noted that the similar mammalian cytochrome P-450 also catalyzes reductive dehalogenation (reviewed by Macdonald [127]). The major function known for P-450 cam is the catalysis of camphor hydroxylation, allowing *Pseudomonas putida* to grow on that substrate. Dehalogenation by P-450 cam appears to be a clear example of a fortuitous activity, since it is very unlikely that the enzyme evolved from selective pressure for DDT dechlorination activity. This study of P-450 cam compared the activities of a deuteroheme (an active-site model), the pure enzyme, and whole cells. This enzyme did appear to be responsible for the activity observed in vivo. It was found that the enzyme and whole cells had lower dechlorination rates and reduced substrate ranges, apparently because of steric effects on substrate binding and uptake limitations. This is a clear example of how the biological environment of the catalyst may affect reductive dehalogenation activity and hence may complicate attempts to establish structure-activity relationships which model that activity (above).

Transition Metal Complexes

Transition metal complexes common to many enzymes have been found, in the presence of a reductant, to catalyze alkyl and some aryl dehalogenations. These findings strongly suggest that such complexes are involved in dehalogenation in living systems. However, conclusions based on cell-free systems must be extrapolated to living systems with caution because of the above-mentioned differences in steric and uptake limitations. Vogel et al. (206) summarize proposed mechanisms for both dehydrogenolyses and vicinal reductions catalyzed by transition metals. Essentially, both involve a one-electron transfer from the transition metal to a halo-substituted carbon atom, forming an alkyl radical and releasing a halide ion (Fig. 18). If the alkyl radical scavenges a hydrogen atom from its environment, or if another one-electron transfer occurs in the presence of a proton, dehydrogenolysis results. If a carbon adjacent to the alkyl radical is also halo-substituted and similarly forms an alkyl radical, the two carbon atoms can form an additional bond and vicinal reduction results.

Iron porphyrins. The ability of ferrohemes [Fe(II) porphyrins] to reductively dechlorinate DDT was recognized as early as 1964 (35). Ferrohemes can catalyze alkyl reductive dehalogenation of a number of compounds including DDT (35, 142, 216), mirex (94), toxaphene (101, 174), and alkyl solvents (37, 38, 78, 105). For halomethanes, evidence supports the above reaction mechanism involving a one-electron transfer (Fig. 18) (38). The frequency of heme-containing enzymes in microorganisms is consistent with the large number of undefined communities and pure cultures which catalyze alkyl dehalogenations. Hematin can also catalyze the dehalogenation of HCB (78) but appears to be

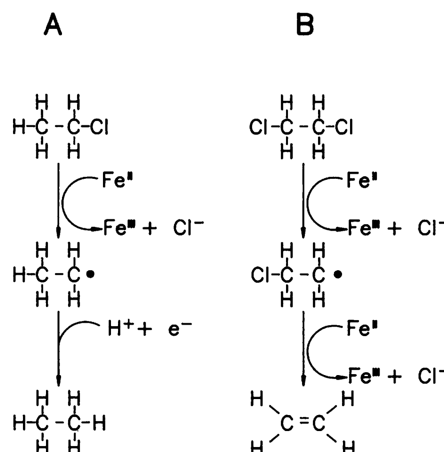

FIG. 18. Proposed reaction mechanisms for reductive dehalogenation catalyzed by a ferroheme. (A) Hydrogenolysis of chloroethane to ethane; (B) vicinal reduction of 1,2-dichloroethane to ethene. (Based on reference 206.)

generally limited in its ability to dehalogenate other aryl halides (15, 78). This observation may indicate that aryl halides are more resistant than are alkyl halides to dehalogenation by heme-containing enzymes, but this generalization requires further substantiation.

Although heme-containing enzymes may catalyze reductive dehalogenation, it is also possible that free ferrohemes catalyze reductive dehalogenation in natural environments. Zoro et al. (216) concluded that iron porphyrins released from decomposing organisms could, when chemically reduced in anaerobic environments, dechlorinate DDT. In this scheme, the catalyst is nonliving but of biological origin and the reductant is a living anaerobic community. Dehalogenation by both this quasi-biological system or by living cells could be inhibited by autoclaving and stimulated by growth substrates, as observed in undefined anaerobic communities (see above). The quasi-biological system would be consistent with these characteristics because of its requirement for the reduced chemical environment provided by a living anaerobic community. It is noteworthy that such a quasi-biological system would not be subject to the same steric and uptake constraints as living cells.

Co- and Ni-containing porphyrins. Recently corrinoids (Co containing) and factor F_{430} (Ni containing) have been shown to catalyze reductive dehalogenation. Corrinoids are widely distributed in bacteria (including any with vitamin B_{12} coenzymes), whereas F_{430} occurs only in methanogenic bacteria. Reductive dehalogenation by these porphyrins also requires the presence of a strong reductant such as Ti(III) or dithionite. Krone et al. (111–113) reported that several corrinoids and F_{430} catalyze dehalogenation of halomethanes including freons and chloronitromethane. Holliger et al. (92) found that vitamin B_{12} (a corrinoid) and F_{430} catalyze the dechlorination of 1,2-dichloroethane, vitamin B_{12} catalyzing both hydrogenolysis to monochloroethane (Fig. 1B) and apparent vicinal reduction to ethene (Fig. 1C) and F_{430} catalyzing only hydrogenolysis. Gantzer and Wackett (78) found that vitamin B_{12} and F_{430} catalyze the dechlorination of PCE and HCB and that vitamin B_{12} catalyzes dechlorination of PCP. These findings suggest that corrinoids may have greater potential than other biological transition metal complexes to dehalogenate aryl halides. This greater potential would be

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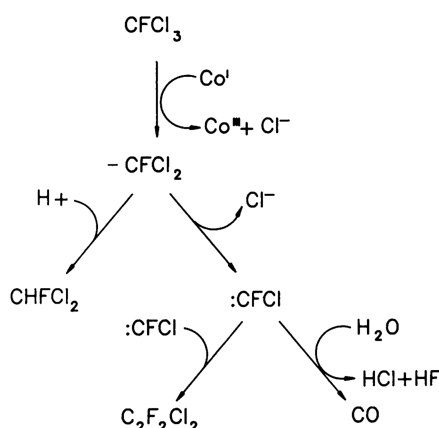

FIG. 19. Proposed reaction mechanism for degradation of Freon 11 catalyzed by a corrinoid yielding fluorodichloromethane (via hydrogenolysis), carbon monoxide, and 1,2-difluoro-1,2-dichloroethene. (Based on reference 113.)

consistent with the relatively low redox potentials of transition metal complexes containing Co (206). The action of Co- and Ni-containing porphyrins may explain the ability of certain bacteria to catalyze alkyl reductive dehalogenation. The activity of F_{430} is consistent with the ability of many methanogenic bacteria to catalyze alkyl reductive dehalogenation (Table 4), and the activity of corrinoids is consistent with the ability of organisms that use the acetyl-CoA pathway to catalyze this activity (above).

In a comparative study, Gantzer and Wackett (78) found the relative rates of dehalogenation of the following groups by vitamin B_{12} and F_{430} were as follows: halomethanes > haloethenes > halobenzoates > halophenols. Corrinoids and F_{430} were found to catalyze each step in the sequential dehalogenation of tetrachloromethane to methane (111, 112) and of PCE to ethene (Fig. 5) (78). Rates were relatively higher for more highly chlorinated (more highly oxidized) congeners of each group. These rate differences may account for the common observation of only partial reductive dehalogenation in various systems.

Krone et al. (113) found that reductive dehalogenation of tetrahalomethanes (carbon tetrachloride and several Freons) by corrinoids was accompanied by formation of carbon monoxide. The formation of this and other products suggested hydrogenolysis via a two-electron transfer, rather than via a one-electron transfer as described above. One reaction mechanism suggested by Krone et al. (113) is shown in Fig. 19. A two-electron transfer from the transition metal to a halo-substituted carbon atom yields a carbanion. Protonation of the carbanion results in hydrogenolysis, while chloride elimination yields a carbene. Hydrolysis of the carbene yields carbon monoxide. Dimerization of two carbenes yields a haloethene, another observed product. Other mechanisms including two-electron transfers and carbene intermediates are possible. In living cells with the acetyl-CoA pathway, carbon monoxide could be oxidized to CO_2 by carbon monoxide dehydrogenase. Thus, the above reaction mechanism explains the observed product formation from tetrahalomethanes by corrinoids and might also explain the production of CO_2 from tetrachloromethanes by undefined and pure cultures.

Conclusion

Transition metal complexes appear to be involved in biological reductive dehalogenation; however, the complete biological systems appear to vary and are not well understood in many cases. Alkyl dehalogenations appear more likely than aryl dehalogenations to be abiologically catalyzed, whereas the aryl dehalogenations appear more likely to be catalyzed by native enzymes. There is some correlation between the occurrence of transition metal complexes in certain microbial groups and dehalogenation activities in those groups. Steric constraints of enzymes and uptake constraints of whole cells appear to limit the activities of transition metal complexes which the enzymes and cells contain. For most reductive dehalogenation activities, such constraints have yet to be characterized. Some evidence exists for more than one reaction mechanism of alkyl hydrogenolysis catalyzed by transition metal complexes. Reaction mechanisms of aryl hydrogenolysis have received very little attention. It would be of great interest to further examine the ability of transition metal complexes, especially those with Co and Ni, to catalyze aryl reductive dehalogenation. Likewise, it would be of interest to test possible dehalogenation activities of purified enzymes known to contain transition metal complexes, as was done with P-450 cam. The environmental significance of dehalogenation by transition metal complexes released from decomposing organisms is another important subject which remains to be addressed.

GENERAL CONCLUSIONS

In agreement with results of earlier studies, recent results indicate that reductive dehalogenation is involved in the biological degradation of a variety of compounds, including aromatic ones, which are toxic and are not otherwise known to be biodegraded. Reductive dehalogenation most readily occurs in undefined anaerobic communities, and the responsible organisms may be obligate syntrophs. Anaerobic dehalogenating communities appear to vary significantly in composition, as they respond differently to environmental factors, particularly the availability of various electron acceptors. Many pure cultures are known to catalyze alkyl reductive dehalogenation, but few are known to catalyze aryl reductive dehalogenation, suggesting that the former activity is more common in microbes. At least one anaerobe, *Desulfomonile tiedjei*, can gain energy from reductive dechlorination, and the ability of others to do so is consistent with observed enrichment of this activity. Reductive dehalogenation also appears to be used by aerobes in the degradation of some haloaromatic compounds which are not initially susceptible to oxidative degradation. Reductive dehalogenation can be catalyzed by a number of transition metal complexes whose occurrence in bacteria is sometimes correlated with the ability of the bacteria to catalyze the activity. In some cases reductive dehalogenation is catalyzed abiologically by transition metal complexes, but in other cases, particularly with aryl halides, inducible enzymes with substrate specificity appear to be required.

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