Chapter 2

Degradation of Haloaromatic Compounds

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Economical and Ecological Relevance of Halogenated Aromatic Hydrocarbons (HAHs)

An ever increasing number of halogenated organic compounds has been produced by industry in the last few decades. These compounds are employed as biocides, for synthetic polymers, as solvents, and as synthetic intermediates. Production figures are often incomplete, and total production has frequently to be extrapolated from estimates for individual countries.

Hexachlorobenzene production, for instance, was estimated at about 10 000 tonne yr^{-1} in the 1970s (Rippen and Frank 1986). Total polychlorobenzene production, since its introduction to industrial use, has been calculated at roughly 750 000 tonne (Reineke and Knackmuss 1988). About two thirds of this overall production are accredited to have entered closed processes. Consequently, 250 000 tonne must have been released more or less directly into the environment. Around 1980, world production of pentachlorophenol was reported as 40 000 tonne yr^{-1} most of which can be supposed to have entered into the environment. 1,4-Dichlorobenzene production, at the same time, was estimated at around 100 000 tonne yr^{-1} (Rippen *et al.* 1984). Total 2,4-dichlorophenol production at the end of the 1970s was calculated in excess of 70 000 tonne yr^{-1} (Behret 1989).

Compounds of this type as a rule are highly persistent against biodegradation and belong, as "recalcitrant" chemicals, to the class of so-called xenobiotics. This term is used to characterise chemical substances which have no or limited structural analogy to natural compounds for which degradation pathways have evolved over billions of years. Xenobiotics frequently have some common features, e.g. high octanol/water partitioning coefficients and low water solubility which makes for a high accumulation ratio in the biosphere (bioaccumulation potential) (Johnson and Kennedy 1973).

Recalcitrant compounds therefore are found accumulated in mammals, especially in fat tissue, animal milk supplies (Frank and Braun 1989), and also in human milk (Collins *et al.* 1982; Ip and Phillips 1989; Skaare and Polder 1990). Highly sophisticated analytical techniques have been developed for the detection of organochlorines at the trace and ultratrace level (Burse *et al.* 1990).

Chlorinated aromatic compounds in many cases display acute or chronic toxicity for humans, with the probability also of mutagenic, cancerogenic (Nesnow *et al.* 1986), and teratogenic effects (Magnus-Francis 1990). Moreover, HAHs frequently are contaminated with highly pernicious polychlorinated dibenzofurans and dibenzodioxins (Hagenmaier 1986). Because of extreme biological effects at very low concentration (Whitlock 1990, Ryan *et al.* 1990, Safe 1990), many HAHs have been included in the list of "Priority Pollutants". This list expresses the public concern about uncontrolled use of these chemicals (Keith and Telliard 1979), and at the same time recognises the tremendous waste disposal problems (Levine and Chitwood 1985).

For a possible microbial clean-up of environmental contamination, one has to differentiate clearly between "point source" and "dispersed" pollution. In the first case, large amounts of chemicals, in high concentration, are present in one location (landfills, waste dumps, industrial effluents, accidental spills). In dispersed pollution, chemical concentration is low but spread over a large area or volume. Different strategies of bioremediation are required for these two types of pollution.

There are also diverse natural sources for HAHs. Marine aquifers, for example, produce a number of brominated compounds (Siuda and DeBernardis 1973, Neidleman and Geigert 1987, Wannstedt *et al.* 1990). The presence of such chloro and bromo compounds, especially in marine environments, has presented a challenge to microorganisms which sometimes develop astonishing degradative capabilities for these halogenated structures.

Biodegradation of HAHs is covered in a number of reviews (Reineke and Knackmuss 1988; Reineke 1986; Ribbons *et al.* 1987; Häggblom 1990; Ghosal *et al.* 1985; Commandeur and Parsons 1990; Rochkind-Dubinsky *et al.* 1986; Leisinger and Brunner 1986; Neilson 1990; Rasul-Chaudry and Chapalamadugu 1991) which concentrate *inter alia* on single classes of HAHs, on ecological aspects, on genetics or on bioremediation techniques. In the present review, special emphasis is placed on the mechanisms by which halide is liberated from organic molecules (Table 2.1).

In sharp contrast to aliphatic halides (Knackmuss 1981), the carbon-halogen bond in aryl halides is not susceptible to hydrolysis by nucleophilic displacement reactions. Frequently, halide elimination is observed only as a secondary process after metabolic transformation of the HAHs to non-aromatic, i.e. aliphatic or olefinic intermediates ("late" elimination). In the course of an alternative mechanism, enzyme-mediated addition of hydroxyl groups or other nucleophiles or of electrophiles (e.g. hydratation, epoxidation, dioxygenation) is followed by elimination of H-Hal ("early" elimination). Reductive halide displacement represents a third mechanistic possibility.

The term "(bio)degradation" will be used in this review only when HAHs are completely mineralised, with stoichiometric release of halide, i.e. synonymous with biomineralisation. In contrast, processes will be characterised as "(bio)transformation" which result in some structural alteration only of the substrate. Frequently, only the disappearance of the substrate is monitored without establishing either the carbon balance or the stoichiometry of the overall reaction. This is likewise subsumed under the term "(bio)transformation" which thus may also cover incomplete metabolisation.

There is reason to assume that such biotransformation processes (also called "cometabolism") represent a major way for the removal of recalcitrant compounds from

Table 2.1. Representative examples for bacterial degradation or transformation of haloarenes

Class of compounds	Bacterial strain/genus	Mechanism	Reference
1. Halobenzoates			
4-chlorobenzoate	Pseudomonas	H	Klages and Lingens 1980
3-chlorobenzoate	Pseudomonas sp. B13	E	Dorn *et al.* 1974
polyhalobenzoates	Pseudomonas aeruginosa JB2	?	Hickey and Focht 1990
2-chlorobenzoate	Pseudomonas	E	Hartmann *et al.* 1989
3-, 4-, and 3,5-dichlorobenzoate	Pseudomonas	E	Hartmann *et al.* 1979
2-halobenzoates	Pseudomonas putida CLB250	O	Engesser and Schulte 1989
4-chlorophenylacetate	Pseudomonas	O	Klages *et al.* 1981
2,4-dichlorobenzoate	Alcaligenes denitrificans	R/H	van den Tweel *et al.* 1987
4-chlorobenzoate	Nocardia	H	Klages and Lingens 1979
2 Halophenoxyalkanoates (Halo-PAs)			
4-chloro-2-methyl-PA	Pseudomonas	E	Gaunt and Evans 1971a,b
2,4-dichloro- / 4-chloro-2-methyl PA	Alcaligenes eutrophus	E	Pieper *et al.* 1988
3. Halophenols			
monochlorophenols	Pseudomonas	E	Knackmuss and Hellwig 1978
polychlorinated phenols	Rhodococcus	H,R,O	Apajalahti + Salkinoja-S.'87
dichlorophenols	Pseudomonas putida	E	Spain and Gibson 1988
monochlorophenols	Rhodococcus sp. AN117, AN213	E	Janke *et al.* 1989
polychlorinated phenols	Flavobacterium	O?, R	Saber and Crawford 1985
polychlorinated phenols	Mycobacterium	H?, O?, B	Häggblom *et al* 1988
4. Halobenzenes			
dichloro- and trichlorobenzenes	Pseudomonas	E/O?	van der Meer *et al.* 1991
1,2-dichlorobenzene	Pseudomonas	E	Haigler *et al.* 1988
1,4-dichlorobenzene	Alcaligenes	E	Schraa *et al.* 1986
5 Haloanilines			
monochlorinated anilines	Pseudomonas acidovorans	E	Loidl *et al.* 1990
monochlorinated anilines	Pseudomonas ?	E	Latorre *et al.* 1984
6. Polychlorobiphenyls (PCB)			
PCBs	Alcaligenes/Acinetobacter	(B*)	Furukawa *et al.* 1978
PCBs	Alcaligenes eutrophus	(B*)	Bedard *et al.* 1987
PCBs	Acinetobacter	?	Adriaens and Focht 1990
PCBs	Enrichment cultures	R(B)	Quensen *et al.* 1988
7 Halobiarylethers			
4-fluoro-4'-carboxybiphenylether	Pseudomonas	(P*)	Engesser *et al.* 1990
chlorinated dibenzofurans	Alcaligenes	?	Parsons *et al.* 1990
8. Miscellaneous			
Diethylsimazine	Rhodococcus corallinus	H	Cook and Hütter 1986
DDT	Aerobacter aerogenes	E/R	Wedemeyer 1967
5-chloro-2-hydroxynicotinic acid	Mycobacterium	E/H?	Tibbles *et al.* 1989b
Chloridazon	Phenylobacterium immobile	(P)	Lingens *et al.* 1985

H = hydrolytic; 0 = oxygenolytic; E = elimination; R = reductive; P = partial degradation; ? = not established unequivocally; (B*) = no dehalogenation observed; (P*) = no dehalogenation observed

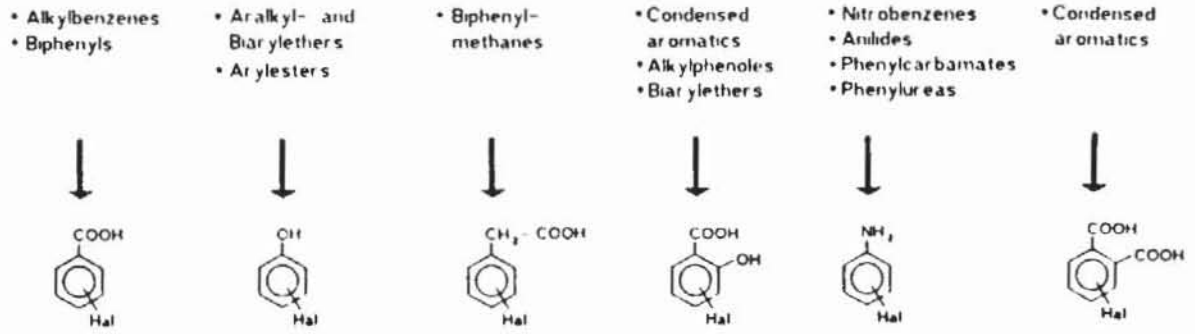

Fig. 2. 1. Central intermediates of haloarene degradation.

the biosphere (Knackmuss 1981). A series of non- or pre-adapted enzymes may transform a substrate stepwise, without energy being derived from the individual reactions. This works only if the co-substrates deliver sufficient energy to maintain the induction and metabolic potential of the active microorganisms. Alternatively, a substrate may be degraded only partially, with the microorganism deriving growth-sustaining energy from these processes, which may be termed "partial degradation".

Channelling of Complex Haloarene Structures into Key Intermediates: the Importance of Model Compounds for Degradation Studies

Among the different haloarenes, produced by industry, there are sometimes extremely toxic compounds such as TCDD (2,3,7,8-tetrachlorodibenzo-ρ-dioxin), PCP (pentachlorophenol), PCBs (polychlorinated biphenyls), and HCB (hexachlorobenzene). Some HAHs are at the same time highly polar and of high molecular weight (chlorinated and sulfonated lignin components), or very insoluble and toxic (halogenated dibenzofurans and dibenzodioxins). In the "early days" of haloarene degradation research, investigations were therefore confined to readily water-soluble mono- and dihalosubstituted benzoates and phenoxyalkanoates. These substrates are easily degradable, and biochemical studies were consequently speeded up.

Figure 2.1 demonstrates that the degradation of many haloarenes, with great structural variety, may be channelled into a limited number of central intermediates (Dagley 1978). A bouquet of so-called "peripheral" enzymes transforms all kinds of haloarenes into a few mononuclear haloaromatic structures, such as benzoic acids, salicylates, anilines, and phenols. Chloro-substituted phenoxyalkanoates, chloronaphthalines, and chlorobiphenyls, for instance, are transformed by these peripheral sequences. Biodegradation of 4,4'-dichlorobiphenyl affords 4-chlorobenzoate as an essential intermediate (Kimbara *et al.* 1988), closely following the degradation pathway for biphenyl (see Fig. 2.1). Chlorinated benzoic acids are also reported as intermediates of polychlorinated biphenyl (PCB) degradation, independent of chlorine content.

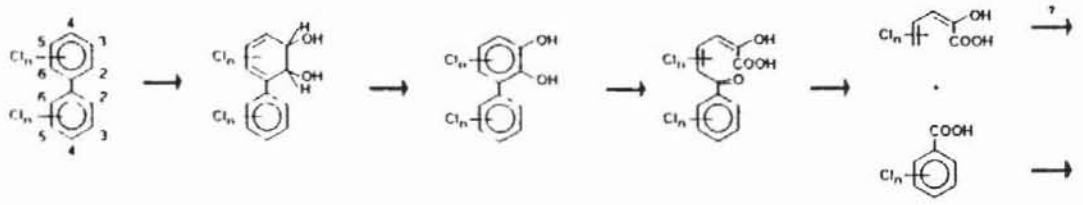

Fig. 2.2. Aerobic degradation of polychlorinated biphenyls by an *Achromobacter* species.

Polychlorinated Biphenyls (PCBs)

Following an early report on the aerobic degradation of polychlorinated biphenyls by an *Achromobacter* species (Ahmed and Focht 1973), an overwhelming amount of information has been presented by many different groups (Ballschmiter *et al.* 1977; Baxter *et al.* 1975; Furukawa and Matsumura 1976; Furukawa *et al.*; 1978, Furukawa *et al.* 1979). The biochemistry of PCB transformation was generally described as analogous to the established pathway of biphenyl catabolism (see Fig. 2.2) (Omori *et al.* 1986; Catelani *et al.* 1971). In a first step, halo-substituted phenylcatechols are formed intermediately by action of biphenyl 2,3-dioxygenases and 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenases. Subsequent meta-cleavage and hydrolysis of the ring cleavage product gives chloro-substituted benzoates from one phenyl ring and 2-oxo-4-pentenoates, with a varying number of chlorines, from th· other (Shields *et al.* 1985; Kimbara *et al.* 1988; Kilpi *et al.* 1988; Adriaens *et al.* 1989). In some cases, (chlorophenyl)glyoxylic acids or chlorinated acetophenones are accumulated, indicating the existence of other marginal biotransformation pathways (Shiaris and Sayler 1982; Bedard *et al.* 1987). From Kanechlor 400^{R} (a technical PCB mixture of mainly tetrachlorobiphenyls), chlorobenzoates, dihydroxy compounds (supposedly chlorocatechols), and meta-cleavage compounds, all with two to four chlorine substituents, are produced during metabolisation by an *Acinetobacter* species (Furukawa *et al.* 1983). More heavily chlorinated PCBs are metabolised only slightly, with dihydroxypentachlorobiphenyl derivatives being detected as sole metabolites.

Aerobic biotransformation of PCBs via biphenyl-related pathways seems to be subject to some structural restrictions which are mostly due to the prevalent 2,3-dioxygenation mode of cometabolic attack on PCBs:

1 An increasing number of chlorine substituents generally decreases biodegradation rates in aerobic systems; consequently, PCB congeners with more than five chlorines are virtually not transformed (Bedard *et al.* 1987).
2 One unsubstituted ring facilitates biotransformation.
3 One ring at least must have one ortho and the adjacent meta position unsubstituted.

Steric screening by the bulky chlorine atoms renders such PCB congeners, where all ortho and/or meta positions are blocked, not or only slightly biodegradable. As mentioned above, PCBs generally are attacked via 2,3-dioxygenation (Safe 1984). Some microorganisms seem to possess a different set of biphenyl degrading enzymes by which PCB congeners are attacked, unexpectedly, in 3,4-position (Bedard *et al.* 1986). Bacteria of this type (e.g. *Corynebacterium* sp MB1 and *Alcaligenes eutrophus* H850) are able to oxygenate, in addition to lower chlorinated PCBs, even 2,5,2',5'-tetrachloro-, 2,4,5,2',5'-pentachloro- and, albeit slower, 2,4,5,2',4',5'-hexachlorobiphenyl, with unusual metabolites such as chlorinated acetophenones being formed.

All microorganisms, described so far as competent for PCB transformation, do not use PCBs with more than two chlorine substituents as sole source of carbon (Bedard *et al.* 1986; Adriaens *et al.* 1989; Shields *et al.* 1985). The respective transformations thus must be termed cometabolic. For one mixed culture, growing solely on a PCB cocktail containing mostly tetrachlorobiphenyls, it has not been clarified whether the microorganism in fact used the higher chlorinated congeners for growth (Kimbara *et al.* 1988).

If biphenyl is added to facilitate any energy-consuming biotransformation process, PCBs under such relaxed conditions are more susceptible to microbial attack (Focht and Brunner 1985; Brunner *et al.* 1985). For example, after addition of both an *Acinetobacter* strain P6 and biphenyl to PCB-containing soil, up to 27% of labeled PCB was recovered as $^{14}CO_2$. Addition of the microorganism alone, without biphenyl, did not improve biodegradation (Brunner *et al.* 1985). Likewise, degradation of 4-chlorobiphenyl, as a PCB model, was greatly enhanced by addition of the 4-chlorobiphenyl-degrading bacterium *Alcaligenes* A4 (Hill *et al.* 1989). The strain survives sufficiently long in soil to offer the opportunity for its application in bioremediation processes.

In most of the experiments reviewed above, only PCB substrate disappearance was monitored. Due to the very low biotransformation rates (Bedard *et al.* 1987) and the difficulty of measuring chloride in soil matrices, no stoichiometry of chloride release is given as a rule. There are indications, however, of cometabolic chloride release, after meta-cleavage of chloro-substituted phenyl catechols, being a feasible though highly inefficient process (Adriaens and Focht 1990).

The halobenzoates, haloacetophenones or other metabolic intermediates, generated by these cometabolic PCB-degrading processes, should in principle be converted to halocatechols. It cannot be ruled out, however, that other pathways are involved.

Chlorinated N-Heterocycles

Enzymes of biphenyl-degrading organisms have been found to be rather unspecific. Accordingly, heterocyclic biphenyl analogues like Antipyrin and Chloridazon are partially degraded by e.g. *Phenylobacterium immobile* and similar strains since the bacteria can utilise at least one phenyl ring as a source of carbon and energy (Lingens *et al.* 1985). The hete-rocyclic moiety is not degraded at all, and is accumulated quantitatively. Comparative analysis of some 16S-ribosomal nucleic acid partial sequences showed that this microorganism constituted a new genus even though the transformation pathway for the heterocyclic substrates is identical, except for one enzyme, with that of the biphenyl-degrading organisms (Ludwig *et al.* 1984). Intensive

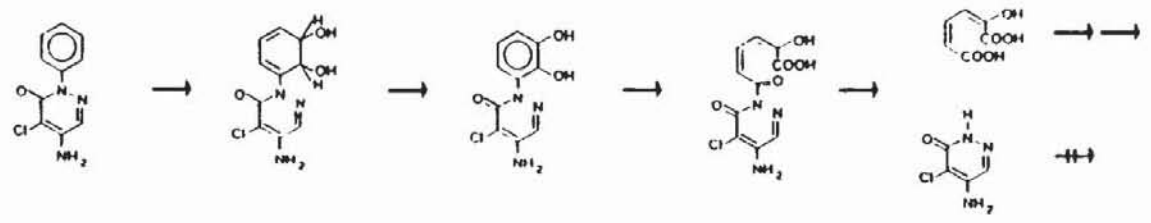

Fig. 2.3. Chlorinated *N*-heterocycle degradation by *Phenylobacterium immobile*.

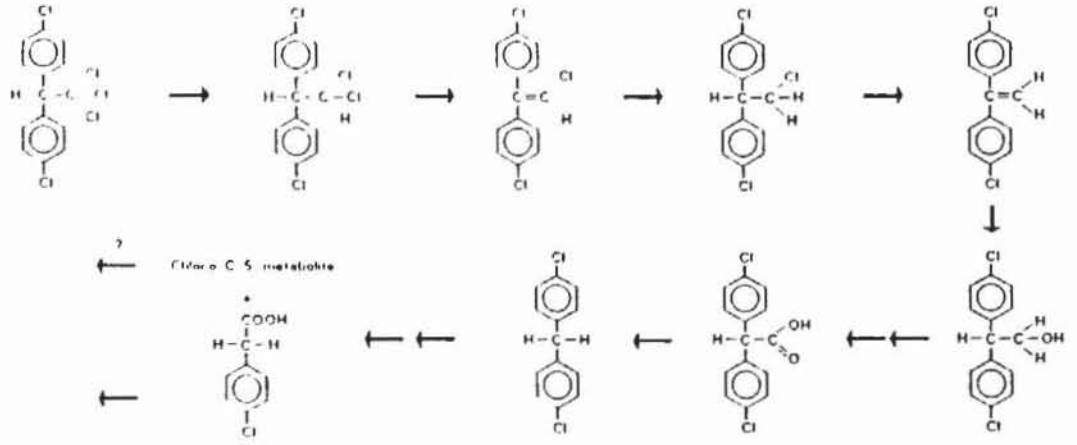

Fig. 2.4. Co-metabolism of DDT by a *Klebsiella (Aerobacter) aerogenes* strain.

characterisation of the N-heterocycle pathway enzymes (see Fig. 2.3) revealed this to be an amidase which releases the C-6 metabolite, 4-oxalocrotonate (2-hydroxymuconate), instead of the C-5 metabolite 2-oxo-4-pentenoate, generated by regular hydrolase activity (Schmitt *et al.* 1984; Sauber *et al.* 1977). These Chloridazon-degrading bacteria also possess a hydrolase-type enzyme, the presence of which could be demonstrated after growth on cinnamic acid (Tittmann *et al.* 1980).

A similar process has been described for the transformation and partial utilisation of 3-chloroquinoline-8-carboxylic acid by *Pseudomonas* spec. EK III. The heterocycle is degraded to the dead-end product 5-chloro-2-hydroxynicotinic acid and pyruvate which serves as the principal carbon source (Tibbles *et al.* 1989a). Another bacterium can utilise this nicotinic acid derivative for growth (see Hydrolytic Displacement of Halogen).

1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT)

DDT was transformed cometabolically (see Fig. 2.4) by a *Klebsiella aerogenes (Aerobacter aerogenes)* strain, *via* reductive dechlorination, to 1,1-dichloro-2,2-bis (4-chlorophenyl)ethene (DDD). In a second step, HCl is eliminated from the DDD and the double bond hydrogenated. The 1-chloro-2,2-bis(4-chlorophenyl)ethane thus formed finally yields 4,4'-dichlorobenzophenone and 4,4'-dichlorodiphenylmethane (Wedemeyer 1967). Other authors have shown these metabolites to be cometabolised, by a diphenylmethane-degrading *Hydrogenomonas* strain, to 4-chlorophenylacetate (Focht and Alexander 1970, Focht and Alexander 1971) which already has been demonstrated to be smoothly degradable (see Oxygenolytic Elimination of Halogen).

In a combined anaerobic/aerobic process, this *Hydrogenomonas* strain was found to first dechlorinate the DDT side chain and, in a second stage, metabolise the 4,4'-dichlorodiphenylmethane to a (4-chlorophenyl)acetate and chlorinated ring cleavage products (see Fig. 2.4). DDT was later also reported to be degraded very slowly in a similar experiment with a *Pseudomonas aeruginosa* strain. With lactate as co-substrate, the trichloromethyl moiety was claimed to be transformed to a carboxyl group under anaerobic or microaerophilic conditions (Golovleva and Skryabin 1981), followed by decarboxylation and reductive dechlorination of both rings. The resulting

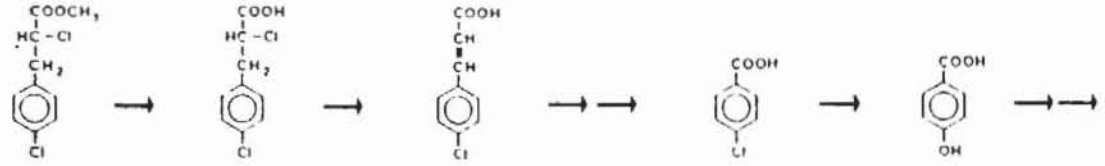

Fig. 2.5. Degradation of the herbicide Bidisin.

biphenylmethane was proposed as source of carbon and energy under aerobic conditions (Golovleva and Skryabin 1981). In some cases, reductive removal of chlorine precedes metabolism by aerobic microorganisms. This will be dealt with separately (see Liberation of Halide by Reductive Mechanisms).

Bidisin

Degradation of the herbicide Bidisin, methyl 2-chloro-3-(4-chlorophenyl)propionate), demonstrates strikingly how a complex structure is channeled into the established catabolic pathway for a basic structure (in this case 4-chlorobenzoate). After hydrolysis of the ester function, the aliphatic chlorine is eliminated, yielding 4-chlorocinnamate (see Fig.2.5). 4-Chlorobenzoate is formed in the ensuing steps according to the regular fatty acid oxidation pathway. This intermediate undergoes hydrolytic dechlorination to 4-hydroxybenzoate (see Hydrolytic Displacement of Halogen) which in turn is smoothly degraded (Köcher *et al.* 1976).

Chloronaphthalines

Knowledge concerning degradation of chloronaphthalines is scarce. Chlorosalicylates have been shown to be intermediates in the transformation of 1- and 2-chloronaphthaline (Morris and Barnsley 1982) and 1,4-dichloronaphthaline (Durham and Stewart 1987). Metabolic sequences dissimilating naphthalene via salicylate obviously are responsible for these reactions which, however, do not sustain growth of the organisms (see also Dehalogenation of Halocatechols after Meta-Cleavage).

Halogenated Biarylethers

As indicated above, little is known about degradation pathways for halogenated biarylethers (Rast *et al.* DBP 1990). Turnover even of the heavily chlorinated 2,3,7,8-tetrachlorodibenzo-p-dioxin was reported although no products of these (generally very slow) processes were characterised (see for example Quensen and Matsumura 1983). This is partially due to the sometimes highly dangerous properties of halogenated biaryl ethers, and to their very limited solubility. Quite recently, degradation of a model compound, 4-carboxy-4'-fluorodiphenyl ether, was described (Engesser *et al.* 1990a), 4-fluorophenol being found to accumulate quantitatively in the growth medium (see Fig. 2.6).

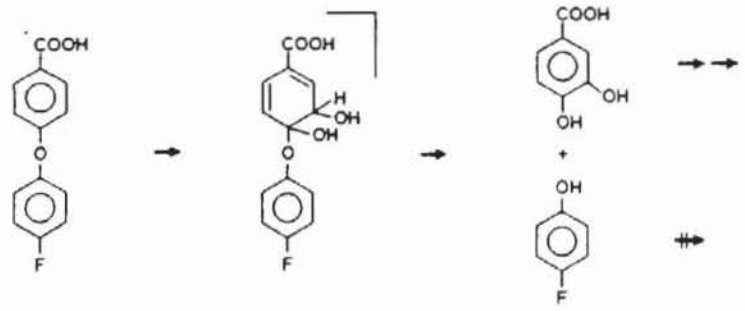

Fig. 2.6. Degradation of a model halogenated biarylether, 4-carboxy-4'-fluorodiphenyl ether.

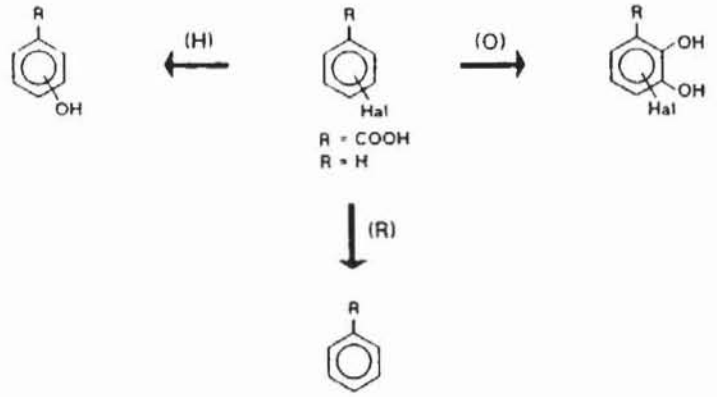

Fig. 2.7. Three routes for the degradation of mononuclear benzene derivatives.

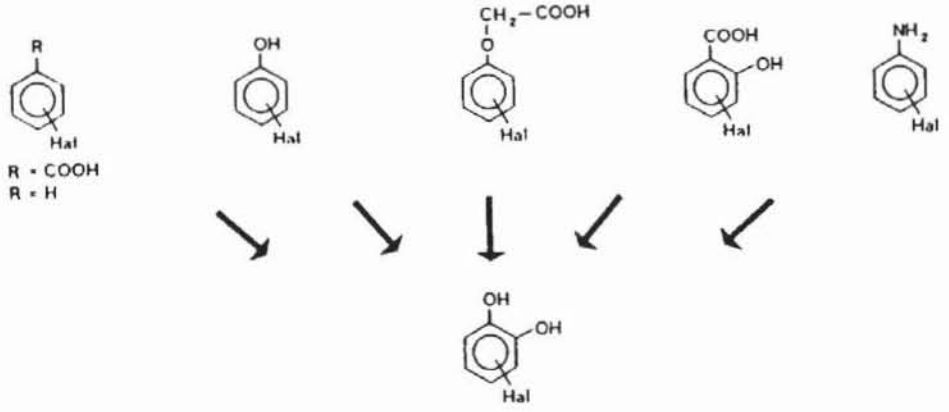

Fig. 2.8. Precursors for the halocatechol branch of mononuclear benzene degradation.

Chlorocatechols as Central Intermediates in Metabolisation of Chlorinated Aromatic Structures

As shown above, many complex haloaromatic structures are reduced to the limited number of basic mononuclear structures collated in Figure 2.1. Further metabolisation of these benzene derivatives proceeds along three different routes (see Fig. 2.7):

1 "hydrolytic" or monooxygenolytic displacement of halide yielding monohydroxy benzene structures (Horvath 1971);
2 reductive liberation of halide (Tweel *et al.* 1987);
3 dioxygenation and subsequent dehydrogenation to halo-substituted dihydroxybenzenes (halocatechols).

For the halocatechol branch, the diversity of the respective "halocatechol precursors" is shown in Figure 2.8.

This mechanistic strategy has been established for chlorobenzoates (Chatterjee *et al.* 1981; Hartmann *et al.* 1989; Hartmann *et al.* 1979; Don *et al.* 1985; Hickey and Focht 1990; Haller and Finn 1979; Horvath and Alexander 1970; Reineke and Knackmuss 1978a,b) as well as for fluorobenzoates (Engesser *et al.* 1990b; Hughes 1965; Vora *et al.* 1988; Horvath and Flathman 1976; Harper and Blakley 1971; Clarke *et al.* 1975; Ali *et al.* 1962). In general, the principle of converging pathways allows for one metabolic sequence to operate on substrates as different structurally as halobenzoates, haloacetophenones (Higson and Focht 1990b), halophenoxyalkanoates, haloanilines, halophenols, and halobenzenes.

3-Chlorobenzoate

A 3-chlorobenzoate(3CB)-utilising bacterium, *Pseudomonas* sp. B13, was isolated in 1974 after pre-enrichment with benzoate (Dorn *et al.* 1974). Of all the isomeric monohalobenzoates, this organism utilises only 3-chloro- and 3-bromobenzoate and, after adaptation, 4-fluorobenzoate. Halosubstituted cyclohexadienediol carboxylic acids are formed in an initial dioxygenation step which are dehydrogenated enzymatically to a mixture of 3- and 4-chlorocatechol or 4-fluorocatechol in the degradation, respectively, of 3-chloro- and 4-fluorobenzoate (Reineke and Knackmuss 1978a,b; Dorn and Knackmuss 1978a,b).

(Chlorophenoxy)alkanoates

Studies on the biodegradation of (2,4-dichlorophenoxy)acetate (2,4-D) and (2-methyl-4-chlorophenoxy)acetate (MCPA) likewise showed chlorocatechols as central intermediates for all strains investigated so far. In the first step, the aryl alkyl ether bond is labilised by action of a monooxygenase. The hemiacetals thus generated are chemically unstable, and rearrange to phenols and glyoxylate or homologous α-keto acids (Loos *et al.* 1967a,b,c; Bollag *et al.* 1967; Tiedje and Alexander 1969; Evans *et al.* 1971a,b; Gaunt and Evans 1961; Gamar and Gaunt 1971). These substituted phenols are subject to a second monohydroxylation reaction yielding catechols (Bollag *et al.* 1968; Gaunt and Evans 1971a,b). One representative enzyme for this process, a 2,4-dichlorophenol hydroxylase, has been purified and characterised as highly specific for chlorophenols (Beadle and Smith 1982; Liu and Chapman 1984). Phenol, on the other hand, is not a substrate for this enzyme.

A direct reductive ether cleavage, not in accord with this general reaction scheme, has been described for the transformation of phenoxyalkanoates with long aliphatic chains (MacRae and Alexander 1963) as well as of (2,4,5-trichlorophenoxy)acetate (2,4,5-T) (Mikesell and Boyd 1985). Hydroxylation of the aromatic nucleus prior to ether cleavage was recognised to yield non-degradable products (Faulkner and Woodcock

1964; Evans *et al.* 1971a). An *Aspergillus niger* species was found to totally degrade 2,4-D with elimination of chloride before cleavage of the ether bond (Shailubhai *et al.* 1983).

As mentioned above, chlorophenols are subject to monohydroxylation yielding chlorocatechols. This transformation can be effected by unspecific phenol hydroxylases (Knackmuss and Hellwig 1978) as well as by highly specialised chlorophenol hydroxylases. Among these, the enzyme coded on the plasmid pJP4 has been shown to strictly prefer 2,4-dichloro- and 2-methyl-4-chlorophenol over monochlorophenols, phenol not being transformed at all (Liu and Chapman 1984; Pieper *et al.* 1988). The respective enzyme from an *Acinetobacter* behaved essentially similar. Phenols, although not transformed, acted as uncoupling agents, i.e. caused H_2O_2 production from NAD(P)H (Beadle and Smith 1982). The phenomenon of preferential attack of chlorinated compounds over the natural substrate is encountered to a lesser degree also in chlorocatechol degradation.

Chloroanilines

Chlorinated anilines are transformed to chlorocatechols in a one-step reaction, two hydroxyl groups being inserted geminal and ortho to the amino function by an aniline dioxygenase. The unstable hemi-aminal thus formed eliminates NH_3 yielding catechols (Bachofer *et al.* 1975; Janke *et al.* 1984). Isomeric halocatechols are produced in varying percentage, depending on the strain employed, from unsymmetrically substituted anilines (Reber *et al.* 1979; Schukat *et al.* 1983; Zeyer *et al.* 1985; Ihn *et al.* 1989; Janke *et al.* 1989; Surovtseva *et al.* 1980; Surovtseva *et al.* 1986; Latorre *et al.* 1984; Loidl *et al.* 1990), as in the case of unsymmetrically substituted halobenzoates (Engesser *et al.* 1990b). Thus, 2- and 4-chloroaniline are transformed, respectively, to 3-chloro- and 4-chlorocatechol only. From 3-chloroaniline, 4-chlorocatechol is formed preferentially.

Chlorosalicylates

Chlorosalicylates, like chlorophenols, can be monooxygenated yielding chlorocatechols (Rubio *et al.* 1986a). Chlorosalicylate-degrading organisms have been constructed by introducing genes encoding a salicylate 1-hydroxylase into a strain with chlorocatechol degrading capacity. Turnover rates vary considerably between the different isomeric chlorosalicylates, with the 3-chloro isomer being the most difficult to degrade (Rubio *et al.* 1986a,b). Degradation of 3,6-dichloro-2-methoxybenzoic acid (Dicamba) *via* 3,6-dichlorosalicylate was reported, though without any details on the further metabolisation of the halosalicylate.

A *Bacillus brevi* strain, originally described to degrade 5-chlorosalicylate, was reported to also dissimilate 3,5-dichlorosalicylate (Krueger *et al.* 1989), 5-chlorosalicylate being proposed as the substrate of a ring-cleaving gentisate 1,2-dioxygenase. This would constitute one of the very few cases of direct ring cleavage of chlorosubstituted monohydroxy arenes (not dihydroxyarenes or catechols) (Crawford *et al.* 1979). The fact that the 5-chlorosalicylate-degrading *Bacillus* dissimilates only 3,5-, but not 3,6-dichlorosalicylate indicates that this compound is not degraded *via* ring cleavage of 3,6-dichlorosalicylate. Rather, transformation of the substrate to a catechol by action of a salicylate monohydroxylase precedes degradation.

Halobenzenes

These are metabolised frequently by dioxygenases yielding halo-substituted dienediols which in turn are dehydrogenated to halocatechols (Reineke and Knackmuss 1984; Sperl and Harvey 1988; Gibson *et al.* 1968). Di- and trihalobenzenes have also been described to be metabolised *via* halocatechols (Bont *et al.* 1986; Meer *et al.* 1991; Spain and Nishino 1987; Schraa *et al.* 1986; Oltmanns *et al.* 1988). Even *o*-dichlorobenzene which is hardly attacked by most haloarene degraders is metabolised by a *Pseudomonas* strain *via* initial dioxygenase attack (Haigler *et al.* 1988). Chlorotoluenes were reported to be degraded despite two mutually "incompatible" substituents on the same aromatic nucleus (see Degradation of Mixtures of Halo and Alkyl Arenes). Degradation of *p*-chlorotoluene followed the normal chlorobenzene metabolism route, i.e. 2,3-dihydroxylation and formation of both chloro- and methyl-substituted catechols. Subsequently, chlorine is eliminated from the respective muconic acid derivative after cycloisomerisation while the methyl substituent is retained in a methyl-substituted dienelactone (Haigler and Spain 1989). 2,6-Dichlorotoluene degradation was once claimed for a landfill-isolate *via* a catechol pathway (Vandenbergh *et al.* 1981) but this preliminary report was not followed up.

The alternative reductive dehalogenation mechanism is discussed later (see Liberation of Halide by Reductive Mechanisms).

Biochemistry of Halocatechol Degradation

4-Fluorocatechol

Catechol is degraded in many bacteria along the well-established ortho or 3-oxoadipate pathway (Ornston and Stanier 1966; Ornston 1966). A catechol 1,2-dioxygenase generates *cis,cis*-muconic acid. This is cycloisomerised to (+)-muconolactone and muconoenollactone which in turn is hydrolyzed to 3-oxoadipate. This is degraded finally to acetyl-CoA and succinate (Fig.2.9).

This mechanism was found to be effective, however, only for the catabolism of 4-fluorocatechol (Clarke *et al.* 1975; Engesser *et al.* 1990b; Engesser *et al.* 1980; Schreiber *et al.* 1980) which is cleaved, in contrast to other halocatechols, by regular catechol 1,2-dioxygenases at sufficiently high rates to allow growth on 4-fluorobenzoate (Dorn and Knackmuss 1978b; Schreiber *et al.* 1980). Ring cleavage of halocatechols generally forms the bottleneck of haloarene degradation via ortho pathways. The astonishing reactivity of 4-fluorocatechol can be rationalised in terms of the strong mesomeric electron release of the parafluorine substituent (Engesser *et al.* 1988). For all other halocatechols, the inductive electron-withdrawing effect of the halogen drastically decreases reaction rates of the regular oxygen-dependent ortho-pyrocatechases (Dorn and Knackmuss 1978b).

Ortho-cleavage of 4-fluorocatechol yields 3-fluoromuconate which can be smoothly cycloisomerised to 4-fluoromuconolactone (Harper and Blakley 1971). This lactone which, under physiological conditions, is chemically reasonably stable, may be transformed enzymatically by regular enollactone hydrolases to maleylacetate with concomitant formation of fluoride (Schlömann *et al.* 1990a,b). This pathway is likely to operate also in the Gram-negative strain FLB300 which showed high maleylacetate reductase activity (Engesser *et al.* 1990b). The reductive step, catalyzed by this enzyme,

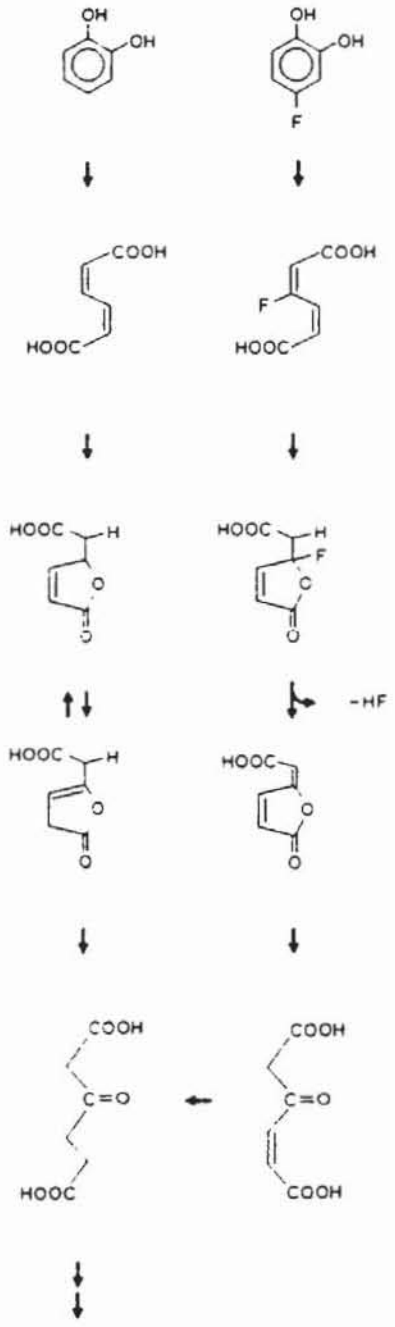

Fig. 2.9. Degradation of catechol and fluorocatechol via the "ortho" pathway.

has the 4-fluorolactone degradation converging with the normal 3-oxoadipate pathway. For dissimilation of4-fluorocatechol *via* the ortho pathway, only one additional enzyme is required over that already present in strains degrading catechol *via* the 3-oxoadipate pathway (see Fig. 2.9). It should be mentioned, however, that in many strains degradation of 4-fluorolactone appears much more complex both as to the enzymes involved and to stability and structure of the transformation products (Schlömann *et al.* 1990a,b).

No productive, i.e. growth-sustaining degradation process, for 3-fluorocatechol has been described so far. Either this substrate is hardly transformed at all, or the 2-fluoromuconic acid intermediate is resistant against the cycloisomerases of all

bacterial sources tested (Engesser *et al.* 1980; Schmidt and Knackmuss 1980; Schmidt *et al.* 1980).3-Fluorocatechol thus represents a typical non-biodegradable haloaromatic compound.

Very recently, anaerobic consortia were described to reductively dechlorinate polychlorocatechols to lesser chlorinated derivatives (Allard *et al.* 1991) (see Liberation of Halide by Reductive Mechanisms). The metabolism of Fluorocatechols, however, was not investigated.

Specialised Enzymes for Halocatechol Catabolism: Three Reasons for Non-biodegradability

As mentioned above, the simple degradative mechanism for 4-fluorocatechol does not work for 4-chlorocatechol. Firstly, the chlorine substituent severely retards ring cleavage by regular ortho pyrocatechase (Dorn and Knackmuss 1978b; Engesser *et al.* 1988). Cycloisomerisation of 3- and especially 2-chloromuconic acid (see also Fig. 2.10) is severely retarded as well since conventional enzymes convert substituted muconic acids only very slowly because of steric hindrance by the rather bulky chlorine and methyl substituents. This illustrates the second major constraint of haloarene catabolism by regular, non-specialised enzymes which often lack the unspecificity to cope with bulky substrates.

The product of 3-chloromuconate cycloisomerisation, unlike 4-fluoromuconolactone (Harper and Blakley 1971; Schlömann *et al.* 1990b), is chemically unstable and eliminates halide, thus forming a doubly unsaturated lactone (dienelactone) in the final degradative step (Schmidt and Knackmuss 1980). This dienelactone is a dead-end product for which no enzymatic activity is present in any particular bacterium not specialised for halocatechol degradation. Cometabolic degradation of haloarenes thus suffers from a third constraint, the formation of dead-end products.

Halocatechol Degradation via Specialised Ortho Pathway Enzymes

Halocatechols are intermediates in the degradation of halobenzoates, halobenzenes, haloanilines, halophenols, and other halogenated arenes. These intermediates are effectively degraded by many strains (Dorn *et al.* 1974; Bollag *et al.* 1968; Pieper *et al.* 1988; Ditzelmüller *et al.* 1989; Horvath *et al.* 1990; Don *et al.* 1985; Evans *et al.* 1971a,b). Specialised catechol 1,2-dioxygenases generally attack mono- or dihalogenated catechols, in preference over catechol (Dorn and Knackmuss 1978a,b; Pieper *et al.* 1988), in an intradiol fashion with formation of substituted muconic acids. Of the various isomeric dichlorocatechols, only 3,5- and 3,6-dichlorocatechol are transformed at excellent rates to, respectively, 2,4- and 2,5-dichloromuconic acid (Pieper *et al.* 1988; Hartmann *et al.* 1979; Bont *et al.* 1986; Spain and Nishino 1987; Schraa *et al.* 1986). Dioxygenation of 3,4- and 4,5-dichlorocatechols proceeds even slower than with catechol (Pieper *et al.* 1988; Ditzelmüller *et al.* 1989; Schraa *et al.* 1986; Loidl *et al.* 1990). Further metabolism of the corresponding chloromuconic acids seems to be severely impeded as well. At high biomass concentrations, however, release of chloride indicated a slow albeit productive metabolism (Pieper 1986; Furukawa *et al.* 1979).

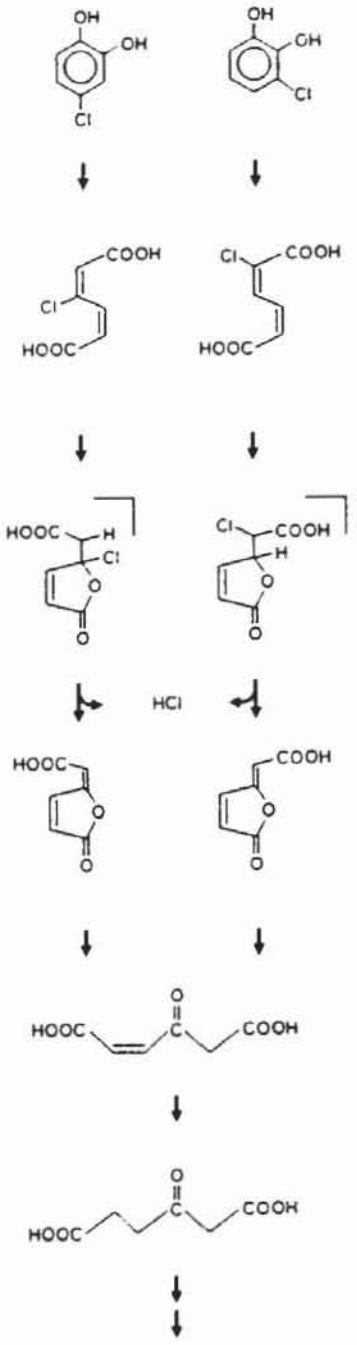

Fig. 2.10. Degradation of chlorocatechols involving cycloisomerisation and spontaneous elimination of hydrogen chloride.

Chlorinated muconic acids are cycloisomerised by more or less specialised chloromuconate cycloisomerases (Schmidt and Knackmuss 1980). Some highly specialised enzymes transform only 2,4-dichloro- and 3-chloromuconic acid at high rates, with 2-chloro- and unsubstituted muconic acid being attacked rather slowly (Pieper *et al.* 1988; Kuhm *et al.* 1990).

As shown in Figure 2.10, cycloisomerisation of 2-chloro-*cis*, *cis*-muconate affords an unstable butenolide which was proposed to form a doubly unsaturated lactone, *trans*-4-carboxymethylenebut-2-ene-4-olide (*trans*-dienelactone), by spontaneous anti-

elimination of hydrogen chloride. Cycloisomerisation of the corresponding 3-chloro-*cis, cis*-muconic acid, upon HCl elimination, yielded the *cis*-4-carboxymethylenebut-2-en-4-olide (*cis*-dienelactone) (Schmidt and Knackmuss 1980; Bollag *et al.* 1968).

This is the first halide liberation step in the course of the ortho pathway. At this point, the degradation pathways of catechol and of the halo analogues are branching mechanistically. Only on the 3-oxoadipate level (see below and Fig. 2.9), the two degradation pathways converge again. The chlorocatechol-related pathways have been shown in some cases, however, to have evolved from the enzymes of regular ortho pathways.

Different muconate cycloisomerases, e.g. chloromuconate cycloisomerase from *Pseudomonas putida* (pAC27) and dichloromuconate cycloisomerase of *Alcaligenes eutrophus* JMP134, were shown to be closely related (Yeh *et al.* 1982; Aldrich *et al.* 1987; Ghosal and You 1988), and may even have evolved from common ancestors. This clearly demonstrates nature to be able to cope with new challenges by evolving new activities from pre-existing though inefficient entities. All these enzymes were concluded to follow the same mechanistic pathway, liberating halide from an enzyme-bonded anionic intermediate instead of eliminating HCl from the free chloro-substituted cycloisomerisation product (Schlömann *et al.* 1990b). Nevertheless, these enzymes should not be mistaken with true halidohydrolases which actively remove halide by substitution with either hydroxide or glutathione (Goldman *et al.* 1968).

Enzymes hydrolyzing these dienelactones are termed dienelactone hydrolases. They have been described for many organisms and seem to play an important role in the degradation not only of chloro-, but also of fluoro- and bromoarenes. One enzyme has been purified to homogeneity and characterised by X-ray diffraction (Ngai *et al.* 1987; Pathak and Ollis 1990; Pathak *et al.* 1988).

The products of these reactions are maleylacetate or its ß-chloro derivative (from 3- and 3,5-dichlorocatechol, respectively). Maleylacetates are readily transformed, by a maleylacetate reductase (MAR), to 3-oxo- or ß-ketoadipates whence this pathway was named. The point at which the second chlorine is removed has not yet been established unequivocally. From experiments with an *Arthrobacter* strain which likewise transforms chlorosuccinate to succinate (Duxburry *et al.* 1970), it was deduced that chloromaleylacetate is reduced to 2-chloro-4-oxoadipate followed by hydrolysis to acetyl-CoA and chlorosuccinate which in turn yields succinate. Accordingly, ring-labeled (2,4-dichlorophenoxy)acetate was metabolised to succinate (Tiedje *et al.* 1969). A maleylacetate reductase from a different source was later shown to dehalogenate (ß-chloromaleyl)acetate to chloride and 3-oxoadipate, thereby excluding chlorosuccinate as an intermediate in 3,5-dichlorocatechol degradation. The authors reported that chloromaleylacetate reduction required twice as much NADH as maleylacetate turnover. They concluded that the product of maleylacetate reductase action, chloro-substituted ß-ketoadipate, spontaneously eliminated chloride to maleylacetate which in a second step was reduced to 3-oxoadipate. There remains some doubt as to this mechanism since no purified enzymes were employed. It is doubtful, for instance, whether 2-chloro-4-oxoadipate in fact is sufficiently unstable to spontaneously eliminate chloride, forming maleylacetate. Nevertheless, chlorocatechol degradation can be considered as clearly established from the work on 3-chlorobenzoate and (2,4-dichlorophenoxy)acetate degradation in the last 25 years.

The pathways, detailed above, allow for metabolisation of mono- and dihalo catechols. There is no indication, however, that the enzymes involved can productively metabolise tri- or even more heavily substituted halocatechols. Polychlorinated aromatic substrates with ≥3 chlorine atoms per ring therefore must be metabolised *via*

different routes. Two exceptions to this rule have recently been proposed. For 1,2,4-trichlorobenzene (1,2,4-TCB), aerobic transformation *via* 3,4,6-trichlorocatechol and subsequent metabolisation along a specialised ortho pathway was suggested (Meer *et al.* 1991). The authors could not exclude the possibility of initial oxygenolytic chloride release (see Oxygenolytic Elimination of Halogen), and did not present any evidence on what happened to the third chlorine of the 1,2,4-TCB substrate. Likewise, aerobic degradation of 1,2,4,5-tetrachlorobenzene has been reported to proceed *via* chlorocatechols (Springer and Rast 1988; Wittich *et al.* 1989). Once again, one cannot exclude initial dechlorination prior to ring cleavage *via* 3,5,6-trichlorocatechol as an intermediate.

Degradation of Mixtures of Halo and Alkyl Arenes

The metabolism of halocatechols *via* so-called meta pathways as a rule is unproductive, resulting in production of dead-end metabolites, slow and non-stoichiometric liberation of halide, and inactivation of the key enzymes, the catechol-2,3-dioxygenases (Bartels *et al.* 1984; Engesser *et al.* 1989a) (for the only exception reported so far, see Dehalogenation of Halocatechols after Meta-Cleavage). This situation still holds today despite many efforts (Engesser KH, unpublished results; Pfeiffer F, personal communication). Alkylcatechols, on the other hand, are generally degraded by enzymes of the meta pathway. Metabolism via ortho-cleavage as a rule leads to accumulation of methyllactones as dead-end metabolites (Engesser *et al.* 1989a). Mixtures of halo- and alkylcatechols therefore cannot be degraded quantitatively since the pathways for these two substrates are mutually incompatible. All efforts have failed so far to isolate bacteria which can productively degrade halocatechols *via* meta pathways, i.e. not in a cometabolic manner. A new selection technique was therefore developed to identify microorganisms with modified ortho pathways. From enrichment experiments with methyllactones, methylcatechol-degrading organisms were isolated which, preferentially or even exclusively, utilise ortho pathways for complete mineralisation of halo and alkyl arene mixtures. The meta-cleaving enzyme was inactivated by transposon insertion to avoid misrouting of halocatechols. Alternatively, enzymes of the methylcatechol ortho pathway were cloned into strains, free of meta pathways (Engesser *et al.* 1989a).

This goes to show that non-biodegradability of otherwise easily metabolisable compounds may also be due to misrouting of substrates. Very recently, a *Pseudomonas* strain was described to degrade a mixture of chlorobenzene and toluene (Pettigrew *et al.* 1991). Chloro- and methylcatechols were found to be accumulated in the culture medium; this did not happen with a mutant of this strain which had been blocked in the gene encoding the meta-pyrocatechase enzyme. Both methyl- and chlorocatechol were metabolised *via* an ortho pathway.

Oxygenolytic Elimination of Halogen

It has been shown in the chapters above that metabolism of haloarenes *via* halocatechols and subsequent ortho cleavage is a useful tool in microbial degradation of these compounds. Such ortho-cleaving routes are restricted, however, to mono- and disubstituted halocatechols. Even these compounds may be rendered slowly

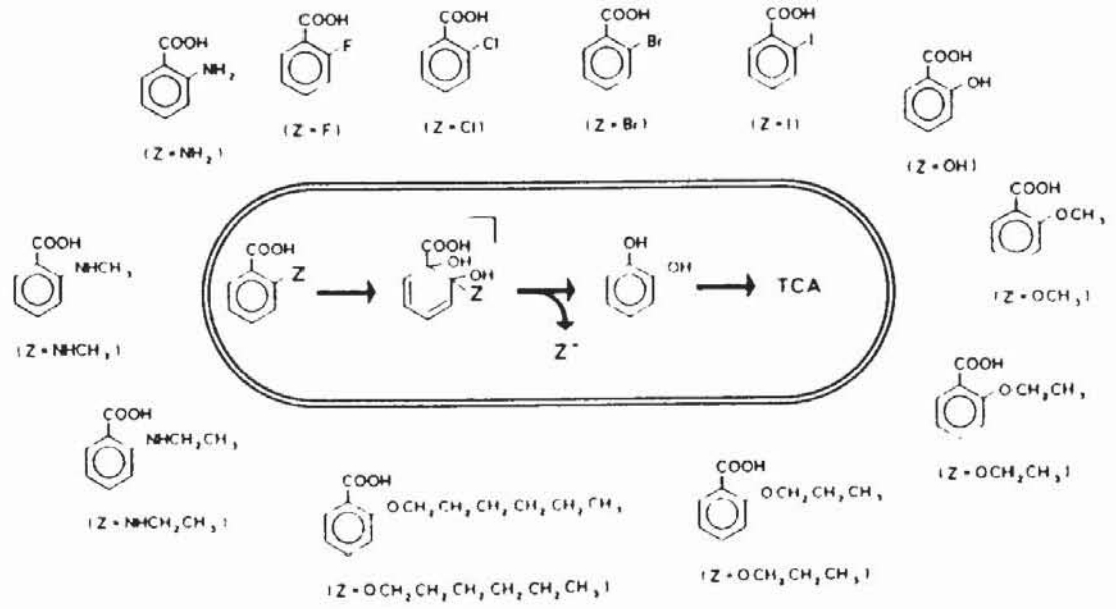

Fig. 2.11. Degradation of various aromatic substrates by initial dioxygenation in *Pseudomonas* putida CLB 250.

biodegradable by unfavourable substitution patterns, e.g. vicinal dihalo substitution. Biomineralisation therefore is greatly facilitated if some mechanism exists by which halo substituents are eliminated at an early metabolic stage.

2-Fluoro- and 2-chlorobenzoate are frequently employed as model substrates for oxygenolytic substituent removal. Dioxygenase attack yields geminal halohydroxy compounds which spontaneously rearomatise to unsubstituted catechol, with concomitant liberation of halide (Engesser *et al.* 1980; Hickey and Focht 1990; Engesser and Schulte 1989; Vora *et al.* 1988). This oxygenolytic mechanism apparently also works for 2-bromo- and, after some mutation, for 2-iodobenzoate (Engesser and Schulte 1989). The halogen-free catechols thus formed are further metabolised along ortho pathways. The 1,2-dioxygenation mechanism was established unequivocally by analysis of the 2-methylbenzoate cometabolism products. For degradation of 2-chlorobenzoate by *Pseudomonas* sp. strain B300, however, oxygenolytic elimination of halide was not confirmed with certainty, and a degradation pathway *via* 3-chlorocatechol could not be definitely excluded (Sylvestre *et al.* 1989).

Interestingly, the 2-chlorobenzoate-degrading enzyme system in *Pseudomonas* sp. CLB 250 (Engesser and Schulte 1989) proved to be functional also for various other 2-substituted benzoates with substituents which may be eliminated as anions (see Fig. 2.11), e.g. alkoxy, amino, and alkylamino groups. Dioxygenases thus could be demonstrated to effectively cleave ether molecules. This capacity was extended even to the degradation of halogenated dibenzofurans and dibenzodioxins (see Strategies for Degradation of Chlorinated Dibenzofurans and Dibenzodioxins).

Fluoride elimination from 3,5-difluoro-4-hydroxybenzoate, with accumulation of 5-fluoro-3,4-dihydroxybenzoate (5-fluoroprotocatechuate), was effected by the monooxygenating 4-hydroxybenzoate-3-hydroxylase of *Pseudomonas fluorescens* (Husain *et al.* 1980). Dehalogenation of 3-substituted fluorobenzenes by a toluene dioxygenase has recently been reported (Renganathan 1989); this rather unspecific enzyme at the same time produced varying amounts of fluorocatechols, however, depending on the size of the substituent in 3-position.

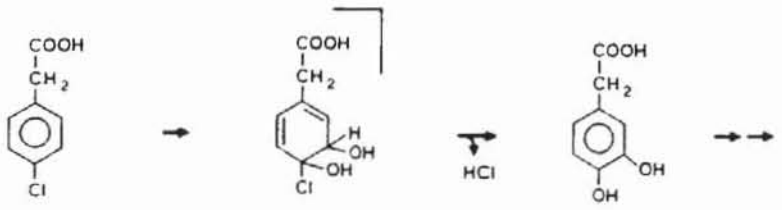

Fig. 2.12. Degradation of 4-chlorophenyl acetate by a *Pseudomonas* species strain CBS 3.

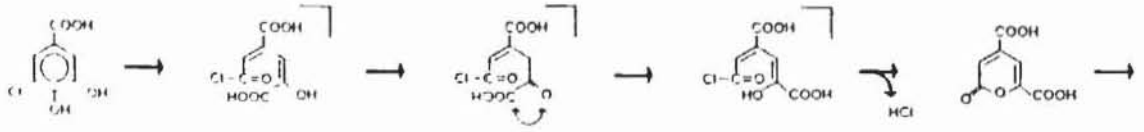

Fig. 2.13. Bacterial dehalogenation of 5-chlorovanillate.

A *Pseudomonas* sp. strain CBS3 has been suggested to dioxygenate 4-halophenyl acetates to unstable *cis*-dihydrodiols (Klages *et al.* 1981; Markus *et al.* 1984) which, after rearomatisation and elimination of halide, afforded homoprotocatechuate (see Fig. 2.12). The two enzyme components catalyzing the dehalogenation were purified, and the reaction was demonstrated to require NADH and Fe^{2+}. No final proof for a dioxygenation mechanism was presented, however, e.g. by product analysis of unproductive alkyl-analogue turnover or by employing mixtures of $^{18}O_2/^{16}O_2$. The homoprotocatechuate formed is metabolised *via* another meta pathway.

Dehalogenation of Halocatechols after Meta Cleavage: the 5-Chlorovanillate Case

In the course of the bacterial catabolism of lignin model compounds, 5-methoxyvanillate was transformed into 5-methoxyprotocatechuate, and the methoxy substituent then removed in the course of the dioxygenation catalyzed by a protocatechuate 4,5-dioxygenase (Kersten *et al.* 1985). Surprisingly, the chlorine in 5-chlorovanillate is removed analoguously after ring cleavage of 5-chloroprotocatechuate (see Fig. 2.13), with a pyrone being formed (2-pyrone-4,6-dicarboxylate). Dehalogenation seems to be gratuitous in this case; it may offer a tool, though, for the evolution of new dehalogenating enzymes from ether-cleaving oxygenases. There are indications, on the other hand, that chloride is eliminated from 4-chlorobenzoate in the first metabolic step of a 4-carboxybiphenyl ether-cleaving dioxygenase (Engesser *et al.* 1990a). As yet, the productive meta-cleavage of halocatechol derivatives, described above, has rarely been found in nature, and seems to be restricted to ortho-halosubstituted catechols.

Consequently, 30% only of the organic halogen was released as halide (measured reliably by a halogen electrode) in the course of cometabolism of the 4-halocatechols produced from 4-chloro- and 4-fluorobenzoate by *Pseudomonas putida* mt-2. A chlorinated semialdehyde was characterised extensively, and the proximal mode of ring

cleavage established unequivocally for 4-halocatechols (Schacht *et al.* submitted for publication). Slow cometabolic degradation *via* meta pathway was claimed for 4-chlorocatechol derived from 4-chlorophenol, with 85% of the stoichiometric amount of halide reputedly being released. Halide detection was based on a spot test, though, and could not be reproduced in our laboratory with the original strain. Also, no ring-cleavage products were characterised (Janke and Fritsche 1979).

Chloro-substituted products of chlorocatechol ring cleavage accumulated in the culture broth during degradation of 1- and 2-chloronaphthalin (Morris and Barnsley 1982). The authors speculated about the toxicity of these compounds without any firm structural data. No growth was observed with chloronaphthalenes, and chloride release was not measured.

Despite many efforts, a productive, i.e. growth-sustaining metabolism of halogenated catechols *via* meta pathways has not been verified so far. Cometabolic liberation of halide, however, may occur to varying extent.

Strategies for Degradation of Chlorinated Dibenzofurans and Dibenzodioxins

Chlorinated dibenzofurans and dibenzodioxins are among the most pernicious chemicals. Up to now, no single microorganism has been found which was able to degrade higher chlorinated dibenzofurans or dibenzodioxins. In devising a strategy for rendering these recalcitrant molecules biodegradable, it is essential to dissect the tricyclic compounds into individual structural elements such as the biarylether linkage and the vicinal dichloro-substitution pattern. Some knowledge has been accumulated in the last few years on the degradation of 1,2-dichloroarenes (Meer *et al.* 1991; Haigler *et al.* 1988; Allard *et al.* 1991). Little information is found in the literature on degradation of biaryl ethers in general, and of cyclic biaryl ethers in particular. Efforts have been concentrated, therefore, on the microbial, especially bacterial degradation of biaryl ethers. Once the two isolated problems have been resolved, the single degradative capabilities must be combined either in a single strain or in a mixed culture; this has been shown a useful tool in biodegradation (Engesser *et al.* 1989a).

As described above, few competent model systems existed for the dioxygenolytic cleavage even of alkyl aryl ethers (Kersten *et al.* 1982), and none for diaryl ethers (Engesser and Schulte 1989). In the meantime, dioxygenolytic ether cleavage surprisingly has been realised even with cyclic biarylethers. Degradation of 3- and 4-carboxybiphenylether (CBPE) (Wittich 1990; Engesser *et al.* 1990a) is triggered by initial 1,2-dioxygenation, shown by isolation and unequivocal characterisation of dead-end products of ether analogues (Engesser *et al.* 1990a). Dioxygenase attack on aryl ether bonds yields chemically labile hemiacetals which, in the case of CBPE, decompose to protocatechuate and phenol both of which are easily biodegradable. This clearly demonstrates the superiority of an early cleavage of those bonds, constituting the xenobiotic structure, over cleavage at a later stage.

Dibenzofuran- and dibenzodioxin-attacking bacteria have been described quite recently (Engesser *et al.* 1989b; Fortnagel *et al.* 1990). A detailed investigation of the DBF metabolism revealed initial dioxygenolytic cleavage of the aryl ether bond once again to be the crucial step in the overall degradation (Engesser *et al.* 1989b). The product of dibenzofuran ether cleavage, 3-(2-hydroxyphenyl)catechol (HPC), was shown to suffer

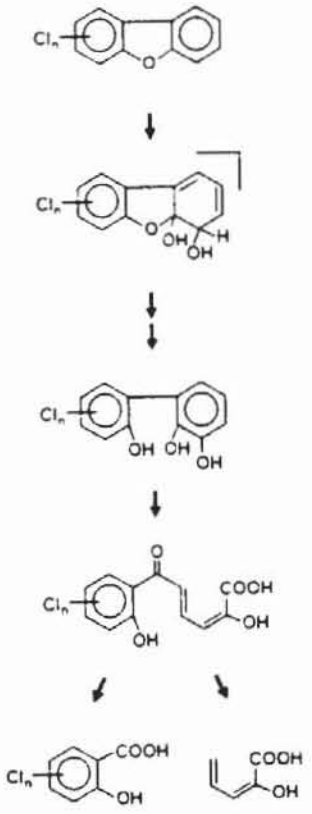

Fig. 2.14. Degradation of chlorinated dibenzofuran ethers by bacteria.

meta cleavage (Strubel *et al.* 1991). In an ensuing step, hydrolase activity produces salicylate and 2-oxo-4-pentenoate, with the same pathway being proposed for chlorinated analogues (Fig. 2.14). Unsubstituted salicylate is metabolised *via* catechol which then is cleaved in meta fashion and subsequently degraded by enzymes of the meta pathway; the same mechanism holds for the oxopentenoate metabolite. Recently, oxidation of 2-chloro- and 2,8-dichloro-DBF has been claimed (Parsons *et al.* 1990) without, however, establishing the biochemistry of the process.

Even though degradation of dibenzodioxin and its chlorinated analogues is complicated by the second ether bond, organisms have been described which effectively transform the parent compound (Harms *et al.* 1990). Mono-, di-, and, to a limited extent, trihalogenated DBDs were already shown to be metabolised substantially by several bacterial species, pre-grown on dibenzofuran. No products, however, were given (Rast *et al.* DBP 1989). There clearly is a pressing need for research in this field in order to identify bacteria which are able to effectively degrade dibenzodioxins and finally the chloro derivatives. The extremely limited "bioavailibility" of these compounds may have to be overcome by applying two-phase systems.

Hydrolytic Displacement of Halogen

Chlorobenzoates

Hydrolytic liberation of halide, at an early stage of the overall metabolism, greatly enhances the biodegradability of the target compounds, as in the case of the oxygenolytic removal of halosubstituents. Such hydrolytic dechlorination was first shown with an *Aspergillus niger* strain transforming 2-chloro- to (2-hydroxyphenoxy)acetate (Faulkner and Woodcock 1961). Later, degradation of 3-chlorobenzoate *via* 3-hydroxy- and 3,6-dihydroxybenzoate (gentisate) was described (Johnston *et al.* 1972), which definitely proceeded without any chlorocatechol production. The stoichiometry of 3-hydroxybenzoate excretion and substrate turnover was not determined, however, and the proposed hydrolytic mechanism of chloride substitution could not be established unequivocally.

In the following period, many different cases of hydrolytic halogen removal were presented. In one case, hydrolytic liberation of halide from 4-chlorobenzoate was reported even for alkalophilic conditions (Shimao *et al.* 1989). Besides 4-fluorobenzoate (Oltmanns *et al.* 1989), 4-chlorobenzoate was frequently employed as substrate; it is dechlorinated by various *Arthrobacter* (Ruisinger *et al.* 1976; Marks *et al.* 1984b), *Nocardia* (Klages and Lingens 1979), *Pseudomonas* (Klages and Lingens 1980), *Acinetobacter* (Adriaens *et al.* 1989; Adriaens and Focht 1991), and *Alcaligenes* species (Tweel *et al.* 1986). If, in the degradation by *Alcaligenes*, oxygen concentration was reduced to 1.2 % of the normal saturation concentration, accumulation of 4-hydroxybenzoate was observed which the authors had suggested as the direct product of hydrolysis. No definite proof was given, though, for the hydrolytic and non-oxygenolytic nature of halide liberation, e.g. by ^{18}O-labeling experiments. In all the transformations described so far, 4-hydroxybenzoate was metabolised first to 3,4-dihydroxybenzoate which then was degraded along either ortho (Klages and Lingens 1980) or meta pathways (Ruisinger *et al.* 1976; Klages and Lingens 1979).

Incorporation of ^{18}OH, from ^{18}O-labeled H_2O, definitely proves that dehalogenation proceeds as a hydrolytic process (Müller *et al.* 1984; Marks *et al.* 1984a). No ^{18}O label was incorporated into the 4-hydroxybenzoate formed in a negative control experiment under incubation with $^{18}O_2$ gas (Marks *et al.* 1984a). The enzyme from *Pseudomonas* CBS3 (Klages and Lingens 1980) in crude extracts was found to be activated by Mn^{2+} and Co^{2+}. In contrast to the enzyme from *Arthrobacter*, 4-fluorobenzoate could not be converted (Thiele *et al.* 1987). The polar or even ionic carboxyl group seems to be requisite for substrate binding (Thiele *et al.* 1988) since methyl 4-chlorobenzoate was not a substrate. Cloning experiments at first seemed to indicate that more than one gene was involved in 4-chlorobenzoate hydrolysis (Savard *et al.* 1986); recently, it was demonstrated that, in a first step, the 4-chlorobenzoate is transformed to the CoA ester which is the effective substrate for the action of a regular molecular-weight hydrolase (Löffler *et al.* 1991). The key step accordingly is a nucleophilic attack at the chloro-substituted C-4 carbon atom of the 4-chlorobenzoate CoA-ester (see Fig. 2.15).

A recent report (Adriaens and Focht 1991) on the mechanism of hydrolysis of the aryl chloro bond, based on results of the 3,4-dichlorobenzoate transformation by an *Acinetobacter* strain 4-CB1, may be interpreted in favour of an addition-elimination mechanism (see Fig. 2.16). In a first step, the para-Cl is replaced by OH. Water then is added in 3,4-position followed by elimination of HCl. Subsequent enzymatic processes

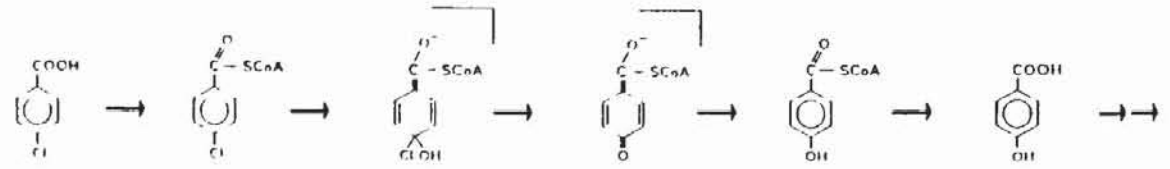

Fig. 2.15. Chloroarene dehalogenation mechanism involving nucleophilic attack at the chloro-substituted Cl atom.

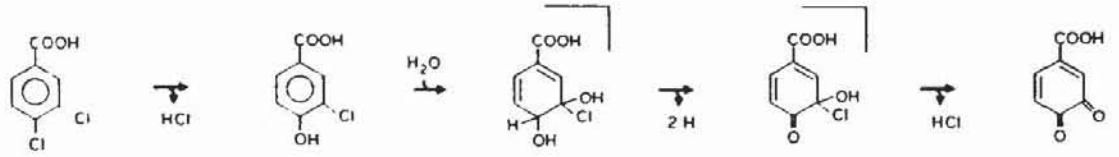

Fig. 2.16. Addition-elimination mechanism for the hydrolysis of the aryl chloro bonds in 3, 4-dichlorobenzoate.

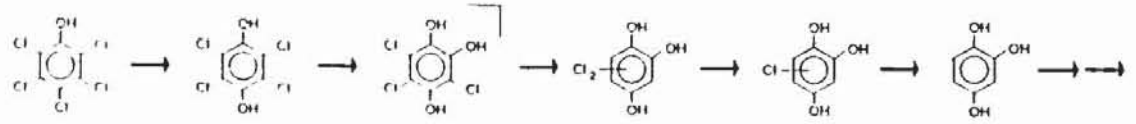

Fig. 2.17. Hydrolytic degradation of pentachlorophenol involving two substitition and three reductive dehalogenation steps.

yield 4-carboxy-*o*-quinone which may be the product of anaerobic cometabolism of 3,4-dichlorobenzoate by the 4-chlorobenzoate-grown cells. No matter which enzymatic mechanism actually prevails, the aromatic π system of the chloroarene substrate has to be broken up prior to hydrolysis of the carbon-halogen bond.

A combination of reductive and hydrolytic attack was reported for 2,4-dichlorobenzoate (Zaitsev and Karasevich 1985; Tweel *et al.* 1987), the initial product of reductive dechlorination, 4-chlorobenzoate, being metabolised *via* hydrolytic halide replacement (see Liberation of Halide by Reductive Mechanisms). For a *Pseudomonas aeruginosa* strain, recently reported to dehalogenate 2-bromobenzoate, hydrolytic displacement of halogen is indicated by accumulation of salicylate in oxygen-depleted cells (Higson and Focht 1990a).

Pentachlorophenol (PCP

For hydrolytic pentachlorophenol (PCP) degradation (Häggblom *et al.* 1989; Apajalahti and Salkinoja-Salonen 1987), initial substitution of chlorine by OH to 2,3,5,6-tetrachlorohydroquinone was proposed, a second substitution step yielding 3,5,6-trichloro-1,2,4-trihydroxybenzene (see Fig. 2.17). Further degradation proceeded *via* three reductive dehalogenation steps (see Liberation of Halide by Reductive Mechanisms). The hypothesis that the *p*-chlorine substituent is displaced hydrolytically

not only in pentachlorophenol but also in 2,3,4,5- and 2,3,4,6-tetrachlorophenol, seems to be confirmed by labeling experiments with $H_2{}^{18}O$ for which ^{18}O-labelled hydroquinone intermediates were established (Apajalahti and Salkinoja-Salonen 1987). However, the hydrolytic displacement reaction worked only in the presence of NADH; additionally, at least traces of oxygen were required. This prompted other researchers to carefully re-examine the first dechlorination step in the *Arthrobacter* strain ATCC 33790 (Schenk *et al.* 1989; Schenk *et al.* 1990). Labeling experiments with $H_2{}^{18}O$ and $^{18}O_2$, as possible oxygen donors, revealed that para-^{18}OH groups were introduced only when the enzyme extract was incubated with NADH/O_2 as ^{18}OH equivalents. However, since the ^{18}OH label was also non-enzymatically incorporated into tetrachlorohydroquinone from $H_2{}^{18}O$, no differentiation is possible between hydrolytic and oxygenolytic removal of halogene. These results, i.e. the requirement for oxygen and a reduced pyridine dinucleotide being present, allow the assumption that the first step in PCP degradation is an oxygenolytic attack also for the *Rhodococcus* strain (see Apajalahti and Salkinoja-Salonen 1987). Interestingly, PCP degradation potential seems to be rather wide-spread, having been demonstrated for many bacteria (Rott *et al.* 1979; Saber and Crawford 1985; Suzuki 1977; Watanabe 1973; Liu *et al.* 1981; Klecka and Maier 1985; Pignatello *et al.* 1983). A *Flavobacterium* sp. has been demonstrated to also attack triiodophenols after growth on PCP (Xun and Orser 1991).

N-Heterocycles

s-Triazines which are widely used as herbicides have been described to be biodegradable in both aerobic and anaerobic systems (Cook and Hütter 1981; Jessee *et al.* 1983). These compounds, just as some chlorinated derivatives like 6-amino-2-chloro-4-ethylamino-1,3,5-triazine, serve as source of nitrogen. Since the transformation was carried out anaerobically in cell extracts, the authors concluded the dehalogenation to follow a hydrolytic mechanism. Degradation was triggered by removal of the chlorine substituent, yielding 2-amino-4-ethylamino-1,3,5-triazine-6(5H)-one. Two dehalogenases have been proposed which differ in specificity towards the two alkylamino functions in these triazine agrochemicals (Cook and Hütter 1986). The subject of s-triazine degradation is covered in an excellent review (Cook 1987).

In the breakdown of 5-chloro-2-hydroxynicotinic acid, a dead-end product of 3-chloroquinoline-8-carboxylic acid partial degradation (see Channelling of Complex Haloarene Structures into Key Intermediates), chloro-substituted maleic or fumaric acid were proposed as intermediates which in turn are subject to hydrolytic dehalogenation (Tibbles *et al.* 1989b).

Liberation of Halide by Reductive Mechanisms

Molecular oxygen was initially considered to be essential for the activation of arenes by hydroxylation. Non-activated benzene derivatives, with three or more halogen substituents, would be expected, from simple mechanistic considerations, to be more or less non-biodegradable by enzymes which employed an electrophilic hydroxylation mechanism. Unexpectedly, polyhalophenols were found to still be degraded in aerobic systems, most probably by action of monooxygenating enzymes. This seemed to invalidate the "dogma" that heavily chloro-substituted arenes are not subject to aerobic

microbial attack. One might argue, however, that the electrophilic displacement of the first chlorine substituent in polyhalophenols is facilitated by the mesomeric effect of a para-hydroxyl group. This is exemplified e.g. by the formation of tetrachloro-*p*-hydroquinone from pentachlorophenol discussed above (Häggblom *et al.* 1989; Apajalahti and Salkinoja-Salonen 1987).

Still more surprisingly, dioxygenolytic liberation of chloride from tetrachlorobenzenes was claimed in two cases (Springer and Rast 1988; Wittich *et al.* 1989). These substrates are deactivated towards electrophilic attack by four chlorines without the remedy of an activating OH-substituent. The situation is problematical, though, only as long as an electrophilic mechanism of hydroxylation is assumed; for a possible attack by radical species, these objections would no longer hold.

Dehalogenation of an aromatic nucleus was reported even in the absence of oxygen (Ide *et al.* 1972; Kuwatsuka and Igarashi 1975; Murthy *et al.* 1979). Accumulation of lesser chlorinated phenols like 2,3,5- and 2,4,5-tri-, 3,4-di- and 3-chlorophenol from pentachlorophenol indicated a reductive mechanism of chlorine removal without additional hydroxylation steps. Many reductive dehalogenation reactions have been described since these early reports, e.g. for pesticides such as techlofthalam (Kirkpatrick *et al.* 1981), thiobencarb (Moon and Kuwatsuka 1984), chlornitrofen (Yamada and Suzuki 1983), 2,4,5,6-tetrachloroisophthalonitrile (Sato and Tanaka 1987), and diuron (Attaway *et al.* 1982; Stepp *et al.* 1985). Partial metabolism was observed in many cases, with only one chlorine atom being removed and lesser chlorinated metabolites being accumulated.

Polyhalophenols are metabolised, after initial attack presumably by monohydroxylases (Apajalahti and Salkinoja-Salonen 1987), by hydrolytic as well as reductive processes, with complete liberation of the organic halogen as halide. A cell free extract of a *Rhodococcus* species (Häggblom *et al.* 1989) was found to catalyze formation of 1,2,4-trihydroxybenzene from 2,3,5,6-tetrachlorohydroquinone (see Fig. 2.16), following a reductive mechanism. This same mechanism seems to operate in the degradation of 2,3,5,6-tetra-, 2,3,6- as well as 2,4,6-tri-, and 2,6-dichlorophenol by a *Flavobacterium* species isolated with pentachlorophenol as a selection substrate. The dehalogenation enzymes preferentially dehalogenate 2,6-disubstituted halophenols (Steiert *et al.* 1987).

Purely reductive dechlorination systems have been described in addition to the combined action of hydrolytic/oxygenolytic on one, and reductive dechlorination systems, on the other hand. Partial dechlorination of pentachlorophenol to lesser chlorinated phenols was found to depend on pre-enrichment with monochlorophenols (Mikesell and Boyd 1986). A combination of these enrichment cultures could be demonstrated to totally dehalogenate pentachlorophenol, with some formation of methane. For pentachlorophenol, uniformly labeled with ^{14}C, 70% of the radioactive carbon was recovered in form of $^{14}CO_2$ and $^{14}CH_4$.

Aquatic sediments were found to liberate halogen from mono- and dichlorophenols (Sharak-Genthner *et al.* 1989a), 4-chlorophenol generally being degraded with the slowest rate. Degradation of 3- and 4-chlorobenzoate in some cases was nitrate-dependent, suggesting nitrate respiration as the energy-providing process (Sharak-Genthner *et al.* 1989b). A 2-chlorophenol-mineralising anaerobic mixed culture was isolated from sewage sludge which dehalogenated also 2-bromo- and 2,6-dichlorophenol reductively. 2,4-Dichlorophenol in this system was transformed only to 4-chlorophenol which once again proved to be hardly degradable under anaerobic conditions (Dietrich and Winter 1990). Recently, however, 2,4-dichlorophenol was

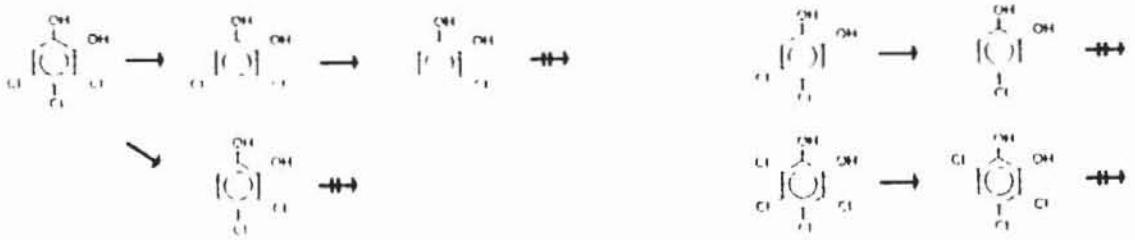

Fig. 2.18. Anaerobic dechlorination of chlorinated catechols.

demonstrated to be totally dechlorinated *via* 4-chlorophenol and phenol; this finally was carboxylated to benzoate (Zhang and Wiegel 1990).

A 2,4-dibromophenol-degrading consortium was described, without commenting on the energetics of the dehalogenation step (King 1988), which consists of dehalogenating organisms and sulfate-reducing bacteria. The latter did not directly attack the halophenol but rather utilised the dehalogenated phenol.

Quite recently, a rare case of anaerobic dechlorination was reported for chlorinated catechols, i.e. 1,2-dihydroxybenzenes (Allard *et al.* 1991). Cultures enriched with vanillins, catechin, and phloroglucinol as substrates exhibited dechlorinating activity with chloro catechols. The process is highly specific, yielding, for example, exclusively 3-chlorocatechol from 3,4,5-trichlorocatechol. Chloride removal was observed also with other isomeric chlorocatechols as shown in Figure 2.18.

Anaerobic transformation of 3-bromo-4-hydroxybenzaldehyde to 4-hydroxybenzoate and, ultimately, phenol has been described to be catalyzed by cultures enriched from sediments (Neilson *et al.* 1988).

A rare case of phototrophic metabolism of 3-chlorobenzoate (3CB) was recorded for *Rhodopseudomonas palustris* WS17 (Kamal and Campbell-Wyndham 1990). Of the radioactive label from 3-chlorobenzoate, 75 % was incorporated into the biomass, 25% being liberated as $^{14}CO_2$. The authors claimed chloride release to occur during reductive degradation of the cosubstrate, benzoate, without presenting any detailed evidence, however, for this pathway.

Biotransformation of polyhalogenated biphenyls probably represents the most important instance of reductive dehalogenation. As described above (see Channelling of Complex Haloarene Structures into Key Intermediates), aerobic metabolism of polychlorinated biphenyls (PCBs) frequently leads to accumulation of chlorinated intermediates which may be bound chemically to soil components (Brunner *et al.* 1985) thus feigning total degradation. Intensive dehalogenation, prior to aerobic metabolism, would render PCBs much more susceptible to total breakdown into CO_2 and chloride, the ultimate goal of biodegradation. Sediments from the Hudson River, New York, were investigated for anaerobic degradation potential towards PCBs. Mono- and di-chlorinated biphenyls were degraded most efficiently, biphenyls with four or more chloro substituents were not. Trihalogenated biphenyls were transformed only when the chlorine substituents were scattered over both rings; 2,3-dichloro substitution, for example, strongly reduced transformation rates (Chen *et al.* 1988).

Other investigators found, however, that even Arochlor 1260^R, with an average of six chlorine atoms per molecule, was attacked in Hudson river sediments. Since different patterns of lesser chlorinated PCBs were formed, different microbial consortia seemed to participate in these transformations. Dehalogenation reactions were clearly biological, no significant chemical reduction taking place (Brown (jr.) *et al.* 1987). In a separate investigation, dechlorination of Arochlor 1242^R was demonstrated at a

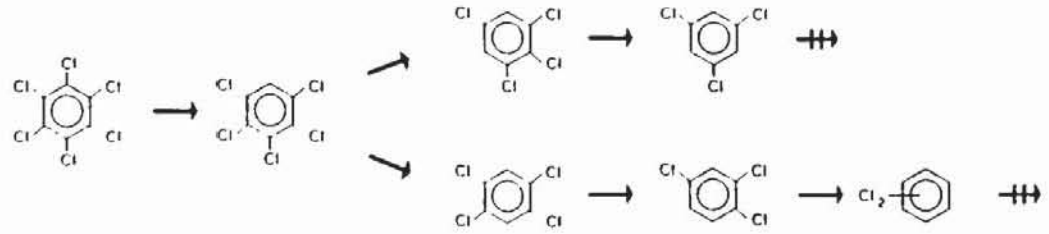

Fig. 2.19. Reductive dechlorination of hexachlorobenzene.

concentration of as high as 700 mgl^{-1} sediment. Within 16 weeks, half of the organic chlorine was metabolised, with the percentage of mono- and dichloro biphenyls increasing from 9% to 88%. The para and meta chlorines were displaced preferentially (Quensen (III) *et al.* 1988). Arochlor^{R}1260, in contrast, proved to be more recalcitrant, and in laboratory degradation experiments required far longer adaptation times. After one year, only 15% of the meta and para chlorines had been removed. Lesser chlorinated biphenyls again proved to be very susceptible to reductive dechlorination in these systems (Quensen(III) *et al.* 1990).

Reductive dechlorination by microorganisms has been described also for hexachlorobenzene (Fig. 2.19). The final products, 1,3,5-tri- and isomeric dichlorobenzenes, were not transformed further; penta- and tetrachlorobenzenes, however, were accumulated only transiently (Fathepure *et al.* 1988). Different organisms, in soil columns, were shown to reductively dechlorinate tri- and dichlorobenzenes to chlorobenzene as the final metabolite (Bosma *et al.* 1988). These dechlorination reactions seem to be mediated by rather unspecialised enzymes since bacteria of many different species, isolated from intestine and not previously exposed to haloarenes, were able to catalyze dechlorination of 1,2,4-trichlorobenzene to chlorobenzene (Tsuchiya and Yamaha 1984). As enzymatic reactions, these dehalogenations still show considerable specificity. Another hexachlorobenzene-degrading consortium was shown to accumulate 1,2,3- and 1,2,4-tri- as well as 1,3- and 1,2-dichlorobenzene (Mousa and Rogers 1990). Partial dechlorination of hexachlorobenzene by glutathione addition has been demonstrated for mammals, penta- and tetrachlorobenzene being formed besides 2,3,5,6-tetrachlorophenylmethylmercaptan (Renner and Nguyen 1984).

Halosubstituted anilines have also been described to be dehalogenated reductively. For example, 2,4- and 3,4-dichloroaniline are transformed to monochloroanilines. These dichloroaniline-transforming cultures degrade 3,4-dichlorophenol without any lag phase; thus, cross acclimation seems to have taken place between phenol- and aniline-transformation processes (Struijs and Rogers 1989). 2,3,4,5-Tetrachloroaniline was reductively dechlorinated to 2,3,5-tri- and 3,5-dichloroaniline (Kuhn and Suflita 1989) (Fig. 2.20); the principle that chlorine substituents in ortho or para position to an electron-releasing group are removed preferentially, seems to hold also for anilines.

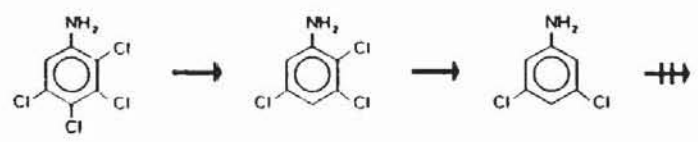

Fig. 2.20. Reductive dehalogenation of chlorosubstituted anilines.

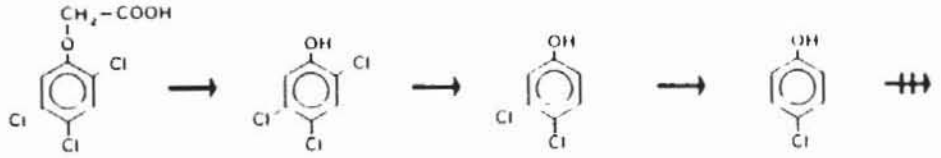

Fig. 2.21. Cleavage of the aryl alkyl ether bond and subsequent reductive dechlorination of chlorinated phenoxyacetate.

In pond sediments, reductive dechlorination of Diuron^R (3,4-dichlorophenyl-1,1-dimethylurea) in 4-position yielded 3-chlorophenyl-1,1-dimethylurea in stoichiometric amount (Attaway *et al.* 1982). Structurally related herbicides also suffer reductive dehalogenation (Stepp *et al.* 1985).

Chlorinated phenoxyacetates were demonstrated to be reductively dechlorinated after cleavage of the aryl alkyl ether bond. A 3-chlorobenzoate-enriched consortium selectively substituted the para-chlorine in 2,4,5-T (2,4,5-trichlorophenoxyacetate), yielding (2,5-dichlorophenoxy)acetate (Suflita *et al.* 1984). 2,4,5-T, after cleavage of the ether bond and removal of the ortho chlorine, was transformed to 3,4-dichlorophenol in anaerobic sewage sludge. Further removal of the meta chlorine resulted in the accumulation of 4-chlorophenol which could not be degraded further (Mikesell and Boyd 1985) (see Fig. 2.21).

A 3- and 4-chlorobenzoate-degrading consortium was found to degrade the halobenzoates anaerobically, inevitably requiring nitrate. The authors speculated about a reductive dechlorination of halobenzoates to benzoate which in turn was proposed to be degraded with nitrate as an electron acceptor (Sharak-Genthner *et al.* 1989a).

A reductive dechlorination of DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) was reported to be catalyzed by a *Pseudomonas aeruginosa* strain (see Channelling of Complex Haloarene Structures into Key Intermediates).

There is only scattered knowledge on the mechanism of reductive dechlorination reactions despite their tremendous potential for bioremediation of contaminated sites. One crucial question is whether the microorganisms can derive energy from the dehalogenating reaction and thus may be kept in pure culture.

The best studied model system is the dehalogenation of 3-halo-substituted benzoates (Suflita *et al.* 1982; Horowitz *et al.* 1983). For a 3-chlorobenzoate-degrading consortium, the individual organisms were separated, and a strain DCB-1 was recognised as principal dechlorinating organism (Shelton and Tiedje 1984). It could be shown from thermodynamic calculations that the reductive dechlorination step in strain DCB-1 is exergonic, supplying energy to the cell by a novel type of chemotrophic mode of growth (Dolfing 1990; Mohn and Tiedje 1990).

Degradation of 2-fluorobenzoate by benzoate-dissimilating, denitrifying bacteria was reported (Schennen *et al.* 1985). When cells were grown anaerobically on benzoate, they transformed 2-fluorobenzoate without any lag period, under concomitant defluorination. A benzoyl-CoA-synthetase, the key enzyme especially of anaerobic benzoate degradation, was demonstrated to be active also towards 2-fluorobenzoate. The authors speculated about fortuitous elimination of fluoride from reduced intermediates, no clear-cut evidence, however, being presented for the reductive nature of the defluorination mechanism. This holds also for the anaerobic defluorination of 2- and 4-fluorobenzoate, catalyzed by other denitrifying bacteria (Taylor *et al.* 1979).

In general, however, the exact mechanism of reductive dechlorination remains uncertain. The reaction may, for one, proceed in form of direct substitution by hydride.

Alternatively, a two-step process may be envisaged, e.g. by substitution of halogen by a hydroxyl group which would have to be followed by reductive dehydroxylation. The fact that reductive dehalogenation is an energy-yielding process would eventually be met also by an intermediate dehydroxylation step which from calculations was estimated to be an energy-conserving reaction (Szewzyk *et al.* 1985). An especially attractive mechanism would follow the addition-elimination process analoguous to that discussed for the hydrolytic removal of halogen by haloarene hydrolases (see Hydrolytic Displacement of Halogen and Figure 2.16) (Adriaens and Focht 1991)). Further in-depth investigations are definitely necessary to clarify this issue. Although the first step in reductive transformation of benzene derivatives generally is strongly endergonic (Evans and Fuchs 1988), the overall reaction may be exergonic due to the elimination of halide.

Conclusions

An important application of HAH biodegradation is bioremediation, i.e. the clean-up of polluted environmental compartments by microbiological methods. On-site trials have shown pentachlorophenol (PCP) to be amenable to biodegradation, with PCP concentration being reduced from 200 mg kg^{-1} to 15 mg kg^{-1} soil upon addition of a *Rhodococcus* species (Valo and Salkinoja-Salonen 1986). Soil contaminated with 2,4,5-T (2,4,5-trichlorophenoxyacetate) was decontaminated after supplementation with a *Pseudomonas* strain, the herbicide concentration being reduced from an initial 20 g kg^{-1} to 1-2 g kg^{-1} soil (Kilbane *et al.* 1983). In these cases, organisms were available which derived energy from the biodegradation processes. Transformation of polychlorinated biphenyls (PCBs), in contrast, seems not to yield energy, and thus does not sustain growth. To overcome this limitation, unsubstituted biphenyl was added to soil heavily contaminated with PCBs. Thus, the cometabolic transformation potential was enhanced considerably although still no total degradation was observed (Brunner *et al.* 1985).

The generally very lipophilic HAHs tend to be absorbed on soil particles (Sabljic 1989). Therefore, the "bioavailability", i.e. the accessibility of these substrates for microorganisms otherwise very competent for degradation, is severely reduced. Addition of emulsifiers or of organic phases (Viney and Bewley 1990; Brink and Tramper 1985) may greatly improve degradation efficiency (Harvey *et al.* 1990). Alternative microorganisms were described producing their own bioemulsifiers (Ramsay *et al.* 1988; Morgan and Watkinson 1989). Research on and application of bioremediation techniques is nicely covered by a recent review (Morgan and Watkinson 1989).

Biodegradation can effectively aid in clean-up of contaminated soils and aquifers. To overcome its limitations, microbiology, biochemistry, chemistry, genetics, and process engineering must collaborate in developing microorganisms with improved degradative capacities when natural evolution does not produce the desired activities.

Acknowledgement

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