Microbial degradation of synthetic organochlorine compounds

by K. Motosugi and K. Soda[1]

Research and Development Center, Unitika Ltd, Uji, Kyoto-Fu 611 (Japan), and Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611 (Japan)

Synthetic organochlorine compounds as pollutants

Although pesticides are designed to be selective, that is, more toxic to harmful organisms than to man and beneficial organisms, their selectivity is not always complete. Most pesticides are degraded chemically and biologically in nature. Their chemical degradation in water and soil is influenced by several factors such as pH, temperature, sunlight, moisture and coexisting inorganic and organic compounds. Examples of organochlorine compounds that have been used as pesticides are shown in figure 1. Because of their persistence in the environment, the use of organochlorine pesticides has been minimized in developed countries.

For four decades, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and its derivatives have been widely used as herbicides, especially for the control of brush, jungle and aquatic weeds. The 1968 annual production of these compounds exceeded 60 million pounds in USA[50], and residues of the substances have been detected in crops, soil, the atmosphere, rainwater and surface water. Technical 2,4,5-T contains traces of the highly toxic compound 2,3,7,8-terachlorodibenzo-p-dioxin (TCDD) as an impurity[2]. In addition, about 0.0002% of 2,4,5-T is converted to TCDD when wood or brush containing 2,4,5-T is burned. The spraying of the herbicide Orange (butyl esters of 2,4-D and 2,4,5-T in an equal amount) over jungle in Southeast Asia has resulted in the accumulation of TCDD in soil at a mean level of 1.9 ppm with maximum levels of 47 ppm. Pollution by TCDD has developed into a cause of continuing public anxiety throughout the world[10].

As summarized in two recent reviews[14,28], the toxity of organochlorine pesticides and their effects on microorganisms have been studied extensively.

Organochlorine compounds used in industry and as pesticides are often found in drinking water. Approximately 80 different organic halogen compounds have been demonstrated in drinking water in the United States[49]. Several of these are listed in table 1. Trihalomethanes such as bromodichloromethane, chlorodibromomethane and chloroform, which have been demonstrated ubiquitously in chlorinated water and which are suspected as carcinogens[13], are found even in drinking water. Chlorination of water is used widely for disinfecting water since it is the most effective method for killing waterborne pathogens

Table 1. Organic halogen compounds identified in drinking water[49]

Aldrin
Bromodichloromethane
Chlorobenzene
Chlorodibromomethane
Chloroethene (vinyl chloride)
Bis(2-chloroisopropyl)ether
p-Chlorotoluene
Dichlorobenzene
Dichloroethane
Dichloroethene
Dichloromethane
2,4-Dichlorophenol
Dieldrin
Hexachloroethane
Nitrotrichlotoethane
Pentachloroethane
Tetrachloroethene
Tribromomethane
Trichloroethane
Trichloromethane

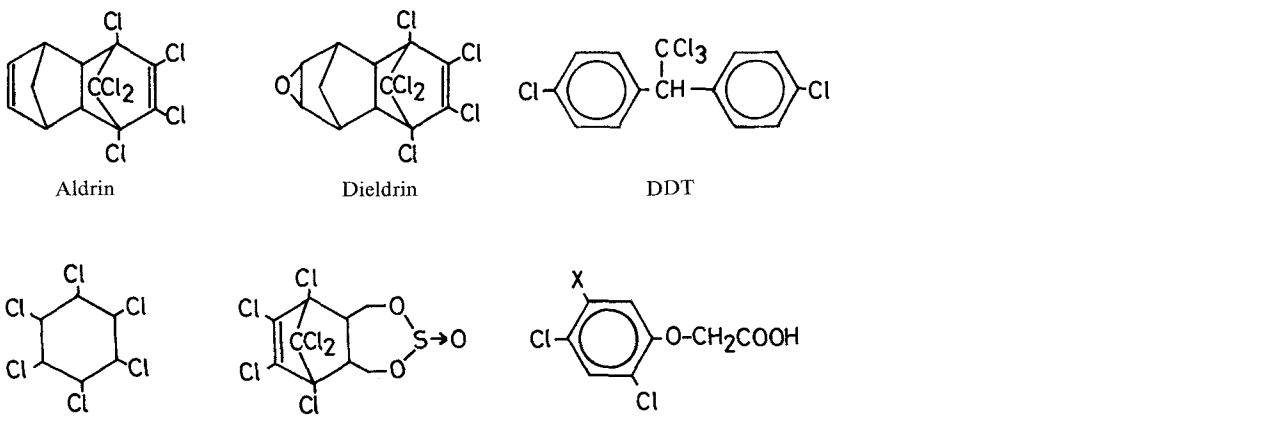

Figure 1. Structure of some organochlorine pesticides.

and for maintaining disinfectant activity in the distribution system[43]. However, the finding that chlorination results in the formation of trihalomethanes and other halogenated hydrocarbons has prompted the development of alternative procedures for the disinfection of water[34].

The formation of volatile bromiated hydrocarbons was shown to be catalyzed by a bromoperoxidase from marine algae[47]. Thus, the pollution of the sea and of the upper atmosphere with halomethanes and possibly also with other volatile halometabolites[30] is caused at least in part by biological halogenation. The following pathway for the enzymatic formation of halometabolites has been detected in an extract of the green marine alga, *Penicillus captatus*. 3-Oxooctanoic acid is brominated to form 2,2-dibromo-3-octanoic acid, and this unstable compound decarboxylates readily to yield 1,1-dibromo-2-heptanone. Dibromoheptanone is then further brominated to form 1,1,1-tribromo-2-heptanone. The tribromoketone undergoes nonenzymatic hydrolysis to liberate bromoform[3]. Bromoperoxidase has been isolated and purified from marine algae[32]. The enzyme has a mol. wt of 97,600 and is composed of two subunits. The heme prosthetic group of bromoperoxidase is ferriprotoporphyrin IX. In terms of catalytic property, comparative data places bromoperoxidase closer to horseradish peroxidase than to chloroperoxidase and catalase.

Degradation of chloroaromatics

Despite the natural abundance of halogen atoms (chlorine is the 11th most common element), only a few halogen-containing metabolites occur in living organisms. Halogen compounds generally have physiological activity. Biochemical aspects of microbial halometabolites have been reviewed[41].

The initial step in the animal metabolism of chlorine-containing compounds involves the introduction into the molecules of reactive groups such as amino, carboxyl and hydroxyl groups, followed by conjugation of the modified compounds and their excretion. The introduction of the groups necessary for conjugation is usually achieved by oxidation, reduction or hydrolysis. These enzymatic reactions are carried out mainly in the liver; the enzymes have low substrate specificity and have not been well characterized[8].

Many microorganisms metabolize or utilize halogen-containing compounds as carbon sources when they are grown in media containing organic halogen compounds. Halogen containing aromatic compounds such as chlorocatechols are toxic for bacteria, and in general are dehalogenated after ringcleavage by dioxygenases. Microbial degradation of a few halogen aromatic compounds is described here.

Polychlorinated biphenyls (PCB)

Polychlorinated biphenyls (PCB) are physically and chemically stable, and various PCB were used widely in transformer oils, capacitor oils and pesticide extenders. Furukawa et al.[16] have studied the degradation of various isomers of PCB by bacteria of the genera *Alcaligenes* and *Acinetobacter*. The relationship between chlorine substitution and degradation of PCB was reported to be as follows: 1. The rate of degradation decreases with an increase in the number of chlorine substituents. PCB isomers containing more than four chlorine atoms in a molecule are not susceptible to bacterial degradation. 2. PCB that contain two chlorine atoms in the ortho position on a single ring (e.g. 2,6-dichloro biphenyl) or on both rings (e.g. 2,2′-dichloro biphenyl) are degraded only slowly. 3. PCB in which chlorine atoms are bound to the same phenyl group are generally degraded faster then those containing chlorine atoms bound to both phenyl groups. 4. Preferential ring fission of the molecules occurs on a nonchlorinated or on a lesser chlorinated ring. 5. 4′-Chloro-substituted PCB are metabolized to form a yellow intermediate, which has not been identified. 6. Significant differences between the two organisms with respect to the ability to degrade PCB were not observed except for the degradation of 2,4,6-trichlorobiphenyl.

The initial step in the oxidation of PCB has not been elucidated enzymologically. A probable pathway for the degradation of PCB is shown in figure 2[15]. Two atoms of molecular oxygen are thought to be incorporated into the 2′ and 3′ positions of the lesser chlorinated ring, and a cis-dihydrodiol compound (**2** in fig. 2) is produced via a cyclic peroxide. Then, compound **2** is dehydrogenated to yield a 2′, 3′-dihydroxy compound (**3**). The meta-cleavage occurs at the 1′ and 2′ positions of the compound **3** to form

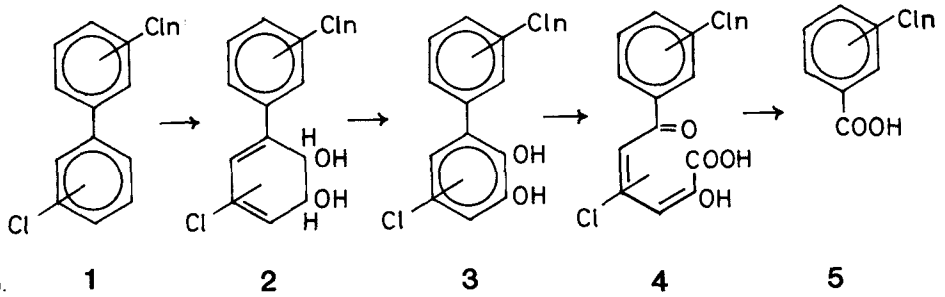

Figure 2. Major pathway for the microbial degradation of PCB (n = 1-4).

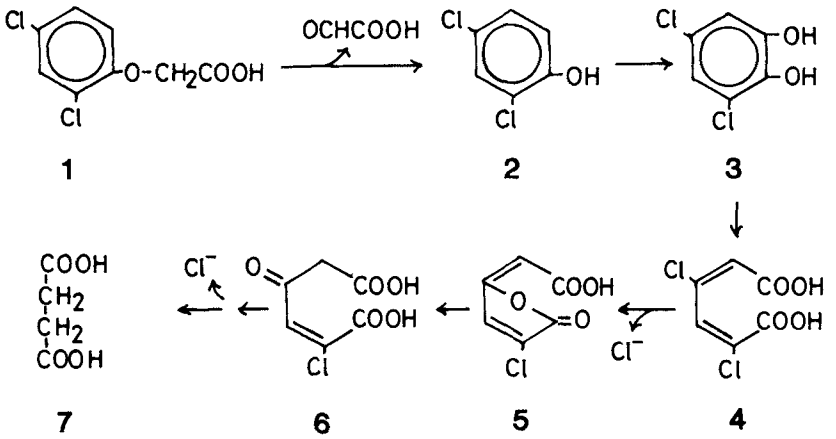

Figure 3. Degradation pathway of 2,4-D by enzymes from *Arthrobacter* sp.

yellow products, which in general are rapidly converted to chlorinated benzoic acids (**5**).

2,4-Dichlorophenoxyacetic acid (2,4-D)

The bacterial degradation of the herbicide 2,4-dichlorophenoxyacetic acid has been studied with *Flavobacterium peregrium*, *Achromobacter* sp.[45] and *Arthrobacter* sp. The degradative pathway is shown in figure 3. The ether linkage of 2,4-D (**1**) is cleaved to yield 2,4-dichlorophenol (**2**) and also probably glyoxylic acid. The former is hydroxylated in the *ortho* position by a mixed function oxidase to 3,5-dichlorocatechol (**3**)[5,6]. The ring of the catechol is then cleaved to form cis, cis-2,4-dichloromuconic acid (**4**). This compound is cycloisomerized to the hypothetical intermediate 2-chloro-4-carboxymethylenebut-2-enolide (**5**), with a spontaneous release of chloride. The ring of butenolide is hydrolyzed to chloromaleylacetic acid (**6**). This is dechlorinated enzymatically to succinic acid (**7**)[46].

2-Fluorobenzoic acid

The metabolism of 2-fluorobenzoic acid by a pseudomonad has been studied (fig. 4). 3-Fluorocatechol (**2**) and 2-fluoromuconic acid (**3**) have been isolated from the growth medium containing 2-fluorobenzoic acid as a sole carbon source[18]. Studies with $^{18}O_2$ showed that catechol (**6**) and 3-fluorocatechol (**2**) contained either 2 atoms of $^{16}O_2$ or 2 atoms of $^{18}O_2$ after growth in an atmosphere consisting of $^{16}O_2$ and $^{18}O_2$[33]. This result ruled out the possibility that the oxygen atoms were introduced by successive hydroxylation reactions which would have produced a random mixture of both oxygen isotopes in the catechols. The incorporation of both oxygen atoms from a molecule of oxygen can be explained by postulating a cyclic peroxide intermediate. In this mechanism of oxygenation, a cis configuration of the reduced diol (**5**) is required and this configuration permits a low energy transition state leading to the spontaneous elimination of HF and CO_2.

Enzymological aspects of haloaromatic degradation

Enzymological studies on the degradation of chloroaromatics have been performed with the 3-chlorobenzoic acid-utilizing *Pseudomonas* sp. B13[11]. Two forms of catechol dioxygenase have been found in cells of this strain. Pyrocatechase I was induced in a medium containing benzoate as the sole carbon

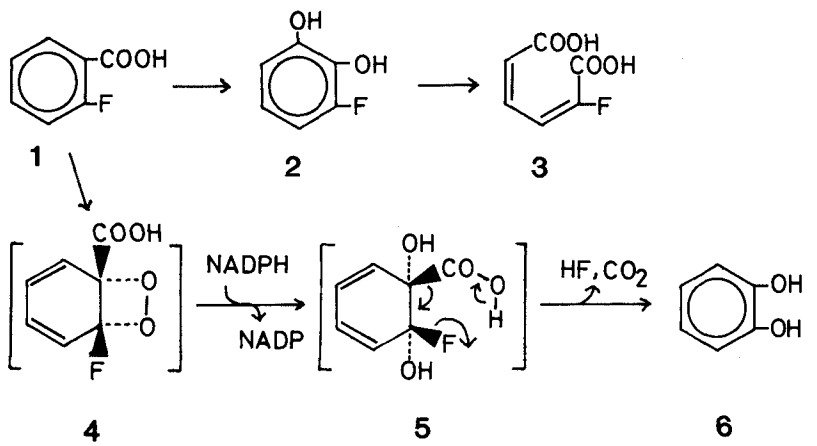

Figure 4. Degradation pathway of 2-fluorobenzoic acid.

source. It has been purified 29-fold. The other pyrocatechase, pyrocatechase II, was induced by chlorobenzoate and showed higher activity for 3- and 4-chlorocatechol than for catechol. Pyrocatechase II was very labile, and has not been purified highly. Pyrocatechase I catelyzed the extradiol cleavage of 3-methylcatechol in addition to intradiol fission at a ratio of 1:14. Pyrocatechase II, however, showed no activity in extradiol cleavage.

The influence of halogen substituents on the 1,2-dioxygenation of catechols has been investigated with pyrocatechases from *Pseudomonas* sp. B13 and *Alkaligenes eutrophus* B9[12]. The substituents on catechol interfere with O_2 binding to the enzymes, and the occurrence of electrophilic substituents such as halogen atoms decreases the K_m-values for catechols and the rate of catechol 1,2-dioxygenation. The rate of benzoate 1,2-dioxygenation is also decreased owing to electrophiles such as halogen in the substrates[42].

An enzyme which catalyzes the chlorine-eliminating cycloisomerization of 2- and 3-chloro-cis, and cis-muconic acid has been isolated from cells of *Pseudomonas* sp. B13 and was designated muconate cycloisomerase II[44]. The enzyme is distinct from an ordinary muconate cycloisomerase on the basis of its K_m- and V_{max}-values. The latter enzyme functions in benzoate catabolism and exhibits low activity for the chlorinated substrates. Muconate cycloisomerase II catalyzes the formation of trans- and cis-4-carboxymethylenebut-2-en-4-olide from 2- and 3-chloro-cis, cis-muconic acid, respectively, with concomitant dechlorination. A conversion of 4-carboxymethylenebut-2-en-4-olide into maleoylacetic acid was shown with enzyme preparations from chlorobenzoate-grown bacterial cells.

Degradation of chlorinated aliphatics

The microbial degradation of 2-haloalkanoic acids, some of which are used as herbicides and pesticides, has been extensively studied[4,26,27,31,48]. The enzymes that release halogen ions from organic halogen compounds were named 'dehalogenase' by Jensen[21]. They play an important role in the microbial degradation of 2-haloalkanoic acids. All the dehalogenases release halogen ions from 2-haloalkanoic acids, but none acts on other haloaliphatics so far studied. The mechanism of degradation of 2-haloalkanoic acids has been investigated with purified dehalogenases. However, little information has been obtained on the microbial degradation of haloalkanes, especially on the enzymological aspects[7,38,39].

Dehalogenases have been classified into haloacetate dehalogenases (EC 3.8.1.3) and 2-halo acid dehalogenases (EC 3.8.1.2)[22]. Haloacetate dehalogenases act exclusively on haloacetate, whereas 2-halo acid dehalogenases catalyze the dehalogenation of several 2-haloalkanoic acids.

Haloacetate dehalogenase

There are two distinct types of haloacetate dehalogenases; one acts on the carbon-fluorine bond of fluoroacetate and the other does not. Fluoroacetate is very toxic and its toxicological mechanism has been reviewed[40]. Goldman found a carbon-fluorine bond cleaving enzyme from cells of a pseudomonad which utilizes fluoroacetate as a sole carbon source and purified it 30-fold to study its properties[17]. The enzyme was formed in cells grown in a fluoroacetate medium. The enzyme had a pH optimum at 9.4, and the K_m-values for fluoroacetate and chloroacetate were 2.4 mM and 20 mM, respectively. The V_{max}-value for fluoroacetate was 6 times higher than that for chloroacetate. Equimolar amounts of glycolic acid and fluoride were formed from fluoroacetate. 3-Fluoropropionate, difluoroacetate, trifluoroacetate and 2-, 3- and 4-fluorobenzoate were inert. The function of a sulfhydryl group of the enzyme active site in the catalysis was studied.

A defluorinating dehalogenase was purified about 40-fold and crystallized from the cells of *Pseudomonas* sp. strain A which produces the enzyme constitutively[25]. The molecular weight of the enzyme was approximately 42,000 and 33,000, when determined by gel filtration and SDS-polyacrylamide gel electrophoresis, respectively. The sedimentation coefficient was 5.17 S, and the isoelectric point was pH 5.4. The enzyme showed maximum reactivity at pH 9.0 and highest affinity for monofluoroacetate (K_m; 2.0 mM). The relative activities of the enzyme in the dehalogenation of various 2-haloalkanoic acids are presented in table 2 (column A). The enzyme acted on monohaloacetates exclusively, monofluoroacetate being the prefered substrate.

Two different dehalogenases were found also in cells of *Moraxella* sp., which utilizes fluoroacetate as a sole carbon source[23]. One (H-1) acts on fluoroacetate and is inducible, whereas the other (H-2) dehalogenates chloroacetate, but not fluoroacetate and is formed

Table 2. Substrate specificity of haloacetate dehalogenases

	Relative activity (%)	
	A*	B**
Monofluoroacetate	100	0
Monochloroacetate	21	100
Monobromoacetate	14	160
Monoiodoacetate	0.03	150
Dichloroacetate	±	3.3
Trichloroacetate	0	0
2-Chloropropionate	±	9.5
3-Chloropropionate	0	0
2,2-Dichloropropionate	0	0.1
2-Chlorobutyrate	0	0
3-Chlorobutyrate	–	0
4-Chlorobutyrate	–	0

*A, Defluorinating enzyme of *Pseudomonas* sp.[25]; **B, H-2 Dehalogenase of *Moraxella* sp.[24]; –, Not determined. ±, Slight release of chloride ions detected.

constitutively. Both enzymes are determined by a plasmid in the organism. The H-2 dehalogenase was purified to homogeneity and some of its properties were investigated[24]. The mol. wt of the enzyme was determined to be 43,000 by gel filtration but 26,000 by SDS-polyacrylamide gel electrophoresis. It was suggested that the discrepancy between these values was due to differences in the experimental conditions employed in the two methods of molecular weight determination. For example, the enzyme may exist in two interconvertible forms depending on the ionic strength of the buffer used or it may not behave as an ordinary globular protein. The sedimentation coefficient is 4.1 S, and the isoelectric point was pH 5.2. Maximum activity was observed at pH 9.5. The substrate specificity of the enzyme is shown in table 2 (column B). The reactivity of monohaloacetates was monobromoacetate, monoiodoacetate and monochloroacetate in this order. Although 2-chloropropionate and 2,2-dichloropropionate were dehalogenated very slowly, the enzyme acted preferably on monohaloacetates with the exception of fluoroacetate. It was inhibited by thiol reagents such as p-chloromercuribenzoate and $HgCl_2$.

2-Halo acid dehalogenase

2-Halo acid dehalogenases (EC 3.8.1.2) catalyze the dehalogenation of 2-haloalkanoic acids with carbon chains of C2–C4, among which haloacetate and 2-monohalopropionate are the best substrates. None of the dehalogenases in this category cleave the carbon-fluorine bond of halo acids such as monofluoroacetate. With the exception of the DL-2-halo acid dehalogenase described below, these enzymes specifically dehalogenate L-2-halo acids. The enzymatic dehalogenation proceeds with an inversion of configuration: L-2-chloropropionate, for example, yields exclusively D-lactate.

Two different dehalogenases were found in a pseudomonad. One was induced by chloroacetate and the other by dichloroacetate[19]. Both enzymes were partially purified. They catalyzed the hydrolytic dehalogenation of various 2-halo acids; the halogen atom was replaced enzymatically by an ^{18}OH group from $H_2{}^{18}O$. The dehalogenation proceeds in an inversion mode.

2-Halo acid dehalogenase was purified approximately 200-fold from cell-free extracts of *Pseudomonas dehalogenans* NCIB 9061[29]. The mol. wt was determined by gel filtration to be about 15,000. As shown in table 4, the highest activity was observed at pH 9.4. The dehalogenation of monochloroacetate (1 mM) was inhibited by the following organic acids (10 mM); glycolate, 29%; DL-2-chlorophenylacetate, 29%; trichloroacetate, 19%; monofluoroacetate, 10%; phenylacetate, 5%; and acetate, 1.5%. The enzyme dehalogenated 2-haloalkanoic acids specifically with an inversion of the configuration. The enzyme was inactivated by iodine, and by photooxidation in the presence of methylene blue. A thiol group did not directly participate in catalysis, but probably a histidine residue plays an important role. These observations led to the proposal of the reaction mechanism shown in figure 5. Two groups of 2-chloropropionate-assimilating bacteria were isolated. One group of bacteria utilizes both D- and L-2-chloropropionates while the other assimi-

Table 3. Substrate specificity of 2-halo acid dehalogenases

Substrate (product)	Relative activity[a] A[b]	B[c]	C[c]
Monoiodoacetate (glycolate)	286	255	81
Monobromoacetate (glycolate)	400	358	240
Monochloroacetate (glycolate)	286	184	28
Monofluoroacetate (glycolate)	0	0	0
Dichloroacetate (glyoxylate)	50	30	7
Trichloroacetate (oxalate)	0	0	3
L-2-Chloropropionate (D-lactate)	100	100	100
D-2-Chloropropionate (L-lactate)	0	0	71
DL-2-Bromopropionate (lactate)	–	247	320
2,2-Dichloropropionate (pyruvate)	26	10	35
DL-2-Bromobutyrate (2-hydroxy butyrate)	–	98	186
DL-2-Bromovalerate (2-hydroxy valerate)	–	0	15

[a] Values are expressed relative to the activity with L-2-chloropropionate as a substrate (100%). [b] Relative rate of acid production. [c] Relative rate of halogen release. A, Enzyme of *Pseudomonas dehalogenans*[29]; B, enzyme of *Pseudomonas putida*[36]; C, enzyme of *Pseudomonas* sp. strain 113[37].

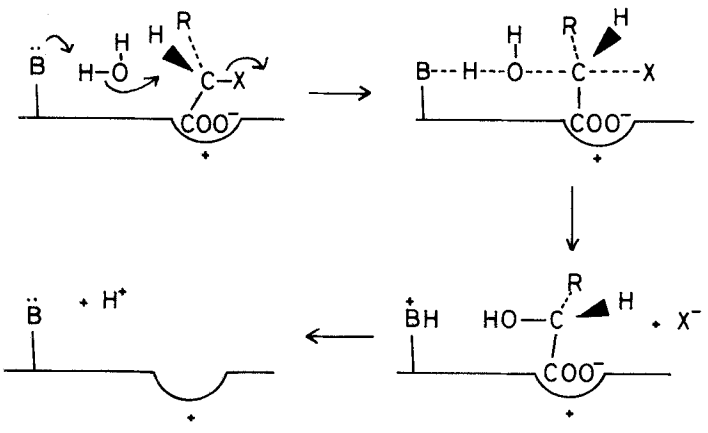

Figure 5. Proposed dehalogenation mechanism of 2-halo acid and dehalogenase from *Pseudomonas dehalogenans*[29]. B, histidine residue.

Table 4. Some properties of 2-halo acid dehalogenases

2-Halo acid dehalogenases	Molecular weight	pH optimum	K_m (mM) Monochloroacetate	L-2-Chloropropionate
Pseudomonas dehalogenans NCIB 9061 enzyme[29]	15,000	9.4	0.24	0.3
Pseudomonas putida enzyme[36]	34,000	10.5	1.0	3.8
Pseudomonas sp. strain 113 enzyme[37]	68,000	9.5	5.0	1.1

lates only the L-isomer[35]. No strains assimilating exclusively D-2-chloropropionate were isolated. The best assimilators of DL- and L-2-chloropropionate were identified as *Pseudomonas* sp. strain 113 and *Pseudomonas putida*, respectively.

An enzyme acting specifically on the L-isomer, L-2-halo acid dehalogenase, is inducibly formed by *Pseudomonas putida*. It was purified to homogeneity from the cell extract[36]. Its mol. wt as determined by gel filtration was approximately 34,000. The mol. wt was estimated to be 25,000 by SDS polyacrylamide gel electrophoresis. This suggests a monomeric structure of the enzyme, which was confirmed by the examination of subunit structure with a cross-linking reagent. The substrate specificity of the enzyme is shown in table 3 (column B). D-2-Chloropropionate, 3-chloropropionate, 2-bromo-n-valerate, 2-chloroiso-butyrate, chloroacetamide and chloroacetaldehyde are inert. Thus, the enzyme acts specifically on L-2-haloalkanoic acids with a carbon chain length of 2–4.

The other enzyme without stereospecificity, DL-2-halo acid dehalogenase was produced constitutively by cells of *Pseudomonas* sp. strain 113. The enzyme was purified to homogeneity and characterized[37]. The enzyme had a mol. wt of approximately 68,000 and was composed of two subunits identical in mol. wt (35,000). The enzyme acted on D-2-chloropropionate at a reaction rate 70% of that for the L-isomer, and the K_m-values for D- and L-2-chloropropionates were 4.8 mM and 1.1 mM, respectively. All enzymes so far studied that work on a chiral carbon of substrates act exclusively on one enantiomer, except racemases. The dehalogenation catalyzed by the enzyme proceeded with an inversion of the configuration:

$$\text{D(L)-CH}_3\text{CHClCOOH} + \text{H}_2 \rightarrow \text{L(D)-CH}_3\text{CH(OH)COOH} + \text{HCl}.$$

The enzyme showed a lower structural specificity for the substrates than the L-2-halo acid dehalogenase of *Pseudomonas putida* (table 3). It is the first enzyme that catalyzes the dehalogenation of trichloroacetate and 2-bromo-n-valerate. All of the monohaloacetates tested, except monofluoroacetate were dehalogenated effectively. The order of reactivity was as follows: bromoacetate > iodoacetate > chloroacetate. Chloroacetamide and chloroacetaldehyde were not dehalogenated, indicating that a free carboxyl group is necessary for a compound to be a substrate. Also the halogen must be at C-2 for a compound to be a substrate; 3-chloropropionate was not accepted as substrate.

The enzymatic elimination of halogen atoms proceeds through an SN_2-type reaction which follows the inversion of configuration. In contrast, nonenzymatic dehalogenation of 2-haloalkanoic acids proceeds in a retention mode, though not fully stereospecificically. For example, L-2-chloropropionate is nonenzymatically dehalogenated by the heating of an aqueous solution, and the configuration of the lactate formed is almost fully retained. The nonenzymatic dehalogenation probably proceeds through a double inversion of the configuration (fig. 6)[9]. The mechanism operating in such a case is called the neighboring-group mechanism and consists essentially of two SN_2 substitutions, each causing an inversion, so that the net result is retention of the configuration.

Recently, Weightman et al.[51] have reported that the reactions of the two different dehalogenases found in the cells of *Pseudomonas putida* PP3 proceed through different mechanisms. One catalyzes an SN_2-type dehalogenation and the other an SN_1-type dehalogenation (the retention of configuration).

All the dehalogenases listed in table 4 are produced by bacteria of the same genus *(Pseudomonas)*, but different in their properties and functions from each other. These variations in dehalogenases have been investigated systematically and discussed from the evolutionary viewpoint[20].

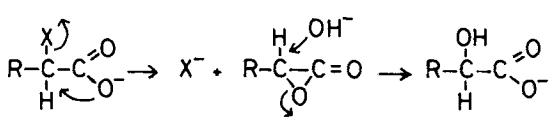

Figure 6. Nonenzymatic dehalogenation (retention).

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