Stereospecificity of 2-Monochloropropionate Dehalogenation by the Two Dehalogenases of *Pseudomonas putida* PP3: Evidence for Two Different Dehalogenation Mechanisms

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Pseudomonas putida PP3 grew on DL-2-monochloropropionate (2MCPA) with a release of chloride ions consistent with the dechlorination of both isomers. The organism grew on either D- or L-2MCPA. Dehalogenase activity in cell-free extracts showed that both D- and L-2MCPA were dehalogenated. *Pseudomonas putida* PP3 contains two dehalogenases, and studies with the separated enzymes revealed that the fraction I enzyme used both D- and L-2MCPA, the rate of dechlorination of L-2MCPA being 80% of the rate of D-2MCPA dechlorination. The product of the reaction, lactate, retained the same optical configuration as the substrate provided. The fraction II dehalogenase also dechlorinated D- and L-2MCPA, with the same difference in rates as for the fraction I dehalogenase, but the lactates produced were of the opposite configuration to their precursors. The two dehalogenases showed further differences with respect to inhibition by two sulphydryl-blocking agents, *N*-ethylmaleimide and *p*-chloromercuribenzoate. Fraction I dehalogenase was considerably more sensitive to these two reagents compared with the fraction II dehalogenase. Dithiothreitol partially protected the fraction I dehalogenase from *N*-ethylmaleimide inhibition. The results are discussed in terms of the possible evolutionary relationships of the two dehalogenases.

INTRODUCTION

Several studies have indicated that bacteria contain a number of different dehalogenases particularly with respect to their substrate specificities, electrophoretic mobilities and inhibition by sulphydryl-blocking agents (Davies & Evans, 1962; Goldman *et al.*, 1968; Little & Williams, 1971; Berry *et al.*, 1979; Slater *et al.*, 1979; Hardman & Slater, 1981; Kawasaki *et al.*, 1981 *a*, *b*). It has been found that *Pseudomonas putida* PP3, isolated by Senior *et al.* (1976) from a microbial community growing on 22DCPA, contains two dehalogenases with different substrate specificities and thermal stabilities (Weightman *et al.*, 1979). None of these previous studies sought to resolve the relationship, or otherwise, between these different enzymes. It was suggested that the fraction I and II dehalogenases of *P. putida* PP3 may have evolved from a common ancestral enzyme (Weightman *et al.*, 1979), a conclusion tentatively drawn from the two enzymes' substrate specificities.

The aim of the present work was to examine those properties of the two *P. putida* PP3 enzymes which might indicate whether or not the enzymes were mechanistically related. This was examined by investigating their dehalogenation of the optically active isomers of 2MCPA and their response towards sulphydryl-blocking agents.

METHODS

Isolation, maintenance and growth of bacteria. Pseudomonas putida PP3 was isolated from a microbial community grown on the herbicide Dalapon (Senior *et al.*, 1976) and maintained and grown as previously described (Slater *et al.*, 1979). *Pseudomonas putida* PP411-006 and PP412-019 were spontaneous mutants of *P. putida* PP3 resistant to MCA and DCA, isolated on solid defined medium containing succinate (0·5 g C l⁻¹) as the carbon and energy source and DCA (1·0 g C l⁻¹) (A. J. Weightman, A. L. Weightman & J. H. Slater, unpublished observations). Both mutants grew with DL-2MCPA as the carbon source and differed in that *P. putida* PP411-006 contained only dehalogenase fraction I and *P. putida* PP412-019 contained only dehalogenase fraction II as determined by polyacrylamide gel electrophoresis. *Pseudomonas putida* PP411-006 and PP412-019 were maintained on defined medium containing DL-2MCPA (0·5 g C l⁻¹) and were regularly checked for resistance to MCA and DCA (A. J. Weightman, A. L. Weightman & J. H. Slater, unpublished observations).

Synthesis of D- and L-2MCPA. D- and L-2MCPA were synthesized from D- and L-alanine, respectively, as described by Fu *et al.*, (1954). The purity of the final distilled fractions was examined by ¹³C-NMR spectroscopy at 22·63 MHz in a Bruker WH90 instrument; the spectra indicated the absence of any other chlorinated compounds.

Measurement of dehalogenase activity of cell-free extracts. Dehalogenase activity was measured in cell-free extracts as previously described (Weightman & Slater, 1980; Hardman & Slater, 1981). One unit of dehalogenase activity was defined as the amount of enzyme catalysing the conversion of 1 μmol MCA min⁻¹ (mg protein)⁻¹. The principle of the assay involved measuring free chloride ions, released during the assay period, by using a Marius Chlor-O-Counter (Labo International, The Netherlands).

Measurement of L-lactate dehydrogenase. L-Lactate dehydrogenase (EC 1.1.1.27) (Sigma) was measured according to Hohorst (1965). The amount of L-lactate converted to pyruvate was calculated on the basis that 0·02 μmol L-lactate corresponded to an absorbance change of 0·123 at 340 nm in a 1·0 ml assay mixture. The assay was also used to determine the amount of L-lactate produced during the dechlorination of known amounts of DL-, D- and L-2MCPA by fraction I or fraction II dehalogenases.

Polyacrylamide gel electrophoresis. The two dehalogenases of *P. putida* PP3 were separated by discontinuous polyacrylamide gel electrophoresis of cell-free extracts as previously described (Weightman & Slater, 1980; Hardman & Slater, 1981).

The electrophoretic method was used to prepare sufficient quantities of a solution containing only fraction I dehalogenase for subsequent analysis. A complete gel was loaded with a total of 0·45 units of dehalogenase in a cell-free extract of *P. putida* PP3. After electrophoresis, the position of fraction I dehalogenase was located in one of the tracks of the gel by the standard assay procedure (Weightman & Slater, 1980). The appropriate regions of the remainder of the gel were cut out and the sections were disrupted by manual homogenization in 16·0 ml 20 mM-Tris/sulphate buffer pH 7·0. The homogenate was used to prepare samples of lactate as described below.

The preparative electrophoretic method could not be used for the fraction II dehalogenase because of this enzyme's greater instability (Weightman *et al.*, 1979). Instead, the fraction II dehalogenase in extracts of *P. putida* PP412-019 grown on DL-2MCPA was used. The extract was used to prepare lactate samples as described below.

Preparation of lactate samples from the two dehalogenases. For the fraction I dehalogenase, the gel homogenate was treated as follows: 5·0 ml homogenate was incubated with 1·7 ml 0·6 M-Tris/sulphate buffer pH 7·9 and 0·19 ml 20% (w/v) 2MCPA. Controls demonstrated that polyacrylamide did not influence the enzyme's activity. The mixture was incubated for 12 h at 30 °C, then centrifuged, and the supernatant was decanted and stored frozen before assaying for the lactate produced.

For the fraction II dehalogenase, the cell-free extract was incubated under standard assay conditions (Weightman & Slater, 1980) for 1 h at 30 °C. Longer incubation times were not used because of the enzyme's instability. The mixture was deproteinized by boiling for 2 min, a procedure which did not cause any further 2MCPA breakdown. The supernatant was decanted and stored frozen before assaying for the lactate produced.

Measurement of DL-2MCPA dehalogenation in the presence of D_2O. Cell-free extracts of strains PP411-006 and PP412-019 were prepared and incubated in dehalogenase assay mixture enriched with D_2O. The mixture contained 0·90 ml 0·6 M-NaH_2PO_4/Na_2HPO_4 buffer pH 7·9 in D_2O, 0·6 ml cell-free extract and 50 mM-2MCPA, made up to 3·30 ml with D_2O (Aldrich Chemical Co., Gillingham, U.K.). After 15 h incubation at 30 °C the assay mixture was evaporated to dryness, and the residue was resuspended in 3·3 ml water and filtered. The lactate concentration was determined as previously described and found to be between 12 and 15 mM. The extracted lactates were analysed by ²H-NMR spectroscopy at 61·4 MHz in a Bruker WH400 instrument.

RESULTS

Growth of P. putida PP3, PP411-006 and PP412-019 on D-, L- and DL-2MCPA and relative dehalogenase activities

Pseudomonas putida PP3 grew on both optical isomers and the racemic mixture with similar specific growth rates of 0·20–0·22 h⁻¹. Strain PP411-006, which contains only the fraction

Table 1. *Specific growth rates and relative dehalogenase activities for various substrates for P. putida strains PP411-006 and PP412-019*

		Specific growth rate (h^{-1})		
		Substrate:		
Strain	Dehalogenase expressed	D-2MCPA	L-2MCPA	DL-2MCPA
PP411-006	I	0·23	0·20	0·22
PP412-019	II	0·10	0·18	0·18

		Dehalogenase activity relative to MCA				
				Substrate:		
		MCA	DCA	D-2MCPA	L-2MCPA	DL-2MCPA
PP411-006	I	1·0	0·17	0·46	0·37	0·49
PP412-019	II	1·0	1·50	0·12	0·09	0·20

I dehalogenase, also grew at similar rates on the optical isomers and the mixture (Table 1), and these rates were comparable with the growth rates of *P. putida* PP3. The dehalogenase relative activities for MCA, DCA and DL-2MCPA were those expected for a strain containing only the fraction I dehalogenase. The rate of L-2MCPA dehalogenation was only 80% of the rate of D-2MCPA dehalogenation (Table 1). In contrast, for *P. putida* PP412-019 which contains only the fraction II dehalogenase, the specific growth rates were considerably lower, especially with D-2MCPA as the substrate (Table 1). Measurement of dehalogenation by the fraction II dehalogenase showed that the rate of L-2MCPA dehalogenation was 75% of the rate of D-2MCPA dehalogenation.

Stereospecificity of fraction I dehalogenase

The fraction I dehalogenase expressed by *P. putida* PP3 grown on 2MCPA was separated from the fraction II dehalogenase by disc gel electrophoresis. An adequate amount of enzyme was prepared by this method for three portions to be incubated with DL-2MCPA, D-2MCPA and L-2MCPA respectively for 12 h, resulting in approximately 50% dehalogenation and the production of lactate (Table 2). The amount of L-lactate produced from the three chlorinated substances was determined by the specific L-lactate dehydrogenase. Little L-lactate was generated when D-2MCPA was the substrate, with the small percentage observed probably due to background amounts of L-lactate in the assay system. By contrast, when L-2MCPA was the substrate for the fraction I dehalogenase practically all the lactate produced was the L-isomer (Table 2). As expected, 50% of the lactate produced from a racemic mixture of 2MCPA was L-lactate (Table 2). These results showed that both D- and L-2MCPA were dechlorinated by the fraction I dehalogenase and that the reaction product, lactate, retained the same configuration as the enzyme's substrate.

Stereospecificity of fraction II dehalogenase

The fraction II dehalogenase could not be prepared in sufficient quantities using the same method as that employed for the fraction I enzyme because the second enzyme showed considerable thermal instability (Weightman *et al.*, 1979). The activity of the enzyme was rapidly lost during the relatively lengthy gel electrophoresis procedures. Instead, a mutant strain, *P. putida* PP412-019, derived from *P. Putida* PP3, expressing only the fraction II dehalogenase, was used to enable a rapid production of suitable amounts of the enzyme for lactate production. However, again because of instability, no further dechlorination was obtained after incubating separately the three portions with the three substrates for 1 h. Thus, the amount of dechlorination obtained was much less than for the fraction I dehalogenase

Table 2. *Production of L-lactate from DL-, D- and L-2MCPA by P. putida PP3 fraction I dehalogenase (separated by disc gel electrophoresis) and by the fraction II dehalogenase of P. putida PP412-019*

The dehalogenases were incubated with each substrate (50 mM starting concentration) for 12 h (fraction I) or 1 h (fraction II). The errors quoted are standard deviations of three determinations for the chloride release values and two determinations for the L-lactate concentrations.

	Fraction I dehalogenase			Fraction II dehalogenase		
	Substrate (50 mM):			Substrate (50 mM):		
	DL-2MCPA	D-2MCPA	L-2MCPA	DL-2MCPA	D-2MCPA	L-2MCPA
Chloride release during lactate preparation (mM)	23·20 ± 0·83	27·63 ± 1·00	26·02 ± 0·36	13·00 ± 0·17	6·02 ± 0·08	5·42 ± 0·14
Amount of L-lactate formed (mM)	12·13 ± 0·27	0·62 ± 0·03	25·39 ± 0·12	6·30 ± 0·10	5·83 ± 0·06	0·49 ± 0·05
Percentage of dechlorinated 2MCPA yielding L-lactate	52·2	2·2	97·7	48·5	96·7	9·3

(Table 2). The results indicated a reversal of the stereochemical results obtained with the fraction I dehalogenase. In common with the fraction I enzyme, fraction II dehalogenase dechlorinated both isomers of 2MCPA, and 50% of the DL-2MCPA mixture produced L-lactate (Table 2). However, L-lactate was, in this case, generated from D-2MCPA. Dechlorination of L-2MCPA failed to yield significant amounts of L-lactate, although the amount of L-lactate detected in the lactate dehydrogenase assay was higher than in the dechlorination of D-2MCPA by the fraction I dehalogenase. One reason for this is that *P. putida* PP3 and its mutants are capable of using D-lactate as a growth substrate, possibly because of the presence of lactate racemase or D-lactate dehydrogenase. The incubations with fraction II dehalogenase included crude cell-free extracts in contrast to the system used for the fraction I dehalogenase, which may have enabled significant conversion of D-lactate to L-lactate to have occurred before deproteinization and L-lactate determination. Thus, the mechanism of dehalogenation by the fraction II dehalogenase resulted in the reversal of the stereochemical configuration.

Effect of N-ethylmaleimide and p-chloromercuribenzoate on fraction I and fraction II dehalogenases

The effect of NEM and PCMB on the two dehalogenases of *P. putida* PP3 was examined by exposing a number of identical cell-free extract gels to 1 mM-NEM or 0·01 mM-PCMB for various periods of time before assaying for enzyme activities (Fig. 1). The two enzymes were differentially inactivated and, furthermore, the fraction I dehalogenase showed different responses to the two sulphydryl-blocking agents.

The thermally unstable fraction II dehalogenase was affected much less by either NEM or PCMB than was the fraction I dehalogenase (Fig. 1 *a, c*). There was no loss of activity after 10 min treatment with PCMB (Fig. 1 *c*) and only a slight loss of activity after 20 min exposure to NEM (Fig. 1 *a*). By contrast, 2 min exposure to 0·01 mM-PCMB caused complete loss of fraction I dehalogenase activity (Fig. 1 *c*) and its activity was barely detectable after 5 min exposure to 1 mM-NEM (Fig. 1 *a*). The presence of 1 mM-DTT afforded some protection against the inhibition caused by NEM but did not significantly affect the response of the fraction II dehalogenase towards NEM (Fig. 1 *b*).
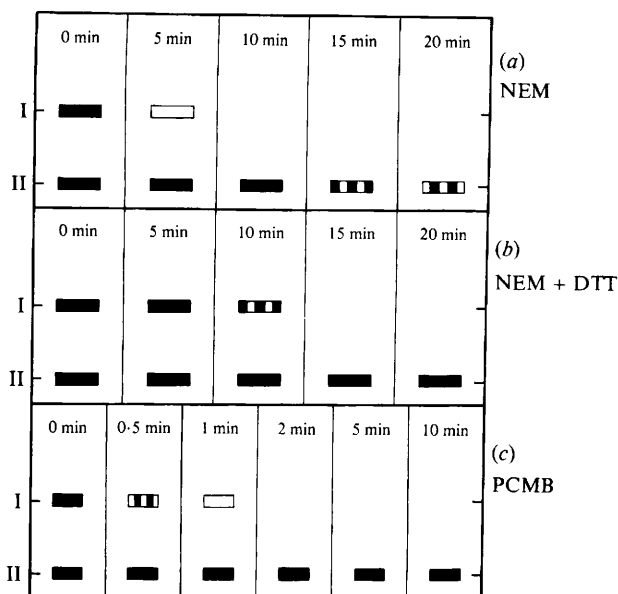
Fig. 1. Effect of NEM (*a*), NEM + DTT (*b*) and PCMB (*c*) on the activity of fraction I and fraction II dehalogenases (denoted I and II, respectively) from *P. putida* PP3 separated by disc gel electrophoresis. The bars represent semi-quantitative assessments of enzyme activity with MCA as the substrate ranging from high to low in the sequence: ■■■ ▌▌▐ ▐▌▌ ▭

^{2}H-NMR spectra of lactate produced in the presence of D_2O

The lactates produced from 2MCPA by extracts containing either fraction I dehalogenase (from *P. putida* PP411-006) or fraction II dehalogenase (from *P. putida* PP412-019) in the presence of D_2O were shown not to contain ^{2}H since the appropriate peaks were absent from ^{2}H-NMR spectra.

DISCUSSION

The stoichiometry of chloride release from DL-2MCPA suggested that *P. putida* PP3 used both enantiomers during growth (Slater *et al.*, 1979). The results presented here show that both the dehalogenases of this organism can dehalogenate both isomers (Tables 1 and 2). To our knowledge this is the first demonstration that dehalogenases can metabolize both D- and L-2MCPA. The two enzymes of *P. putida* PP3 and related mutants clearly contrasted with the halidohydrolase studied by Little & Williams (1971), which only dechlorinated L-2MCPA. Similarly, the two halidohydrolases found in a soil pseudomonad growing on either MCA or DCA (Goldman *et al.*, 1968) were only capable of using L-2MCPA and L-2-chlorobutyrate; no halide release was detected from the D-isomers.

Previous studies have shown that the fraction I dehalogenase has greater activity towards chlorinated propionates compared with the fraction II dehalogenase (Weightman *et al.*, 1979) and this pattern was maintained with respect to both enantiomers (Table 1). Furthermore, the ratio of activity towards D- and L-2MCPA showed that both enzymes dehalogenate the D-isomer at a significantly higher rate than the L-isomer (Table 1). Thus, on these criteria, there appears to be considerable similarity between the two enzymes which has led to the suggestion that the two dehalogenases evolved from a common parent enzyme, perhaps involved initially only with MCA dehalogenation (Weightman *et al.*, 1979).
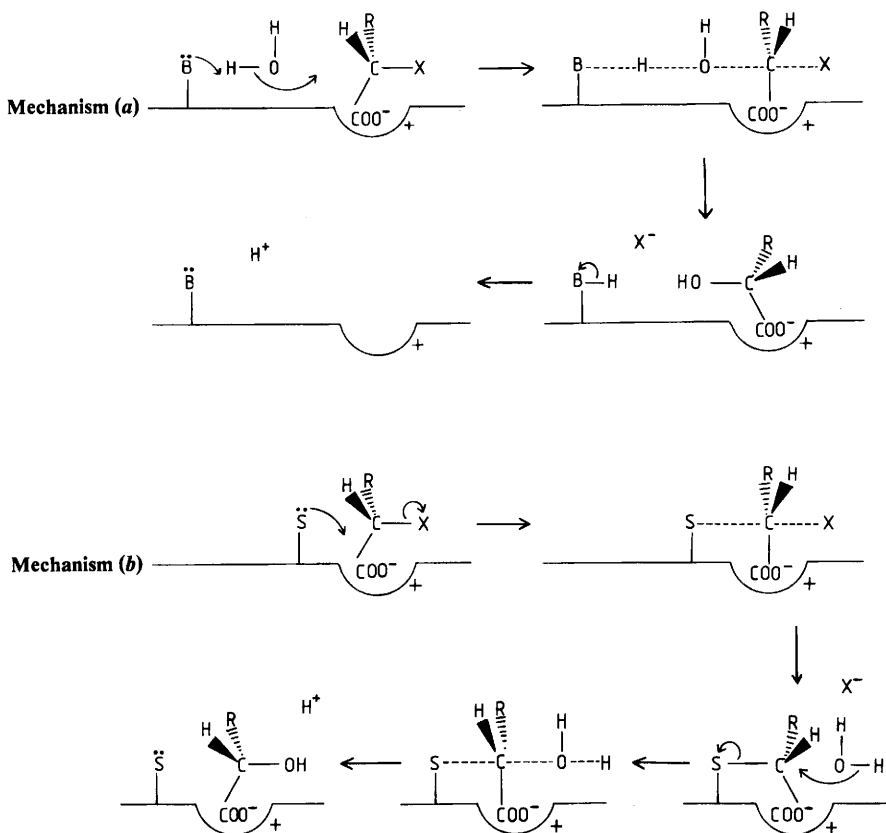
Fig. 2. Two mechanisms for the dehalogenation of 2MCPA: (a) a mechanism resulting in the inversion of configuration; (b) a mechanism resulting in retention of configuration. X = halide; R = alkyl group. [After Goldman et al. (1968) and Little & Williams (1971)].

However, this interpretation is most probably valid only if it can be demonstrated that the mechanism of dehalogenation is the same for both enzymes, notwithstanding the variations between them with respect to substrate affinities. The results presented in this paper make it clear that the two dehalogenases operate different mechanisms (Fig. 2) and therefore raise the question of the closeness of the evolutionary relationship between the two enzymes. The enzymes studied by Goldman et al. (1968) and Little & Williams (1971) converted L-enantiomers to products with inverted optical configurations. The mechanisms proposed were similar to that shown in Fig. 2(a) and involved an enzyme active site with an electron-donating group. Furthermore, the mechanisms ought to be insensitive to sulphydryl-blocking agents and, for the enzymes of Goldman et al. (1968) and Little & Williams (1971), this was indeed the case. The results presented in this paper show that the fraction II dehalogenase behaved in a similar fashion: there was a single inversion of configuration (Table 2) and relative insensitivity to sulphydryl-blocking agents (Fig. 1). The fraction II enzyme dehalogenase is not, however, identical to those of Goldman et al. (1968) and Little & Williams (1971), since it uses D-2MCPA as substrate.

Goldman (1965) had earlier studied a halidohydrolase in a Pseudomonas sp. capable of growing on fluoroacetate, which was extremely sensitive to 10 μM-NEM. A mechanism was therefore suggested which involved a sulphydryl group in the enzyme's active site and an enzyme-intermediate of the thioether form. This was subsequently elaborated by Goldman et al. (1968) who proposed a scheme similar to that shown in Fig. 2(b). This mechanism was

Dehydro-
dehalogenation

Hydration

$$H_3C \overset{H}{\underset{Cl}{—C—}} COOH \xrightarrow{\quad H^+Cl^- \quad} H_2C \overset{H}{=\!=} C\!—\!COOH \xrightarrow[H_2^*O]{\quad} H_3^*C \overset{H}{\underset{OH^*}{—C—}} COOH$$
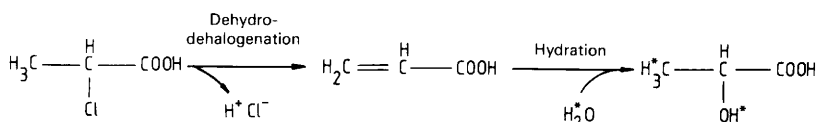
Fig. 3. A third mechanism for the dehalogenation of 2 MCPA involving two steps: a dehydro-dehalogenation followed by hydration.

rejected by Goldman *et al.* (1968), however, since it involved a double inversion resulting in the retention of substrate configuration by the product, a result which was not observed in the two dehalogenases of the pseudomonad. The mechanism could not be tested with the pseudomonad isolated by Goldman (1965) since the enzyme either did not have, or was not examined for, activity towards optically active substrates. Little & Williams (1971) further criticized this mechanism on the grounds of the inherent stability to hydrolysis of thioethers. We wish to propose that the mechanism outlined in Fig. 2 (*b*) accounts for the observed properties of the fraction I dehalogenase of *P. putida* PP3: the retention of configuration through a double inversion involving a thioether intermediate (Table 2; Fig. 2*b*) and the highly sensitive response to sulphydryl-blocking agents (Fig. 1).

Finally, a mechanism involving first a dehydrodehalogenation followed by a hydratase reaction (Fig. 3) had to be considered. It may have been that the two dehalogenases of *P. putida* PP3 both carried these capabilities with the retention or conversion of product configuration depending on the direction of attack by the hydroxyl group in the hydratase reaction. This reaction was considered unlikely since both enzymes were capable of dehalogenating chloroacetates. In fact, ^{2}H-NMR spectra of products from the incubation of DL-2MCPA with fraction I and fraction II dehalogenases in the presence of D_2O demonstrated that this mechanism did not operate since the spectra failed to show ^{2}H incorporation into the lactates produced.

Growth of *P. putida* PP3 on D-2MCPA or L-2MCPA could have been due to the utilization of L-lactate produced by at least one of the enzymes, whichever substrate was supplied. However, growth studies with the two mutants *P. putida* PP411-006 and PP412-019 showed that growth on D-lactate, presumably via the activity of a lactate racemase or D-lactate dehydrogenase, was possible. For strain PP411-006, there was no significant difference in the rate of growth (Table 1). However, for strain PP412-019 containing the fraction II dehalogenase, which inverts the product's configuration, the substrate which produced L-lactate resulted in much slower growth than the substrate which produced D-lactate (Table 1). This is unlikely to be due to differences in the rate of uptake of the two enantiomers since *P. putida* PP411-006 was capable of high growth rates on D-2MCPA. The lower growth rates of strain PP412-019 on D- or L-2MCPA, compared with the growth rates with strains PP3 or PP411-006 was due to the fact that fraction II dehalogenase had much slower activity towards chlorinated propionates (Table 1; Weightman *et al.*, 1979).

These results, together with those of Goldman *et al.* (1968) and Little & Williams (1971), suggest that there are at least three types of dehalogenase recognizable on the basis of the mechanism of dehalogenation in contrast to other properties, such as pH dependence (Slater *et al.*, 1979), electrophoretic mobility (Hardman & Slater, 1981) and substrate affinities (Slater *et al.*, 1979; Berry *et al.*, 1979). The first type dehalogenates only L-isomers in a reaction which results in inversion of product configuration and which is insensitive to sulphydryl-blocking agents (Goldman *et al.*, 1968; Little & Williams, 1971). The second type is similar to the first in terms of product inversion of configuration and insensitivity to sulphydryl-blocking agents but differs in that both D- and L-isomers are substrates (fraction II dehalogenase of *P. putida* PP3). The third type reacts with D- and L-substrates producing products with the same configuration in a reaction which involves a sulphydryl group accounting for its extreme sensitivity to compounds such as PCMB and NEM (fraction I dehalogenase of *P. putida* PP3).

The results presented in this paper reduce the probability of the two *P. putida* enzymes having evolved from a common ancestral dehalogenase. Rather, it now appears that two different mechanisms evolved independently resulting in the parallel evolution of the present dehalogenases. It is conceivable that the enzymes studied by Goldman *et al.* (1968) and Little & Williams (1971) are a variation of *P. putida*'s fraction II dehalogenase, evolving with minor modifications to deal with L-isomers. Similarly, the halidohydrolase sensitive to sulphydryl-blocking agents described by Goldman (1965) could be related to the type exemplified by the fraction I dehalogenase.

It is clear from other studies that a common regulatory mechanism controls both dehalogenases of *P. putida* PP3 (Slater *et al.*, 1979; Weightman *et al.*, 1979; Weightman & Slater, 1980). The genes encoding both fraction I and fraction II dehalogenases have been co-transferred by the chromosome-mobilizing plasmid R68.45 into different bacterial hosts (J. H. Slater, A. J. Weightman, P. C. Gowland, A. L. Weightman and A. H. Filipiuk, unpublished results) which may indicate a close linkage on the *P. putida* chromosome. This raises two intriguing questions. Firstly, at what stage in the evolution of these two enzymes did a common regulatory mechanism arise and, secondly, when did the two dehalogenase genes become located in a similar region of the chromosome?

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