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BIOCHEMICAL CHARACTERIZATION OF A HALOALCOHOL DEHALOGENASE FROM *ARTHROBACTER* sp H10a

by

Helena Maria Serrado Assis

A thesis submitted to the Faculty of Natural Sciences of the University of Kent

for the degree of Ph.D. in Microbiology

The Biological Laboratory, October 1993

To Manuel

DECLARATION

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

28 October 1993

Helena M. S. Assis

Helena Assis

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LIST OF ABBREVIATIONS

1,3-DBP	1,3-dibromo-2-propanol
1,3-DCA	1,3-dichloro acetone
1,3-DCP	1,3-dichloro-2-propanol
1,3-DHP	1,3-dihalo-2-propanol
1,4-DBB	1,4-dibromo-2-butanol
1-MCP	1-chloro-2-propanol
1-MHP	1-halo-2-propanol
2,2DCPA	2,2-dichloropropionic acid
2,3-DBP	2,3-dibromo-1-propanol
2,3-DCP	2,3-dichloro-1-propanol
2,3-DHP	2,3-dihalo-1-propanol
2-MCP	2-chloro-1-propanol
2-MHE	2-haloethanol
2D-PAGE	two dimensional polyacrylamide gel electrophoresis
2MBPA	2-chloropropionic acid
2MCB	2-chlorobutanoic acid
2MCPA	2-monochloropropionic acid
ACN	acetonitrile
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
APS	ammonium persulphate
ATP	adenosine 5'-triphosphate
BCP	1-bromo-3-chloro-2-propanol
BSA	bovine serum albumin
CAA	3-chloroacrylic acid
CAH	3-chloroacrylic acid hydratase
CAPS	3-cyclohexylamino-1-propanosulphonic acid
CFE	cell free extracts
CMC	1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide

CMPA	3-chloro-2-methylpropionic acid
CPD	3-chloro-1,2-propanediol
DCA	dichloroacetic acid
DCCD	dicyclocarbodiimide
DCE	dichloroethane
DCM	dichloromethane
DEAE	diethylaminoethyl
DEP	diethylpyrocarbonate
DTT	dithiothreithol
e.e.	enantiomeric excess
EBH	epibromohydrin
ECH	epichlorohydrin
EDTA	ethylenodiamine tetracetic acid
ELISA	enzyme linked immunosorbant assay
FPLC	fast protein liquid chromatography
GDL	glycidol
GSH	glutathione (reduced form)
GST	glutathione-S-transferase
HAD	haloalkane dehalogenase
HCD	halocarboxylic acid dehalogenase
HPD	3-halo-1,2-propanediol
HPLC	high performance liquid chromatography
ID	internal diameter
IgG	immunoglobulin G
kDa	kilodaltons
K_R	retardation coefficient
MBA	bromo acetic acid
MBE	2-bromoethanol
MCA	chloro acetic acid
MCE	2-chloroethanol
MeCMPA	3-chloro-2-methylpropionate methyl ester

MFA	fluoro acetic acid
MIA	iodo acetic acid
min	minute
MOPS	3-(morpholino)propanesulphonic acid
MW	molecular weight
NAD	nicotinamide-adenine dinucleotide (oxidized form)
NADH	nicotinamide-adenine dinucleotide (reduced form)
NADP	nicotinamide-adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide-adenine dinucleotide phosphate (reduced form)
NEM	N-ethylmaleimide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCE	tetrachloroethylene
PEP	phosphoenol pyruvate
PES	phenazine ethosulphate
PMB	para-chloromercuric benzoate
R_f	relative mobility
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TFA	trifluoroacetic acid
TRICINE	N-tris(hydroxymethyl)methylglycine
TRIS	2-amino-2-hydroxymethyl-1,3-propanediol
UV	ultra violet
V	voltage
VC	vinyl chlorine
X	halogen

Other abbreviations are explained where appropriate in the text.

ABSTRACT

Haloalcohols are an important class of industrial pollutants some of which are believed to be carcinogenic. Bacteria that are capable of efficient degradation of these compounds have been isolated. In our laboratory several soil isolates have been isolated by enrichment on 1,3-dichloro-2-propanol and have been subject of biochemical and physiological characterization. In order to shed more light on the biochemical and molecular mechanisms of dehalogenation an *Arthrobacter* sp H10a was chosen for this study.

Arthrobacter sp strain H10a possesses two enzymes capable of dehalogenating halohydrins (haloalcohol dehalogenases). These dehalogenases designated as Deh1 and Deh2 are expressed constitutively but levels of enzyme activity can be increased 3 to 4 fold in the presence of glycidol. The Deh1 enzyme showed higher activity towards 1,3-DCP while the Deh2 dehalogenase showed higher activity towards CPD. The analysis of the ratio of dehalogenation rate for both CPD and 1,3-DCP showed that addition of haloalcohols to the growth medium resulted in an increase in the Deh2 enzyme activity in relation to the Deh1 activity, whilst epoxides had an opposite effect. In an attempt to understand the mechanisms of dehalogenation the Deh1 haloalcohol dehalogenase was purified and characterized. This enzyme is constituted by two subunits of 31.5 and 34 kDa molecular weight, that associate with other proteins to form a large protein complex of 200 kDa. Peptide mapping with different proteases and amino acid micro-sequence analysis of tryptic digests showed 100% identity between the two Deh1 subunits.

The Deh1 haloalcohol dehalogenase catalyzed the conversion of vicinal halohydrins to epoxides and the reverse reaction in the presence of an excess of halogen. This enzyme showed maximum activity at 50°C and a broad pH optimum from 8.5 to 10.5. The apparent K_m and V_{max} values for dehalogenation of 1,3-DCP and CPD were 0.11 mM, 236 µmol min^{-1} mg^{-1} and 2.02 mM, 1.55 µmol min^{-1} mg^{-1}, respectively. The enzyme activity was inhibited by MCA and DCA. The inhibition pattern suggested a mixed type inhibition predominantly uncompetitive. Amino acid modifying experiments have shown that one or more cysteine and arginine residues may be involved in catalysis or play important roles in maintenance of the enzyme structure. Modification of histidine, lysine, aspartic and glutamic acids had no effect on the dehalogenase activity.

Studies of the stereospecificity of the epoxides formed by the Deh1 haloalcohol dehalogenase revealed that (R)-ECH was selectively produced from 1,3-DCP. During the reverse reaction (R)-ECH was stereoselectively halogenated to 1,3-DCP if the halogen in the reaction mixture was chloride. However, if chloride was substituted by bromide, the (S)-isomer was halogenated preferentially. Although the enantiomeric excess and the yields of ECH obtained were low it was shown that it is possible to produce both isomers of ECH if the reaction conditions were optimized.

An antibody raised against the Deh1 enzyme was used to screen other bacterial isolates in the laboratory culture collection. This antibody showed immunocross-reactivity with a haloalcohol dehalogenase from strain H10c. This enzyme revealed the same electrophoretic mobility as the Deh1 protein under both native and denaturing conditions. The Deh1 antibody also showed cross-reaction with a 31.5 kDa protein from strain H10f. No immunological cross-reactivity was found between this antibody and the total protein extracts from haloacid and haloalkane degrading bacteria.

CHAPTER 1

INTRODUCTION

1.1. Introduction

Halogenated aliphatic hydrocarbons are widely employed in the chemical industries as solvents and synthons in chemical synthesis, degreasing agents, flame retardants and other specialist applications. They can also find wide spread use as pesticides and therefore are directly introduced into the environment. The intensive use of these industrial chemicals combined with their improper disposal has contributed to their accumulation in terrestrial and aquatic environments. Among these compounds, chlorinated alkanes and alkenes that contain between one and three carbon atoms are of main concern as they are suspected of being human carcinogens.

The growing environmental awareness has stimulated interest in studying processes which can eliminate halogenated compounds (and pollutants in general) from the environment. The development of treatment biotechnologies requires an understanding of the organisms, their metabolic mechanisms, and for halocompounds, the catalysis of dehalogenation. The study of the physiological and the kinetic aspects of these biotransformations are therefore of significant importance.

A large number of microorganisms capable of dehalogenating a wide range of compounds have been reported in the literature (Vogel *et al.*, 1987; Hardman, 1991; Janssen *et al.*, 1991). The conversion of these compounds to mineral products and cell components requires the activity of an efficient mechanism of dehalogenation. Hence significant effort has been made to identify the dehalogenase enzymes and study their characteristics.

1.2. Dehalogenation mechanisms

The study of the biodegradation of halogenated aliphatic compounds by a wide variety of bacteria has revealed that the mechanisms of dehalogenation are mainly of four

types: hydrolysis, elimination, reduction and oxidation. The first two types of reactions are catalyzed by enzymes, designated dehalogenases; enzymes that specifically cleave the carbon halogen bond. The last two dehalogenation reactions are the result of catalysis by an enzyme that, due to its relaxed substrate specificity, is able to reduce or oxidize the halogenated hydrocarbons and cause the elimination of the halosubstituents.

1.2.1. Hydrolysis of the C-X bond

The most extensively studied dehalogenation reaction is the hydrolytic cleavage of the carbon halogen bond, catalyzed by halidohydrolase-type dehalogenases (Hardman, 1991). Dehalogenation of certain haloacids and haloalkanes is catalyzed by these enzymes which generate hydroxy analogues and free halogen ions (Figure 1.1 A). Although the end products are similar, several types of enzymes can be distinguished according to the substrate specificity. For example, halidohydrolases are either active towards halogenated acids or alkanes. Within these groups halidohydrolases also show different substrate specificities, electrophoretic mobilities under nondenaturating conditions and inhibition by substrate analogues or specific amino acid modifying reagents (e.g. thiol reagents). There are also hydrolytic dehalogenases which require cofactors (eg: glutathione) for activity (Stucki *et al.*, 1981).

The specificity of hydrolytic dehalogenases depends on the chain length, position and type of halogen substituent. In general the specific activity decreases in the order F<Cl<Br<I.

1.2.2. Elimination of the HX

Enzyme-catalyzed elimination of HX (Figure 1.1 B) has been found only in the dehalogenation of halohydrins. These enzymes, designated as halohydrin epoxidases (Castro & Bartnicki, 1968) or haloalcohol dehalogenases (van den Wijngaard *et al.*, 1989) are capable of converting vicinal haloalcohols to the corresponding epoxides with the associated liberation of inorganic halogen. These enzymes also catalyze the reverse reaction of halogenation.

Haloalcohol dehalogenases have recently received particular attention due to their stereospecificity features. These enzymes can produce preferentially one of the epoxide isomers, or preferentially assimilate one of the halohydrin isomers. Either way, the resulting products are chiral glycerol derivatives which are important chiral building blocks for the synthesis of chiral pharmaceuticals.

1.2.3. Reduction of halogenated hydrocarbons

Reductive dehalogenation in most cases takes place under anaerobic conditions, and involves the removal of a halogen substituent from a molecule with concurrent addition of electrons. This can occur either by (i) hydrogenolysis, where the halogen substituent is replaced with a hydrogen atom (Figure 1.1 C); or by (ii) vicinal reduction or dihaloelimination, where two halogen substituents from adjacent carbon atoms are removed and an additional bond between the carbon atoms is formed. Reductive dehalogenation has been attributed to nonspecific reduction by transition metals associated with certain cofactors such as corrinoids, iron porphyrins and P_{450cam}, (Mohn & Tiedje, 1992).

Although reductive dehalogenation is not exclusively linked to methanogenesis (diStefano *et al.*, 1982) organisms of this genus may play an important role in anaerobic biotransformation of polychlorinated ethylenes. Dechlorination of tetrachloroethylene (PCE) to vinyl chloride (VC) and ethylene under anaerobic conditions is achieved through sequential hydrogenolysis. In general, the rates of reductive dehalogenation are greater for the more highly substituted ethylenes resulting in the accumulation of the lesser chlorinated analogues, which are often more toxic than the original compounds (eg: vinyl chloride).

1.2.4. Oxidation of halogenated hydrocarbons

Oxidation of halogenated hydrocarbons is a consequence of fortuitous catalysis by an oxygenase with a broad substrate specificity and results in the production of the corresponding alcohol, aldehyde or epoxide (Figure 1.1 D); dehalogenation occurs by spontaneous degradation of the oxidized product. Although co-oxidation often results in incomplete degradation of the halogenated compound, this type of biodegradation can play a major part in the decomposition of halogenated alkenes in the environment. Several studies revealed that cytochrome P_{450} (Miller & Guenguich, 1982), ammonia monooxygenase (Arceiro *et al.*, 1989; Vannelli *et al.*, 1990), toluene oxygenases (Nelson *et al.*, 1987; Nelson *et al.*, 1988; Wacket & Gibson, 1988), propane monooxygenase (Wacket *et al.*, 1989), propene monooxygenase (Ensign *et al.*, 1992), and methane monooxygenase (Little *et al.*, 1988; Tsien *et al.*, 1989; Janssen *et al.*, 1988b) were capable of oxidizing halogenated alkenes. The differential degradation of chlorinated alkenes by different bacterial strains reflects (a) the affinity of the oxygenase towards the substrate, (b) the inhibitory effects of the oxidation products on the enzymes and (c) the constraints on uptake of chlorinated alkenes into the cell. In general, the rates of oxidation are higher for the lesser chlorinated ethylenes and none

of the oxygenases have shown activity towards tetrachloromethane and tetrachloroethylene.

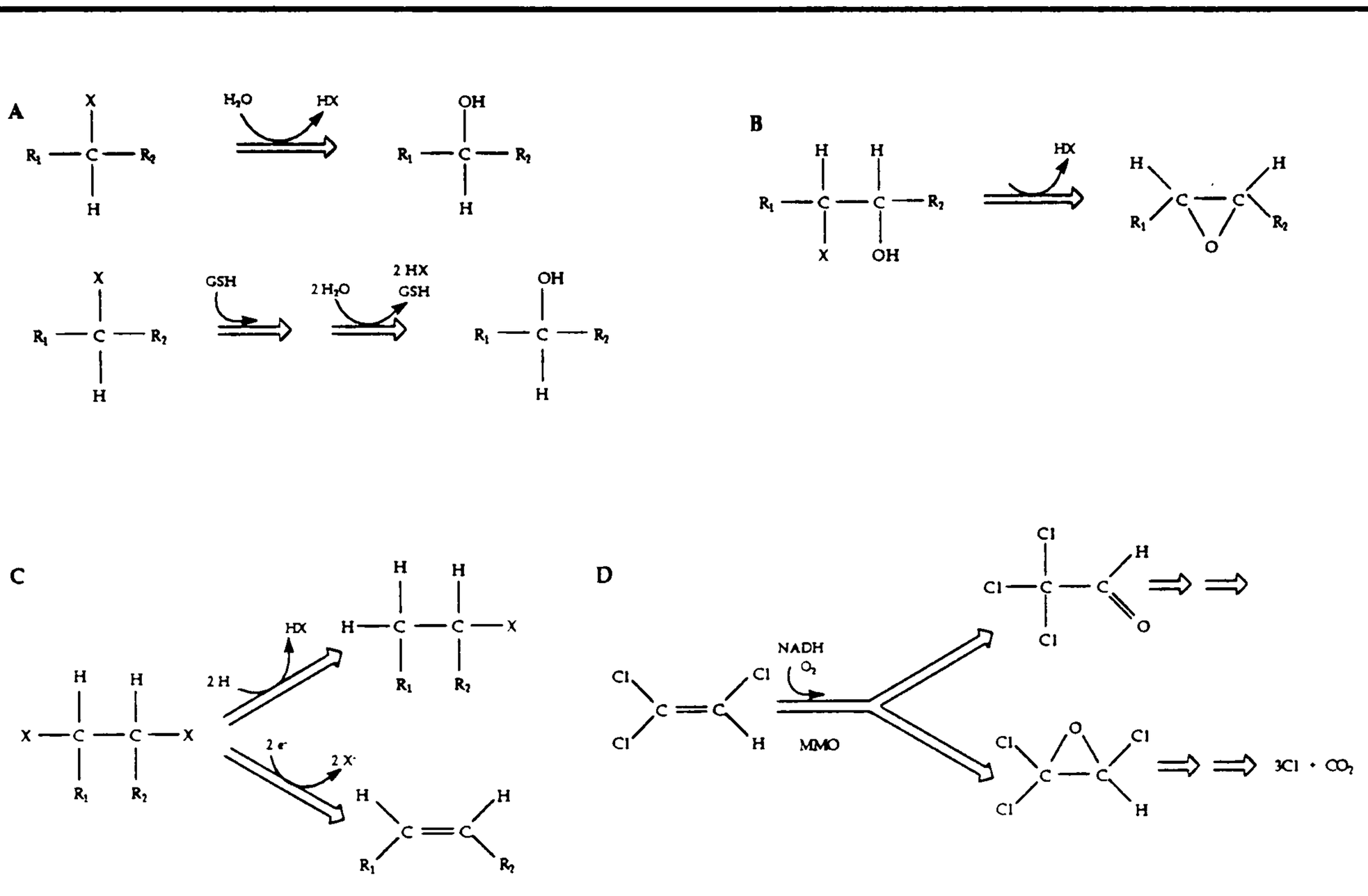


Figure 1.1: Examples of dehalogenation; (A) hydrolysis; (B) elimination; (C) reduction; (D) oxidation of trichloroethylene. R_1 and R_2, alkyl groups; X, halogen.

Although the oxygen requirements of reductive dehalogenation and oxidation of halogenated ethylenes are opposite, these processes may complement each other in the removal of such pollutants from the environment. In general, the oxidation rates are higher with the less chlorinated compounds while the rates of reduction are lower. Furthermore, reductive dehalogenation of highly substituted ethenes leads to the accumulation of vinyl chlorine (VC); under aerobiosis, VC is rapidly oxidized by a large number of oxygenases. Moreover, tetrachloroethylene is not a substrate for the oxygenases studied so far, but this compound is rapidly dehalogenated to less

substituted ethylenes under anaerobic conditions. An understanding of the pathways and the degradation products of halogenated aliphatic compounds by microorganisms can be useful in evaluating contamination patterns and in selecting the most appropriate remediation process.

1.3. Biotransformation of short chain halogenated aliphatic compounds, physiological pathways and dehalogenating enzymes

In the next section an overview of the dehalogenation pathways with a special emphasis on the enzymes that catalyze the removal of halogens from halogenated hydrocarbons is presented.

1.3.1. Biodegradation of halogenated alkanoic acids

1.3.1.1. 2-haloalkanoic acids

The wide spread use of the herbicide Dalapon, the active ingredient of which is 2,2'-dichloropropionic acid, prompted the search for microorganisms capable of degrading halogenated alkanoic acids. Several bacterial genera are able to use these compounds as carbon and energy sources (Hardman, 1991), the most commonly found belonging to the genus *Pseudomonas*. Haloacid dehalogenases are the most studied group of dehalogenating enzymes. These enzymes do not require co-factors and catalyze the hydrolytic removal of the halogen from alkanoic acids, and are therefore designated as halidohydrolases. The dehalogenation products of these enzymes are either intermediates of the central metabolism or can easily be converted to such (Figure 1.2).

Haloacid halidohydrolases have been divided into two main groups according to their substrate specificity: haloacetate dehalogenases (EC 3.8.1.3) and 2-haloacid dehalogenases (EC 3.8.1.2). The former, are only active towards halogenated acetic acid and can be further classified into two different types, the ones that are (Goldman *et al.*, 1968; Kawasaki *et al.*, 1981b) and are not (Davies & Evans, 1962; Kawasaki *et al.*, 1981a and 1981b) active towards fluoroacetate.

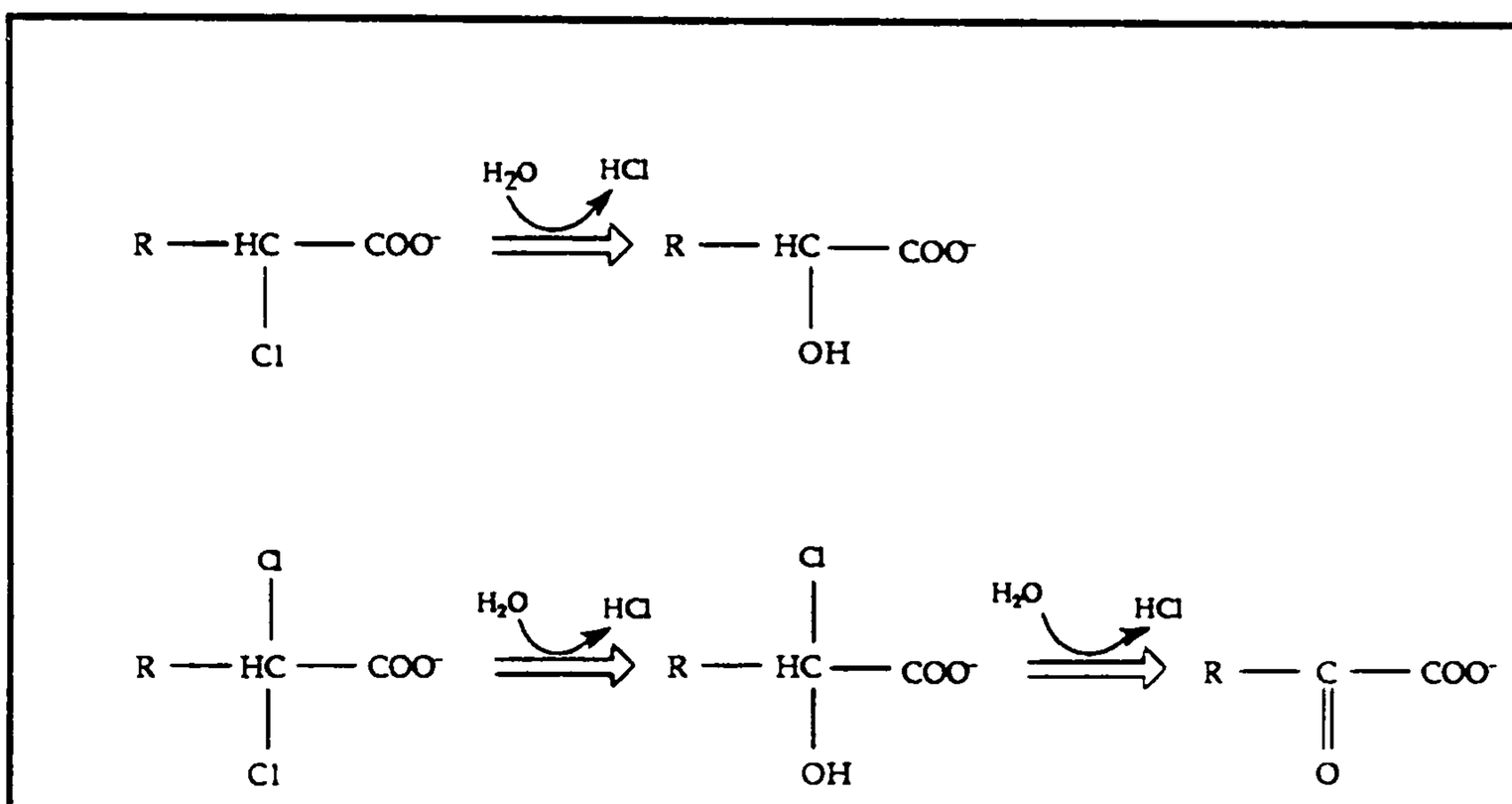


Figure 1.2: Dehalogenation of mono- and di- substituted 2-haloacids.

The 2-haloacid halidohydrolases are specific towards haloalkanoic acids of short chain length ($<C_5$), with a free primary carboxylic group and the reactive halogen in the carbon 2 position. Four different types of enzyme have been distinguished according to the configuration of the substrate and product, and sensitivity to thiol blocking reagents. Type 1 halidohydrolases are active only towards the L-isomer of 2-monochloropropionic acid (2-MCPA), and the reaction products have opposite optical configuration; these enzymes are insensitive to -SH reagents (Goldman *et al.*, 1968; Little & Williams, 1971; Leigh *et al.*, 1988; Tsang *et al.*, 1988). Type 2 enzymes dehalogenate both D- and L-isomers, once more inverting the configuration of the products and are also insensitive

to thiol modifying reagents (Motosugi *et al.*, 1982c; Weightman *et al.*, 1982; Leigh *et al.*, 1988). Type 3 enzymes dehalogenate both D- and L-isomers, but the products have the same optical configuration and these enzymes are sensitive to thiol reagents (Weightman *et al.*, 1982). Type 4 enzymes act only on the D-isomer, invert the optical configuration of the substrate and are insensitive to thiol blocking reagents (Leigh *et al.*, 1988; Smith *et al.*, 1990).

Based on these findings Weightman *et al.* (1982) proposed two mechanisms of dehalogenation that might occur with different types of 2-haloacid halidohydrolases. The dehalogenation mechanism proposed in Figure 1.3 A for the Type 1, 2 and 4 enzymes is in agreement with the reaction features observed for these enzymes, inversion of the product optical configuration and insensitivity with thiol reagents. The differences of stereospecificity observed for these three types of dehalogenase may be related to the type and/or position of the amino acid that coordinates the acid group of the substrate. The mechanism outlined in Figure 1.3 B accounts for the properties observed for the Type 3 2-haloacid halidohydrolases; the optical configuration of the substrate is maintained through a double inversion involving a thioether intermediate.

In an attempt to shed more light on the mechanism of dehalogenation of 2-haloacid halidohydrolase Asmara *et al.* (1992), carried out experiments involving chemical modification of the Type 1 haloacid halidohydrolase of *Pseudomonas cepacia* MBA4 (Tsang *et al.*, 1988) and random and site directed mutagenesis. They identified two amino acid residues, His_{20} and Arg_{42}, as the key residues for catalytic activity. These residues are located on the regions 9-20 and 40-50 in the amino acid sequence of the *dhl*Vla enzyme, and have been previously shown to be highly conserved among L-2-haloacid halidohydrolases (van der Ploeg *et al.*, 1991; Murdiyatmo *et al.*, 1992). Asp_{18} was also implicated in the reaction mechanism, possibly by positioning the correct tautomer of His_{20} in the enzyme substrate complex (Figure 1.4).

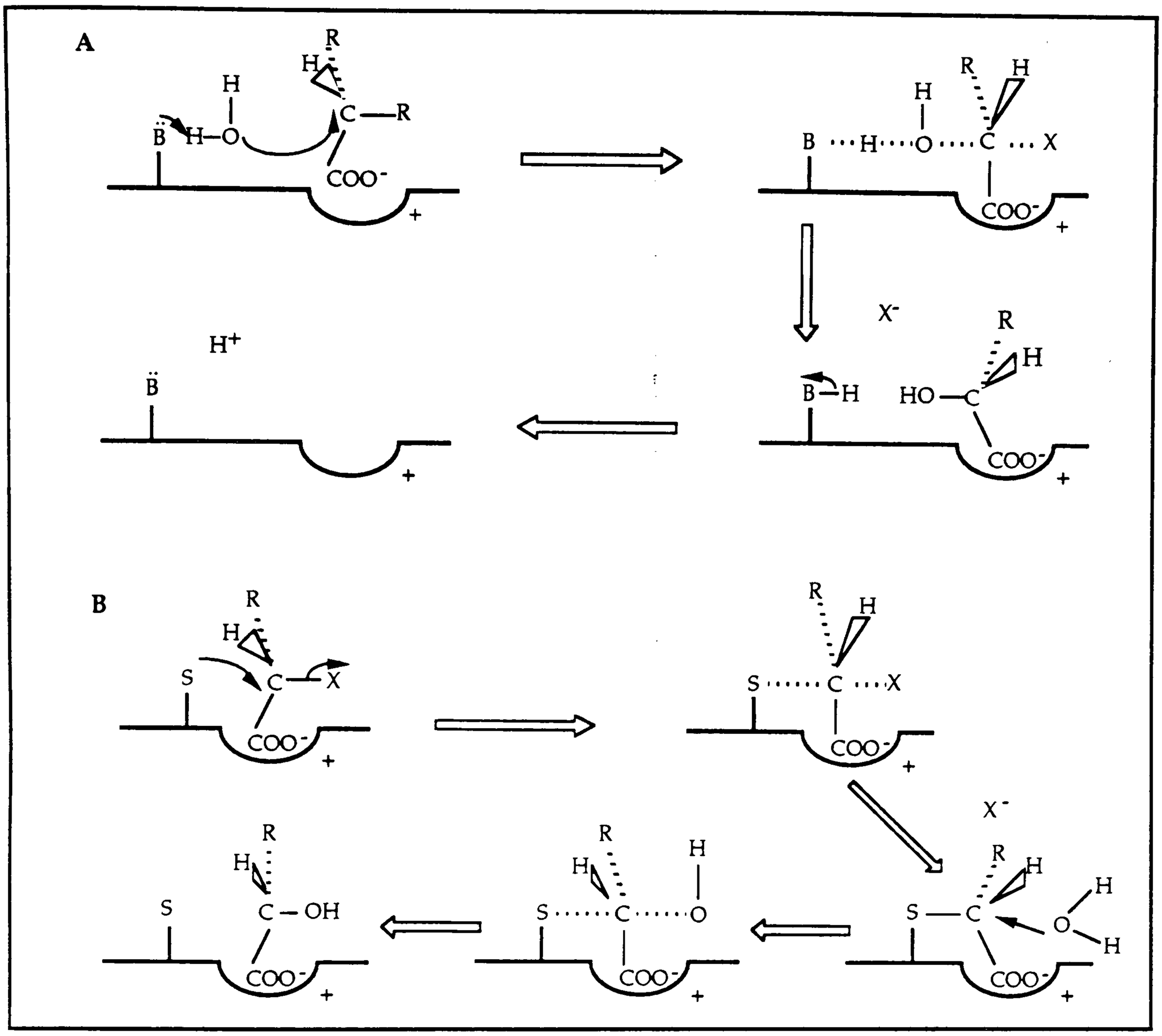


Figure 1.3: Proposed mechanisms of dehalogenation of 2-haloacids. (A) a mechanism resulting in the inversion of optical configuration; (B) a mechanism resulting in retention of configuration. X=halide, R=alkyl group (After Goldman *et al*, 1968 and Little & Williams, 1971).

The halidohydrolases are cytoplasmic enzymes normally induced by their own substrates or analogues thereof. Although they show a wide substrate specificity the apparent K_m values are normally in the mM range indicating poor substrate affinity. In general, the rates of dehalogenation decrease with increasing chain length and number of halogens substitutes; brominated compounds are dehalogenated faster than the chlorinated analogues (Table 1.1). 2-Haloacid halidohydrolases are monomers or dimers of a single type of subunit with a molecular weight ranging between 15,000 and

33,000 with an acidic pI (4.9-5.4). The *Pseudomonas putida* AJ1/23 enzyme differs from the other halidohydrolases in its tetrameric structure and high molecular weight (134,500) (Smith *et al.*, 1990). These enzymes show a basic optimum pH (9-10.5) and an optimum temperature of 45° to 50°C.

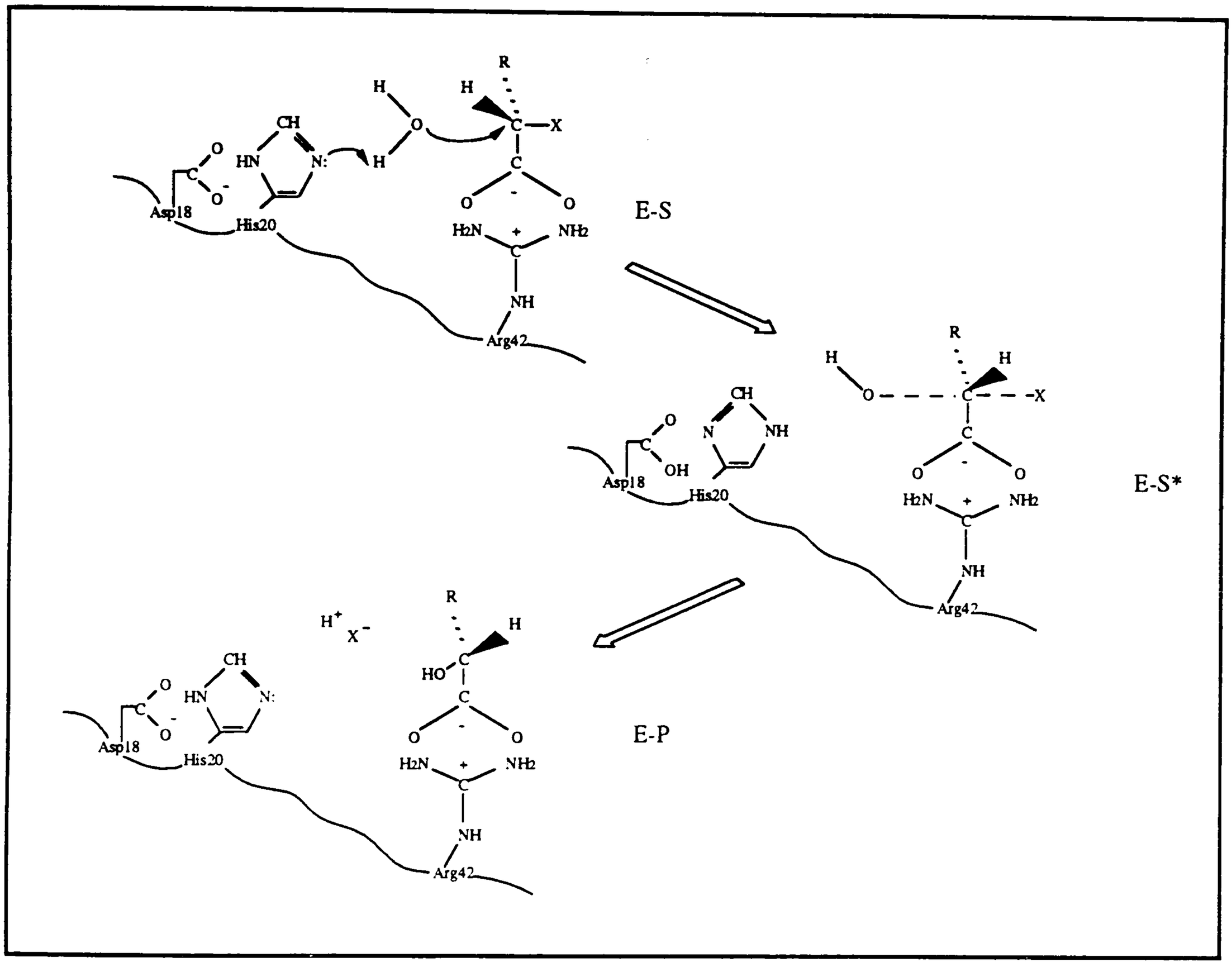


Figure 1.4: Proposed dehalogenation mechanism for *P. cepacia* MBA4 halidohydrolase IVa (After Asmara *et al*, 1992).

The evolution of halidohydrolase activities has puzzled many researchers since many of the halogenated compounds of concern are man-made and were introduced into the environment only relatively recently. The existence of transposons associated with halidohydrolases would enable translocation and expression of previously cryptic genes and/or duplication of these genes (Slater *et al.*, 1985). The presence of natural

plasmids encoding halidohydrolase enzymes would provide another route for acquisitive evolution by gene transfer mechanisms. Kawasaki *et al.* (1981b & 1981c) isolated and characterized two large plasmids, pOU1 and pOU2 from *Moraxella* sp strain B and *Pseudomonas* sp strain C respectively, that encoded two haloacetate halidohydrolases. Furthermore, four *Pseudomonas* and two *Alcaligenes* species contain large plasmids (5 plasmids ranging in size from 149 to 285 Kb) which have been correlated with dehalogenase activities of the host cells (Hardman *et al.*, 1986).

Table 1.1: Relative activities towards 2-haloalkanoic acids of different halidohydrolases. (1) Tsang *et al.*, 1988; (2) Motosugi *et al.*, 1982a; (3) Motosugi *et al.*, 1982c; (4) Smith *et al.*, 1990; (5) Jones *et al.*, 1992; (6) Little & Williams, 1971; (7) Leigh *et al.*, 1988; (8) Weightman *et al.*, 1982; (9) Goldman *et al.*, 1968; (10) van der Ploeg *et al.*, 1991; (11) Strotman *et al.*, 1990; (12) Kawasaki *et al.*, 1981a; (13) Kawasaki *et al.*, 1981b; (14) Kocabiyik & Türkoglu, 1989; (15) Davies & Evans, 1962.

	Type	MIA	MBA	MCA	MFA	DCA	2MBPA	2MCP	2,2 DCPA	2MCB
Pseudomonas cepacia MBA4 (1)	1		2.40	1.00		0.23	0.85	0.13		
Pseudomonas putida 109 (2)	1	2.55	3.58	1.84	0.00	0.30	2.47	1.00	0.10	0.01
Pseudomonas sp 113 (3)	2	0.96	2.80	0.33	0.00	0.08	3.80	1.00	0.42	0.18
Pseudomonas putida AJ1 HadL (4)	4		4.05	1.40	0.00		3.64	1.00	0.07	0.03
HadD (5)	1							1.00	0.30	0.75
Pseudomonas dehalogens NCIB 9061 (6)	1	1.00	1.40	1.00	0.00	0.18		0.35	0.09	
Rhizobium (7) I	1			0.00		5.01		10.2	0.00	
I I	2			8.36		7.41		4.86	2.58	
I I I	4			2.04		0		3.83	0.00	
Pseudomonas putida PP3 (8) I	3			1.00		0.17		0.49		
I I	2			1.00		1.50		0.20		
Pseudomonad (9) I	1	1.5		9.00	0.00	1.00		5.00		0.70
I I	1	0.5		0.50	0.00	1.00		0.60		0.40
Xanthobacter autotrophicus GJ10 (10)	1		0.98	1.00		3.71		0.92	0.05	0.03
Pseudomonas putida US2 (11)	3			1.00		0.16		0.27	0.16	0.00
Pseudomonas sp A (12)	MFA	>0.01	0.14	0.21	1.00			>0.01	0.00	0.00
Moraxella sp (13)	MCA	1.5	1.60	1.00	0.00	0.03		0.09	>0.01	0.00
Pseudomonas sp 19S (14)	MCA	0.67	0.17	1.00						
Pseudomonas sp K (15)	MCA	1.00	1.00	1.00	0.00	0.17		0.00	0.00	

More than one halidohydrolase isoenzyme has been found in the same microorganism and often these isoenzymes showed different substrate specificities. The *Pseudomonas* sp CBS3 2-haloacid halidohydrolases, which show 70% amino acid sequence homology, might have arisen from gene duplication of an ancestral gene and subsequently evolved slightly different substrate specificities (Schneider *et al.*, 1991). However, the amino acid sequence of the H-1 and H-2 dehalogenases of *Moraxella* sp B (Kawasaki *et al.*, 1992) and *Pseudomonas putida* AJ1 HadD and HadL (Barth *et al.*, 1992) enzymes show no significant homology between the two isoenzymes of each bacteria, ruling out the possibility of gene duplication as outlined above.

1.3.1.2. 3-Haloalkanoic acids

Isolation of bacteria that specifically dechlorinate ß-halogenated carboxylic acids, and the lack of 2-haloacid halidohydrolase activity towards the ß-substituted analogues suggests that the enzymes involved in the two dehalogenations are completely different. A *Micrococcus denitrificans* strain was found to dehalogenate 3-chloropropionic acid with the formation of acrylic acid, suggesting that the chlorine was removed by a dehydrohalogenation-type of reaction (Bollag & Alexander, 1971).

Hartmans *et al.* (1991a) and van Hylckama *et al.* (1991) isolated two Coryneform strains (CAA2 and FG41) that were able to use both isomers of 3-chloroacrylic acid (CAA) as a sole carbon and energy source and a *Pseudomonas cepacia* sp strain CAA1 capable of growth with only the *cis*-isomer. Dehalogenation of CAA by strain CAA2 took place after an enzyme catalyzed hydration of the double bond. The unstable 3-chloro-3-hydroxypropionic acid so formed spontaneously decomposed to malonate semialdehyde (Figure 1.5) (Hartmans *et al.*, 1991). In contrast with the halidohydrolases, the enzymes responsible for dehalogenation of CAA, designated as

hydratases (Hartman *et al.*, 1991a) or dehalogenases (van Hylckama & Janssen, 1991), have the pH optima in the range of pH 7.3 to 8.0, and show very high substrate specificities, suggesting that they represent a distinct type of enzyme.

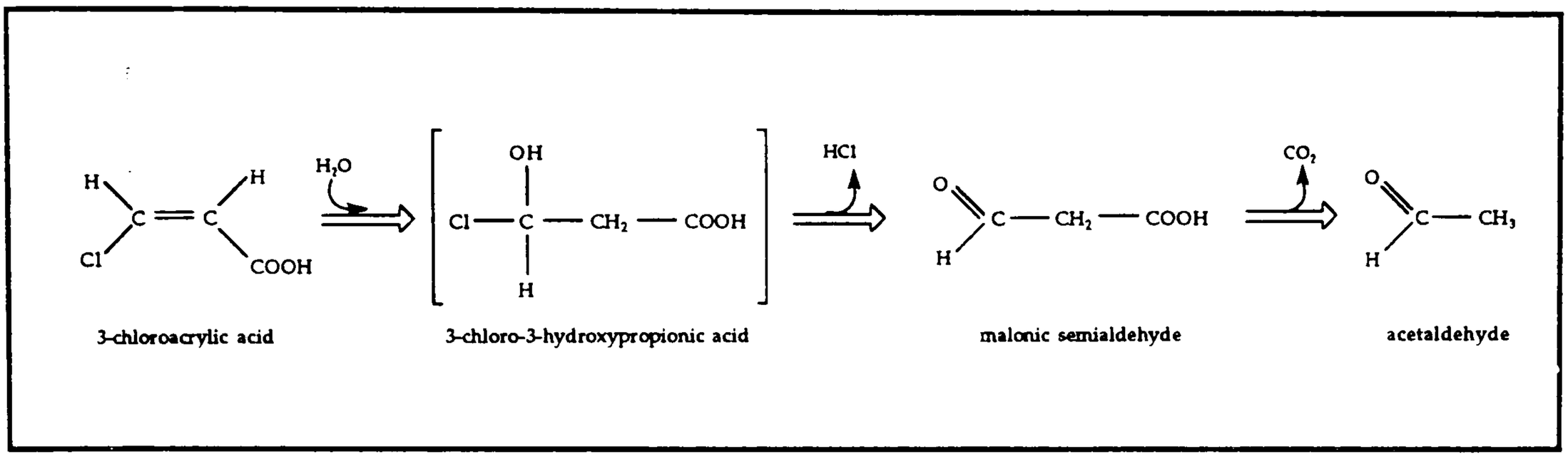


Figure 1.5: Proposed pathway for *cis*-CAA degradation in *Pseudomonas cepacia* strain CAA1 and the Coryneform strain CAA2 (After Hartmans *et al.*, 1991a). CAH, chloroacrylic acid hydratase.

Degradation of ß-chlorinated four carbon fatty acids such as 3-chlorobutyrate, *cis*-3-chlorocronate and *trans*-3-chlorocronate, by *Alcaligenes* sp strain CC1 is dependent on CoA, ATP and Mg^{2+} (Kohler-Staub & Kohler, 1989). Activation of the acids to their CoA derivatives prior to their dehalogenation is probably catalyzed by a broad substrate specificity acyl-CoA synthetase involved in ß-oxidation of fatty acids. The mechanism of dechlorination the CoA ester derivative is not yet understood, but probably the chlorine is removed by nucleophilic displacement by an hydroxyl group, as shown in Figure 1.6. A similar dehalogenation mechanism was observed for the degradation of 3-chloro-2-methylpropionic acid (CMPA) and its methyl ester (MeCMPA) by *Xanthobacter* sp CIMW99 (Smith *et al.*, 1991).

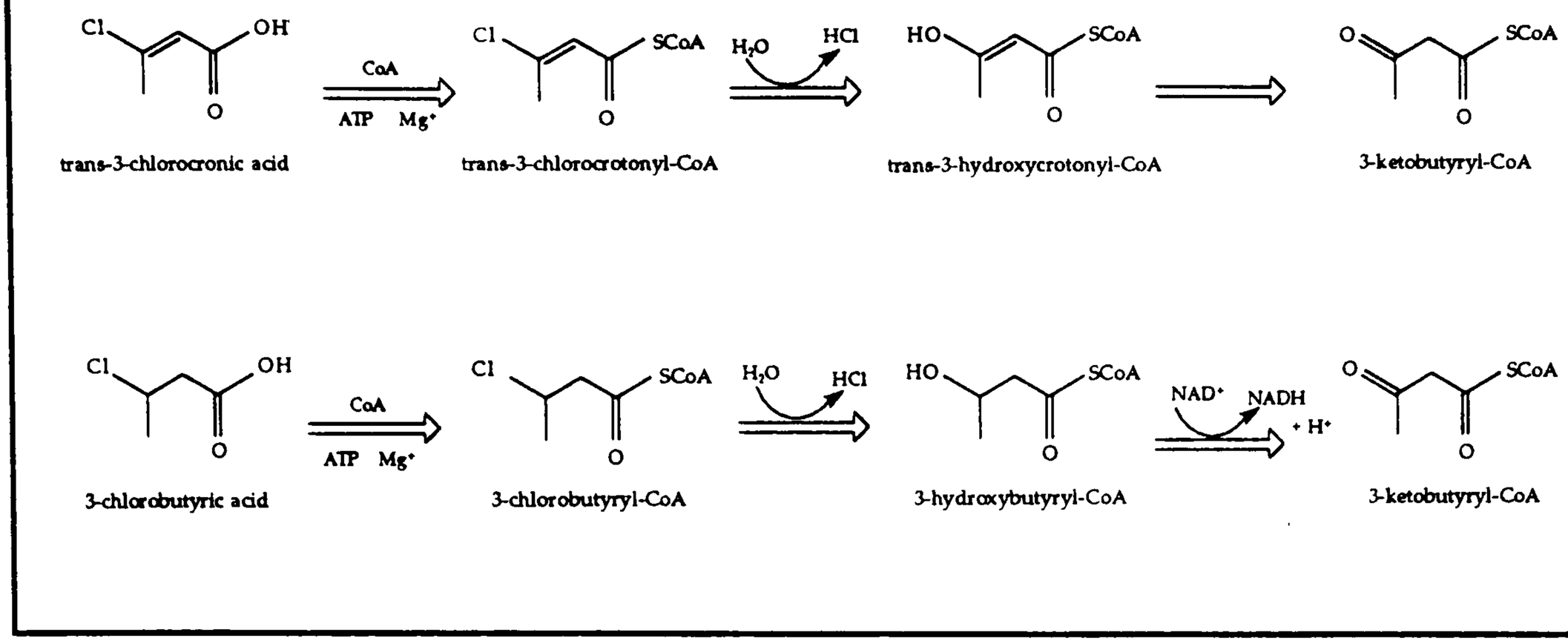


Figure 1.6: Proposed catabolic pathway of *trans*-3-chlorocronate and 3-chlorobutyrate by *Alcaligenes* sp strain CC1 (After Kohler-Staub & Kohler, 1989).

1.3.2. Biodegradation of halogenated alkanes

Due to great toxicity of halogenated alkanes only a small number of microorganisms capable of using these compounds as a single carbon and energy source have been isolated. The number of biochemical studies of haloalkane metabolism are therefore fewer than for haloacids.

1.3.2.1. Halogenated methanes

Halomethanes are widely used in chemical industries as intermediates in chemical synthesis or as solvents in a variety of industrial processes. Halogenated methanes are degraded anaerobically by methanotrophs, by reductive dehalogenation to less substituted methanes, and by substitutive and oxidative mechanisms to CO_2 (Egli *et al.*, 1988). The ability to anaerobically transform CCl_4 has been correlated with the presence of the acetyl-CoA pathway for the degradation or synthesis of acetate (Egli *et*

al., 1990). Anaerobic degradation of halomethanes is in fact a chemical process that arises from non-specific dehalogenation catalyzed by the metal prosthetic groups of some enzymes.

Under aerobic conditions dichloromethane (DCM) is degraded to formaldehyde by a *Pseudomonas* strain DM1 (Brunner *et al.*, 1980). Further studies on the mechanism of dehalogenation of DCM by some facultative methylotrophs (Stucki *et al.*, 1981, Galli & Leisinger, 1988) revealed that the first step in the degradation of this xenobiotic substrate is catalyzed by an inducible glutathione dependent dichloromethane dehalogenase. The dehalogenation of DCM to formaldehyde involved a glutathione S-transferase which forms an S-chloromethyl glutathionine conjugate. This intermediate undergoes non-enzymatic hydrolysis to yield S-hydroxymethyl glutathione which decomposes to formaldehyde and GSH (Figure 1.7).

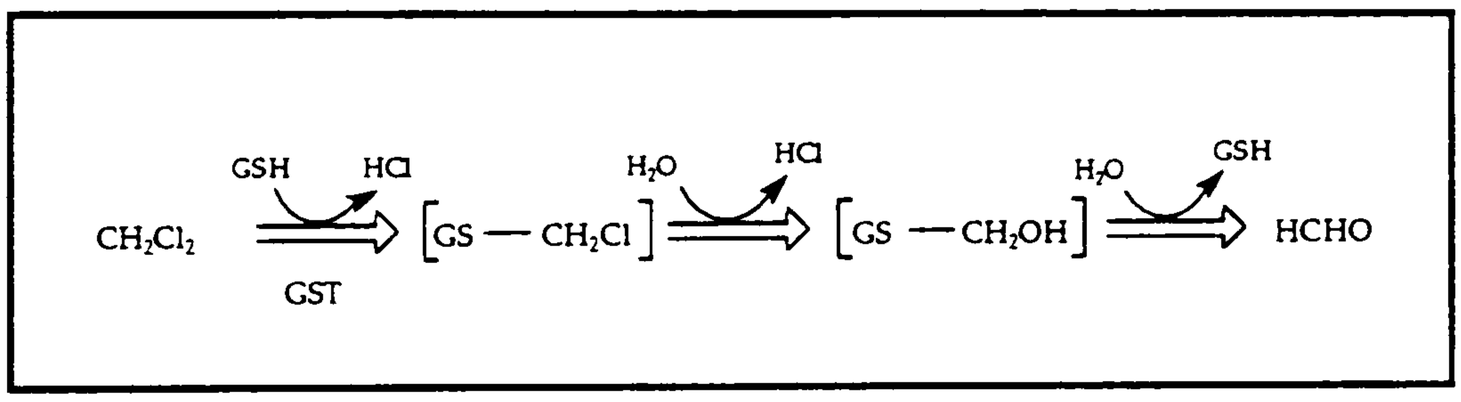


Figure 1.7: Proposed dehalogenation pathway for the degradation of dichloromethane by *Hyphomicrobium* DM2 (After Kohler-Staub & Leisinger, 1985). GST, glutathione-S-transferase.

The dichloromethane dehalogenases found in *Hyphomicrobium* sp DM2 (Kohler-Staub & Leisinger, 1985), *Methylobacterium* sp DM4, *Hyphomicrobium* sp GJ21, *Pseudomonas* sp DM1 and *Hyphomicrobium* sp DM2 (Kohler-Staub *et al.*, 1986) are identical with respect to substrate specificity, molecular weight and N-terminal amino acid sequence and they also showed immunological cross-reactivity. These observations, together with the fact that the DCM-degradation phenotype might be correlated with the possession of 120 Kb plasmid found in *Methylobacterium* sp DM4

(Galli & Leisinger, 1988), suggest that the dichloromethane dehalogenase gene was horizontally distributed among facultative methylotrophic bacteria. Sequence analysis of the *dcm*A gene from strain DM4 has shown conserved regions common with eukaryotic GSH S-transferases, indicating that DCM-dehalogenase belongs to the glutathione S-transferase supergene family.

The DCM dehalogenase of the fast growing methylotrophic bacterium strain DM11 shows some kinetic differences from the strains DM1, DM2, DM4 and GJ21 enzymes (group A) (Scholtz *et al.*, 1988b). The DM11 dehalogenase (group B) shows a 5.6 fold higher V_{max} than the group A enzymes; however, it represents only 8% of the total soluble protein whereas the enzyme in the slow growing strains constituted 16% of the total soluble protein. Both types of enzymes are glutathionine dependent but the group B dehalogenase showed a positive cooperativity in GSH binding while the group A enzymes showed a hyperbolic saturation with the co-substrate. Allosteric effects in enzymes offer greater metabolic control over reaction rates and this may constitute an evolutionary step in DCM-degradation. Although the two enzyme groups show a very similar subunit molecular weight (34,000-35,000), they are not related immunologically and the N-terminal amino acid sequences show no homology, suggesting that the two DCM dehalogenase groups represent distinct enzymes.

1.3.2.2. Halogenated ethanes

Dehalogenation of 1,2-dichloroethane (DCE) in the environment was found to be catalyzed by a number of bacteria using a variety of mechanisms. Reductive dehalogenation of DCE to ethylene and chloroethane by *Methanosarcina barkeri* strain DMS2948 was considered to be via a co-metabolic activity of enzymes containing corrinoids or factor F_{430} as their prosthetic groups (Hollinger *et al.*, 1992).

A number of methylotrophic bacteria that are capable of utilizing 1,2-dichloroethane (DCE) as a single carbon and energy source have been isolated (Stucki *et al.*, 1983; Janssen *et al.*, 1984; Yokota *et al.*, 1986; van den Wijngaard *et al.*, 1992). Initially Stucki and co-workers (1983) proposed that DCE degradation by strain DE2 started with oxidation, yielding an unstable intermediate, 1,2-dichloroethanol, which spontaneously decomposed to 2-chloroacetaldehyde and hydrochloric acid. The former compound was oxidized to 2-chloroacetate by a NAD-dependent chloroacetaldehyde dehydrogenase and later dehalogenated by a 2-haloacid halidohydrolase to produce glycolate. Subsequent studies indicated that degradation of DCE proceeds via 2-chloroethanol, chloroacetaldehyde, chloroacetate and glycolate (Figure 1.8) and involves two different halidohydrolases, one specific towards haloalkanoates and another specific towards haloalkanes, and two different dehydrogenases (Janssen *et al.*, 1985).

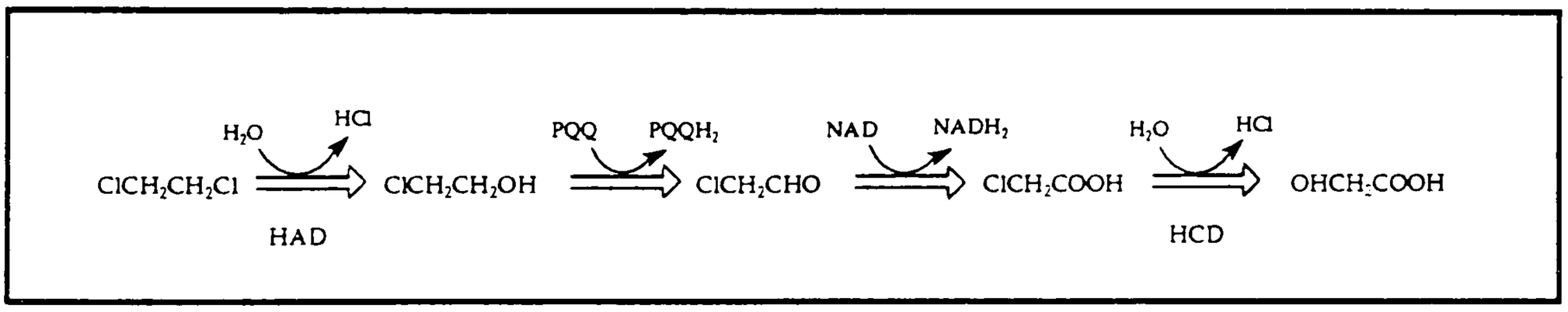


Figure 1.8: Catabolic route for 1,2-dichloroethane degradation of *Xanthobacter autotrophicus* GJ10 (After Janssen *et al.*, 1989). HAD, haloalkane dehalogenase; HCD, halocarboxylic acid dehalogenase.

The *Xanthobacter autotrophicus* strain GJ10 haloalkane halidohydrolase is the best studied haloalkane dehalogenase as it has been purified to homogeneity and characterized (Keuning *et al.*, 1985), the gene has been cloned (Janssen *et al.*, 1989) and the three dimensional structure determined (Franken *et al.*, 1991). This enzyme catalyses the hydrolytic cleavage of the chlorine from 1,2-dichloroethane and a number of other short chain halogenated n-alkanes (C_1-C_5), to produce 2-chloroethanol or the corresponding alcohols and halide ions (Janssen *et al.*, 1985). The GJ10 haloalkane

halidohydrolase consists of a single polypeptide chain with a molecular weight of 36,000. Thiol reagents, such as $HgCl_2$, iodoacetamide, p-chloromercuric benzoate and N-ethylmaleimide have been shown to strongly inhibit the enzyme (Keuning *et al.*, 1985).

The haloalkane halidohydrolase genes (*dhl*A) of *Ancylobacter* AD20, *Ancylobacter* AD25, *X. autotrophicus* GJ11 and *X. autotrophicus* GJ10 have identical sequences (van den Wijngaard *et al.*, 1992). A large plasmid implicated in DCE dehalogenation and carrying the haloalkane halidohydrolase and chloroacetaldehyde dehydrogenase genes has been detected in *X. autotrophicus* GJ10 (Tardif *et al.*, 1991). The six DCE-degrading bacteria (*X. autotrophicus* strains GJ10 and GJ11, *Ancylobacter* strains AD20, AD25 and AD27, and strain RB8) have the *dhl*A gene located on a DNA restriction fragment with the same molecular weight, indicating that the whole *dhl*A region might be derived from a common ancestral bacterial strain which was transferred horizontally (van den Wijngaard *et al.*, 1992). The *dhl*A sequence showed no overall similarity to other proteins, however, later studies have shown some N-terminal homology with the monofluoroacetate dehalogenase H-1 of *Moraxella* sp (Kawasaki *et al.*, 1992).

The three dimensional analysis of the GJ10 haloalkane halidohydrolase has shown that the enzyme is a spherical molecule composed of two domains: domain I has a α/β type structure with a central eight-stranded mainly parallel ß-sheet (Figure 1.9) and domain II lies like a cap on the top of domain I and consists of α-helices connected by loops (Franken *et al.*, 1991). The putative active site is completely buried in an internal hydrophobic cavity located between the two domains. Verschueren *et al.* (1993) have found that dehalogenation of 1,2-DCE occurs by a two step mechanism in which the Asp_{124} starts the reaction by acting as a nucleophile. In a second step a water molecule hydrolyses the covalent ester intermediate formed (Figure 1.10).

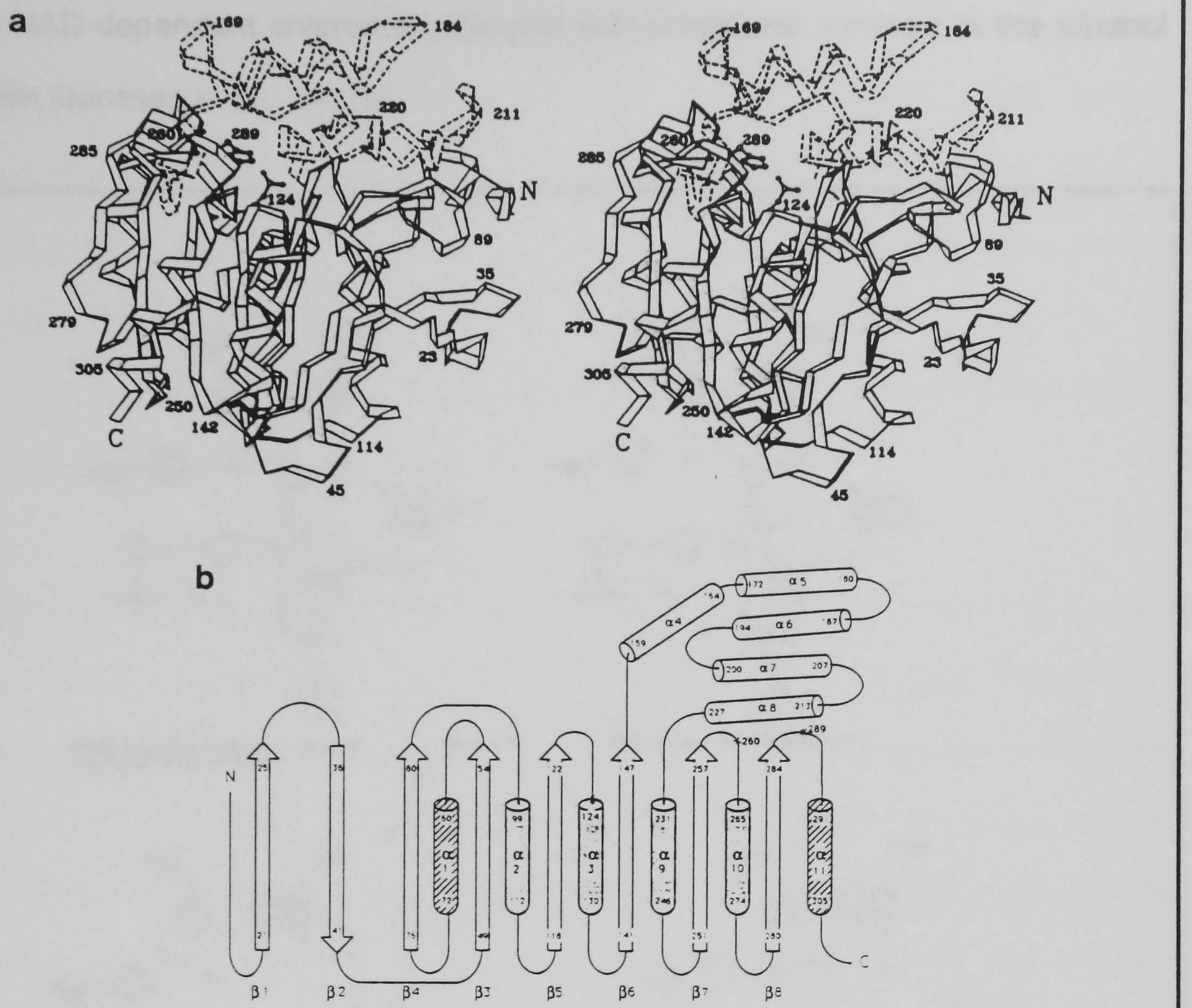


Figure 1.9: Three dimensional structure of the *X. autotrophicus* GJ10 haloalkane halidohydrolase. (A) Stereo view of the α-carbon chain; domain I is shown with solid lines and domain II with dashed lines. The putative active site residues are indicated and selected residues are labelled. (B) Topology of the secondary structure elements; α-helices are represented as cylinders and ß-strands as arrows; helices in one side of the ß-sheet are dashed, the ones on the other side are shaded and the ones in domain II are unfilled. (After Franken *et al.*, 1991).

Oxidation of 2-chloroethanol to chloroacetate in the DCE degrading *X. autotrophicus* GJ10 was catalyzed by two different dehydrogenases (Figure 1.8). The chloroethanol dehydrogenase appeared to be an inducible pyrrolo-quinoline (PPQ)-containing alcohol dehydrogenase, generally found in methylotrophs and responsible for methanol oxidation. This enzyme, due to its broad substrate specificity, was found to be active towards 2-chloroethanol (Janssen *et al.*, 1987b). The second dehydrogenase was a

inducible NAD-dependent chloroacetaldehyde dehydrogenase involved in the ethanol metabolism (Janssen *et al.*, 1987b).

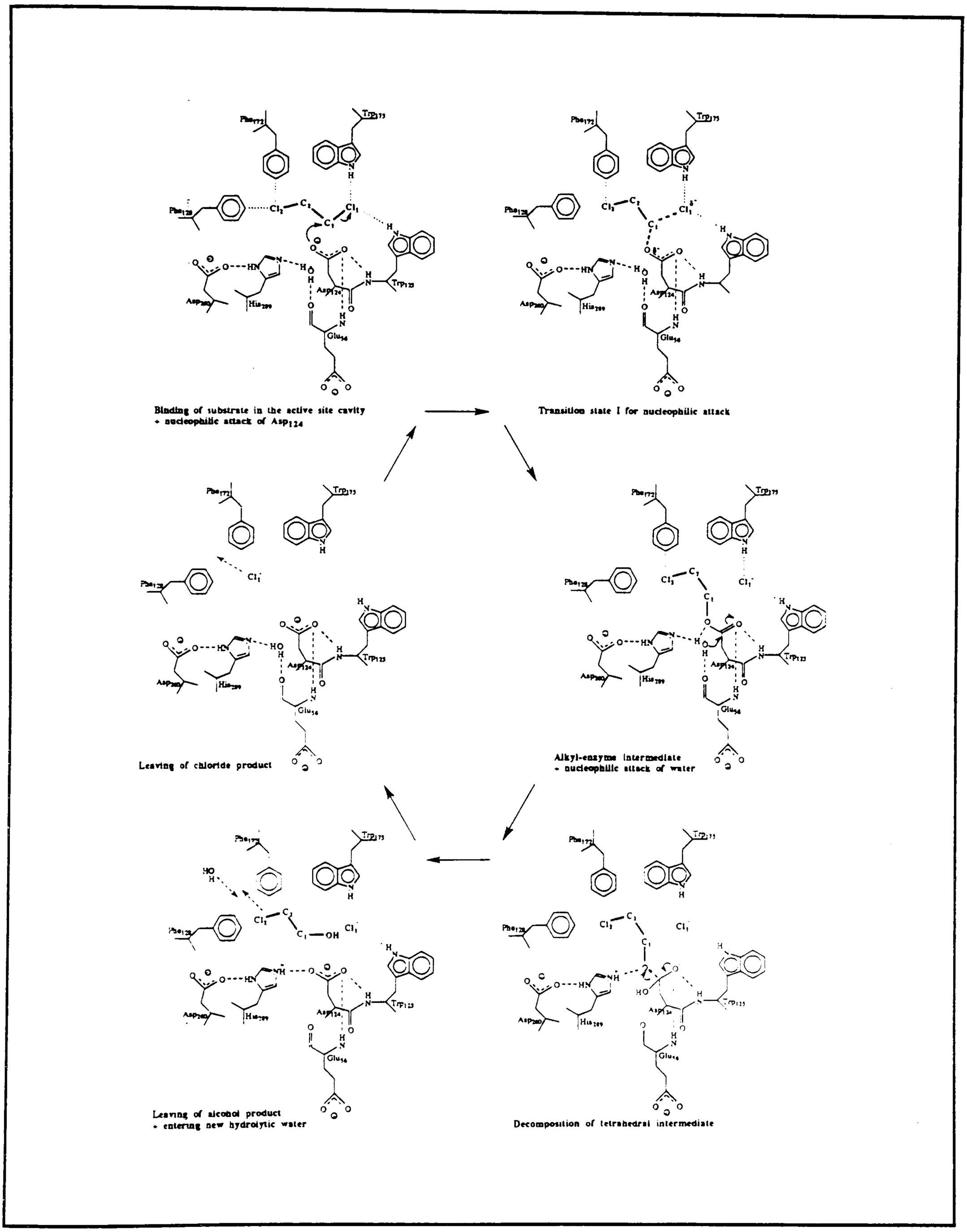


Figure 1.10: Possible mechanism of dehalogenation of haloalkanes by the *X. autotrophicus* GJ10 haloalkane dehalogenase (After Verschueren *et al.*, 1993).

The last dehalogenation step is catalyzed by a 2-haloacid dehalogenase. The *X. autotrophicus* GJ10 enzyme has a molecular mass of 28,000 and is relatively insensitive to thiol reagents. The amino acid sequence encoded by *dhl*B gene showed considerable homology with other Type 1 2-haloacid halidohydrolase, the two *Pseudomonas* sp strain CBS3 (Schneider *et al.*, 1991) and the *Pseudomonas cepacia* MBA4 (Murdiyatmo *et al.*, 1992) enzymes.

Although DCE degradation by these bacteria involves very similar enzymes, major differences were found in their expression levels and regulation (van den Wijngaard *et al.*, 1992). The high levels of haloalkane dehalogenase activity found in *Ancylobacter* AD20 (2 to 3-times) and in *Ancylobacter* AD25 (15-20 times) were due to over expression of these enzymes in these strains. Regulation of DCE-degradation pathway in strains AD20 and AD25 takes place at the level of the alcohol and aldehyde dehydrogenases. In strain AD25 the amount of the toxic metabolite, 2-chloroacetaldehyde in the medium is maintained at low levels by repression of the alcohol dehydrogenase during early stages of growth on DCE (Table 1.2). Moreover, the levels of chloroacetaldehyde dehydrogenase detected in strains AD20 and AD25 were at least 10-times higher than the one found in strain GJ10 (Table 1.2). This type of regulation enables these microorganisms to demonstrate high rates of DCE degradation, whilst avoiding the growth inhibitory effects of 2-chloroacetaldehyde (van den Wijngaard *et al.*, 1992).

The four genes of the enzymes involved in the metabolism of 1,2-dichloroethane were isolated on four different DNA segments, indicating that these genes are not closely linked (Janssen *et al.*, 1989). The difficulty in isolating DCE degrading bacteria might not only be due to its toxic effects, but also due to its dependence on the existence of the genetic information necessary to encode the four different enzymes. Also, all of them must be inducible by the halogenated substrates.

Table 1.2: Activities of the enzymes involved in the conversion of DCE to glycolate, of the strains AD20, AD25 and GJ10 grown on DCE (After van den Wijngaard *et al.*, 1992).

Enzyme and substrate	Enzyme activity (mU/mg of protein)		
	AD20	AD25	GJ10
Haloalkane dehalogenase/DCE	541	4293	232
Alcohol dehydrogenase/2-chloroethanol	487	260	480
Aldehyde dehydrogenase/chloroacetaldehyde	1387	1872	109
Chloroacetate dehalogenase/MCA	910	1035	416

Dehalogenation of 2-chloroethanol by *Pseudomonas* sp CE1 (Stucki & Leisinger, 1983) and *Pseudomonas putida* US2 (Strotman *et al.*, 1990) proceeds through 2-chloroacetaldehyde and 2-chloroacetic acid (Figure 1.8), as in the degradation of DCE, although some differences were observed in the enzymes involved in this pathway. Oxidation of 2-chloroethanol in strain US2 is carried out by a phenazine ethosulphate (PES)-dependent dehydrogenase and the 2-haloacid halidohydrolase is induced by 2-chloroethanol and chloroacetic acid to 50 times higher levels of activity than usually found in other 2-haloacid utilizing-bacteria (Stucki & Leisinger, 1983 and Janssen *et al.*, 1985). Although the GJ10 and US2 2-haloacid halidohydrolases have a similar molecular weight (28,000), the US2 enzyme is inhibited by thiol reagents while these compounds had little effect on the GJ10 enzyme, suggesting that they are indeed different enzymes.

1.3.2.3. Halogenated alkanes with more than two carbons

In general, hydrocarbons with 10 to 18 carbons are very easily degraded by bacteria, while a smaller number of microorganisms have been isolated which are able to

degrade hydrocarbons with less than 9 carbons. Furthermore the presence of halogen atoms on these molecules makes them even less susceptible to microbial degradation. Co-metabolism of certain halogenated compounds such as dichlorononane and 6-bromohexanoate has been demonstrated in bacteria that are able to grow on the non-halogenated analogues, n-undecane and benzoate respectively (Omori & Alexander, 1978). In this case it is possible that the capacity to remove halogens from these compounds is not a function of a specific dehalogenase but is the result of catalysis by an enzyme with broad substrate specificity.

Microorganisms capable of utilizing halogenated alkanes with a chain length of C_3 to C_{16} as single carbon and energy source have been isolated (Yokota *et al.*, 1986; Scholtz *et al.*, 1987; Janssen *et al.*, 1987; Sallis *et al.*, 1990). These bacteria produced inducible dehalogenases capable of hydrolytic removal of the halogen from a number of halogenated alkanes, such as mono- and di-halogenated n-alkanes, chloroalkanes with a side chain, 2-chloroalkanes and some haloalcohols.

Although these bacteria have been isolated from geographically separate industrial sites, the haloalkane halidohydrolases of *Rhodococcus sp* m15-3, *Corynebacterium* m2C-32 (Yokota *et al.*, 1986), *Arthrobacter* sp HA1 (Scholtz *et al.*, 1987a) and *Rhodococcus erythropolis* Y2 (Sallis *et al.*, 1990) show a large number of similarities. These enzymes are monomeric proteins with a molecular weight from 34,000 to 36,000 and their N-terminal amino acid sequences showed 100% homology. The halidohydrolases had similar pH optima (pH 9.2 to 9.5), the K_m values towards 1-chlorobutane fell in the same range (0.06-0.26 mM) and, where determined, they have been seen to have similar activation energies (40-42,9 KJ/mol) and isoelectric points (4.5 to 4.7). Although the *Arthrobacter* GJ70 haloalkane halidohydrolase had a similar substrate profile to that of the other enzymes described above, it showed a more acidic pH optimum (pH 8.0-9.0), a higher activation energy (59 KJ/mol) and a significantly lower molecular weight (28,000).

Thiol modifying reagents have different effects on these four halidohydrolase, whereas the GJ70 and Y2 enzymes are sensitive to these reagents, they have little effect on the HA1 enzyme. Optimum temperature of dehalogenation of 1-chlorobutane ranged from 30-37°C for the GJ70, m15-3 and the Y2 halidohydrolases while for the HA1 enzyme the optimum temperature was much higher, 50°C.

Janssen *et al.* (1988) proposed that the *X. autotrophicus* GJ70 halidohydrolase catalyzed the removal either via nucleophilic attack involving a carboxyl residue or via general base reaction (Figure 1.11).

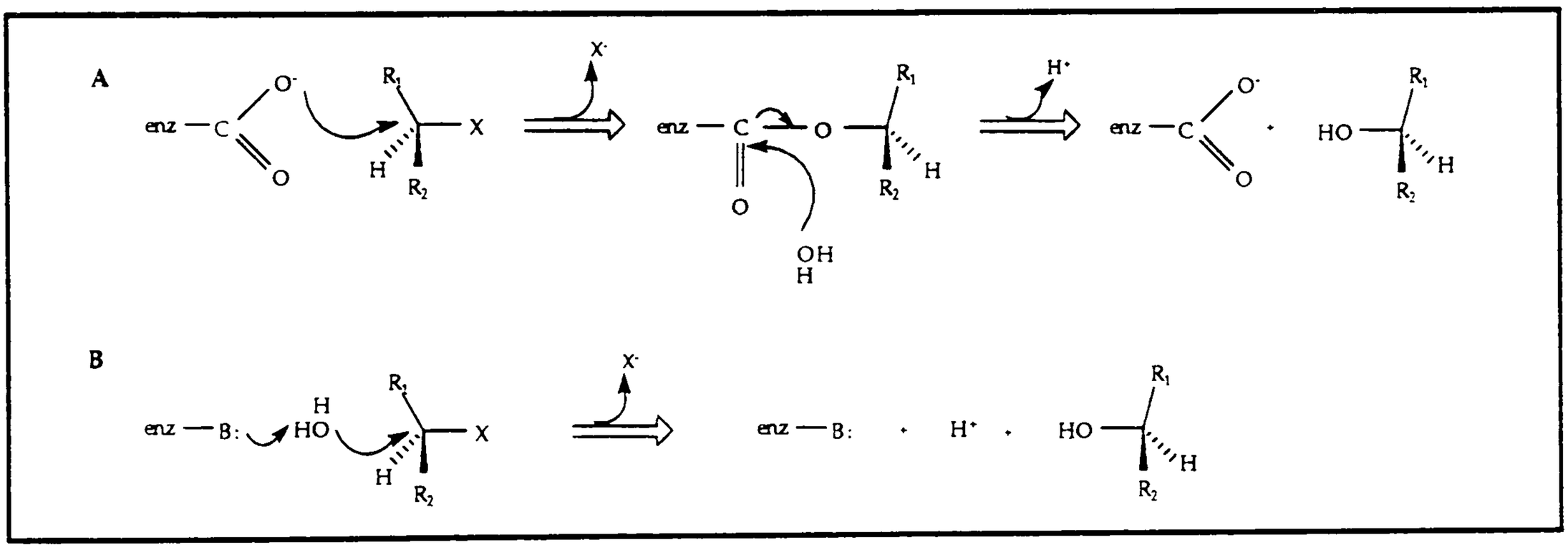


Figure 1.11: Possible mechanisms for the haloalkane dehalogenase; (A) nucleophilic involving a carboxyl residue (B) general base catalysis (After Janssen *et al.*, 1988a). R_1 and R_2 alkyl groups. X, halogen.

These types of dehalogenases are markedly different from the ones isolated from microorganisms that degrade dichloroethanes. The substrate specificity of haloalkane halidohydrolases is very broad. These enzymes are capable of dehalogenating C_1 to C_{16} mono and dihaloalkanes, and some halogenated ethers and alcohols, whereas the haloethane halidohydrolases showed only activity towards short chain haloalkanes (C_1-C_5). Although the molecular weight of both types of dehalogenases was similar (28-37

kDa) it is possible to separate the two groups of enzymes according to the N-terminal amino acid sequence (Figure 1.12).

Haloethane halidohydrolase

Met-Ile-Asn-Ala-Ile-X-Tyr-Pro-Asp-Glx-

Haloalkane halidohydrolase

Ser-Glu-Ile-Gly-Thr-Gly-Phe-Pro-Phe-Asp-Pro-His-Tyr-Val-Glu-Val-Leu-Gly-Glu-Arg-

Figure 1.12: Amino acid sequences of the haloethane and haloalkane halidohydrolase. X, unknown residue.

1.3.3. Biodegradation of halogenated alcohols

Until quite recently there was relative little information about microbial degradation of halogenated alcohols, although several of them are important pollutants. Dehalogenation of haloalcohols by crude extracts of bacteria that possess a haloalkane halidohydrolase has been widely found. However, often these bacteria are unable to use these compounds as carbon and energy sources for growth (Scholtz *et al.*, 1987a; Yokota *et al.*, 1987; Janssen *et al.*, 1988a; Sallis *et al.*, 1990; van den Wijngaard *et al.*, 1992). This type of enzyme dehalogenated haloalcohols with chain length C_2-C_6 and produced the corresponding diols.

Some halogenated alcohols, such as chloroethanol (Stucki & Leisinger, 1983; Strotman *et al.*, 1990) (section 1.2.2.2), 2-chloroallylalcohol (van der Waard *et al.*, 1993) and 3-bromopropanol (Castro & Bartnicki, 1965) (section 1.2.1.2) can serve as growth substrates for bacterial mono-cultures, but dehalogenation takes place only after oxidation of the haloalcohols to the corresponding acids.

Degradation of 2,3-dibromo-1-propanol (2,3-DBP) via epibromohydrin, 3-bromo-1,2-propanediol, glycidol and glycerol (Figure 1.13) was first found in *Flavobacterium* sp (Castro & Bartnicki, 1968). Recently, bacteria that are capable of using 2,3-dichloro-1-propanol (2,3-DCP) (Kasai *et al.*, 1990; Kasai *et al.*, 1992a), 1,3-dichloro-2-propanol (1,3-DCP) (Nakamura *et al.*, 1992), 3-chloro-1,2-propanodiol (CPD) (Suzuki & Kasai, 1991; Suzuki *et al.*, 1992) and epichlorohydrin (ECH) (van den Wijngaard *et al.*, 1989) as a single carbon and energy source have been isolated. Conversion of halopropanols to glycerol is catalyzed by two enzymes, an haloalcohol dehalogenase and an epoxide hydrolase. The first enzyme catalyzes the removal of the halogen and the formation of the epoxide ring, in a second step, the epoxide ring is opened by an epoxide hydrolase and the corresponding alcohol is formed (Figure 1.13). If a second halogen is present the haloalcohol is further metabolized in the same way.

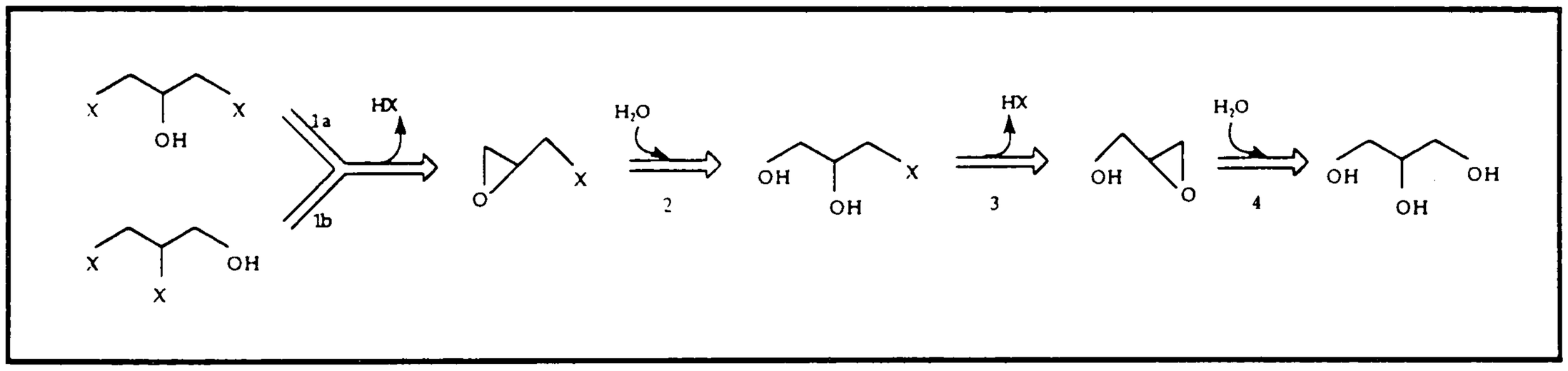


Figure 1.13: Proposed route for the dehalogenation of vicinal haloalcohols. Steps 1(a), 1(b) and 3 are catalyzed by an haloalcohol dehalogenase; steps 2 and 4 are catalyzed by an epoxide hydrolase. (Adapted from Kasai *et al.*, 1990).

Van den Wijngaard *et al.* (1989) isolated three strains able to use epichlorohydrin as a single carbon and energy source. Two of these strains, *Pseudomonas* AD1 and coryneform bacteria AD3, degraded epichlorohydrin to 3-chloro-1,2-propanodiol (CPD) very rapidly. In contrast, the *Arthrobacter* strain AD2 was a slow utilizer of epichlorohydrin. A study of the epoxide hydrolase and dehalogenase activities in crude extracts of strains AD1 and AD2 revealed that epichlorohydrin is converted by strain AD1 via CPD and glycidol by the action of an epoxide hydrolase and a dehalogenase

respectively. The same route of dehalogenation takes place in the strain AD2, which is dependent on the chemical hydrolysis of epichlorohydrin and glycidol rather than via an enzymatic conversion.

The dehalogenation step of 3-chloro-1,2-propanodiol to glycidol in *Arthrobacter* AD2 was catalyzed by a haloalcohol halogen-halide lyase (van den Wijngaard *et al*, 1991). This enzyme converted C_2 and C_3 haloalcohols, with the halogen in the vicinal position to the hydroxy group, to the corresponding epoxides. The dehalogenase was also active towards chloroacetone and 1,3-dichloroacetone. The AD2 haloalcohol dehalogenase was a dimeric protein (65,000), consisting of two monomers of identical molecular weight, 29,000. The enzyme showed a broad substrate optimum around 8.5 and the optimum temperature for activity of 50°C. Thiol reagents partially inhibited the enzyme activity, although no cysteines or cystine residues were found during amino acid analysis. Haloalkanoic acids, such as chloroacetic acid acted as competitive inhibitors.

The AD2 haloalcohol dehalogenase antibody showed immunological cross-reactivity with the AD3 crude extract but not with the AD1 enzymes. Furthermore, the AD2 and AD1 dehalogenases had different substrate profiles indicating that they are completely different enzymes. The enzymatic properties of the *Arthrobacter* AD2 haloalcohol dehalogenase are similar to the ones found for the enzyme I_a of *Corynebacterium* sp N-1074 (Nagasawa *et al.*, 1992) except that the latter enzyme is a tetrameric protein with a molecular weight of 105,000. Nevertheless, the molecular weight of the subunits of both enzymes (28 and 29 kDa) as well as the N-terminal amino acid sequence are very similar (Nagasawa *et al.*, 1992). The haloalcohol dehalogenase I_b of *Corynebacterium* N-1047 is a completely different enzyme, showing no immunological cross-reactivity with the antibody against the I_a enzyme. Furthermore, the enzyme I_b is a 115 kDa protein composed of two different subunits, 35 and 32 kDa. The two enzymes differ mostly in their substrate specificity towards 1,3-DCP, CPD, 1,3-DBP and

bromoethanol. The specific activity of the dehalogenase I_b for 1,3-DCP was about 30-fold higher than that of the enzyme I_a, but it showed very little activity towards CPD; the dehalogenation rates of the brominated compounds are more than 100 times higher than the chlorinated analogues or of the same order if the reaction is catalyzed by the enzyme I_a or I_b, respectively (Nakamura *et al.*, 1992).

The haloalcohol dehalogenase of *Flavobacterium* sp in the presence of a nucleophile was capable of opening the epoxide ring. The qualitative capacity of the enzyme to transfer halide or water to epichlorohydrin or epibromohydrin lies in the following order: $Cl^- > Br^- > OH^-$ (Bartnicki & Castro, 1969). The addition of hydroxide was a very slow process and hence did not account for the rapid degradation of 2,3-DBP by the *Flavobacterium* sp strain. As observed with the haloalcohol dehalogenase of *Flavobacterium* sp, the enzymes of *Arthrobacter* AD2 and *Corynebacterium* N-1074 in the presence of 0.1 M of halide were also capable of transhalogenation but not of hydrolysis of epoxides (van den Wijngaard *et al.*, 1991; Nagasawa *et al.*, 1992). Further studies on the catalytic function of the *Corynebacterium* sp N-1074 haloalcohol dehalogenase I_a revealed that this enzyme was capable of transforming epoxides to the corresponding β-hydroxynitriles in the presence of cyanide (Nakamura *et al.*, 1991a). Nitto-Chem claimed several patents for the preparation of 4-halo-3-hydroxybutyronitrile, a useful intermediate in the synthesis of L-carnitine (vitamin B_T), by converting 1,3-dihalo-2-propanols or epihalohydrin in the presence of alkali cyanide, using either a microorganism or a dehalogenating enzyme (Kasai & Sakaguchi, 1992; Nitto-Chem., 1991a and 1991b).

Bartnicki and Castro (1969) studied the stereochemistry of epoxide formation of the two forms of 3-bromo-2-butanol catalyzed by the *Flavobacterium* sp halohydrin epoxidase. They suggested that the epoxide formation and probably transhalogenation was a stereospecific *trans* elimination of HBr in which a positively (Z) and a negatively (Q) charged group is involved (Figure 1.14).

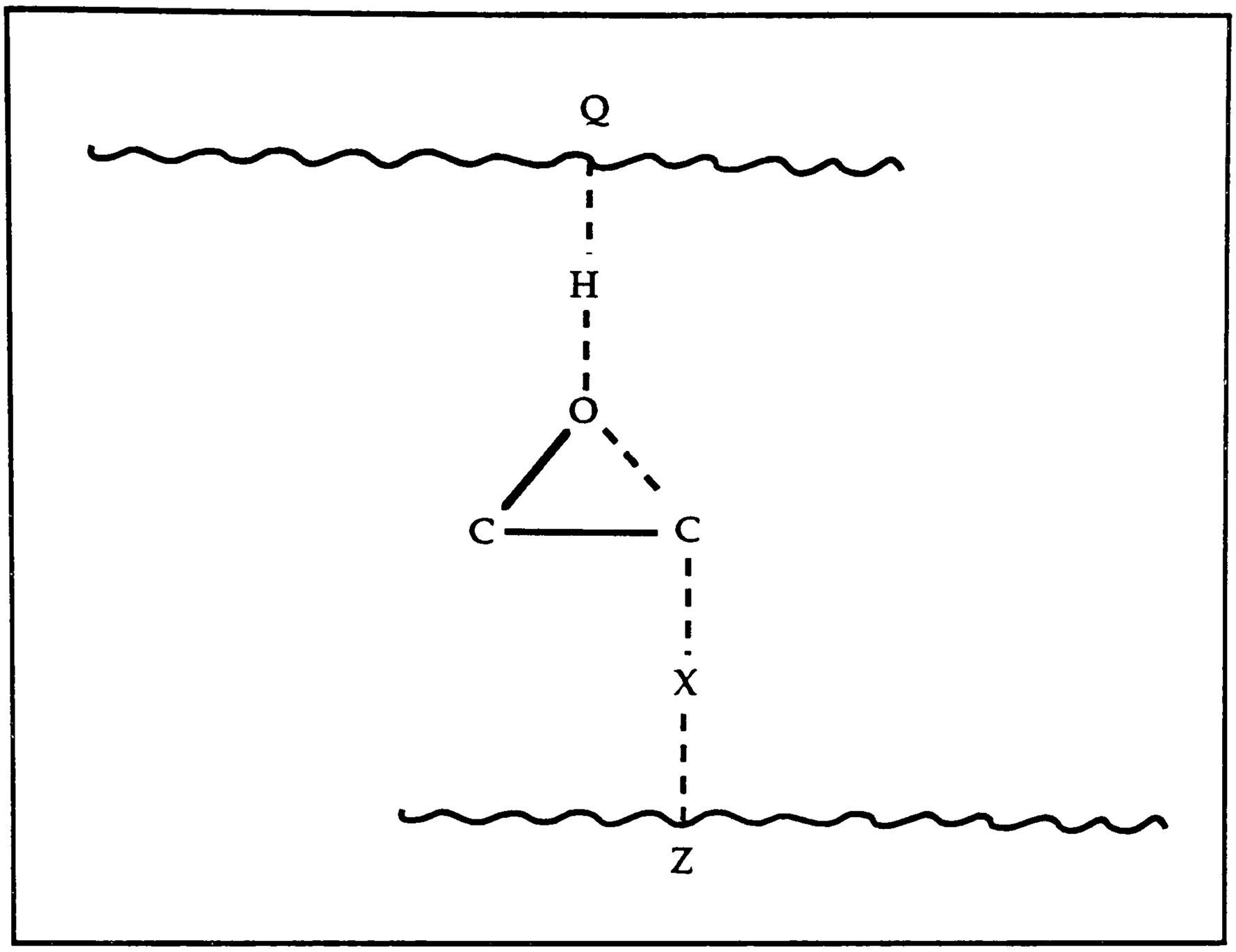


Figure 1.14: Putative dehalogenation mechanism of the halohydrin epoxidase of *Flavobacterium* sp showing a positive and a negative group on the enzyme (After Bartnick & Castro, 1969).

With the aim of producing chiral glycerol derivatives, synthons in the chemical synthesis of pharmacological compounds, several other microorganisms able to stereospecifically transform prochiral and chiral halohydrins and epoxides have been isolated (Kasai *et al.*, 1990; Nakamura *et al.*, 1991b; Suzuki & Kasai, 1991; Kasai *et al.*, 1992a; Suzuki *et al.*, 1992). Although no detailed study exits on the enzymes that transform these compounds, all the strains possessed epoxide hydrolase and haloalcohol dehalogenase activities, suggesting that conversion of the haloalcohols proceeds in a similar mode to that previously described. It is worth noting that the relative activity values obtained from crude extracts of different bacterial strains indicated that the haloalcohol dehalogenases and epoxide hydrolases of these organisms were not identical (Table 1.3). As such we can distinguish three types of dehalogenases, the ones that (i) show low activity towards CPD as the *Flavobacterium* sp (Castro & Bartnicki, 1968) and the enzyme I_b of *Corynebacterium* N-1074 (Nakamura *et al.*,

1992); (ii) dehalogenases that show no activity towards 1,3-DCP as in *Alcaligenes* DS-S-7G (Suzuki *et al.*, 1992); (iii) and the alcohol dehalogenases that show no or very low activity towards 2,3-DCP found in *Pseudomonas* AD1, *Arthrobacter* AD2 (van den Wijngaard *et al.*, 1989), *Corynebacterium* N-1074 (Nagasawa *et al.*, 1992; Nakamura *et al.*, 1992) and *Flavobacterium* sp (Castro & Bartnicki, 1968).

Only two different types of epoxide hydrolase can be distinguished; ones that show no activity towards epibromohydrin as found in *Alcaligenes* DS-S-7G (Suzuki *et al.*, 1992) and ones that show no activity towards non-substituted epoxides, in *Alcaligenes* strains DS-K-S38 (Kasai *et al.*, 1992a) and DS-S-7G (Suzuki *et al.*, 1992).

Table 1.3: Relative activities of haloalcohol dehalogenases and epoxide hydrolases from haloalcohol degrading bacteria. (1) Kasai *et al.*, 1990; (2) Kasai *et al.*, 1992a; (3) Suzuki *et al.*, 1992; (4) van den Wijngaard *et al.*, 1989; (5) Nagasawa *et al.*, 1992; (6) Nakamura *et al.*, 1992; (7) Castro and Bartnick, 1968.

	Haloalcohol dehalogenase						Epoxide hydrolase					
	1,3-DCP	1,3-DBP	2,3-DCP	2,3-DBP	CPD	BPD	ECH	EBH	GDL	PO	BO	REF
Pseudomonas OS-K-29	1.00		0.10	0.19	0.33		15.6	21.7	7.70	7.80	6.0	1
Alcaligenes DS-K-S-38	1.00		0.47	0.56	1.06		2.53	0.25	0.09	0.00		2
Alcaligenes DS-S-7G	0.00		0.36	0.39	1.00	0.73	0.30	0.00	0.39	0.00	0.0	3
Pseudomonas AD1	1.00	17.5	0.00		0.07		1.92	2.98	0.37	10.5		4
Arthrobacter AD2	1.00	134	0.00		0.19		<0.01					4
Corynebacterium N-1074 I_a	1.00	125	0.03		0.38	0.09						5
I_b	1.00	1.7	0.001		0.01							6
Flavobacterium		1.0		0.06	0.00	0.09						7

Other differences found in the relative activities of epoxide hydrolases and alcohol dehalogenases (Table 1.3) may be due to the presence of isoenzymes in single microorganisms. In *Pseudomonas* sp AD1 the hydrolysis of the epoxides is performed by two enzymes, one active towards epichlorohydrin but not towards glycidol, and the other active only towards glycidol (Jacobs *et al.*, 1991). The *Corynebacterium* N-1074 possesses two haloalcohol dehalogenases and two epoxide hydrolases. The haloalcohol dehalogenase I_b showed high activity towards 1,3-DCP but very low activity towards CPD. Although the enzyme activity of dehalogenase I_a towards 1,3-DCP is 30 times lower, it showed higher specificity towards CPD than the enzyme I_b (Nakamura *et al.*, 1992). The existence of more than one enzyme with different affinities towards substrate analogues can be advantageous to the microorganism, as it can enhance the ability of the strains to utilize these compounds as growth substrates, under different environmental conditions.

The epoxide hydrolase responsible for the degradation of epichlorohydrin to CPD in *Pseudomonas* AD1 has been purified and characterized (Jacobs *et al.*, 1991). The enzyme was produced constitutively, however, its production could be further induced by the presence of epichlorohydrin. This hydrolase appears to be a monomeric protein of 35 kDa which catalyzed the conversion of epoxides without requirement of cofactors. The enzyme activity was inhibited by sulphydryl reagents ($HgCl_2$, PMB and iodoacetamide) and the maximum hydrolase activity was obtained at pH 8.4-9.0 and at 50°C. The enzymatic epoxide hydrolysis proceeded by nucleophilic displacement with water at the primary carbon atom of the epoxide ring. Comparison of the amino acid sequence of the N-terminus and some of the CNBr peptides, showed no significant homology between this and other proteins. This enzyme showed no activity towards glycidol and differences in substrate specificity compared to the ones found in AD1 crude extracts, suggested that another epoxide hydrolase is responsible for glycidol conversion in strain AD1 during growth on epichlorohydrin.

1.4. Synthesis of optically pure epoxides and halohydrins

Most commercial drugs with a chiral centre in the molecule are sold as a racemic mixture of enantiomers although the stereoisomers often differ in their pharmacological activities. There is however, an increasing demand for the production of drugs in an enantiomericaly pure form. The preparation of a compound in such a form is often more expensive than the synthesis of the corresponding racemate, since the chemical synthesis route has to employ expensive natural optically active starting materials (synthons) or extra steps are required to resolve a particular building block into the optically active form. Great emphasis has been given to the development of chemical and biochemical methods for the generation of chiral synthons in order to offer the chemical industries an easy and cheap method of producing such chiral compounds.

The use of biocatalysts for organic synthesis takes advantage not only of the mild reaction conditions and the low frequency of side reactions but mostly of the enantioselectivity of certain enzymes . The range of biocatalytic applications in organic synthesis is immense and various (Santaniello *et al.*, 1992). Molecular biological techniques and the continuous discovery of new substrates and enzymes will open doors for the synthesis of a even wider range of chiral molecules.

Epoxides are cyclic ethers, but due to the characteristics imposed by their structure (i.e. the strain inherent to the three member ring, combined with the polarity arising from the electronegativity of the oxygen) they demonstrate considerably higher reactivities than that normally associated with ethers. This makes these compounds versatile intermediates in organic synthesis as they react with a wide range of compounds, including nucleophiles, electrophiles, acids, bases, reducing agents and some oxidizing agents (Figure 1.15). Substituted epoxides that possess an extra reactive site, such as epichlorohydrin and glycidol, have found wide use in the manufacture of polymers and

in organic synthesis of fine chemicals (Klunder *et al.*, 1986; Koosterman *et al.*, 1988; Avignon-Tropis *et al.*, 1991; Cimitiere *et al.*, 1991; Crosby, 1991; Hanson , 1991; Lai *et al.*, 1992; Kasai & Sakaguchi, 1992).

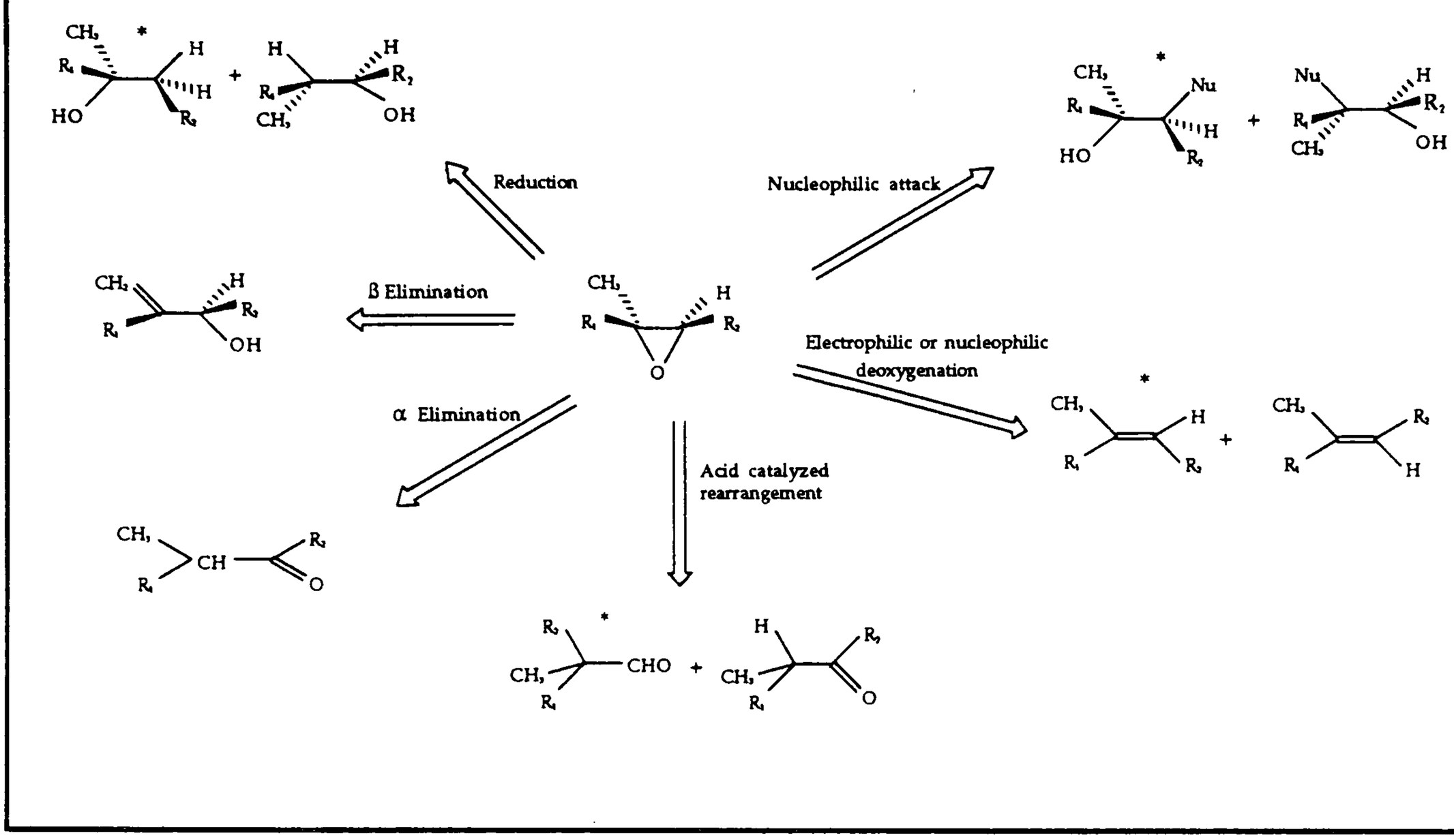


Figure 1.15: Some synthetical useful reactions of epoxides (After Leak *et al.*, 1992). The * indicates major products; R_1 and R_2, alkaly groups.

1.4.1. Chemical synthesis of optically pure epoxides

Racemic mixtures of epoxides are manufactured by direct oxidation of the corresponding alkenes while chemical synthesis of pure enantiomers in general involves numerous steps which are fairly specific for an identified compound isomer. In spite of the availability and number of methods for the synthesis of non-racemic epoxides, most of them are fairly complicated and only result in a low enantiomeric excess (e.e.).

The earliest approach to the synthesis of non-racemic glycidol and epichlorohydrin was based on the cleavage of the diacetonide of D-mannitol (Figure 1.16 A, Baldwing *et al.*, 1978) while S-glycidol was synthesized from L-serine (Lock *et al.*, 1976) (Figure 1.16 B). Although these methods are reliable and produce enantiomers in high enantiomeric excess (97-99%) they rely upon the natural source of chiral compounds (D-mannitol and L-serine).

The less expensive titanium-catalyzed asymmetric epoxidation of allylic alcohols (Katsuki & Sharpless, 1988) has the disadvantage of a lower optical purity of the resultant epoxides (e.e. >80%) than that observed for the above reactions. However, upon the use of one or the other tartrate isomers it is possible to produce both epoxide isomers (Figure 1.16 C). The commercially available enantiomers of glycidol are produced from allyl alcohol by the Sharpless epoxidation method (Hanson & Sharpless, 1986; Gao *et al.*, 1987).

1.4.2. Microbial production of chiral epoxides and halohydrins

Microbial production of epoxides becomes more attractive when it is possible to obtain these compounds in an optically pure form. Two strategies have been employed for such production, (i) stereoselective degradation of one of the isomers in a racemic mixture or (ii) stereoselective formation of one isomer from a racemic mixture of the substrate or from a prochiral molecule.

Epoxyalkanes are intermediates in the metabolism of alkenes and halohydrins. Because epoxides are highly reactive molecules, organisms that form these compounds often have an enzymatic system responsible for its degradation, as such both approaches for chiral synthesis can be considered.

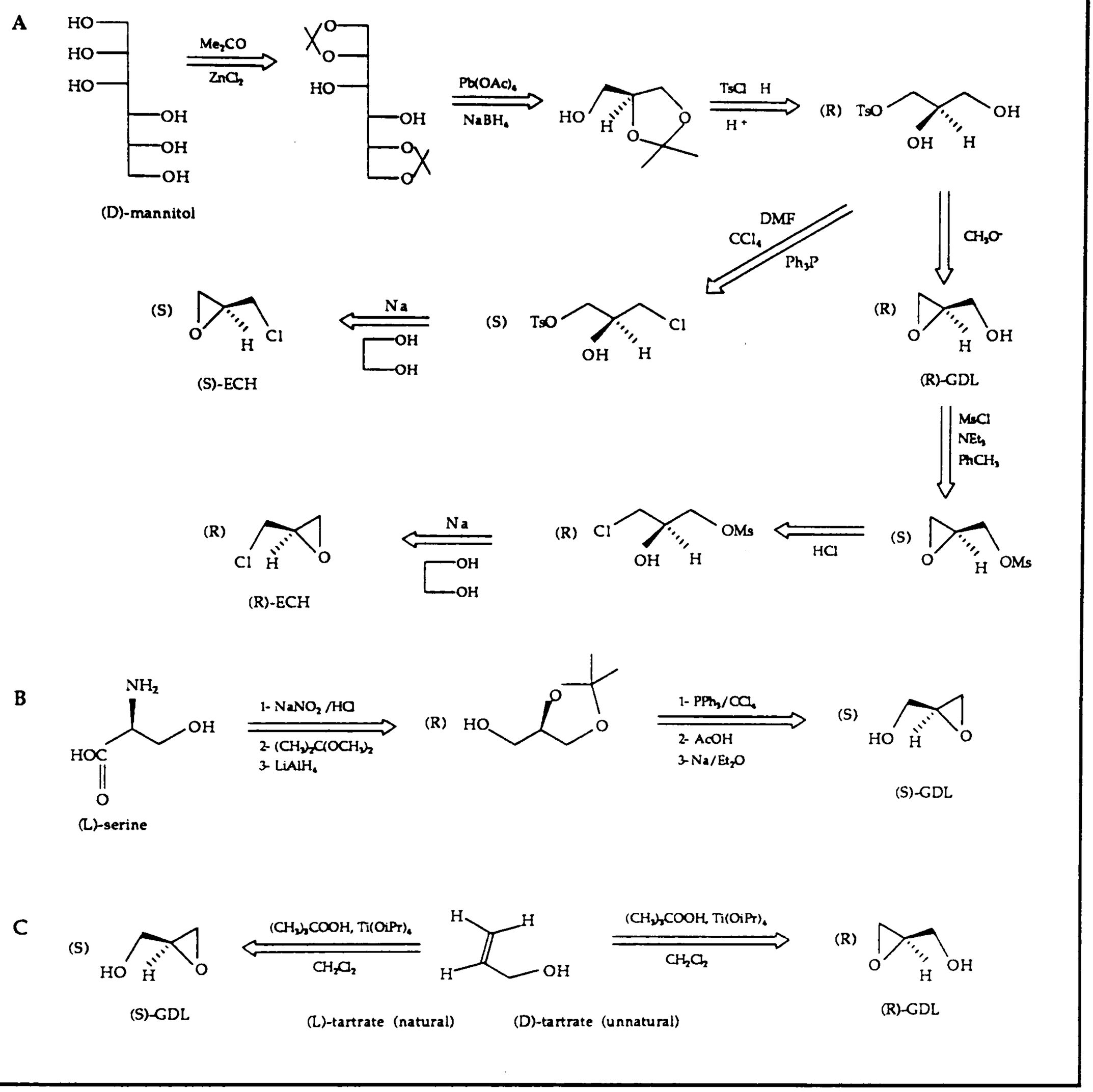


Figure 1.16: Chemical synthesis of optically pure enantiomers of epichlorohydrin and glycidol. From (A) (D)-mannitol; (B), (L)-serine and (C), from both tartrate isomers using the Sharpless epoxidation method.

1.4.2.1. Epoxidation of alkenes

Although ammonia monooxygenase (Hyman & Wood, 1984), methane monooxygenase (Hou *et al.*, 1980; Weijeirs *et al.*, 1988a), alkane monooxygenase

(Hou *et al.*, 1983; Weijeirs *et al.*, 1988a) and alkene monooxygenase (Habets-Crutzen *et al.*, 1985; Habets-Crutzen & de Bont, 1987, Weijeirs *et al.*, 1988a; Mahmoudian & Michael, 1992) were capable of epoxidating alkenes, only the latter produced optical pure epoxides in high enantiomeric excess (Habets-Crutzen *et al.*, 1985; Weijeirs *et al.*, 1988a). Interestingly, ammonia monooxygenase from *Nitrosomonas europea* ATCC 19178, exhibited the opposite stereospecificity compared to all the other strains, although the e.e. values were low.

The alkene-grown cells produced an excess of R-epoxypropane and R-epoxybutane and the S-isomer of 3-chloro-1,2-epoxypropane (epichlorohydrin) (the tetrahedral arrangement of the groups around the carbon 2 of the three epoxides is identical and the denotation of S for epichlorohydrin as compared to the R of the two other epoxides is due only to the priority sequence adopted, Basil *et al.*, 1991). Although the S-epichlorohydrin is formed in high enantiomeric excess (e.e. 99%) the development of a large scale process for its production is not viable because this compound is toxic to the microorganism, and is thus produced only to very low concentrations (Hartmans *et al.*, 1989).

Despite the stereoselectivity of alkene-degrading microorganisms, the existence of a epoxide degrading mechanism represents a draw-back for the use of these bacteria for the production of optically pure epoxides. However, production of epoxyalkanes by exploiting the substrate specificity of the epoxide degrading enzymes (van Ginkel *et al.*, 1987) and use of non-growth substrates, such as 3-chloropropene have been considered. In either case the levels of epoxide production is too low to stimulate industrial interest. Furthermore the instability of the enzyme *in vitro* and the low turnover number of monooxygenase type enzymes, combined with the requirement for co-factor regeneration, makes the use of immobilized enzymes economically impracticable.

1.4.2.2. Stereospecific degradation of epoxides

An alternative approach to the production of optically active epoxides is stereoselective degradation of one of the isomers. Resolution of epoxides in this manner involves the destruction of half of the amount of epoxide. However, it can be an economically feasible method, if the price difference between the racemic mixture and the optically pure isomer is taken into account.

Most of the organisms isolated on aliphatic alkenes or on epoxide alkanes lack chiral selectivity in the degradation of 1,2-epoxyalkanes. However, propene-grown cells of *Norcadia* sp H8 showed some enantioselectivity in the degradation of 1,2-epoxyalkanes, 1,2-epoxybutane and 1-chloro-2,3-epoxypropane, with yields varying between 10 and 19% (Weijeirs & de Bont, 1991). It is interesting that the remaining enantiomer has an optical configuration opposite to the one produced by alkene-oxidizing microorganisms. It seems that this is a complementary and not an alternative process for producing optically active epoxides.

The propene grown *Xanthobacter* sp Py2 contains an alkene monooxygenase that oxidizes 1-alkenes to R-1,2-epoxyalkanes and an epoxide-degrading enzyme(s) that degrades both isomers of 1,2-epoxypropanes but only the 2S,3S- and 2S,3R-isomers of 2,3-epoxybutane, resulting in the accumulation of the 2R-forms of *cis*-2,3-epoxybutane and trans-2,3-epoxybutane (Weijeirs *et al.*, 1988b).

1.4.2.3. Using haloalcohol degrading microorganisms

Halogenated alcohols are cheap materials produced by the petroleum industry, therefore production of chiral glycerol derivatives from these compounds is an attractive option as reflected in the number of patents claims (Table 1.4). Synthetic strategies

using non-racemic mixtures of halohydrins and epoxides using haloalcohol degrading bacteria are based on either the stereospecific assimilation of one of the isomers from a racemic mixture of 2,3-dichloro-1-propanol (2,3-DCP) or 3-chloro-1,2-propanodiol (CPD); on the asymmetric production of optically active CPD by microbial dehalogenation of prochiral 1,3-dichloro-2-propanol (1,3-DCP), or by hydrolysis of epichlorohydrin (ECH) (Table 1.4). Optically pure epoxides, ECH and glycidol (GDL) were produced from the corresponding chiral alcohols by treatment with aqueous NaOH.

Table 1.4: Production of chiral halohydrins and epoxides by using haloalcohol degrading bacteria. R/S-ass, preferential assimilation of the designated isomer; R/S-prod, preferential production of the designated isomer; (1) Kasai *et al* 1990; (2) Osaka-Soda (1989); (3) Osaka-Soda (1990); (4) Kasai *et al*, 1992; (5) Daiso (1991a); (6) Daiso (1991c); (7) Suzuki & Kasai, 1991; (8) Suzuki *et al*, 1992; (9) Daiso (1991b); (10) Nitto-Chem. (1990b); (11) Nakamura *et al*, 1991; (12) Fujio & Wataru (1992a); (13) Nitto-Chem (1990a); (14) Fujio & Wataru (1992b).

Substrate	Method	Catalyst	Product	Yield (%)	%e.e.	2nd reactions	Ref.
2,3-DCP	R-ass	*Pseudomonas* OS-K-29	S-2,3-DCP				1
	R-ass	*Pseudomonas* OS-K-74, OS-K-9, OS-K-76	S-2,3-DCP		99		2
	S-ass	*Pseudomonas* 10520	R-2,3-DCP		99	R-2,3-DCP ——> S-ECH	3
	S-ass	*Alcaligenes* DS-K-S-38	R-2,3-DCP		99.5	R-2,3-DCP ——> S-ECH	4,5
CPD	R-ass	*Alcaligenes* DS-S-7G, DS-S-8S, DS-S-1C	S-CPD	38-44.7	99.4	S-CPD ——> S-GDL	6,7,8
	S-ass	*Pseudomonas* DS-K-9D1, DS-K-2D1, DS-K-14A4	R-CPD		98-99	R-CPD ——> R-GDL	9,7
1,3-DCP	R-prod	*Corynebacterium* *Microbacterium* N-4701	R-CPD	100			10
	R-prod	*Corynebacterium* N-1074	R-CPD	97.3	83.3		11
	S-prod	*Agrobacterium* DH079	S-CPD	100			12
ECH	R-prod	Epihalohydrin hydratase *Corynebacterium* N-2354 *Microbacterium* N-4701	R-CPD	>50	61.3		13
	S-prod	*Agrobacterium* DH079	S-CPD	64			14

Although optical resolution methods have the disadvantage of low yield (less than 50%), the isomers can be obtained in high enantiomeric excess. On the other hand, in spite of the high yields obtained from dehalogenation of 1,3-DCP by *Corynebacterium* sp N-1074 the e.e. of R-CPD (83.3%) was relatively low when considering its use as a chiral synthon (Nakamura *et al.*, 1993). Transformation of 1,3-DCP via ECH into R-CPD by *Corynebacterium* sp N-1074 is catalyzed by four enzymes, two haloalcohol dehalogenases (enzymes I_a and I_b) and two epoxide hydrolases (enzymes II_a and II_b). The enzymes I_a and II_a showed low enantioselectivity for each reaction whereas fractions I_b and II_b exhibited considerable enantioselectivity, yielding R-rich ECH and CPD respectively. Thus the optical purity of R-CPD produced by N-1074 may reflect a conjugated system of at least four enzymes (Nakamura *et al.*, 1992).

1.4.2.4. Other biotransformations

Lipases and esterases are commercially available inexpensive enzymes and as such they have wide application in organic synthesis. The hydrolysis of epoxyesters by lipase constitutes an alternative route to the production of optically active epoxides. However, unless both products are recovered, the maximum theoretical yield is limited to 50%. Andeno-DMS produced R-glycidol and R-glycidyl butyrate from a common substance, by stereoselective hydrolysis of the S-isomer of glycidyl butyrate (Ladner & Whitesides, 1984; Kloosterman *et al.*, 1988).

The most promising method for producing S-1,2-epoxypropane reported to date is the asymmetric reduction of prochiral chloroacetone into chiral 1-chloro-2-propanol by *Rhodotorula glutinis*, which was then chemically converted into the epoxide. The conversion yields obtained were high (96%) with final concentration of S-1,2-epoxyalkanes up to 100 mM with an e.e. of 98% (Weijeirs *et al.*, 1992).

1.5. Aims of the project

The removal of pollutants from the environment has become an important scientific issue. The finding that some of these compounds could be degraded by microorganisms has prompted the search for more efficient biocatalysts with an increased spectrum of substances that can be mineralized. Halogenated aliphatic hydrocarbons constitute an important class of pollutants that can be degraded by a number of different microorganisms. As such, several studies have concentrated on the understanding of the mechanisms by which these organisms have evolved the ability to cleave the carbon-halogen bond as well as on the enzymes involved in the biodehalogenation. These studies have revealed a "new" class of enzymes designated as dehalogenases. Most of the insight on the dehalogenases has arisen from work on haloacid and haloalkane halidohydrolases. Examples of bacteria that degrade halohydrins have only been reported very recently in the literature. Therefore, the study of the mechanisms of biodegradation of these compounds is still in its early stages, nevertheless the data available indicates that the enzymes involved in the catabolism of haloalcohols are different from the others found in haloacid and haloalkane degrading bacteria.

Haloalcohol degrading bacteria have previously been isolated in our laboratory by enrichment in 1,3-dichloro-2-propanol (P.J. Sallis, Bull, A.T. and Hardman, D.J., US Patent). From this bacterial collection, strain H10a was selected for this study due to its ability to degrade haloalcohols very efficiently and because it expressed high levels of dehalogenase activity. The initial aim of this project was to purify and biochemically characterize the haloalcohol dehalogenase(s) produced by this organism in order to shed more light on the regulation and characteristics of such enzymes. Biochemical studies carried out during this project showed that one of the strain H10a dehalogenases dehalogenated haloalcohols stereoselectively. On this basis,

stereospecificity studies were planed in order to evaluate the use of this biocatalyst in the synthesis of valuable chiral epoxides.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial strains

The bacterial strains used in this study are listed in Table 2.1.

Table 2.1: Bacterial strains

Strain	Source
Haloalcohol degrading bacteria	
Arthrobacter sp H10a	Laboratory isolate
Coryneform strain H10f	Laboratory isolate
Agrobacterium biovar one H10e	Laboratory isolate
Agrobacterium tumefaciens HK7	Laboratory isolate
Pseudomonas cepacia H10g	Laboratory isolate
Arthrobacter histidinolovorans HK1	Laboratory isolate
Strain H10c	Laboratory isolate
Haloalkane degrading bacteria	
Arthrobacter sp HA-1	Scholtz *et al.*, 1987
Rhodococcus erythropolis Y2	Sallis *et al.*, 1990
Xanthobacter autotrophicus GJ10	Janssen *et al.*, 1985
Pseudomonas sp E4M	Vienravi, Armfield , Hardman (unpublished)
Haloacid degrading bacteria	
Strain A5-12	Laboratory isolate
Pseudomonas cepacia MBA-4	Tsang *et al.*, 1988
Pseudomonas sp K37	Laboratory isolate
Strain I4	Laboratory isolate
Bacillus sp R1	Laboratory isolate
Strain F1	Laboratory isolate
Strain F10	Laboratory isolate
Pseudomonas putida PP3	Weightman *et al.*, 1982

2.1.2. Chemicals

Halogenated compounds were supplied by Aldrich Chemical Company Ltd. (Gillingham, UK.). The yeast extract was supplied by Oxoid Ltd. (Basingstoke, Hampshire, UK.). All other compounds except those specifically mentioned in the text were either supplied by Sigma Chemical Co. (Poole, Dorset, UK.), BDH Ltd. (Poole, Dorset, UK.) or Fison Plc (Loughborough, UK.).

2.1.3. Other materials

Polaroid films were supplied by Polaroid Corporation (Cambridge, USA). Nitrocellulose membrane was supplied by Scleicher and Schull GmBH (Einbeck, Germany). DEAE-Sephacel and Octyl-Sepharose CL-4B were supplied by Pharmacia Ltd. (Milton Keynes, UK.). Ultrafiltration membranes (PM 30) were supplied by Amicon Corporation (Massachusetts, USA). The gas-chromatography column DB-Wax was supplied by J & W Scientific, Jones Chromatography Ltd. (Hengoed, Mid Glamorgan, UK.). The chiral column FS-cyclodex alpha II/P was supplied by DVK-D. von Keviczki (Langerwehe, Germany). Vials and caps were supplied by Chromacol, BDH, Merck Ltd. (Lutterworth, Leics, UK.).

2.2. Methods

2.2.1. Culture conditions, maintenance and storage

Arthrobacter sp strain H10a was cultured at 30°C in a orbital shaker at 200 rpm, in minimal medium containing (gl^{-1}) Na_2HPO_4,, 2.0 g; KH_2PO_4, 1.4 g; $(NH_4)_2SO_4$, 0.5 g;

$MgSO_4.7H_2O$, 0.2 g; with 20 mg yeast extract and 5 ml of trace elements solution ((gl^{-1}) Na_2EDTA, 12 g; NaOH, 2.0 g; $MgSO_4.7H_2O$, 1.0 g; $ZnSO_4.7H_2O$, 0.4 g; $MnSO_4.7H_2O$, 0.4 g; $CuSO_4.5H_2O$, 0.1 g; H_2SO_4, 0.5 ml; Na_2SO_4, 10.0 g; Na_2MoO_4, 0.1 g; $FeSO_4.7H_2O$), 2 g; 0.36 gl^{-1} of carbon 1,3-dichloro-2-propanol (1,3-DCP) was used as a single carbon source, unless otherwise stated. Large scale bacterial culture was performed on a 12 L (Biotec, LKB) or 30 l (CMF 400 modular fermentor (Chemap) controlled by a CBC control unit (Chemap)) fermentor, with a input of air of 3 L min^{-1} and stirred at a speed of 300 rpm. The pH was maintained at 6.8 by addition of 2 M NaOH.

The other microorganisms (Table 2.1) were grown under the same conditions; 2-monochloropropionic acid and 1-chlorobutane were used as carbon sources for haloacid and haloalkane degrading bacteria respectively. *Xanthobacter autotrophicus* GJ10 was grown with 1,2-dichloroethane as the carbon source. All the strains were stored at -70°C in minimal medium containing 20% of glycerol.

2.2.2. Preparation of the crude extract

Cell free extracts were prepared according to the method described by Hardman and Slater (1981). Late exponential cultures were harvested by centrifugation (11,000xg, 4°C, 20 min) washed and resuspended in 0.02 volumes of ice cold 100 mM TRIS-SO_4, pH 8.0 containing 1.0 mM DTT. Bacterial cells were disrupted by two passages through an Amicon French-pressure cell (SLM Instrument Inc., Urbana I.L.) at 11,500 Psi. For protein purification, the harvested cells were first resuspended in 10 mM TRIS-SO_4, pH 7.0; 1 mM DTT and cell disruption was achieved by passing 10 times through a homogenizer (Microfluidizer 110Y, Microfluidics Corporation) at 25,000 Psi. The

remaining whole cells and cell debris were removed by centrifugation at 48,000xg for 45 min at 4°C. The cell free extracts were stored at -20°C.

2.2.3. Analytical procedures

2.2.3.1. Determination of the protein concentration

Protein was measured by the method of Sedmak and Grossberg (1977) with bovine serum albumin (BSA) used as the standard (0-10 µg of protein). A 0.5 ml aliquot of 0.06% Coomassie Brilliant Blue G (Aldrich, Gillingham, Dorset, UK) in 0.3 M perchloric acid was added to 0.5 ml of protein solution diluted as required. The solution was mixed and the absorbance at 620 nm measured on a Lambda 15 UV spectrophotometer (Perking-Elmer & C., GmBH, Oak Brook, Illinois, USA).

2.2.3.2. Determination of free halide concentration

Dependent upon the concentrations of chloride and bromide in solution the values were determined using either a electrocoulometrically or by a colorimetric method.

For halide concentrations greater than 0.5 mM determinations were carried out using a Marius Chloro-o-Counter (FT Scientific Instruments Ltd., Tewkesbury, UK) as described by Slater *et al.* (1979). The samples were added to 20.0 ml of base solution (9.8% CH_3COOH and 0.2% HNO_3), 1.0 ml of indicator solution (0.6% of gelatin, 0.01% of thymol and 0.01% thymol blue) and the free halide ions were titrated against Ag^+, forming an insoluble precipitate. The appearance of free Ag^+ was detected amperometrically with a silver electrode.

When the halide concentrations were between 0.1-1.0 mM determinations were made according with the method of Bergman and Sanik (1957). Either a 0.02 M solution of KCl or 0.002 M KBr was used as a standard. For each 1.0 ml of halide solution 0.1 ml of reagent A (0.25 M $FeNH_4(SO_4)_2$ in 9 M HNO_3) was added. The mixture was then centrifuged in a microfuge (13,000 rpm 5 min at room temperature) and 1.0 ml aliquot transferred into a 1.5 ml cuvette, mixed with 91 µl of reagent B (95% ethanol saturated with $Hg(SCN)_2$) and the absorbance measured at 460 nm on a Lambda 15 UV spectrophotometer (Perkin-Elmer & C., GmBH).

2.2.3.3. Determination of glucose concentration

Glucose concentration determination was based on the method developed by Bergmeyer *et al.* (1974). The enzyme hexokinase catalyses the phosphorylation of D-glucose to glucose-6-phosphate with the simultaneous conversion of ATP to ADP. Glucose-6-phosphate in then oxidized by $NADP^+$ to gluconate-6-phosphate with the formation of NADPH, in the presence of glucose-6-phosphate dehydrogenase. The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose and the increase in NADPH is measured by means of its absorbance at 340 nm.

The following procedures were carried out according to instructions of the kit manufacturers (Boehringer Mannheim, Lewes, East Sussex, UK.). Samples were incubated at 80°C for 15 min, centrifuged (13,000 rpm, 5 min, room temperature) to remove the cells and precipitate proteins. A 50 µl aliquot was removed and mixed with 1.0 ml of double distilled water and 0.5 ml of buffer coenzyme solution (triethanolamine buffer pH 7.6, 3.3 mM $NADP^+$, 11 mM ATP, $MgSO_4$ and stabilizers). Three minutes later, the absorbance at 340 nm was determined and the reaction was started by the

addition of 0.1 ml of suspension 2 (290 U/ml of hexokinase and 145 U/ml of glucose-6-phosphate dehydrogenase). The mixture was incubated 15 min at room temperature and the absorbance at 340 nm again determined. Glucose concentration was determined using the absorption coefficient of NADPH at 340 nm (6.3 l (mol cm)$^{-1}$).

2.2.3.4. Determination of glycerol concentration

Glycerol concentrations were determined using the 3 step enzymatic assay developed by Eggstein and Kuhlmann (1974). Glycerol is phosphorylated by ATP to L-glycerol-3-phosphate in a reaction catalyzed by glycerol kinase. The ADP formed is recovered by conversion of PEP to pyruvate with the aid of pyruvate kinase. In the presence of lactate dehydrogenase, pyruvate is reduced to L-lactate with the oxidation of NADH to NAD. The amount of NADH oxidized is stoichiometric to the amount of glycerol, NADH concentration is then determined by means of its absorption at 340 nm.

The following assay procedures were carried out as recommended by the kit manufacter (Boehringer Mannheim, Lewes, East Sussex, UK.). The culture samples were incubated at 80°C for 15 min, centrifuged (13,000 rpm, 5 min, room temperature) and a 50 µl aliquot mixed with 1.0 ml double distilled water, 0.5 ml of coenzyme buffer solution (glyglycine buffer pH 7.4, 1 mM NADH, 4 mM ATP, 6 mM PEP, $MgSO_4$ and stabilizers) and 50 µl of suspension 1 (600 U/ml of pyruvate kinase, 550 U/ml lactate dehydrogenase). After 5 min the absorbance at 340 nm was determined. The reaction was started adding 50 µl of suspension 3 (85 U/ml of glycerol kinase). The solution was well mixed and left at room temperature for 10 min. Absorbances at 340 nm of the blank and the samples were read immediately one after the other. Glycerol concentrations were determined by multiplying the absorbance difference measured by the absorption coefficient for NADH at 340 nm (6.3 l (mol cm)$^{-1}$).

2.2.3.5. Determination of 1,3-dichloro-2-propanol and 3-chloro-1,2-propanediol concentration

Samples (0.5 ml) were extracted with 1.0 ml of ethyl acetate and 0.5 ml fractions were collected for analysis on the GC-MS. A 1.0 µl sample was injected by an automatic injector (Model 7673 Hewlett Packard, Workingham, Berks, UK) into a gas-liquid chromatograph (Model 5890 series II, Hewlett Packard, Avondale, USA) with a mass selective detector (Model 5971A, Hewlett Packard) fitted with a 30 m x 0.25 mm capillary glass column (DB-Wax) coated with polyethylene glycol as a non-polar stationary phase. The operating conditions were: helium carrier gas with a flow of 0.500 ml min^{-1}; injection temperature 180°C; with temperature program set to give an initial column temperature of 40°C for 2 min, followed by a temperature gradient of 40-240°C (25°C min^{-1}) and a 4 min holding time at 240°C.

The peaks were identified by the mass spectrum with reference to standards. For routine analyses the mass detector was set to detect only the major ions of each of the compounds (Table 2.2). Calibration curves of 0 to 10 mM of 1,3-DCP and CPD were made by extracting 0.5 ml samples of known concentrations of the chemicals as described above.

2.2.3.6. Determination of epichlorohydrin, epibromohydrin, 1,3-dichloro-2-propanol and 1,3-dibromo-2-propanol concentrations

1 µl samples were prepared and injected onto a gas liquid chromatograph column as described above (section 2.2.3.5). In this instance the temperature programme was set to give: (1) hold at 40°C for 7 min; (2) a temperature gradient of 40-240°C (50°C min^{-1})

and (3) hold at 240°C for 4 min ECH, EBH, DCP and DBP were used as a reference standard and the mass spectrum was selected to detect their major ions (Table 2.2). A calibration curve of 0 to 10 mM was made by extracting of 0.5 ml solutions of known concentrations of the compounds in 100 mM TRIS-SO_4, pH 8.0 as described above (section 2.2.3.5).

Table 2.2: Major ions of halohydrins and epoxides used on the mass selective detector

Compound	Major ion	Other ions
1,3-DCP	79	49
1,3-DBP	125	93
CPD	62	64
ECH	57	62, 49
EBH	57	93

2.2.3.7. Chiral analysis of epichlorohydrin and epibromohydrin

The reaction samples (5.0 ml) were extracted twice with an equal volume of ethyl acetate. then concentrated 10 times by evaporating the solvent and dried with anhydrous sodium sulphate. An aliquot (0.1 to 0.5 µl) was injected (split 1:50) into a gas-chromatograph (AMS, model 93, Pampisford, Cambridge, UK) with a FID detector (AMS) fitted with a 50 m x 0.32 mm FS-Cyclodex alpha II/P column. The operating conditions were: isotherm at 40°C, carrier gas, helium, pressure 11 Psi; injector and detector temperature at 200°C.

2.2.4. Induction studies of the haloalcohol dehalogenase

Induction studies of haloalcohol dehalogenase activities were performed in minimal medium using 1,3-DCP, epichlorohydrin (ECH), 3-chloro-1,2-propanediol (CPD), glycidol (GDL) and glycerol, and glucose as carbon sources. The *Arthrobacter* sp strain H10a was cultivated on minimal medium (section 2.2.1) with one of the above compounds or in combination thereof as the carbon sources, at 30°C in a orbital shaker at 200 rpm. The growth was monitored by measuring the optical density at 600 nm (OD_{600}) and the substrate consumption and chloride release determined as described in analytical procedures sections 2.2.3. At late exponential phase of growth, the cultures were centrifuged and cell free extracts (CFEs) produced (section 2.2.2).

Induction of the haloalcohol dehalogenase activity by glycidol was also tested in the presence of a protein synthesis inhibitor, chloramphenicol. The *Arthrobacter* sp strain H10a was grown to mid exponential phase on minimal medium (section 2.2.1) with 10 mM 1,3-DCP as a single C-source (12 h). At this stage chloramphenicol was added to a final concentration of 50 µg ml^{-1}. Three hours later glycidol was added (5 mM) and the cells were further incubated for a period of 7 h. The bacteria were then harvested and a cell free extract produced (section 2.2.2).

2.2.5. Purification of the haloalcohol dehalogenase

The crude extract was fractionated by stepwise addition of ammonium sulphate and the 45-80% precipitate collected. The precipitate was dissolved in 30 ml 10 mM TRIS-SO_4, pH 7.0, 1 mM DTT and dialyzed over night against the same buffer.

The dialyzed preparation was applied to a DEAE-Sephacel (Pharmacia) column (2.6 by 30 cm) that was previously equilibrated with 10 mM TRIS-SO_4, pH 7.0; 1 mM DTT. The column was washed with 500 ml of this buffer and the protein subsequently eluted with a 600 ml linear gradient of 0-700 mM $(NH_4)_2SO_4$. The active fractions were pooled, concentrated by ultrafiltration (Amicon PM-30 membrane) under nitrogen pressure and dialyzed against 1 l of 50 mM TRIS-SO_4 pH 7.0; 1 mM DTT; 1.17 M $(NH_4)_2SO_4$. The dialyzed preparation was then applied to an Octyl-Sepharose CL-4B (Pharmacia) column (2 by 15 cm) equilibrated with the same buffer. The protein was then eluted with 150 ml negative gradient of 1.17-0 M $(NH_4)_2SO_4$. The fractions containing dehalogenase activity were pooled and concentrated by ultrafiltration (Amicon PM-30 membrane) under nitrogen pressure to a final volume of 19 ml.

The haloalcohol dehalogenase used for immobilization and production of chiral epoxides was first fractionated on DEAE-Sephacel (5.0 by 42 cm) and Octyl-Sepharose CL-4B (3.0 by 38 cm) columns as described above. The eluate was dialyzed against 100 mM TRIS-SO_4 pH 8.0; 1 mM DTT and stored at -20°C.

2.2.5.1. Determination of haloalcohol dehalogenase molecular weight

The native molecular weight of the haloalcohol dehalogenase was determined by gel filtration chromatography using an FPLC system (Pharmacia, Milton Keynes, UK.) with a Superose 12 column equilibrated with 50 mM TRIS-SO_4 pH 8.5. The haloalcohol dehalogenase (50 µg in 50 µl) was applied to the column and eluted with the same buffer (flow rate of 0.5 ml min^{-1}). The column was calibrated with blue dextran, 2,000 kDa; ß-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; bovine carbonic anhydrase, 29 kDa; and horse heart cytochrome C, 12.4 kDa (Sigma).

2.2.6. Enzymatic assays

2.2.6.1. Dehalogenase assay

Dehalogenase assays were based on the method described by Weightman and Slater (1980). Dehalogenase activity was determined at 30°C in 100 mM TRIS-SO_4, pH 8.0 containing 1µl ml^{-1} of substrate. A suitable amount of enzyme solution was added and 0.5-1.0 ml aliquots were removed for determination of halide ions concentration (see section 2.2.3.2). One unit of enzyme activity was defined as the activity that catalyzed the formation of 1 µmol of halide per minute per mg of protein under the conditions used.

2.2.6.2. Epoxide hydrolase assay

Epoxide hydrolase activity towards epichlorohydrin (0.5 µl ml^{-1}) was assayed in 100 mM TRIS-SO_4, pH 8.0 at 30°C. At 4 min intervals 0.5 ml aliquots were removed and epichlorohydrin extracted with 1.0 ml of ethyl acetate. The organic phase (0.70 ml) was removed and loaded into glass vials to be analyzed by gas chromatography (section 2.2.3.6). One unit of enzyme activity was defined as the activity that catalyzed the consumption of 1 µmol of epichlorohydrin per minute per mg of protein under the conditions used.

2.2.6.3. Determination of the optimum temperature for the dehalogenase activity

The haloalcohol dehalogenase activity towards 1,3-DCP at different temperatures was investigated. The assay mixtures were incubated for 10 min at the desired temperature

to allow temperature equilibration. The reaction was performed as described above (section 2.2.6.1). The stability of the substrate at different temperatures was also determined.

2.2.6.4. Determination of the optimum pH for the dehalogenase activity

The pH dependence of the haloalcohol dehalogenase was determined in a reaction mixture containing 200 mM TRIS, 200 mM glycine and 200 mM sodium acetate or in 200 mM CAPS (3-cyclohexylamino-1-propanosulphonic acid) adjusted to the required pH by addition of concentrated NaOH or glacial acetic acid. The stability of 1,3-DCP at various pH values was determined.

2.2.6.5. Kinetic studies

Kinetics constants were determined by measuring the initial velocity of halide production from different substrate (1,3-DCP and CPD) concentrations. K_m and v_{max} values were calculated from the Linweaver-Burk plots.

2.2.6.6. Effect of ions and chelating agents on dehalogenase activity

The effect of cations (Ca^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+} and K^{+}) and metal chelators (EDTA and 1,10-phenanthroline) on the activity of the haloalcohol dehalogenase was determined by measuring the enzyme activity towards 1,3-DCP as described above (section 2.2.6.1) in the presence of these putative inhibitors of dehalogenase activity.

2.2.6.7. Reaction mixtures used for the production of chiral epoxides

The reaction mixtures, set as described in Table 2.3, contained 200 mM TRIS/glycine/acetate buffer, 10 mM of substrate and 0.175 U/ml of partially purified haloalcohol dehalogenase (section 2.2.5). At time intervals 0.5 ml and 5.0 ml samples were removed for quantitative analysis of ECH, EBH, 1,3-DCP and 1,3-DBP (section 2.2.3.6), and chiral analysis of the epoxides (section 2.2.3.7), respectively.

Table 2.3: Reaction mixtures

Reaction	Buffer pH	Substrate	Salt
1	7.5	DCP	
2	8.0	DCP	
3	8.5	DCP	
4	8.0	ECH	10 mM KCl
5	8.0	ECH	50 mM KCl
6	8.0	ECH	200 mM KCl
7	8.0	ECH	50 mM KBr
8	8.0	ECH	200 mM KBr
9	8.0	EBH	50 mM KBr
10	8.0	EBH	200 mM KBr
11	8.0	DCP	200 mM KBr

2.2.7. Chemical modification of amino acid side chain

The selection of the reagents for chemical modification of amino acid side chains in the *Arthrobacter* sp H10a dehalogenase protein was based on the specificity towards a designated amino acid and the reaction conditions. For each amino acid the reagents were selected according to differences in mode of reaction and where appropriate, size and hydrophobicity of the molecule.

Unless otherwise stated, after chemical modification the remaining activity of the haloalcohol dehalogenase towards 1,3-DCP was assayed according to the method described above (section 2.2.6.1). When chemical modification resulted in loss of dehalogenase activity, the modification reactions were repeated in the presence of an excess of 1,3-DCP or 2-chloroethanol.

2.2.7.1. Chemical modification of cysteine residues

2.2.7.1.1. With p-chloromercuric benzoate

Chemical modification of sulphydryl groups by p-chloromercuric benzoate (PMB) was performed according to the method described by Riordan and Valle (1972). A 1.0 mM stock solution of PMB was made by dissolving 8.93 mg of PMB in a minimal volume of 2 M NaOH and diluting to 25.0 ml with double distilled water. Addition of NaOH to PMB the chlorine being substituted by an hydroxy group, a derivative which is more water soluble. The purified haloalcohol dehalogenase (25 µg) was incubated in 0.1 mM PMB in 100 mM TRIS-SO_4, pH 8.0 for 10 min at 30°C.

2.2.7.1.2. With iodoacetamide

Chemical modification of -SH groups with iodoacetamide was achieved using the method described by Gurd (1972). 25 µg of the purified haloalcohol dehalogenase was incubated with iodoacetamide to a final concentration of 2 mM, in 60 mM CAPS (3-cyclohexylamino-1-propanosulphonic acid) pH 8.5. The mixture was incubated at 30°C for 20 min.

2.2.7.1.3. With N-ethylmaleimide

The procedure used for the modification of cysteine with N-ethylmaleimide (NEM) was described by Riordan and Valea (1972). The purified haloalcohol dehalogenase (25 µg) was incubated in 100 mM TRIS-SO_4, pH 8.0 with NEM at a final concentration of 2 mM, for 10 min at 30°C.

2.2.7.2. Chemical modification of histidine residues

Modification of histidine residues with diethylpyrocarbonate (DEP) was achieved according to the method described by Meyer and Cromartie (1980). A 10 mM stock solution of DEP in 30% ethanol was prepared. The purified haloalcohol dehalogenase was dialyzed against 100 mM borate buffer, pH 8.0; 50 µl aliquots were removed and incubated with 2 mM of DEP at 25°C for 20 min.

2.2.7.3. Chemical modification of arginine residues

2.2.7.3.1 With phenylglyoxal

Arginine modification with phenylglyoxal was carried out according to the method described by Takahashi (1968). A concentrated solution of phenylglyoxal, 50 mM in 40% ethanol was prepared. 45 µg of the purified haloalcohol dehalogenase in 40 mM borate buffer, pH 7.0, was incubated with phenylglyoxal to a final concentration of 5.0 mM, at 35°C for 30 min. The remaining dehalogenase activity towards 1,3-DCP was tested in 100 mM borate buffer and in the presence of 5 mM phenylglyoxal.

2.2.7.3.2. With 2,3-butadione

Modification of arginine with 2,3-butadione was based on the method described by Yankeelov (1972). A concentrated solution of 100 mM 2,3-butadione was produced. The reaction was carried out in 100 mM borate buffer, pH 8.0, 10 mM 2,3-butadione with 45 µg of purified haloalcohol dehalogenase in 40 mM borate buffer pH 7.0. The mixture was incubated at 35°C for 30 min. The remaining dehalogenase activity towards 1,3-DCP was tested in 100 mM borate buffer pH 8.0 and 10 mM 2,3-butadione.

2.2.7.4. Chemical modification of lysine residues

2.2.7.4.1. With ethyl acetamide

Lysine residue modification with ethyl acetamide hydrochloride was made according to the method described by Sekiguchi *et al.* (1979). The purified haloalcohol dehalogenase (25 µg) in 40 mM borate buffer, pH 7.0, was incubated with 10 mM ethyl acetamidate in 100 mM borate buffer pH 9.0, at 35°C for 30 min.

2.2.7.4.2. With pyridoxal-5'-phosphate

Chemical modification of lysine residues with pyridoxal-5'-phosphate was performed as described by Rippa *et al.* (1967). The purified haloalcohol dehalogenase (25 µg) was incubated with 10 mM pyridoxal-5'-phosphate in 100 mM borate buffer, pH 8.0, at 28°C for 30 min. Sodium borohydride was added to the final concentration of 1.0 mM and the reaction mixture was further incubated at 0°C for 15 min. The effect of $NaBH_4$ on the dehalogenase activity towards 1,3-DCP was also investigated.

2.2.7.4.3. With formaldehyde

Modification of lysine residues with formaldehyde and sodium borohydride was performed as described by Means and Feeney (1968). 25 µg of the purified haloalcohol dehalogenase was incubated with 4 mM formaldehyde in 0.25 M borate buffer pH 9.0, for 40 min at 0°C. Sodium borohydride was added to a final concentration of 1.0 mM and the mixture was further incubated at 0°C for 20 min. The effect of each of the chemicals on the dehalogenase activity towards 1,3-DCP was separately studied.

2.2.7.5. Chemical modification of glutamic and aspartic acid residues

2.2.7.5.1. With CMC

Modification of carboxylic acids of the H10a haloalcohol dehalogenase with CMC (1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide) was performed as described by Banks *et al.* (1969). A 0.1 M CMC stock solution in 0.1 M KCl was prepared. The pure haloalcohol dehalogenase (20 µg) was mixed with CMC to a final concentration of 10 mM in 70 mM TRIS-SO_4, pH 8.0 and 10 mM KCl. The mixture was incubated at 25°C for 20 min. The effect of KCl on the dehalogenase activity towards 1,3-DCP was also investigated.

2.2.7.5.2. With DCCD

Carboxylic acid residues were modified with DCCD (dicyclocarbodiimide) as described by Arana and Valejos (1981). A 10 mM concentrated solution of DCCD in ethanol was

prepared. 20 µg of the purified haloalcohol dehalogenase in 40 mM MOPS (3-[morpholino]propanesulphonic acid) was mixed with DCCD to a final concentration of 160 µM. The solution was incubated at 30°C for 60 min. The effect of ethanol on the dehalogenase activity was also determined.

2.2.7.5.3. With Woodward's reagent

Modification of carboxylic amino acid residues with the Woodward's reagent (N-ethyl-5-phenylisoxazolium-3'-sulfonate) (Arana and Vallejos, 1981) was achieved in 40 mM TRICINE (N-tris[hydroxymethyl]methylglycine) pH 7.9. The pure haloalcohol dehalogenase (20 µg) was incubated with 1 mM Woodward's reagent for 20 min at 25°C.

2.2.8. Polyacrylamide gel electrophoresis

2.2.8.1. Dehalogenase activity stain polyacrylamide gel electrophoresis (Activity stain-PAGE)

Activity-stain polyacrylamide gels were used to visualize dehalogenases amongst other proteins in cell free extracts (Weightman & Slater, 1980). A discontinuous slab gel with a 5% polyacrylamide stacking gel and an 8 or 10% polyacrylamide resolving gel was used. The final concentrations in the resolving gel were 0.375 M TRIS-SO_4, pH 8.5 and the gels were polymerized by the addition of 0.05% (w v^{-1}) ammonium persulphate (APS) and $7.5x10^{-4}$% (v v^{-1}) TEMED. The stacking gels contained 125 mM TRIS-SO_4, pH 6.9 and were polymerized as above.

The slab gels were pre-run at 100 V on a Mighty Small™ II (7 cm vertical slab unit, Hoefer, Scientific Instruments, California, USA) gel-tank for 1 h to remove unpolymerized acrylamide TEMED and APS which have previously been shown to inhibit the activity of some dehalogenases (Hardman & Slater, 1981). The running buffer contained 0.2 M glycine and 25 mM TRIS, pH 8.5. The samples were prepared by mixing the enzyme preparation with 0.25 volumes of loading buffer (25% (v v^{-1}) glycerol, 0.05% bromophenol blue, 0.02 M TRIS-SO_4, pH 7.9), loaded into preformed wells with a Hamilton syringe and electrophoresed at 100 V at 4°C until the marker dye reach the bottom of the gel (ca. 2-3h).

The gels were incubated in 50 ml of 100 mM TRIS-SO_4, pH 8.0, 10 mM 1,3-DCP (or 50 mM of MCA and DCA or 1,2-dibromoethane for haloacid or haloalkane dehalogenases respectively) at 30°C for 30-60 min. After washing twice with distilled water, the gels were overlayed with 0.1M $AgNO_3$. The free halide ions in the gel resulting from dehalogenation of the substrates formed a white AgCl precipitate, thereby locating the dehalogenases in the gel. The gels were washed as above, incubated for 5 min in 5% (v v^{-1}) acetic acid and thoroughly washed again. The AgCl precipitate became dark brown when exposed to UV light and pictures of the gels were taken by using Polaroid MP4 (Polaroid Corporation, Cambridge, Massacchusetts, USA) camera with Polaroid type 665 black and white film.

2.2.8.2. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels (Laemmli, 1970) used 5% polyacrylamide stacking gels and either 12.5% or 15% polyacrylamide resolving gels depending on the size of the proteins to be studied. The final concentrations in the resolving gel were 0.375 M TRIS-SO_4, pH 8.5, 0.1% SDS and the gels were polymerized by addition of 0.05% (w v^{-1}) APS and

7.5x10^{-4}% (v v^{-1}) TEMED. Stacking gels contained 0.125 M TRIS-SO_4, pH 6.9, 0.1% (w v^{-1}) SDS and were polymerized as for the resolving gels. The running buffer used was 25 mM TRIS, 0.2 M glycine and 0.1% SDS.

The protein samples were mixed with 0.2 volumes loading buffer (125 mM TRIS-SO_4, pH 6.8, 20% (v v^{-1}) glycerol, 10% ß-mercaptoethanol, 0.4% (w v^{-1}) SDS, 0.015% (w v^{-1}) bromophenol blue) and placed in boiling water for 2 min. The samples were loaded into preformed wells and the gel electrophoresed at 200 V in a Mighty Small™ II gel-tank, until the dye marker reach the bottom of the gel (ca. 1-2 h).Bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa) (Dalton mark VII-L™, Sigma, Poole, Dorset, UK.) were used as marker proteins. Photographs of the gels were taken using Polaroid MP4 camera with Polaroid type 665 black and white film.

2.2.8.3. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The first dimension was carried out under non-denaturing conditions (section 2.2.8.1) using a 14 x 16 x 0.15 cm slab gel on a Model 16 vertical gel electrophoresis (BRL). The purified haloalcohol dehalogenase (20 µg) was loaded into preformed wells and the gel was electrophoresed at 30 mA until the dye marker reach the bottom of the gel. The lanes were sliced and one stained with Coomassie Blue (section 2.2.8.6.1) and the second gel slice incubated in 40 ml 5 times diluted gel loading buffer (section 2.2.8.2) for 40 min with gentle agitation.

Molecular weight standards (Dalton Mark VII-L™, Sigma, Poole, Dorset, UK.) and 10 µg of the purified haloalcohol dehalogenase were electrophoresed using a 8%

polyacrylamide SDS-PAGE (section 2.2.8.2) until the marker dye was 1 cm from the top of the gel. The gel slices were cut, incubated in the gel loading buffer, as described above, positioned together with the second gel slice on the bottom of the glass plate and squashed between the two glass plates to remove all air bubbles. The stacking gel was prepared (8% polyacrylamide, 0.125 M TRIS-SO_4, pH 6.9, 0.1% (w v^{-1}) SDS, 0.1% (w v^{-1}) APS, 1.5 x 10^{-4}% TEMED) and poured between the glass plates to cover the gel and 1 cm above it. A 12.5% polyacrylamide gel (section 2.2.7.2) was cast and the gel was electrophoresed at 25 V for 4 h and then at 75 V until the marker dye reach the top of the gel.

Alternatively, the protein bands of the first gel slice were cut, smashed with a pipette tip and incubated over night with gel loading buffer (section 2.2.8.2). The samples were boiled for 2 min and 10 µl loaded onto a 12.5% SDS-PAGE (section 2.2.8.2). The gel was then silver stained (section 2.2.8.6.2).

2.2.8.4. Determination of native haloalcohol dehalogenase molecular weight by nondenaturating gel electrophoresis

Determination of the molecular weight (MW) of proteins by gel electrophoresis is based on the protein mobilities (R_f value) in gels with different polyacrylamide concentrations (Hendrick & Smith, 1968). A semilogarithimic plot of the R_f versus the polyacrylamide concentration provides a line with a slope characteristic of a protein with a specific molecular weight, the retardation coefficient (K_R). A linear relationship between the MW and the K_R value is obtained from a plot of log(-K_R) versus log MW.

MW determination of the multiple bands formed by the H10a haloalcohol dehalogenase on nondenaturating polyacrylamide gels (section 2.2.8.1) was performed according to

instructions of the "Nondenatured protein molecular weight marker kit" (Sigma, Poole Dorset, UK.). A set of running gels of different polyacrylamide concentrations were prepared according to Table 2.4. A 2.5% acrylamide, 0.625% bisacrylamide stacking gel was prepared. The final concentrations were 62.5 mM TRIS-SO_4, pH 6.7, 1.058 (v v^{-1}) TEMED, 2.5% (w v^{-1}) sucrose and the gels were polymerized by addition of $5x10^{-4}$% (w v^{-1}) riboflavin. The running buffer used contained 5 mM TRIS, 38 mM glycine. The protein standards and samples were mixed with the same volume of gel loading buffer (0.167 M TRIS-SO_4, pH 6.7, 33% glycerol, 0.83% bromophenol blue) and loaded into preformed wells. The gels were electrophoresed at 100 V in a Mighty Small™ II gel-tank. Bovine serum albumin 66 kDa monomer and 132 kDa dimer, chicken egg albumin 45 kDa, carbonic anhydrase 29 kDa and bovine milk α-lactalbumin 14.2 kDa (MW-ND-500 kit, Sigma) were used as protein standards. The gels were stained with Coomassie Blue (section 2.2.8.6.1) and the pictures were taken by using Polaroid MP4 camera with Polaroid type 665 black and white film.

Table 2.4: Composition of the nodenaturating polyacrylamide gels.

	% Polyacrylamide gel concentration							
	7.0		8.0		9.0		10.0	
	Volume	Final conc.	Volume	Final conc.	Volume	Final conc.	Volume	Final conc.
3 M TRIS-SO_4, pH 8.9	1.5 ml	0.35 M	1.5 ml	0.35 M	1.5 ml	0.35 M	1.5 ml	0.35 M
0.23% (v v^{-1}) TEMED		0.029%		0.029%		0.029%		0.029%
28% Acrylamide	3.0 ml		3.43 ml		3.86 ml		4.29 ml	
0.74% Bisacrylamide								
5% sucrose	6.75	2.81%	6.32	2.63%	5.89	2.45%	5.47	2.38%
0.8% APS	0.75	0.05%	0.75	0.05%	0.75	0.05%	0.75	0.05%

2.2.8.5. Isoelectric focusing

The isoelectric point of the purified haloalcohol dehalogenase was determined using LKB Ampholine® PAGE plates (Pharmacia). An Ampholine gel (pH 3.5 to 9.5) was placed on the Multiphor (Pharmacia) cooling plate with insulating fluid (silicone fluid) in between. The electrode strips were soaked with electrode solutions (anode solution 1 M H_3PO_4; cathode solution 1 M NaOH) and applied along the edges of the gel. The samples (20 µl) were applied onto the sample application pieces positioned at both ends of the gel. The gel was electrophoresed until the dyed standards were in focus. The gels were then immersed in a fixing solution (11.5% trichloroacetic acid, 3.45% sulphosalicylic acid) for 1 h. The gels were washed with destaining solution (25% ethanol, 8% acetic acid) for 5 min and stained for 10 min with 0.115% Coomassie Blue R 250 in destaining solution which had been preheated to 60°C. The gels were destained until a clear background was obtained. Horse heart cytochrome C, pI 10.6; sperm whale met-myoglobin, pI 8.3; equine met-myoglobin, pI 7.3; porcine met-myoglobin, pI 6.45; trifluoroacetylated porcine met-myoglobin, pI 5.92; *P.aeruginosa* azurin, pI 5.65; *A.nidulans* C. phyconyanin, pI 4.75 (Electran, range 4.7-10.6, BDH, Merk Ltd, Lutterworth, Leics, UK.) were used as protein standards. Photographs of the gels were taken by using Polaroid MP4 camera with Polaroid type 665 black and white film.

2.2.8.6. Polyacrylamide gel stain

The polyacrylamide slab gels were routinely stained with Coomassie Blue, however when protein concentrations were lower then 0.01 mg ml^{-1} the gels were stained using the silver stain method.

2.2.8.6.1. Coomassie Blue stain

The gels were stained with 0.1% Coomassie Blue (Coomassie Brilliant Blue R-250, Bio-Rad, Hemel Hempstead, Hertfordshire, UK.) in 40% (v v^{-1}) methanol, 15% (v v^{-1}) acetic acid for 1 h and destained in 10% (v v^{-1}) methanol, 7.5% (v v^{-1}) acetic acid, until a clear background was obtained.

2.2.8.6.2. Silver stain

Silver staining of polyacrylamide slab gels was based on the method developed by Henkeshoven and Dernick (1965) and carried out according to the kit suppliers instructions (Sigma, Poole, Dorset, UK). The proteins were fixed onto the polyacrylamide net by incubating the gels in a 30% ethanol and 10% acetic acid solution. The slab gels were then washed thoroughly until all the acetic acid was removed, and placed in 50 ml of silver equilibration solution (silver nitrate) for 30 min with gentle agitation. The gels were rinsed for 10-20 sec with 50 ml of distilled water and incubated with 30 ml of development solution (sodium carbonate and formaldehyde). After 5-8 min the development solution was discarded and replaced with fresh solution. The gels were incubated until the darkest protein bands were obtained whilst minimizing darkening of the gel. Development of the gel was stopped by adding 50 ml of 1% (v v^{-1}) acetic acid for 5 min. Background was removed by incubating the gels in 50 ml of reducer solution (potassium ferricyanide, sodium thiosulphate and sodium carbonate) for 10-30 s and then immediately rinsing the gel under running tap water for 1 min. The gels were stored in distilled water.

2.2.9. Immunochemical analysis

2.2.9.1. Antiserum production

Antiserum against the haloalcohol dehalogenase of the *Arthrobacter* sp H10a was raised by injecting a rabbit intramuscularly with 50 µg of the purified enzyme in complete Freund adjuvant. Five subsequent injections in incomplete Freund adjuvant were administered at 10 day intervals. Ten days after the last injection blood was collected and the serum containing the polyclonal antibody was collected by centrifugation (8,000xg, 10 min, 4°C).

2.2.9.2. Determination of antiserum titre

Activity of the serum against the haloalcohol dehalogenase produced by the *Arthrobacter* sp H10a was tested by ELISA (Harlow & Lane, 1968). Microtitre plates (96 wells) were coated overnight at 4°C with 0.1 µg of the pure fraction of the haloalcohol dehalogenase per well in 50 µl of 0.05 M $NaHCO_3$, pH 9.5. The following steps were then taken at room temperature. After three washes with TBS (20 mM TRIS-HCl, pH 7.5, 0.5 M NaCl), 0.05% Tween 20, the wells were blocked with 200 µl of 1% (w v^{-1}) BSA in TBS and the plate was incubated for 2 h. The plates were washed as before and the polyclonal antibody, serially diluted, was added to the wells. The microtitre plates were incubated for 3 h and the wells washed as before. The second antibody (goat anti-rabbit immunoglobulin coupled with alkaline phosphatase) diluted 1:350 in TBS was added and the plates incubated for 2 h. After washing six times with TBS, 0.05% (v v^{-1}) Tween 20, the amount of antibody was detected using 150 µl of substrate buffer (125 mM glycine pH 10.4, 25 mM $ZnCl_2$, 25 mM $MgCl_2$ and 1 mg ml^{-1} of Sigma 104 phosphatase substrate). The microtitre plates were incubated in the dark for 30 min and the

absorbance at 405 nm determined in a plate reader MR500 Dynatch (Dynatch, Guernsey, UK).

2.2.9.3. Antibody purification

An antibody solution rich in IgG (Harlow & Lane, 1988) was prepared by precipitating the serum proteins with caprylic acid, 2.5% (v v^{-1}) final concentration, in 40 mM sodium acetate buffer pH 4.0. The precipitate was removed by centrifugation (5,000xg, 10 min, 4°C) and the supernatant dialyzed 3 times against 2 l of PBS (137 mM NaCl, 2.6 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4, pH 7.2). The antibodies were concentrated by precipitation with 25-50% saturation $(NH_4)_2SO_4$. The precipitate was collected by centrifugation (3,000xg, 30 min) and resuspended in, and dialyzed against PBS.

The haloalcohol dehalogenase antibody was further purified by an immunobloting technique (Olmstead, 1981). A partially purified enzyme solution was electrophoresed in two 12.5% SDS-PAGE (1.5x130x150 mm, section 2.2.8.2) and transferred to a nitrocellulose membrane (Scleicher & Schull, 0.45 µm) in a electroblot apparatus (Bio-Rad, Henel Hempstead, Hertfordshire, UK.) filled with transfer buffer (20 mM TRIS, 150 mM glycine, 20% (v v^{-1}) methanol). Electrophoresis was performed for 5 h at 200 V at 4°C. Further steps were performed at room temperature with gentle shaking. The membranes were stained with 0.2% Ponceu S in 0.3% trichloroacetic acid, and the protein bands corresponding to the *Arthrobacter* sp H10a haloalcohol dehalogenase cut out. The membrane strips were blocked with 3% BSA in TBS, washed with TBS and incubated over night with the antibody solution diluted 1:100 in 1.0% (w v^{-1}) BSA, TBS. The membrane strips were washed five times with 0.5% NaCl, 10 mM sodium phosphate pH 7.4, 0.2% (v v^{-1}) Triton X-100, 1 mM NaN_3, 1 mM Na_2EDTA. The antibody was eluted by incubating the membrane strips with 2 ml of 0.2 M glycine

hydrochloride, pH 2.0, for 10 min. The solution was neutralized with a 1 M TRIS solution and dialyzed over night against 200 ml of TBS.

2.2.9.4. Western blot

Western immunoblot analysis was performed essentially as previously described by Towbin *et al.* (1979). Samples of cell free extracts or purified protein were electrophoresed under native (section 2.2.8.2) or denaturating conditions (section 2.2.8.1) by PAGE and transferred to a nitrocellulose membrane as described above (section 2.2.9.3). The membranes were blocked by incubating for 2 h with 3.0% (w v^{-1}) BSA in TBS and incubated over night with the purified antibody diluted 1:100 in 1.0% (w v^{-1}) BSA, TBS. The nitrocellulose sheet was subsequently washed four times with TBS and the second antibody (alkaline phosphatase coupled to a goat anti-rabbit immunoglobin; Sigma, Poole, Dorset, UK) was added (diluted 1:2,000) and incubated for 2 h. The blot was washed six times with TBS and twice with APB (100 mM TRIS-HCl, pH 9.5; 10 mM NaCl; 5 mM $MgCl_2$). The antibody binding was detected by incubating the membrane with alkaline phosphatase solution (5 mg nitro blue tetrazolium in APB plus 2.5 mg bromo indolyl phosphate in 50 µl dimethylformamide).

2.2.10. Analysis of the Deh1 haloalcohol dehalogenase subunits

2.2.10.1. Polypeptide separation

The 30.5 and 34 kDa monomers were separated by preparative PAGE (Model 491 Prep Cell, Bio-Rad, Henel Hempstead, Hertfordshire, UK.) according to manufacturers instructions. A 5 cm high 10.5% T, 2.67% C resolving gel in 0.375 M TRIS-SO_4, pH 8.8,

and a 2 cm high 4% T, 2.67% C stacking gel in 0.125 M TRIS-SO_4, pH 6.9, were cast in the 28 mm ID gel tube. The resolving gel was polymerized by adding 0.025% (w v^{-1}) APS and 0.025% (v v^{-1}) TEMED and cooled during polymerization by passing a stream of cold tap water through the cooling core. After 2 h, the gel was overlayed with 0.375 M TRIS-SO_4, pH 8.8, buffer and polymerization continued overnight. The overlay buffer was removed, the stacking gel poured and polymerized with 0.05% (w v^{-1}) APS and 0.1% (v/v) TEMED. The elution chamber and the Prep cell were assembled as described in the instruction manual. The upper, lower and elution reservoirs were filled with 25 mM TRIS, 200 mM glycine and 0.05% SDS. During electrophoresis cooling of the gel was achieved by pumping the lower electrophoresis buffer through the cooling core.

A 1.0 ml sample (500 µg of protein) of the Octyl-Sepharose CL-4B eluate (section 2.2.4) mixed with 0.2 volumes of gel loading buffer was loaded on the top of the stacking gel and electrophoresis was carried out at 30 mA for 7 h. The elution buffer flow was set to 1 ml min^{-1} and fraction collection (2.5 ml) was started just before the ion/dye front eluted from the gel. Protein elution was monitored by using a UV monitor (Pharmacia) coupled to a chart recorder 301D Chessell (Chessell Ltd., Worthing, Sussex). The fractions with protein were analyzed on 12.5% SDS-PAGE (section 2.2.8.2) and the gels were silver stained (section 2.2.8.6.2). The fractions containing the pure subunits of the haloalcohol dehalogenase were collected and concentrated by ultrafiltration (Amicon pM-30 membranes) under nitrogen pressure.

2.2.10.2. Amino acid analysis

Tryptic digests of the peptides and amino acid sequencing were made by Jay Tee Biosciences (Herne Bay, Kent, UK).

2.2.10.2.1. Tryptic digestion

The purified haloalcohol dehalogenase subunits (50 µg) (section 2.2.9.1) were digested with trypsin at a ratio of 1:100 enzyme substrate. The mixture was incubated at 37°C over night and the tryptic digests were separated by reverse phase chromatography (SGE, C8 150x2 mm, 300Å) on a HPLC system (Shimadzu, Japan). The column was previously equilibrated with solution A (0.08% trifluroacetic acid (TFA) in distilled water) and the peptides were eluted with the following three step gradient: (a) 1-60% of solution B (0.08% TFA, 90% acetonitrile (ACN) in distilled water) for 30 min; (b) hold at 60% of solution B for 5 min and (c) a gradient from 60-100% of solution B in 10 min. Detection of peptides was achieved using on a UV detector (Shimadzu, Japan) coupled to the HPLC at a wavelength of 214 nm.

2.2.10.2.2. Amino acid sequencing

The purified peptides were subject to N-terminal sequencing in an automated gas liquid phase protein sequenator (Hewick *et al.*, 1981) based on the Edman-degradation reaction (Edman & Beeg 1967). The peptides were loaded onto the sequenator (Model 8710 Protein Sequencer, Jaytee Bioscience, Herne Bay, UK) and the amino acids were separated on a HPLC system with a UV detector at a wavelength of 269 nm.

2.2.10.3. Peptide mapping

Peptide mapping of the haloalcohol dehalogenase subunits was carried out according to the method described by Cleveland *et al.* (1977). A protein fingerprinting kit developed

by Promega (Southampton, UK.) was used. The pure monomers of the haloalcohol dehalogenase obtained by preparative electrophoresis (section 2.2.10.1) were cleaved with endoproteinase Lys-C, endoproteinase Glu-C and alkaline protease. A 15% resolving gel with a 5% stacking SDS-PAGE (section 2.2.8.2) was prepared. The proteins (2.5 µg) were mixed with 5 µl of the diluted protease as in Table 2.5 and loaded into the preformed wells.

Table 2.5: Serial dilutions of the proteases used for peptide mapping.

Protease	Dilution
Endoproteinase Lys-C (0.2 mg/ml)	1:100; 1:10
Endoproteinase Glu-C (0.2 mg/ml)	1:100; 1:10
Alkaline protease (0.2 mg/ml)	1:10,000; 1:1,000; 1:200

The gels were electrophoresed initially at 75 V to stack the proteins at the interface between stacking and resolving gel. The voltage was then interrupted for 20 min to allow digestion and finally a voltage of 150 V was applied until the maker dye reach the bottom of the gel. The slab gels were silver stained (section 2.2.8.6.2).

2.2.11. Immobilization of the Deh1 haloalcohol dehalogenase

2.2.11.1. In hollow fibers

The haloalcohol dehalogenase was immobilized in a hollow fiber reactor as described by Diaz *et al.*, (1989). Romicon P10 fibers (Romicon, Woburn, Massacusetts) were conditionated by incubating for 30 min with 0.05 M H_3PO_4 followed by 30 min in 0.125 M NaOH. Nine hollow fibers (9 cm in length with an diameter of 0.11 cm) with a nominal molecular weigh cut-off of 10,000 kDa, were inserted into the reactor. The enzyme

preparation was loaded into the shell-side space surrounding the fibers and allowed to associate with them by incubating overnight at 4°C. The reactor was extensively washed with TRIS-SO_4, pH 8.0 and the effluent monitored for protein loss (see section 2.2.3.1). The reactor was then submerged in a water bath at 30°C and reaction started by pumping in TRIS buffer containing 10 mM 1,3-DCP. Samples were periodically removed and the concentration of free chloride determined.

2.2.11.2. In alginate beads

Immobilization of the haloalcohol dehalogenase into alginate beads was based on the method of Mattisson (1983). The enzyme solution was mixed with the same volume of 4% alginate (sodium alginate from *Laminaria hyperborea*, BDH, Poole, Dorset, UK). The preparation was loaded onto a 10 ml syringe and slowly extruded into a 0.2 M $CaCl_2$ solution. The size of the beads (1.5 cm) was determined by the diameter of the needle connected to the syringe. The beads were then washed with 100 mM TRIS-SO_4, pH 8.0 saturated with $CaSO_4$ (approximately 10 mM) until the chloride concentration was lower than 1.5 µmol ml^{-1}. The beads were transfered to a water-jacked vessel warmed to 30°C and the reaction started by adding 30 ml of TRIS-SO_4, pH 8.0 saturated with $CaSO_4$. Samples were periodically removed and the concentration of free chloride determined.

CHAPTER 3

DEGRADATION OF HALOALCOHOLS BY THE *ARTHROBACTER* sp H10a

3.1. Introduction

The use of herbicides and pesticides as well as the careless disposal of many man-made chemicals has increased dramatically in the last few decades. Degradation of these compounds in soil is a desirable process since it prevents their accumulation to potential toxic levels. These substances, that can vary from very complex molecules to short chain halogenated hydrocarbons, are often suitable carbon and energy sources for a number of soil microorganisms. Therefore a wide variety of bacterial genera have been found in natural environments that are capable of degrading halogenated hydrocarbons.

As part of an industrial project several bacterial strains that were capable of using haloalcohols as single carbon and energy sources have been isolated. These microorganisms were isolated by batch enrichment in minimal medium containing 1,3-DCP from soil samples collected in a industrial site located in the north of Kent, UK. (P.J. Sallis, Hardman & Bull, unpublished data). Amongst these isolates the fast 1,3-DCP degrading strain H10a was selected for further study of the enzymes involved in the degradation of haloalcohols. The genus of strain H10a was identified by NCIMB (The National Collections of Industrial and Marine Bacteria Ltd, Aberdeen, Scotland) as *Arthrobacter*. The morphological and physiological properties of strain H10a are shown in Table 3.1.

Regulation of the enzyme activity in bacteria is accomplished by induction of the proteins required for growth and repression of the ones that are not necessary. Furthermore, the level of the enzymes of anabolic pathways (e.g. amino acid synthesis) can be regulated by end product repression or attenuation. Alternatively

the enzyme activity in the cell can be controlled by allosteric effects, by feed-back inhibition or even covalent modification.

Table 3.1: Some morphological and physiological properties of *Arthrobacter* sp H10a.

Cell morphology	Irregular
Gram stain	Positive
Spores	Negative
Motility	Negative
Colony morphology	Semi-translucent; round; regular; entire; shiny; low convex; < 0.5 mm diameter
Pigmentation	Pastel yellow
Growth at 37°C	Positive
45°C	Positive
Catalase	Positive
Oxidase	Negative
Fermentative in glucose OF	Negative
Chemotaxonomic analysis	
Mycolic acids	Negative
Cell wall diamino acid	Lysine
13-methyltetradecanoic acid	6.51%
12-methyltetradecanoic acid	71.52%
14-methylpentadecanoic acid	4.14%
Hexadecanoic acid	0.83%
14-methylhexadecanoic acid	16.17
Unknown	0.83%

The genes of inducible enzymes required for a metabolic pathway are often located on the bacterial genome adjacent one to another. Together with an operator and a promoter they may form a regulatory unit, an operon. The transcription of the operon can be induced by the substrate of the first enzyme or by one of the products of the

metabolic pathway. Substrates utilized as a consequence of constitutive enzyme systems (e.g. glucose) can cause catabolite repression of inducible pathways. The transport of these substrates into the cells causes a drastic decrease of the intracellular level of cyclic AMP. The latter, is required for the initiation of RNA synthesis in operons that encode inducible pathways. Therefore the second substrate is only utilized after the first is completely metabolized resulting in a biphasic growth curve designated as diauxic growth by Monod.

The enzymes of many catabolic pathways are synthesized in response to the presence of inducing substrates. Haloacid and haloalkane dehalogenases are usually induced by their substrates and some of its analogues. However, in all DCE degrading bacteria the two dehalogenases involved in the biodegradation of 1,2-dichloroethane are constitutively produced (Janssen *et al.*, 1985; van den Wijngaard *et al.*, 1992). Haloalcohol degrading bacteria, when grown on media that do not contain halogenated carbon sources, have shown low levels of haloalcohol dehalogenase and epoxide hydrolase activity (van den Wijngaard *et al.*, 1989; Jacobs *et al.*, 1991; Nakamura *et al.*, 1993). However, when those bacteria were grown in the presence of halogenated alcohols and epoxides the enzymes activities were increased by a factor of 2.5 to 20.

3.2. Utilization of halogenated compounds

A number of low molecular weight chlorinated alcohols and related epoxides were tested as single carbon sources for the *Arthrobacter* sp H10a (Table 3.2). The strain H10a could degrade halohydrins and epoxides like 1,3-DCP, CPD and ECH. However, it could not use 1-chloro-2-propanol or 2-chloroethanol as a sole carbon

source although some chlorine release was observed when the H10a cells were incubated in minimal medium containing either of these compounds. The cell growth and the percentage of substrate degradation were very similar whether the strain H10a was grown with 1,3-DCP, CPD or with ECH.

Table 3.2: Degradation of chlorinated alcohols and epoxides by *Arthrobacter* sp H10a. The cells were grown in minimal medium (see section 2.2.1) containing the halogenated compound indicated as the sole carbon source, until early stationary phase. The maximum growth (OD_{600}) and the chloride release (see section 2.2.3.2) were determined. The percentage of degradation was calculated from the amount of the halogenated hydrocarbon and the chloride found in the medium.

Carbon sources	Growth (OD_{600})	Halogen release (%)
1,3-DCP	0.882	72.5
CPD	0.811	83.3
ECH	0.772	79.0
1-Chloro-2-propanol	0.037	5.2
2-Chloroethanol	0.037	37.1

3.3. Synthesis of the Arthrobacter sp H10a haloalcohol dehalogenase(s)

Since many of the dehalogenases studied to date are induced either by their substrates or by degradation products an experiment was established to test the effect of several halohydrins and some of their non-halogenated analogues on the synthesis of the *Arthrobacter* sp H10a haloalcohol dehalogenase(s). The following carbon sources were chosen: (1) 1,3-DCP, CPD and epichlorohydrin as the halogenated alcohols and epoxides; (2) glycerol and glycidol as the non-halogenated analogues and putative degradation metabolites and (3) glucose as a control since it is known to be a catabolite repressor for many bacteria.

The *Arthrobacter* sp H10a was grown for 8 consecutive passages on minimal medium (see section 2.2.1) containing 0.36 g carbon l^{-1} of one of the above compounds as the sole carbon source. On the 1st, 4th and 8th passages the maximum cell growth was determined and crude extracts produced (see section 2.2.2). The levels of haloalcohol dehalogenase(s) were similar in the cultures grown on the halogenated alcohols and epoxides, glycerol and glucose. However, when glycidol was used as the carbon source the level of enzyme activities increased 3 fold (Figure 3.1). The growth on medium containing the halogenated carbon sources was similar (OD_{600}, 0.6 to 0.8) but lower than that observed for non-halogenated carbon sources (OD_{600}, 1.3 to 1.4).

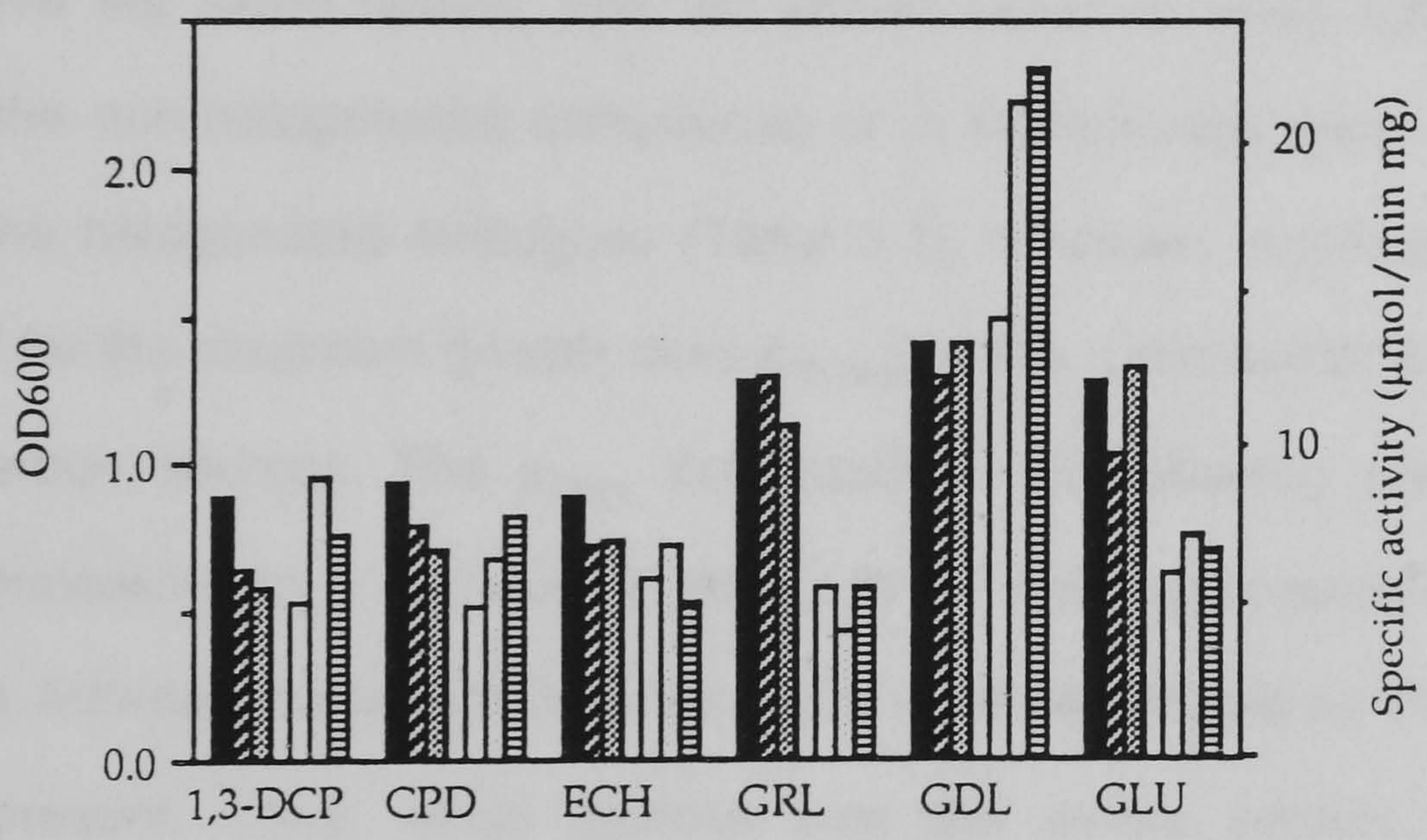


Figure 3.1: Comparison of the maximum growth and haloalcohol dehalogenase activity of the *Arthrobacter* sp H10a when grown for several generations on the same carbon source. Strain H10a was grown on minimal medium (see section 2.2.1) containing 0.36 g of carbon l^{-1} 1,3-DCP, CPD, ECH, glycerol (GRL), glycidol (GDL) or glucose (GLU) as a single carbon source. The cell growth (OD_{600}) obtained on the first 1st (■), 4th (▧) and 8th (▨) consecutive passages on minimal medium contained one of the above compounds as a single carbon sources was determined. The crude extracts of the 1st (□), 4th (▩) and 8th (▤) were prepared as described on see section 2.2.2 and the haloalcohol dehalogenase activity towards 1,3-DCP determined (see section 2.2.3.1).

As seen in Figure 3.1 the variation in the *Arthrobacter* sp H10a cell growth and haloalcohol dehalogenase activity between each experiment was too high to allow statistical analysis of the data. However, a consistent increase in the cell growth was observed when the bacterium was grown with the non-halogenated compounds as substrates and an increase in the dehalogenase specific activity with glycidol grown cells. Therefore all the results that are further considered in this chapter represent one set of data, but given that similar profiles for growth and enzyme activities were obtained for 3 separate of experiments, performed under the same conditions, it was felt that the results so obtained were reliable.

Growth studies of *Arthrobacter* sp H10a on minimal medium containing mixed carbon sources gave the same results. The cell growth obtained when strain H10a was grown on the non-halogenated compounds or in combination were always higher than with the halogenated analogues (Table 3.3). However, significant differences were found for the maximum growth rates (μ_{max}) of the *Arthrobacter* sp H10a on the different carbon sources. The μ_{max} decreased in the following order glucose > glycerol > haloalcohols > epoxides (Table 3.3). In medium containing two carbon sources the *Arthrobacter* sp H10a growth rates were determined by the best growth substrate present. Thus, when glucose was the single carbon source or in combination with any of the others, the growth rates were similar but higher than with 1,3-DCP or glycidol alone. The same results were obtained with the other carbon sources studied (Table 3.3).

Although the cell growth obtained when glycidol was used as a carbon source were similar to the ones obtained with glycerol and glucose, the μ_{max} was much lower. The H10a cells grown on the epoxides also showed a longer lag phase (Figure 3.2). Furthermore, the rate of chloride release did not reflect the amount of dehalogenase

specific activity inside the H10a cells. The bacterium grown on 1,3-DCP plus glycidol showed high levels of dehalogenase activity but the rates of chloride release were very low when compared with the glycerol/1,3-DCP and glucose/1,3-DCP grown cells (Table 3.3 and Figure 3.2 C, E, & H).

Table 3.3: The effect of different carbon sources on the growth and haloalcohol dehalogenase activity of *Arthrobacter* sp H10a. *Arthrobacter* sp H10a was grown to the stationary phase in minimal medium with the indicated carbon sources, the growth (OD_{600}) and the chloride release determined (see section 2.2.3.2). The cell free extracts were produced and the haloalcohol dehalogenase specific activity towards 1,3-DCP and CPD was determined (see section 2.2.6.1).

Carbon source	Growth parameters			Specific Activity (µmol min^{-1} mg^{-1})		
	OD_{600}	μ_{max} (h^{-1})	Cl^- Release (µmol min^{-1})	1,3-DCP	CPD	CPD/DCP x 100
1,3-DCP	0.81	0.19	1.05	4.80	0.11	2.2
CPD	0.90	0.21	0.91	5.17	0.86	1.7
ECH	0.94	0.14	0.55	6.29	0.06	0.9
GDL	1.30	0.16		14.90	0.14	0.9
1,3-DCP + GDL	1.19	0.18	0.40	16.22	0.24	1.5
Glycerol	1.19	0.27		5.87	0.04	0.6
Glycerol + 1,3-DCP	1.05	0.27	0.67	4.43	0.11	2.6
Glycerol + GDL	1.23	0.23		11.04	0.08	0.7
Glucose	1.07	0.34		2.91	0.03	1.1
Glucose + 1,3-DCP	1.17	0.36	0.50	4.67	0.09	2.0
Glucose + GDL	0.90	0.35		13.61	0.10	0.7

Although glucose is a catabolite repressor for a number of metabolic pathways, it did not cause any repression of the haloalcohol dehalogenase activity in strain H10a.

The growth of *Arthrobacter* sp H10a in medium containing glucose and either 1,3-DCP or glycidol was not diauxic, which is characteristic of bacterial growth in the presence of a catabolite repressor and a second substrate that is degraded by inducible enzymes. The rates of chloride release were similar with or without glucose in the growth medium (Figure 3.2 (E & H). Furthermore glucose did not inhibit the increase of the haloalcohol dehalogenase activity observed when glycidol was added to the growth medium (Table 3.3). In all of the substrate mixtures studied both carbon sources were consumed at the same time (Figure 3.2). On medium containing 1,3-DCP alone or in combination with other carbon sources the chloride release occurred simultaneously with growth (Figure 3.2 A, C, E & H).

Whenever glycidol was used as a single carbon source or in combination with any of the other compounds, the haloalcohol dehalogenase specific activity towards 1,3-DCP increased by a factor of 2 to 3.5 (Table 3.3). In order to determine whether the effect of glycidol on the haloalcohol dehalogenase specific activity was the result of enzyme activation or induction of synthesis, the *Arthrobacter* sp H10a was grown in the presence of chloramphenicol, a protein synthesis inhibitor, and glycidol (see section 2.2.4). When chloramphenicol was present in the growth medium the levels of the haloalcohol dehalogenase activity were maintained even upon the addition of glycidol (Table 3.4).

As is seen in Table 3.3, addition of epoxides and haloalcohols to the growth medium had different effects on the H10a haloalcohol dehalogenase activity towards CPD. Whenever one of the epoxides, GDL or ECH, was present the ratio of the dehalogenase activity CPD/DCP decreased to values lower than 0.9. However, if an halogenated haloalcohol was added to the growth medium the ratio increased to values of 1.7 to 2.5.

Table 3.4: The *Arthrobacter* sp H10a was grown on minimal medium containing 0.36g carbon l^{-1} of DCP as the carbon source. Chloramphenicol was added to a final concentration of 50 µg ml^{-1}, at the early exponential phase (c.a. 9 h). One generation time later (c.a. 3 h) 5 mM of glycidol was added and the cells grown for another 6 h. The cell growth (OD_{600}) and the chloride release (see section 2.2.3.2) was measured and the crude extract produced (see section 2.2.2). The dehalogenase activity was determined as described in see section 2.2.6.2. The values represent the average of two experiments.

	DCP	DCP + glycidol	DCP+ chloramphenicol	DCP + glycidol + chloramphenicol
OD_{600} at 18 h	0.390	0.369	0.135	0.125
Cl^- at 18 h (mM)	0.48	0.46	0.22	0.20
Specific activity (µmol/min. mg)	4.81	7.30	4.73	5.80

Alterations in the protein profiles of *Arthrobacter* sp H10a cells grown on different carbon sources was studied by SDS-PAGE (see section 2.2.8.2) (Figure 3.3 (A)). Whenever glycidol was used either as a single carbon source or in combination with any of the others, an increase in the level of protein banding at the 31.5 and 34 kDa region was observed. When the haloalcohols were used as the carbon sources it was not possible to detect any change in protein band intensities. Extracts of glycerol-grown cells of an *Arthrobacter* sp H10a "mutant" that had low haloalcohol dehalogenase activity (0.94 U mg^{-1}), showed a decrease in the intensity of the bands corresponding to the 31.5, 34, 36 and 47 kDa proteins (Figure 3.3 (B)). Furthermore, extracts produced from-glycidol grown cells which possessed high levels of dehalogenase activity showed very high band intensities corresponding to the same proteins, together with a 27 kDa protein. The glucose grown cells showed a decrease on the band intensity corresponding to a protein of 27 kDa (Figure 3.3 (A), lane 11).

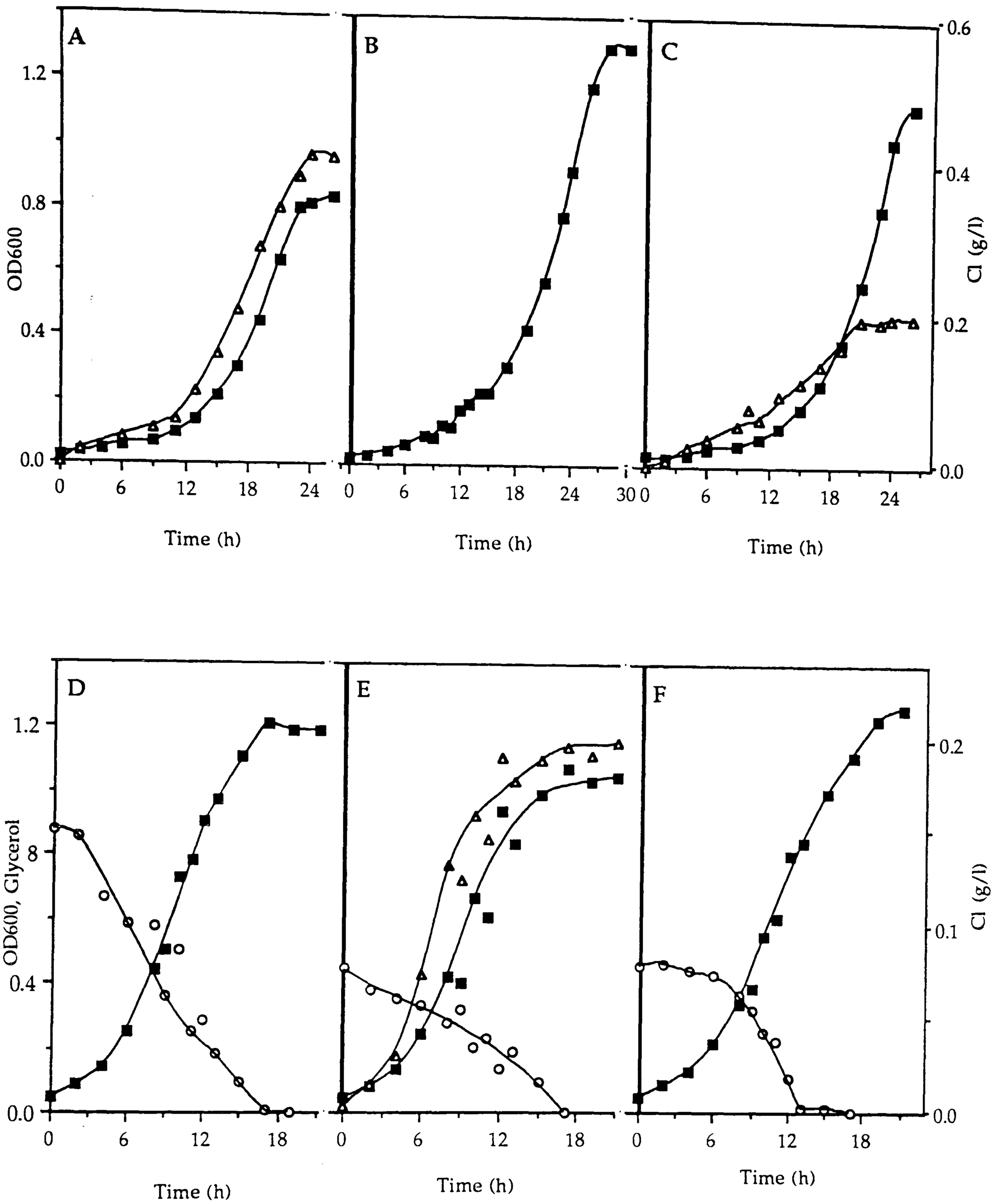


Figure 3.2: Growth curves of the *Arthrobacter* sp H10a grown with differents carbon sources. The strain H10a was grown on minimal medium (see section 2.2.1) containing 0.36 g of carbon l^{-1} of either (A) 1,3-DCP; (B) Glycidol; (C) 1,3-DCP plus glycidol; (D) glycerol; (E) DCP plus glycerol; (F) glycidol plus glycerol; (G) glucose; (H) DCP plus glucose; (I) glycidol plus glucose. The growth (■), chlorine release (Δ), glucose (●) and glycerol (○) consumption were determined (see sections 2.2.3).

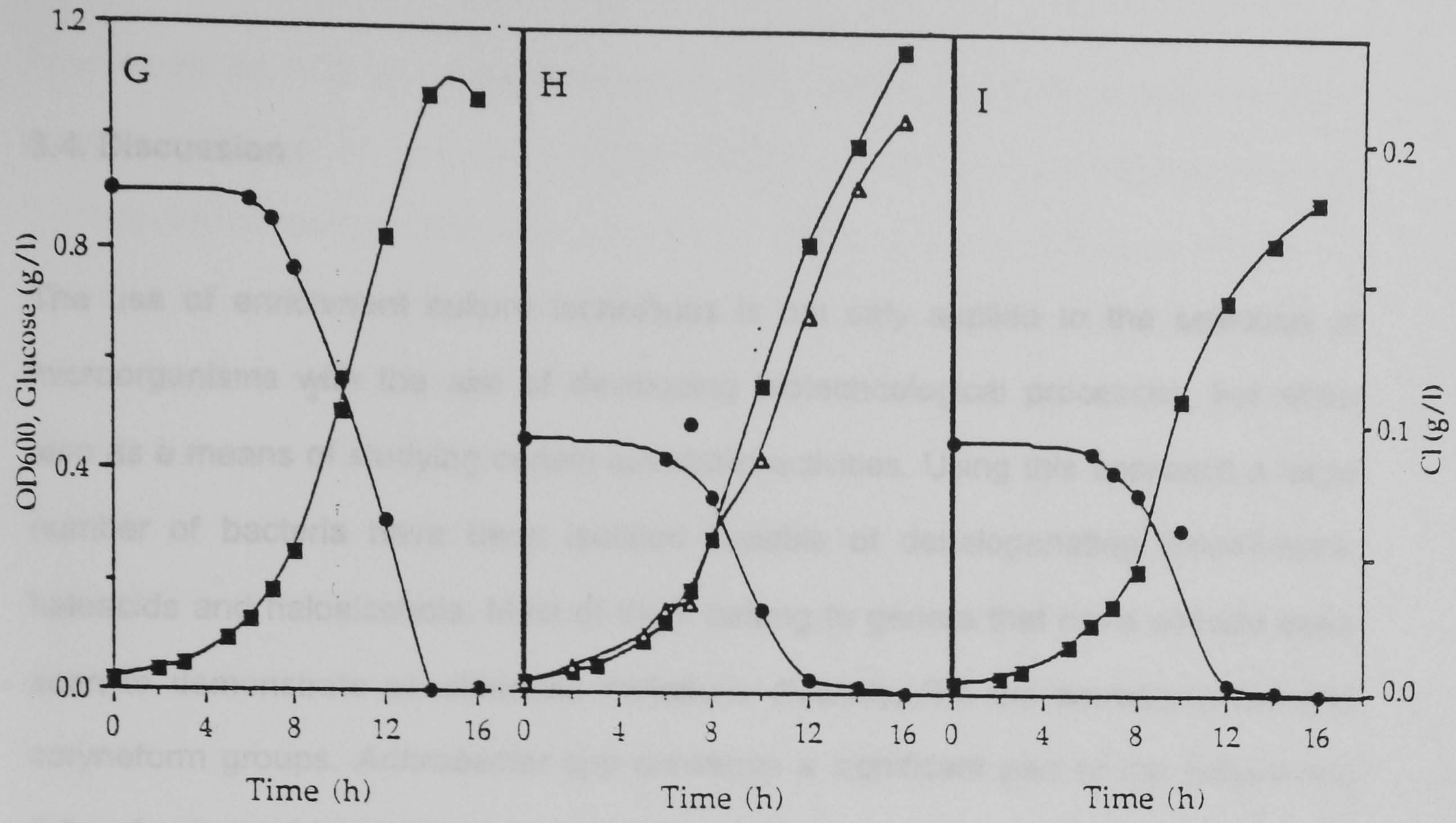


Figure 3.2: Continuation.

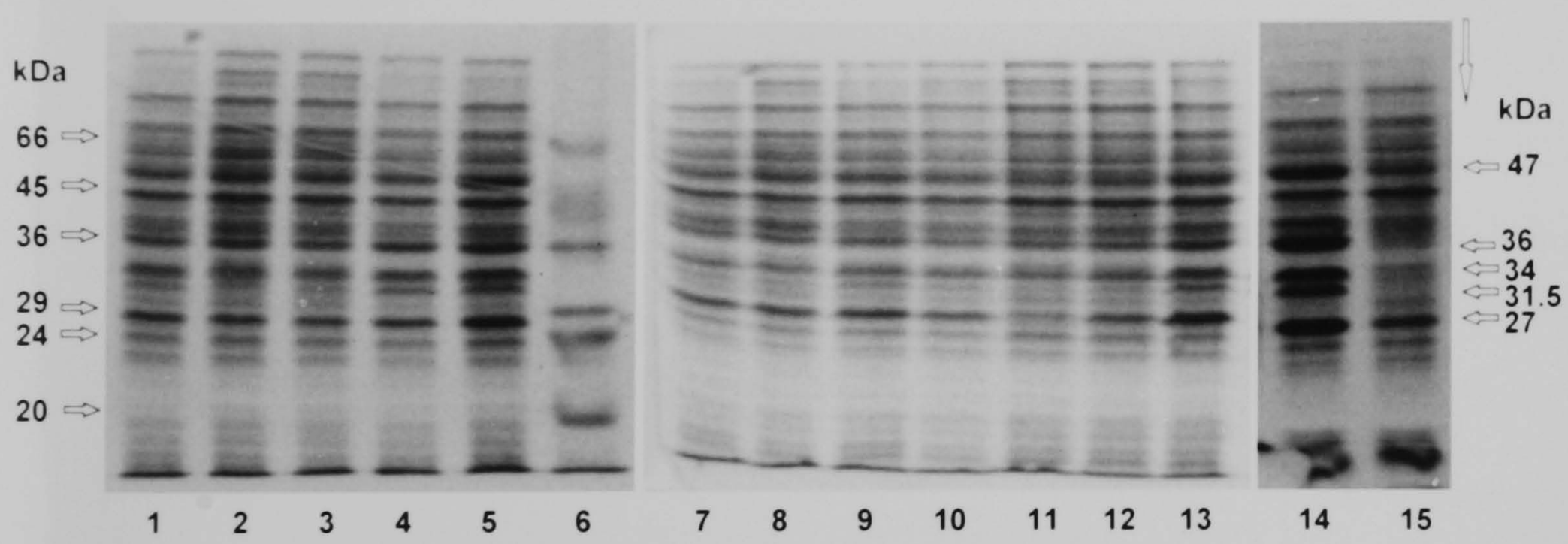


Figure 3.3: Protein profiles of the *Arthrobacter* sp H10a grown on different carbon sources. 12.5% SDS-PAGE (see section 2.2.8.2) of the crude extracts of the bacterium grown in minimal medium containing, lanes: 1, 1,3-DCP; 2, ECH; 3, CPD; 4, GDL; 5, GDL & 1,3-DCP; 6, MW markers; 7, glycerol; 8, glycerol & 1,3-DCP; 9, glycerol & GDL; 10, glycerol & glucose; 11, glucose; 12, glucose & 1,3-DCP; 13, glucose & GDL; 14, high dehalogenase activity cell free extract; 15, low dehalogenase activity crude extract. The arrows on the right side indicate proteins that may be involved in the degradation of halogenated epoxides and alcohols the vertical arrow indicates the direction of the proteins migration.

3.4. Discussion

The use of enrichment culture techniques is not only applied to the selection of microorganisms with the aim of developing biotechnological processes, but often also as a means of studying certain metabolic activities. Using this approach a large number of bacteria have been isolated capable of dehalogenating haloalkanes, haloacids and haloalcohols. Most of them belong to genera that have already been seen to demonstrate considerable metabolic diversity, like the pseudomonad and coryneform groups. *Arthrobacter* spp constitute a significant part of the indigenous flora of soils and because of their innate nutritional versatility, bacteria belonging to this genus are often isolated by enrichment using unusual organic substrates as the sole carbon and energy sources. Amongst the compounds reported to be degraded by *Arthrobacter* spp are hydrocarbons (Leahy & Calwell, 1990) n-alkanes (Klein *et al.*, 1968), halogenated alkanes (Janssen *et al.*, 1987a; Schlotz *et al.*, 1987b) and alcohols (van den Wijngaard *et al.*, 1989; Sallis, unpublished results), chlorobenzoic acids (Pettigrew *et al.*, 1990) and chlorinated biphenyls (Furukawa & Chakrabaty, 1982). Furthermore *Arthrobacter* species have been employed in the production of commercially important substances, like amino acids (Martin, 1989), erythromycin (French *et al.*, 1970) enzymes (Doi *et al.*, 1973; Smith *et al.*, 1991a) and in steroid transformation (Martin, 1977).

Among the compounds tested as carbon sources, not all the chlorinated alcohols could be utilized by *Arthrobacter* sp H10a. Although 2-chloroethanol and 1-chloro-2-propanol were dehalogenated by H10a cell free extracts they could not serve as growth substrates. Since some chloride release was observed, their failure to serve as the sole carbon and energy source may be due to the lack of enzymes that degrade its dehalogenation products or failure to induce the enzymes. The

Arthrobacter sp AD2 and *Pseudomonas* sp AD1 shared the same growth features as strain H10a, as they grew on 1,3-DCP, CPD and ECH but not 2-chloroethanol or 1-chloro-2-propanol (van den Wijngaard *et al.*, 1989). The *Pseudomonas* sp OS-K-29 could use any of the above compounds as single carbon sources (Kasai *et al.*, 1990) while the *Alcaligenes* sp DS-S-7G could only utilize CPD (Suzuki *et al.*, 1992). The *Alcaligenes* sp DS-K-S38, isolated by enrichment on 2,3-DCP, could also use CPD, 2-chloroethanol or 1-chloro-2-propanol, but not 1,3-DCP or ECH (Kasai *et al.*, 1992a). This diversity on the growth profiles among haloalcohol degrading bacteria suggests that a number of different enzymes may be involved in the degradation of these compounds. None of these strains were capable of using haloacids and haloalkanes suggesting that a completely different type of enzyme systems are involved in the degradation of these compounds.

The maximum cell growth obtained when *Arthrobacter* sp H10a was grown on minimal medium containing the halogenated carbon sources was lower than the values obtained when equivalent amounts of glycidol, glycerol and glucose were used (Figure 3.1). When the epoxides were used as carbon sources the growth rates of the *Arthrobacter* sp H10a were lower than the values obtained with the other growth substrates. Furthermore, under these conditions the H10a cells showed a long lag phase (Figure 3.2) and low rates of chloride release, although the levels of haloalcohol dehalogenase specific activity were high (Table 3.3). The observed slow degradation rates may be related to the toxic and mutagenic features of epoxides which were added in high concentrations to the growth medium.

When halogenated carbon sources were absent from *Arthrobacter* sp H10a growth media a constitutive level of haloalcohol dehalogenase activity was still observed (Figure 3.1, Table 3.3). Other haloalcohol degrading bacteria, such as *Arthrobacter* sp AD2, *Pseudomonas* sp AD1 and *Corynebacterium* sp N-1074 have also been

found to possess a constitutive level of dehalogenase activity. However, their activity was increased by addition of halogenated epoxides and alcohols. The levels of haloalcohol dehalogenase activity of strains AD2 and AD1 increased 10 to 20 fold if these bacteria were grown with ECH instead of citrate (van den Wijngaard *et al.*, 1989). CPD induced only a 4 fold increase of the enzyme activity of strain AD2 (van den Wijngaard *et al.*, 1991) and ECH induced a 2.5 fold increase on the epoxide hydrolysis activity of *Pseudomonas* sp AD1 (Jacobs *et al.*, 1991). ECH had no effect on the dehalogenase activity of *Arthrobacter* sp H10a. However, addition of its non-halogenated analogue, glycidol, alone, or in combination with other carbon sources, resulted in a 2 to 4 fold increase in dehalogenase activity towards 1,3-DCP. Addition of glycidol to H10a cells grown in medium containing a protein synthesis inhibitor (chloramphenicol) had no effect on the dehalogenase activity (Table 3.3). These results suggest that glycidol may enhance the expression of a low level constitutive haloalcohol dehalogenase enzyme.

The presence of halogenated alcohols or epoxides in the growth medium of *Arthrobacter* sp H10a had different effects on the level of haloalcohol dehalogenase specific activity towards 1,3-DCP and CPD. This was more noticeable when the specific activity ratio CPD/DCP was compared (Table 3.3). The epoxide-grown cells showed lower CPD/DCP ratios (< 0.9) while, with haloalcohol-grown cells this ratio increased to values of 1.7 to 2.5. These results indicate that the *Arthrobacter* sp H10a may have more than one haloalcohol dehalogenase enzyme. If this were the case then one of the enzyme(s) induced by the epoxides, especially by glycidol, shows a high specific activity towards 1,3-DCP (Deh1). The other dehalogenase(s) (Deh2) would then be induced by the haloalcohols and show a higher specific activity towards CPD than the Deh1 enzyme. Similar results were obtained with *Corynebacterium* sp N-1074 (Nakamura *et al.*, 1992). This strain was found to have two haloalcohol dehalogenases, one enzyme (I_b) that showed high affinity towards

1,3-DCP but low activity towards CPD while the second (I_a) showed a higher affinity towards CPD than enzyme I_b. Unfortunately there is no published data on the induction of these enzymes.

The level of dehalogenase activity of strain N-1074 cells increased 4 to 10 fold when grown on glycerol. Further addition of halogenated alcohols resulted only on a small increase of the enzyme activity (Nakamura *et al.*, 1993). H10a cells grown on glycerol showed no increase in the haloalcohol dehalogenase activity and further addition of 1,3-DCP had no effect on the levels of the enzyme activity towards 1,3-DCP. However, addition of halogenated alcohols to the growth medium resulted in an increase on the ratio CPD/DCP, due to an increase in the synthesis of the enzyme(s) that showed a greater affinity towards CPD.

The increase of the haloalcohol dehalogenase specific activity towards 1,3 DCP, found on glycidol-grown *Arthrobacter* sp H10a crude extracts, might be related to the increase in the protein band intensities of the 31.5 and 34 kDa region, found when this extracts were analyzed on SDS-PAGE (Figure 3.3). These two proteins were later identified as one of the *Arthrobacter* sp H10a haloalcohol dehalogenases (see section 4.2). The study of the protein profiles of *Arthrobacter* sp H10a grown in different carbon sources (Figure 3.3) has revealed other proteins (molecular weight 27, 36 and 47 kDa) that may be involved in the degradation of haloalcohols.

Whenever glucose was present in the growth medium together with any of the other substrates studied they were utilized simultaneously by *Arthrobacter* sp H10a (Figure 3.2). When strain H10a was grown on glucose, repression of a 27 kDa protein which was probably involved in the degradation of halogenated alcohols and epoxides, was observed. Also, *Arthrobacter* sp H10a cells, that have been growing for several generations with glucose, when transferred to medium containing 1,3-

DCP showed a long lag phase (2 days), although were seen to possess high levels of haloalcohol dehalogenase activity (10.20 U mg^{-1} protein). These results suggest that glucose may act as a catabolite repressor for strain H10a, however the mixed substrate growth curves (Figure 3.2) did not show a diauxic type of growth as would be expected. Furthermore, glucose did not hinder the induction of the haloalcohol dehalogenase activity on medium containing glucose and glycidol. The same observations were made with the haloacid degrading bacterium *Pseudomonas putida* P3 (Slater *et al.*, 1979). Although this strain possessed an inducible dehalogenase activity when it was grown in medium containing a combination of a nonchlorinated carbon sources (propionate, lactate, pyruvate and succinate) and 2-MCPA or 2,2-DCPA, both substrates were consumed simultaneously with no apparent diauxic growth. Furthermore, induction of the dehalogenase activity could be achieved upon addition of the halogenated acids to the cells in exponential phase.

In order to further study the enzymes responsible for the dehalogenation of the haloalcohols it was necessary to undertake the purification of the dehalogenase activity(ies) so as to shed more light on the mechanisms of activity and on the evolution of the enzymes involved on the biodegradation of halogenated alcohols and epoxides. The haloalcohol dehalogenase Deh1 purification as well as its physical and biochemical characterization are presented in the next two chapters.

CHAPTER 4

PURIFICATION AND BIOPHYSICAL CHARACTERIZATION OF THE *ARTHROBACTER* sp H10a Deh1 HALOALCOHOL DEHALOGENASE

4.1. Introduction

In order to fully understand and successfully exploit the potential of enzymes it is essential that they are characterized in detail. This requires the proteins to be purified. From studies on pure enzymes it is possible to learn about their substrate specificities, kinetic parameters for the reactions and catalytic mechanisms. In addition, studies of the enzyme structure are only possible with pure proteins. Isolated and purified enzymes also have widespread use as catalysts in a number of industrial applications and in clinical diagnosis.

Over the years the procedures used for protein purification have evolved from selective precipitation to high performance liquid chromatography (HPLC). In the early steps of protein chemistry, differential precipitation constituted the only means of purifying proteins. Proteins can be precipitated either by varying the concentration of chaotropic salts in solution, salting in and salting out, or by addition of water miscible solvents, such as methanol, ethanol, butanol and acetone. Precipitation of a protein is related to its size, shape and number and distribution of charges, and nonionic polar and hydrophobic residues on the surface of the protein. Since in most cases the structure of the protein is unknown the conditions that cause its precipitation have to be determined empirically. With the development of column chromatography, precipitation techniques are being used as concentration steps, with the exception of ammonium sulphate precipitation that is still widely employed on protein purification procedures.

The chromatographic techniques employed on protein purification are based on adsorption to a matrix either by electrostatic forces (ion exchange chromatography), hydrophobic interactions (hydrophobic chromatography) or specific binding (affinity chromatography). Protein separation using gel filtration chromatography is based on the relative size of the protein molecules which will or will not diffuse into matrices of

controlled porosity. This technique has found widespread use for both protein separation and molecular weight determination. As for protein precipitation the performance of the chromatographic technique is related to the size, shape, charge, hydrophobicity and arrangement of functional groups within the protein's three-dimensional structure. The selection of a gel matrix from the wide range of chromatographic materials available, is also empirical and often based on previous experience.

The resolution of chromatographic separation methods was largely improved with the advent of the HPLC. The fractionation principles are the same as for classical chromatographic methods, but the small size of the packing material allowed major advances in resolution and speed of procedure. The use of smaller gel matrices (5 to 10 fold decrease) enhances adsorption-desorption kinetics and diminishes band spreading. Furthermore, the greater mechanical strength of the gels allow the chromatographic processes to be operated at 10-60 times greater mobile phase velocity than with conventional chromatography.

4.2. Purification of the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase

The *Arthrobacter* sp H10a haloalcohol dehalogenase purification procedure was designed having in mind the resources available in the laboratory and stability of the enzyme while aiming to the maximize protein purity and recovery. The *Arthrobacter* sp H10a was grown on minimal medium (see section 2.2.1) containing 1,3-DCP as the sole carbon and energy source. The selection of the growth substrate was based on the fact that the enzymes involved in the degradation of the haloalcohols would be expressed to their maximum levels. As is shown in Table 3.3 the rates of chloride release and the ratio of activity CPD/DCP were high when 1,3-DCP was used as the carbon source. A crude extract (1.5g of protein) was produced (see section 2.2.2) from 42 l of H10a cells grown to late exponential phase. Precipitation with ammonium sulphate was chosen as

the first purification step since it could be used as a purification and concentration procedure. The H10a haloalcohol dehalogenase precipitated between 45 and 80% saturation of ammonium sulphate. During precipitation 43% of the haloalcohol dehalogenase activity was lost but the amount of protein was reduced by 4 fold (Table 4.1).

The H10a haloalcohol dehalogenase was further purified by ion exchange chromatography on a DEAE-Sephacel column (see section 2.2.5). Figure 4.1 shows the elution profile obtained under the chromatographic conditions used. The haloalcohol dehalogenase was localized using the microtitre plate assay as described in section 2.2.6.1. The haloalcohol dehalogenase was eluted as a single peak at $(NH_4)_2SO_4$ concentrations of approximately 0.35 to 0.5 M (Figure 4.1). After this chromatography, 67% of the total dehalogenase activity towards 1,3-DCP was recovered and the enzyme was purified by a factor of 8.1 (Table 4.1).

Figure 4.1: Elution profile of the ammonium sulphate fractionated proteins of *Arthrobacter* sp H10a on a DEAE-Sephacel column. The protein recovered from the 45-80% ammonium sulphate precipitate (0.37 g) in 10 mM TRIS-SO_4 pH 7.0, 1 mM DTT was loaded onto a DEAE-Sephacel column (2.6 x 30 cm) equilibrated with the same buffer. The proteins were eluted with a 600 ml of 0-700 mM $(NH_4)_2SO_4$ gradient. The fractions were collected (10 ml) and the protein (——) concentration (OD_{280}) and the haloalcohol dehalogenase (---) activity towards 1,3-DCP (see section 2.2.6.1) were determined.

The fractions containing dehalogenase activity towards 1,3-DCP were pooled and further fractionated on an Octyl-Sepharose CL-4B column (see section 2.2.5). Under the conditions used the dehalogenase was retained on the column being eluted only at $(NH_4)_2SO_4$ concentrations lower than 0.5M (Figure 4.2). The fractions showing dehalogenase activity were pooled together, dialyzed against 50 mM TRIS-SO_4, pH 8.0, 1 mM DTT to remove all the salt, and then concentrated by ultrafiltration to be stored at -20°C. Unless otherwise specified this enzyme preparation was used for further characterization of the *Arthrobacter* sp H10a haloalcohol dehalogenase. The overall purification factor obtained after these three fractionation steps was 40 fold with 30% of the total dehalogenase activity recovered (Table 4.1).

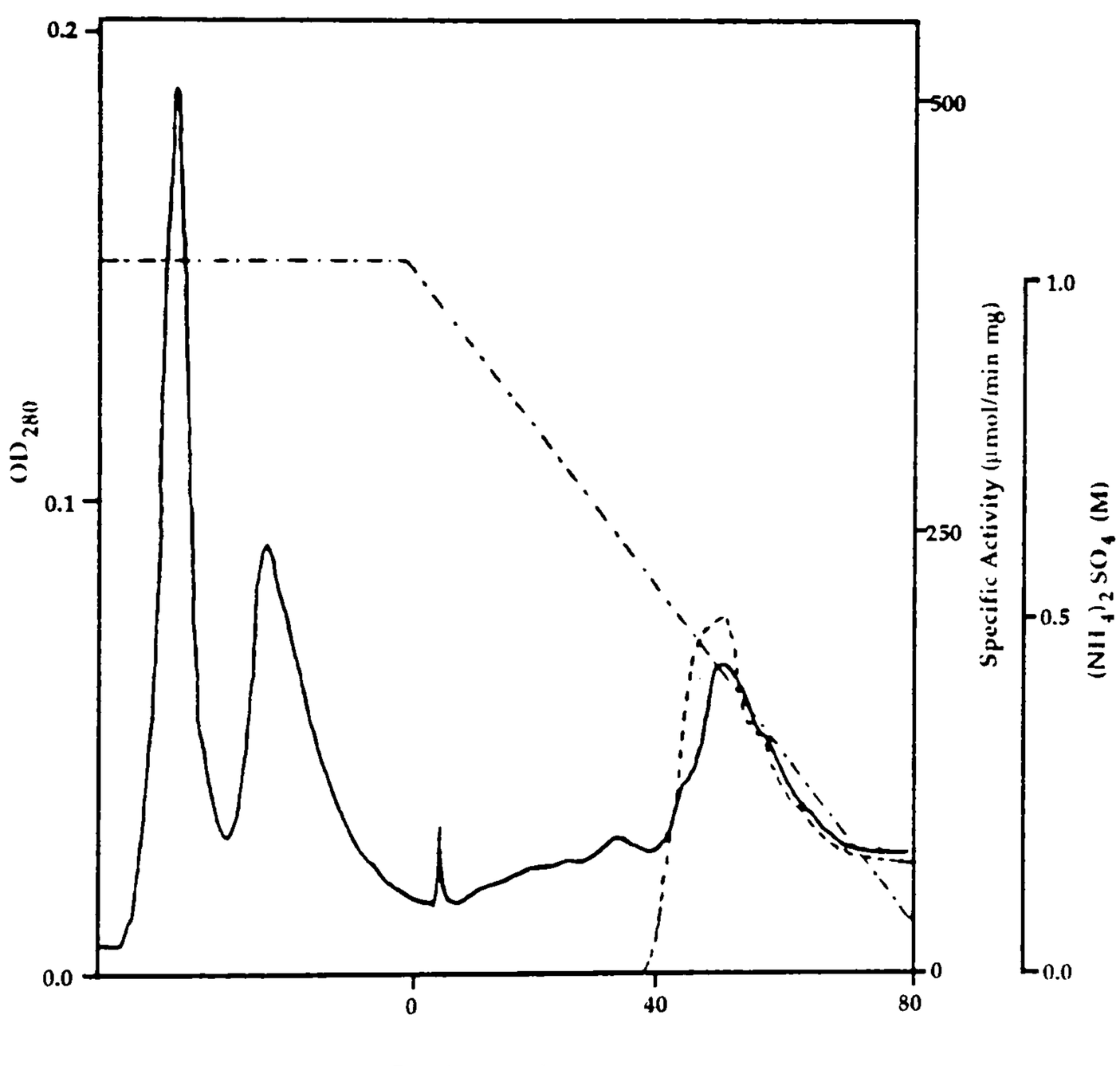


Figure 4.2: Elution profile of the DEAE-Sephacel haloalcohol dehalogenase enriched fraction on the Octyl-Sepharose CL-4B column. The dehalogenase eluted from the DEAE-Sephacel column in 50 mM TRIS-SO_4, pH 7.0, 1 mM DTT, 1.17 M $(NH_4)_2SO_4$, was loaded onto an Octyl-Sepharose CL-4B column (2 x 15 cm) as described in section 2.2.5. The proteins bonding to the gel were eluted with a negative gradient of 1.17 - 0 M $(NH_4)_2SO_4$. The protein (——, OD_{280}) and the haloalcohol dehalogenase (---) towards 1,3-DCP (see section 2.2.6.1) were determined.

To visualize the level of purification obtained at each step of the *Arthrobacter* sp H10a haloalcohol dehalogenase fractionation the protein content was monitored on SDS-PAGE (see section 2.2.8.2). The Octyl-Sepharose eluate showed two major bands of 31.5 and 34 kDa corresponding to the glycidol induced dehalogenase previously designated as Deh1 (see section 3.3) and several minor bands at high sample loading (Figure 4.3). When non-denaturating gels were stained with Coomassie blue it was found that the two polypeptides produced 5 bands of R_f 0.51, 0.55, 0.58, 0.62, and 0.65 corresponding to the five active bands appearing when the gels were activity stained (Figure 4.4). The active band of R_f 0.42, which appeared when the crude extract was electrophoresed under non-denaturating conditions, was not visualized on the partially pure fraction. This dehalogenase was lost during the hydrophobic chromatography step (Figure 4.4).

Table 4.1: Purification of the *Arthrobacter* sp H10a haloalcohol dehalogenase. In order to determine the efficiency of the fractionation steps, the protein (see section 2.2.5) and the haloalcohol dehalogenase activity towards 1,3-DCP (see section 2.2.6.1) were determined at each stage of the strain H10a dehalogenase purification

	Total Protein (mg)	Total Activit (U)	Specific Activity (U (mg protein)$^{-1}$)	Yield (%)	Purification
Crude extract	1470	7518	5.1	100	
$(NH_4)_2SO_4$ 45-80%	364	4288	11.7	57	2.3
DEAE-Sephacel	68	2890	41.4	38	8.1
Octyl-Sepharose CL-4B	11	2234	204.6	30	40.0

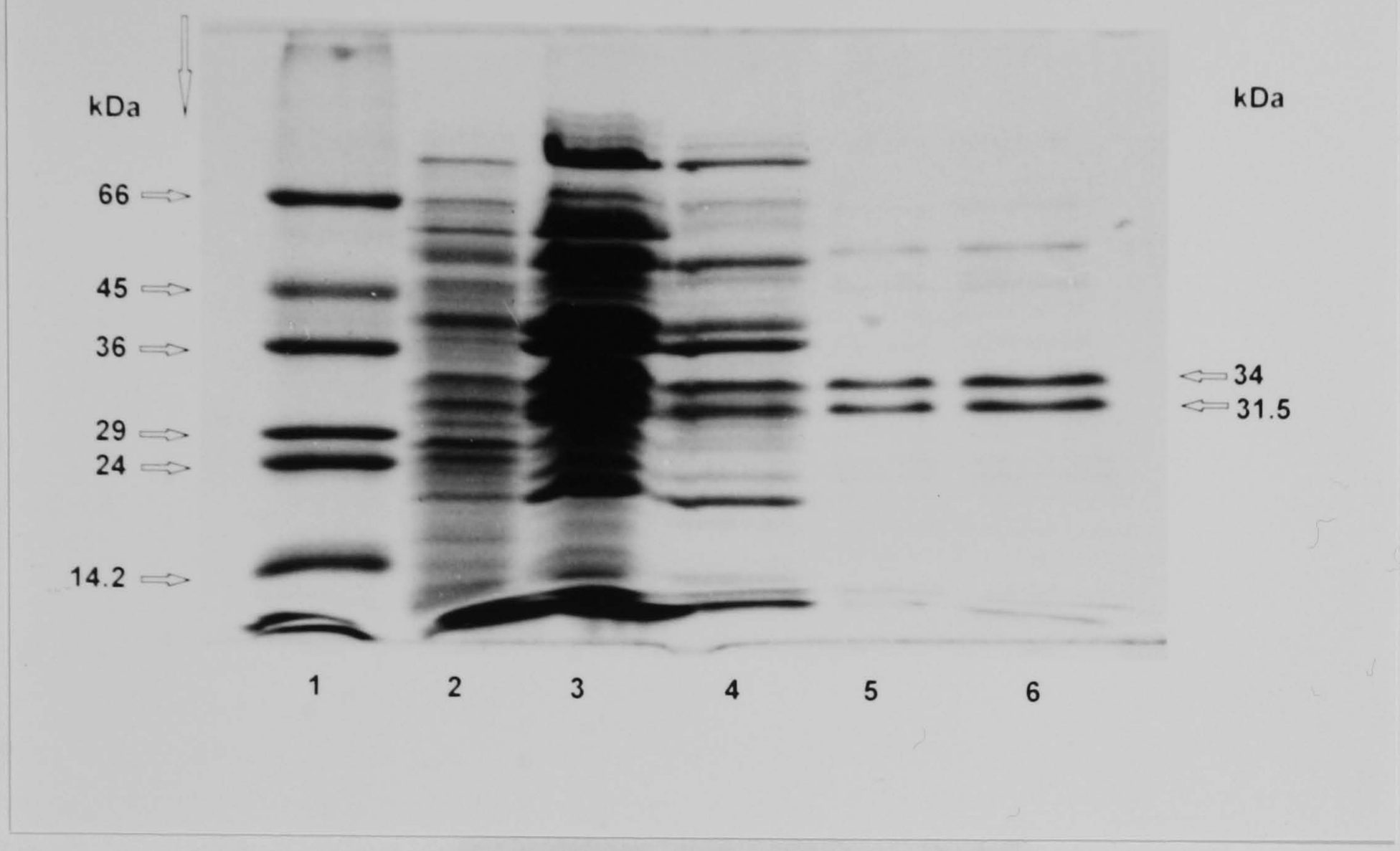


Figure 4.3: SDS-polyacrylamide gel of *Arthrobacter* sp H10a of the cell free extracts at different stages of enzyme purification. A protein sample corresponding to each purification step of the H10a haloalcohol dehalogenase was electrophoresed on a 12.5% polyacrylamide SDS gel (see section 2.2.8.2). Lanes: 1, MW markers; 2, crude extracts (12.5 µg protein); 3, 45-80% ammonium suphate precipitate (12.5 µg protein); 4, DEAE-Sephacel eluate (7.5 µg protein); 5 and 6, Octyl-Sepharose CL-4B eluate (2.5 and 5.0 µg of protein respectively). The vertical arrow indicates the direction of protein migration.

4.3. Physical characterization of the *Arthrobacter* sp H10a Deh1 dehalogenase

4.3.1. Molecular weight determination

The apparent molecular weight of a given protein is perhaps the most often cited characteristic of the molecule. Gel filtration chromatography is the most commonly used method for estimation of native protein molecular mass. This method relies on the comparison of the elution volume of the unknown with those of several protein standards of known molecular weight. The accuracy of this method relies on the shape similarity between the protein under study and the standards used to calibrate the

column. Electrophoretic methods can be also used to determine the apparent size of the native protein. Likewise the accuracy of this method is dependent on the selection of appropriate standards.

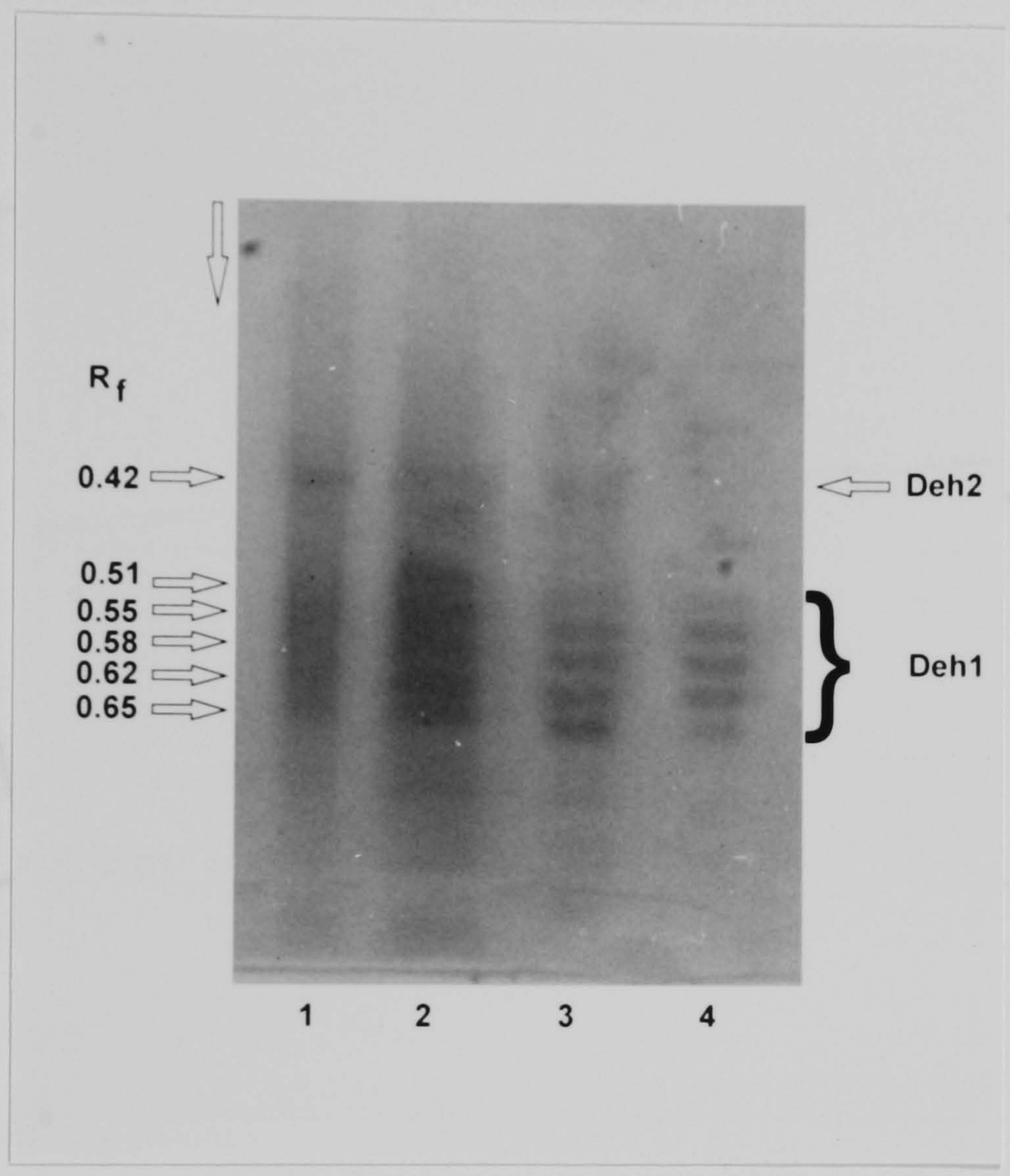


Figure 4.4: Nondenaturating gel electrophoresis of *Arthrobacter* sp H10a cell free extracts at different stages of enzyme purification. A protein sample corresponding to each purification step of the H10a haloalcohol dehalogenase was electrophoresed on a 8% polyacrylamide nondenaturating gel stained for activity towards 1,3-DCP (see section 2.2.8.1). (A) Lanes: 1, crude extract (30 µg protein); 2, 45-80% ammonium sulfate precipitate (30 µg protein); 3, DEAE-Sephacel eluate (20 µg protein); 4, Octyl-Sepharose CL-4B eluate (10 µg of protein). The vertical arrow indicates the direction of protein migration.

Once purified, the molecular weight of the native Deh1 enzyme of *Arthrobacter* sp H10a was determined by gel filtration using a Superose 12 column (see section 2.2.5.1). The column was calibrated with protein standards ranging from 14.2 to 200 kDa. As shown in Figure 4.5 the Deh1 dehalogenase was eluted as a single peak with an v_e/v_o of 1.42

which corresponds to a molecular weight of approximately 200 kDa. Since no other major peaks were found, the Octyl-Sepharose CL-4B contaminants shown on SDS-PAGE must have co-migrated with the Deh1 dehalogenase.

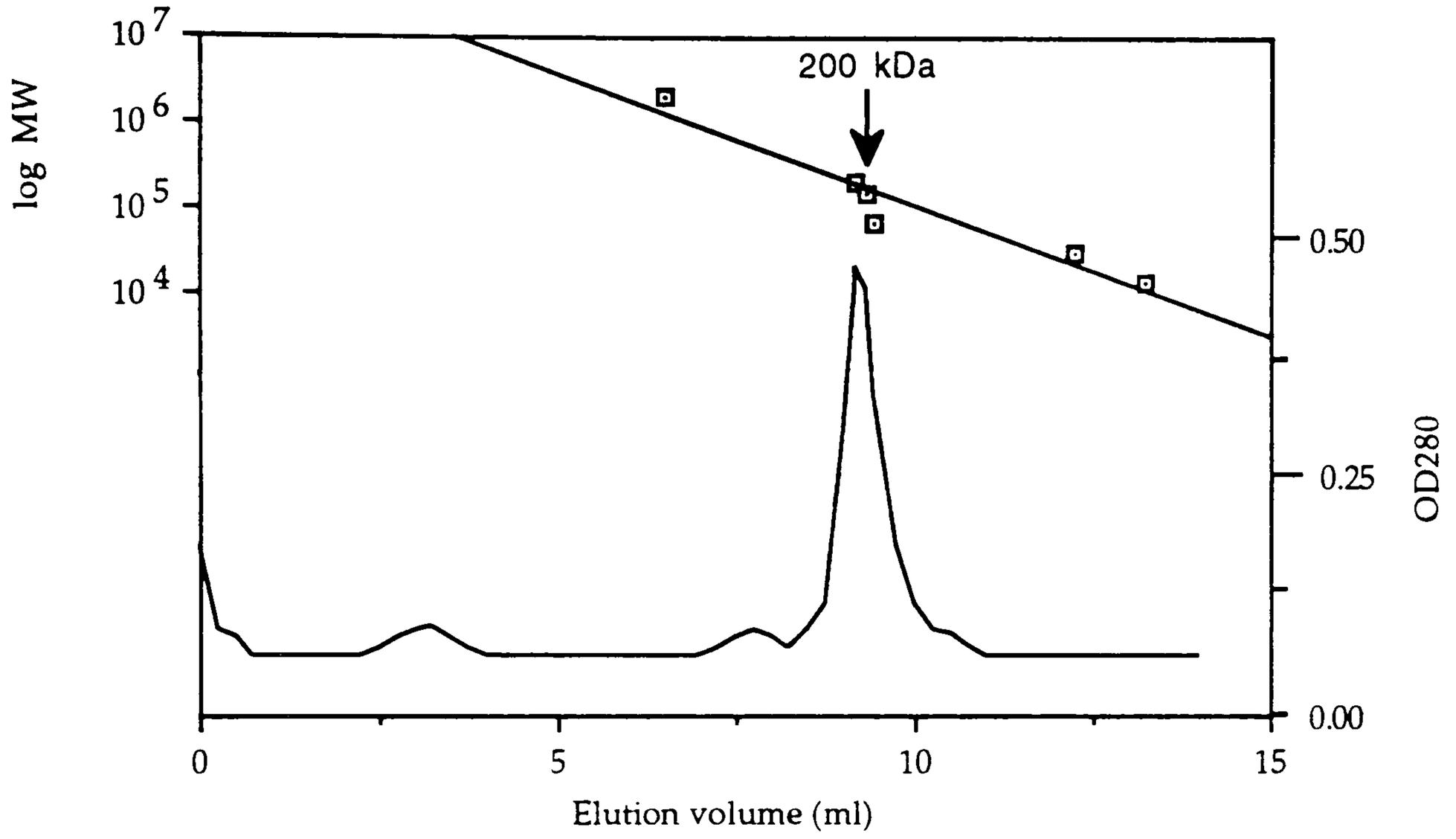


Figure 4.5: Elution profile of the *Arthrobacter* so H10a Deh1 dehalogenase on a FPLC Superose 12 column. The column was equilibrated with 50 mM TRIS-SO_4, pH 8.5 buffer and calibrated with protein standards ranging from 14.2 to 200 kDa (see section 2.2.5.1). The purified Deh1 enzyme (35 µg/50 µl) was applied onto the column and eluted with the same buffer (0.5 ml/min.). The OD_{280} was monitored and the collected fractions (1 ml) were tested for dehalogenase activity towards 1,3-DCP (see section 2.2.6.1).

4.3.2. Studies on the nature of the multiple banding formed on native gels

As a first approach to understand the nature of the multiple banding formed on nondenaturating polyacrylamide gels, their molecular weights were determined using the Ferguson analysis as described in section 2.2.8.4. The slopes of the Ferguson plots (log of the relative mobility [R_f] vs gel concentration) of the five bands were on the range of

7.3 to 8.0 which corresponds to molecular weight of 90.4 to 106 kDa. Due to the proximity of these values it was not possible to determine whether the migration differences were the result of molecular weight or charge isomers. However, the results suggested that the Deh1 haloalcohol dehalogenase separated on native gels as five different trimers.

The isoelectric point of the Deh1 dehalogenase under native conditions (see section 2.2.8.5) was investigated in order to determine if the multiple bands formed when this enzyme was electrophoresed on nondenaturating gels was due to the presence of charge isomers. However, for reasons unknown, it was not possible to obtain a sharp band(s). The Deh1 dehalogenase on IEF gels showed a large streaking protein band that spread from pH 6.1 to 4.9. This type of band streaking is usually due to protein precipitation, especially at its isoelectric point, or to protein modification.

Two dimensional PAGE was used in order to determine the composition of each of the 5 bands formed under native conditions. On a first dimension a sample of the purified enzyme Deh1 (30 µg) was electrophoresed on a 8% polyacrylamide nondenaturating gel. The second dimension was performed on a 12.5% polyacrylamide SDS gel (see section 2.2.8.3). Using this method, the protein bands formed under nondenaturating conditions would be further analyzed by SDS-PAGE for the subunit content and molecular weight. As shown in Figure 4.6, a large proportion of the proteins migrated as high molecular weight aggregates, suggesting that denaturation on the gel slice was incomplete. However, the five bands were resolved into seven spots on the second dimension indicating that the factors determining the formation of the multiple bands of the Deh1 dehalogenase on nondenaturating gels were its subunits molecular weight and charge.

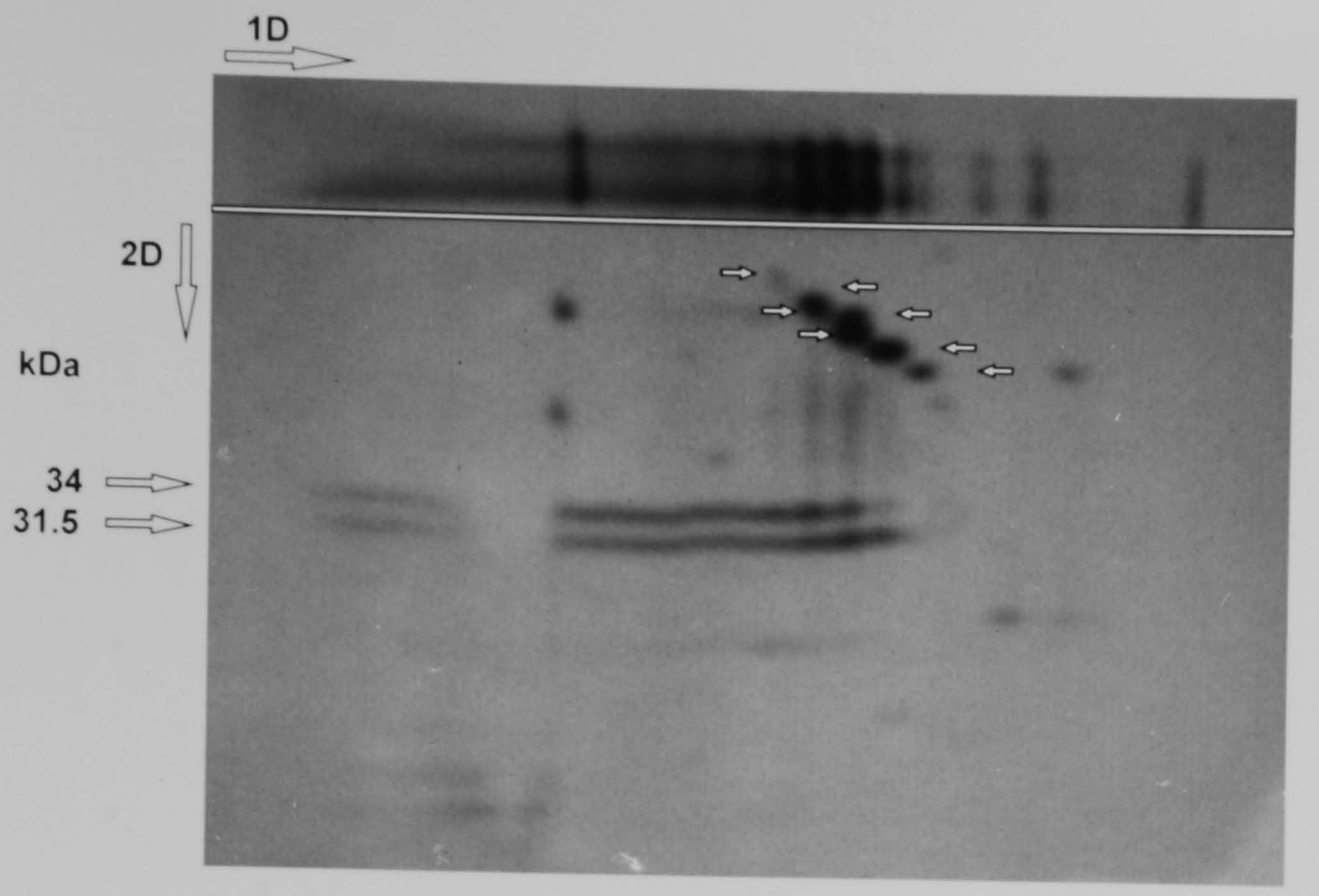


Figure 4.6: Two dimensional polyacrylamide gel of the *Arthrobacter* sp H10a Deh1 dehalogenase. The purified Deh1 enzyme (20 µg) was first separated on a 8% polyacrylamide gel under nondenaturating conditions (see section 2.2.8.3). The proteins on the gel were denaturated and loaded onto a 12.5% polyacrylamide SDS gel as described in section 2.2.8.6.2. The arrows labeled 1D and 2D indicate the direction of protein migration on the first and the second electrophoresis, respectively.

Total denaturation of the dehalogenase Deh1 separated on native polyacrylamide gel was achieved by boiling for 5 min in 10 µl of the gel loading buffer (containing 1% SDS and 10% ß-mercaptoethanol). The gel slices were then analyzed on 12.5% polyacrylamide SDS-PAGE (see section 2.2.8.2). As shown in Figure 4.7, the protein bands that showed haloalcohol dehalogenase activity, labelled Deh1a to Deh1e, had either the 31.5 or the 34 kDa or both proteins but in different proportions. The 34 kDa subunit was predominant in Deh1a whilst the 31.5 kDa protein was predominant in Deh1d and Deh1e. On the bands Deh1b and Deh1c both subunits were present in similar proportions. The most interesting result from this gel is that 5 other proteins appear to be associated with the two subunits. The Deh1d and Deh1e bands showed a 20.0 kDa protein besides the two proteins previously identified as the two haloalcohol dehalogenase subunits. A 24.5 and a 43.5 kDa molecular weight protein were present in

lanes corresponding to Deh1a, Deh1b and Deh1c whilst a 54 kDa protein was shown only on the Deh1a and Deh1b lanes. Furthermore, the Deh1a showed an extra 39 kDa protein.

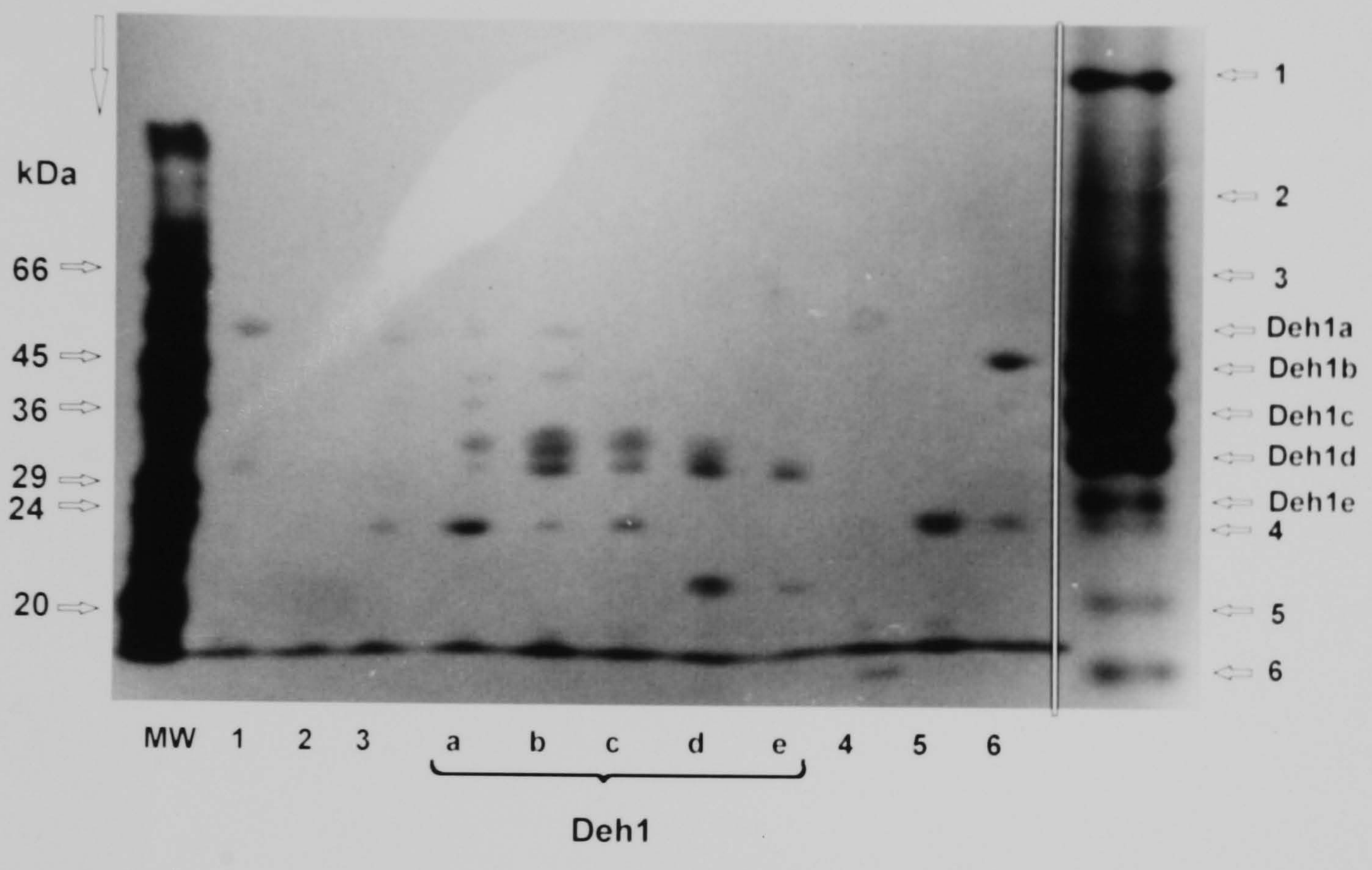


Figure 4.7: SDS polyacrylamide gel of the purified Deh1 dehalogenase protein bands separated on native polyacrylamide gel. The purified Deh1 dehalogenase was electrophoresed on a nondenaturating 8% polyacrylamide gel (see section 2.2.8.1). The gel was briefly stained with Coomassie blue and the slices showing protein excised. The protein was denaturated and the gel slices loaded onto a 12.5% polyacrylamide SDS-PAGE (see section 2.2.8.2). The proteins were silver stained (see section 2.2.8.6.2). The vertical arrow indicates the direction of protein migration.

Similar results were obtained when the fractions of the Octyl-Sepharose CL-4B eluate were analyzed on nondenaturating and SDS polyacrylamide gels (Figure 4.8). The haloalcohol dehalogenase was eluted in a very broad peak and when the corresponding fractions were analyzed on native gels (see section 2.2.8.1) the migration profiles obtained were very different (Figure 4.9). The fractions 50 to 70, containing equivalent

amounts of the two subunits, also had a few high molecular weight contaminants. On native gels these fractions showed the multiple band profile characteristic of the Deh1 dehalogenase. Fractions 86 to 108, which showed on SDS-PAGE decreasing amounts of the 34 kDa protein and large amounts of high molecular weight contaminants, formed predominantly the Deh1c, Deh1d and Deh1e active bands on native gels.

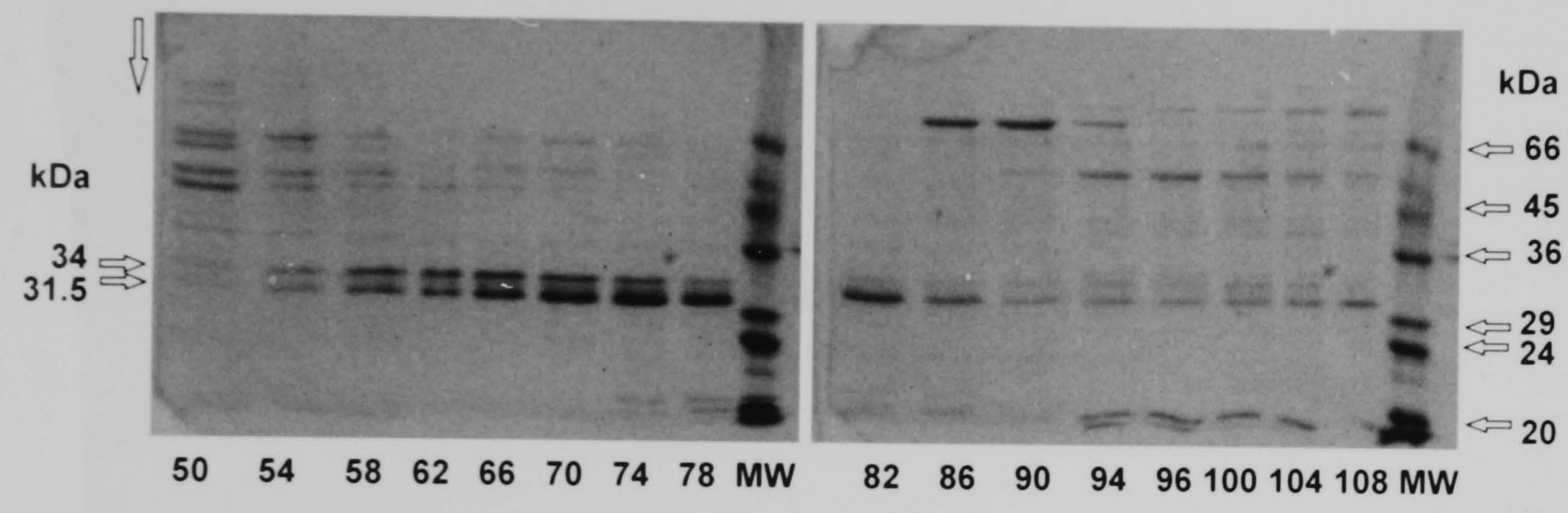


Figure 4.8: SDS polyacrylamide gel of the fractions eluted from the Octyl-Sepharose CL-4B column.
A sample (20 µl) of each fraction (as indicated under each lane) was loaded onto a 12.5% polyacrylamide gel and electrophoresed (see section 2.2.8.2). The gels were stained with Coomassie (see section 2.2.8.6.1). The vertical arrow indicates the direction of protein migration.

The fractions containing large amounts of dehalogenase activity (62 to 78) showed high protein band intensity for the 34 and 31.5 kDa subunits, indicating that the haloalcohol dehalogenase activity might be associated with these two proteins. Furthermore, the fractions that contained higher specific activities (74 and 78) showed only very small amounts of contaminants. These fractions, on native gels, showed high intensity bands for the Deh1d and Deh1e proteins, and some intensity for the Deh1c protein.

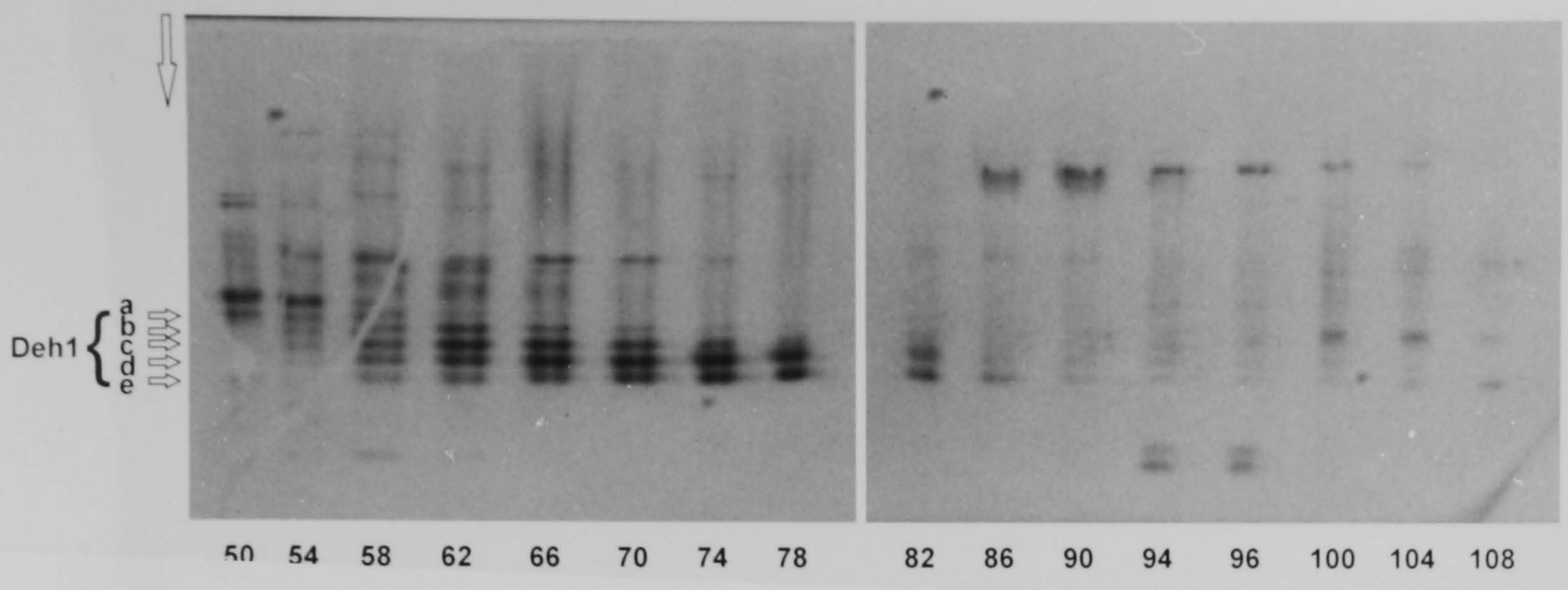


Figure 4.9: Native gel of the fractions eluted from the Octyl-Sepharose CL-4B column. A sample (20 µl) of each fraction (as indicated under each lane) was loaded onto a 8% polyacrylamide gel and electrophoresed under nondenaturating conditions (see section 2.2.8.1). The gels were stained for protein (see section 2.2.8.6.1) and degradation of 1,3-DCP (see section 2.2.8.1). The protein bands with haloalcohol dehalogenase activity were labeled as Deh1a to Deh1e. The vertical arrow indicates the direction of protein migration.

4.3.3. Analysis of the 31.5 and the 34 kDa proteins

In order to investigate homologies between the Deh1 enzyme and others of known protein amino acid sequence, the N-terminal sequence and some internal segments of the 31.5 and 34 kDa proteins were sequenced.

4.3.3.1. Purification of the two subunits

As clear separation of the two subunits was observed on SDS-PAGE, preparative electrophoresis seemed to be the most suitable method for the purification of the 31.5 and 34 kDa proteins. Denaturation of the Deh1 enzyme was achieved by adding SDS to

a final concentration of 0.5%. This sample (500 µg) was then electrophoresed on a 10.25% T/ 2.5% C polyacrylamide gel as described in section 2.2.10.1. The elution profile of the proteins is shown in Figure 4.10. The proteins eluted at the bottom of the gel were collected in 2.5 ml fractions and analyzed on a 12.5% SDS-PAGE (Figure 4.11 A). The lower molecular weight subunit (31.5 kDa) was eluted in fractions 36 to 44 and the larger protein (34 kDa) eluted in fractions 44 to 50. The non-overlapping fractions of each subunit were pooled together and concentrated by ultrafiltration. An unidentified contaminant of approximately 32 kDa was seen in fractions 41 to 45. However, as shown in Figure 4.11 B, this contaminant was present in concentrated fractions in such low amounts that was not detected by Coomassie blue stained SDS-PAGE. The purified proteins were stored at -20°C and later used for amino acid sequencing and peptide mapping.

4.3.3.2. Amino acid sequence

Analysis of the amino acid sequence of the 31.5 and 34 kDa proteins was undertaken by Jay Tee Biosciences (Herne Bay, Kent, UK). Attempts to sequence the N-terminus of the purified Deh1 subunits were not successful, most likely due to modification of the first amino acid. Therefore, both polypeptides were subjected to trypsin digestion and the respective fragments separated by HPLC reverse phase chromatography (see section 2.2.10.2.1). Figure 4.12 shows the elution profiles of the tryptic digests obtained from each subunits. Although a different pattern was obtained for each chromatogram, some peptides showed similar elution volumes, as indicated by the arrows on the Figure 4.12.

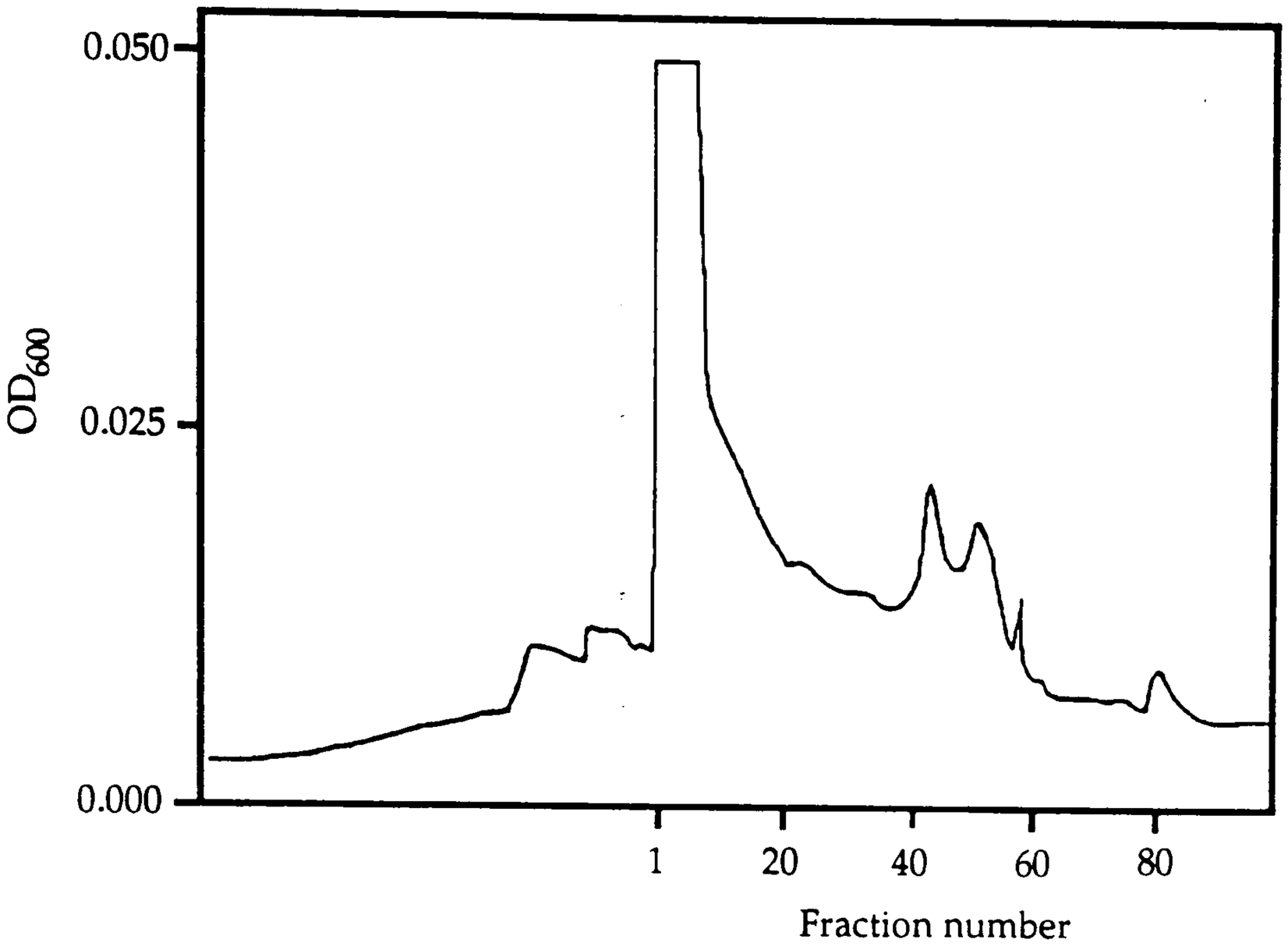


Figure 4.10: Elution profile of the subunits of the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase by preparative gel electrophoresis. The purified Deh1 dehalogenase (500 µg) was denaturated by adding SDS to a final concentration of 0.5%. This protein solution was loaded into a SDS-PAGE, 10.5% T, 2.67% C resolving gel and 4% T, 2.67% C stacking gel as described in section 2.2.10.1. The gel was electrophoresed at 30 mA for 7 h. The elution buffer (25 mM TRIS, 0.2 M glycine, 0.5% SDS) was set to 1 ml min^{-1}. and the fraction collection started just before the ion/dye front eluted from the gel.

The selection of the peptides for amino acid sequencing was based on purity, which was determined by the sharpness and separation of the corresponding chromatographic peaks. Therefore, the selected fractions were: 12 and 22 from the 31.5 kDa tryptic digests; and fractions 12/13, 15 and 36 of the 34 kDa tryptic digests. The amino acid sequence of these peptides is shown in Figure 4.13. Of particular interest is the fact that fractions 12 and 12/13, of the 31.5 and 34 kDa tryptic digests respectively, showed a 100% of homology in 9 out of 10 amino acids. No other peptides from the two subunits with similar elution volumes were sequenced and so the study of the degree of homology between the two proteins was not possible. The amino acid sequence of the peptide eluted in fraction 36 of the 34 kDa tryptic digest showed a highly repetitive sequence. The same results were observed with the amino acid sequence of the fraction 22 obtained from the 31.5 kDa tryptic digest.

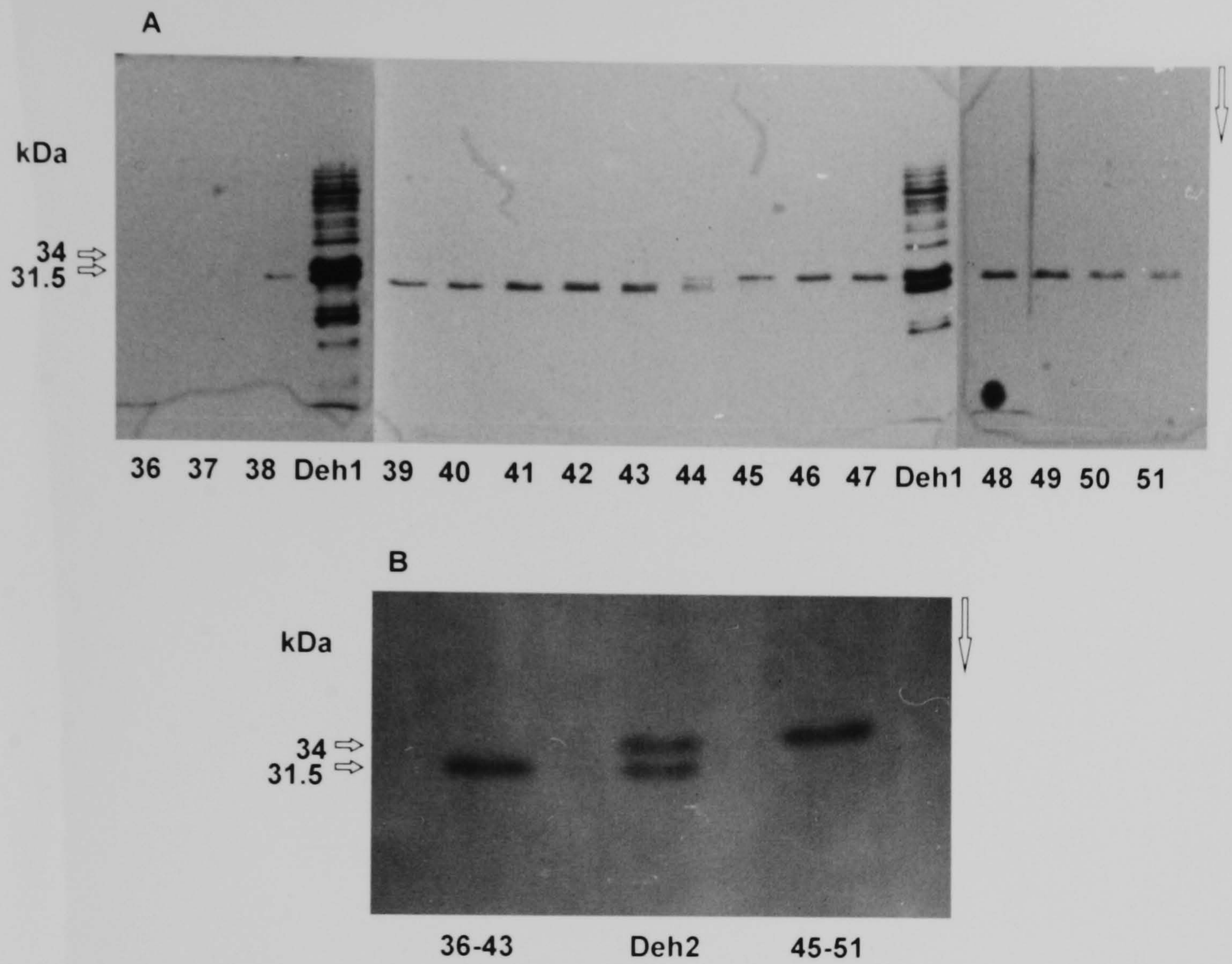


Figure 4.11: SDS polyacrylamide gel of the *Arthrobacter* sp H10a Deh1 dehalogenase fractionated by denaturating electrophoresis. (A) 15 µl sample was electrophoresed on a 12.5% polyacrylamide SDS gel (see section 2.2.8.2). The lane numbers correspond to the fractions collected from preparative electrophoresis. Lane Deh1, 2.5 µg of the purified dehalogenase. The gels were silver stained. (B) The fractions 36-43 and 45-51 were pooled together and concentrated by ultrafiltration. The purity of each fraction (2 µg as indicated above each lane) was investigated on a 12.5% polyacrylamide SDS-PAGE. A Coomassie blue stained gel (see section 2.2.8.6.1) is shown. The vertical arrow indicates the direction of protein migration.

A computer search of the "owl" protein data bank clearly showed the absence of any other proteins with significant similarity to the sequences of each subunit. Furthermore, no homology was found with the other dehalogenases of known sequence.

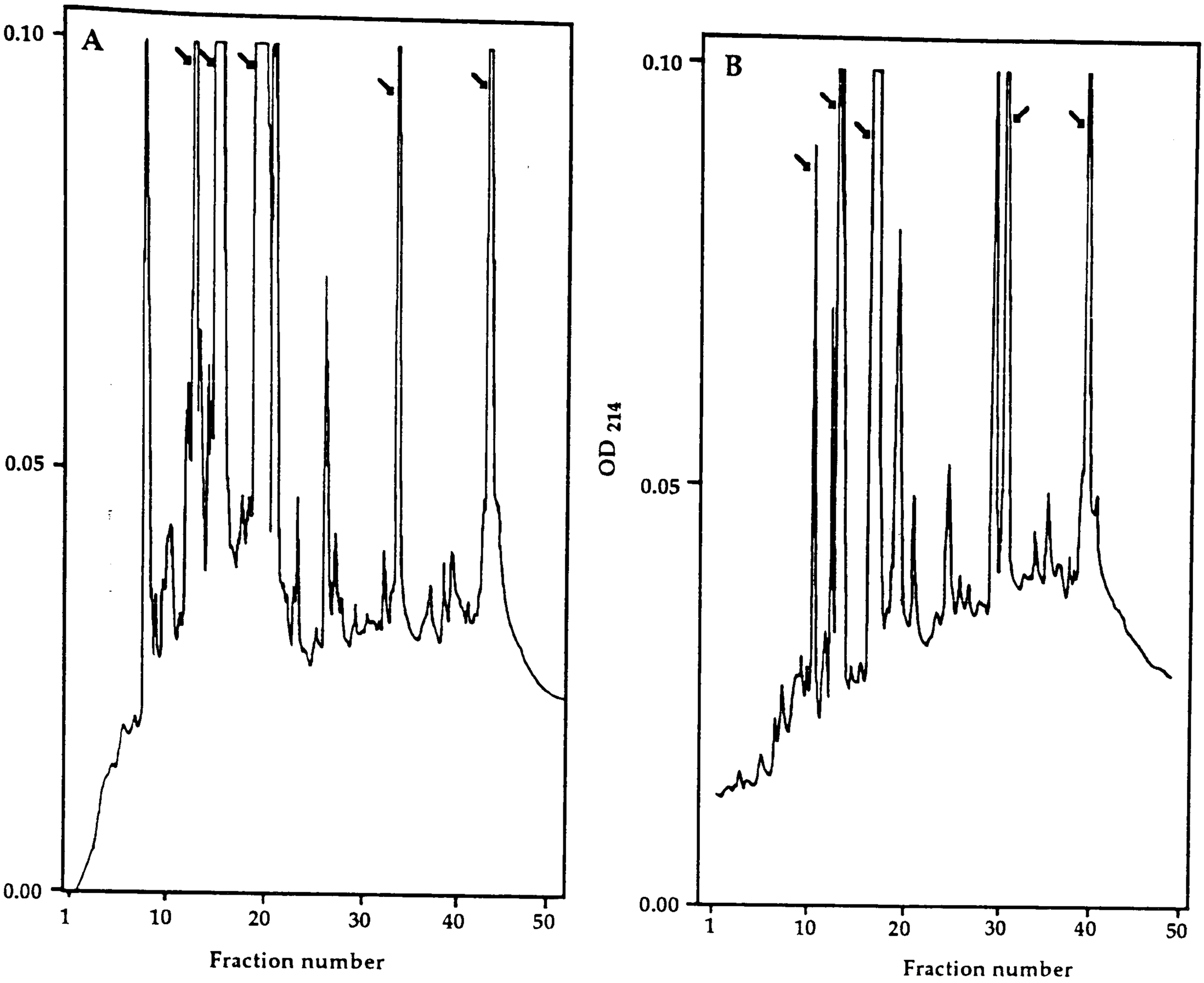


Figure 4.12: Elution profiles of the *Arthrobacter* sp H10a Deh1 dehalogenase subunits by reverse phase chromatography. The purified 31.5 (A) and 34 (B) kDa proteins (50 µg each) were digested with trypsin and the peptides separated by reverse phase column chromatography on a HPLC (see section 2.2.10.2.1). The arrows indicate the peptides eluted at similar elution times.

31.5 kDa tryptic peptides	34 kDa tryptic peptides
Fraction 12	Fraction 12/13
Ser Gln Glu Gly Ala Leu Ala Tyr Ser Thr	Tyr Gln Glu Gly Ala Leu Ala Tyr Ser Thr Ala
Fraction 22	Fraction 15
Asn Val Asn Val Asn Phe Ile Ala Gln	Ser Tyr Val Thr X Leu Lys Pro Glu X Ala
	Fraction 36
	Ala Leu Leu Ala Leu Phe Leu Ala

Figure 4.13: Amino acid sequence of the peptides obtained by trypsin digestion of the 31.5 and 34 kDa proteins. The N-terminal amino acid sequencing of the tryptic digests was performed in an automated gas liquid phase protein sequenator as described in section 2.2.10.2.2. [X , unidentified residue].

4.3.3.3. Peptide mapping of the 31.5 and 34 kDa proteins

The homology between the two subunits of the Deh1 dehalogenase suggested by the sequencing of the tryptic digests (see section 4.3.3.2) was further studied by peptide mapping. The purified 31.5 and 34 kDa subunits obtained by preparative electrophoresis (see section 4.3.3.1) were digested with the endoproteinase Lys-C, endoproteinase Glu-C and alkaline protease (see section 2.2.10.3). As shown in Figure 4.14 (A), the peptides obtained by protease digestion with endoproteinase Lys-C, of the 31.5 and 34 kDa proteins, showed the same number of protein bands and similar apparent sizes. The same results were also found when these two proteins were digested with the endoproteinase Glu-C (Figure 4.14 B) and alkaline protease (Figure 4.14 C), suggesting a high degree of similarity between the two Deh1 subunits. The endoproteinase Lys-C digests showed (Figure 4.14 (A)) only two protein bands on SDS-PAGE suggesting that both subunits have one lysine residue. The endoproteinase Glu-C digests showed 6 peptides (Figure 4.14 (B)), indicating the presence of at least 5 glutamic acid residues on each subunit. The number of hydrophobic and polar residues on the two subunits was high and the proteins digested with low concentrations of alkaline protease showed a large number of fragments, as seen in Figure 4.14 (C).

4.4. Immunochemical analysis

The existence of organisms capable of metabolizing xenobiotic molecules is of considerable evolutionary interest, since these compounds have been introduced in to the environment very recently. Observations on the relationships between the enzymes that catalyze these reactions could shed light on the rates and mechanisms of microbial evolution. In order to investigate whether the Deh1 enzyme of *Arthrobacter* sp H10a was related to other bacterial dehalogenases, a rabbit antibody was raised against the

purified haloalcohol dehalogenase (see section 2.2.9.1).The serum from the rabbit blood was collected by centrifugation (see section 2.2.9.1).

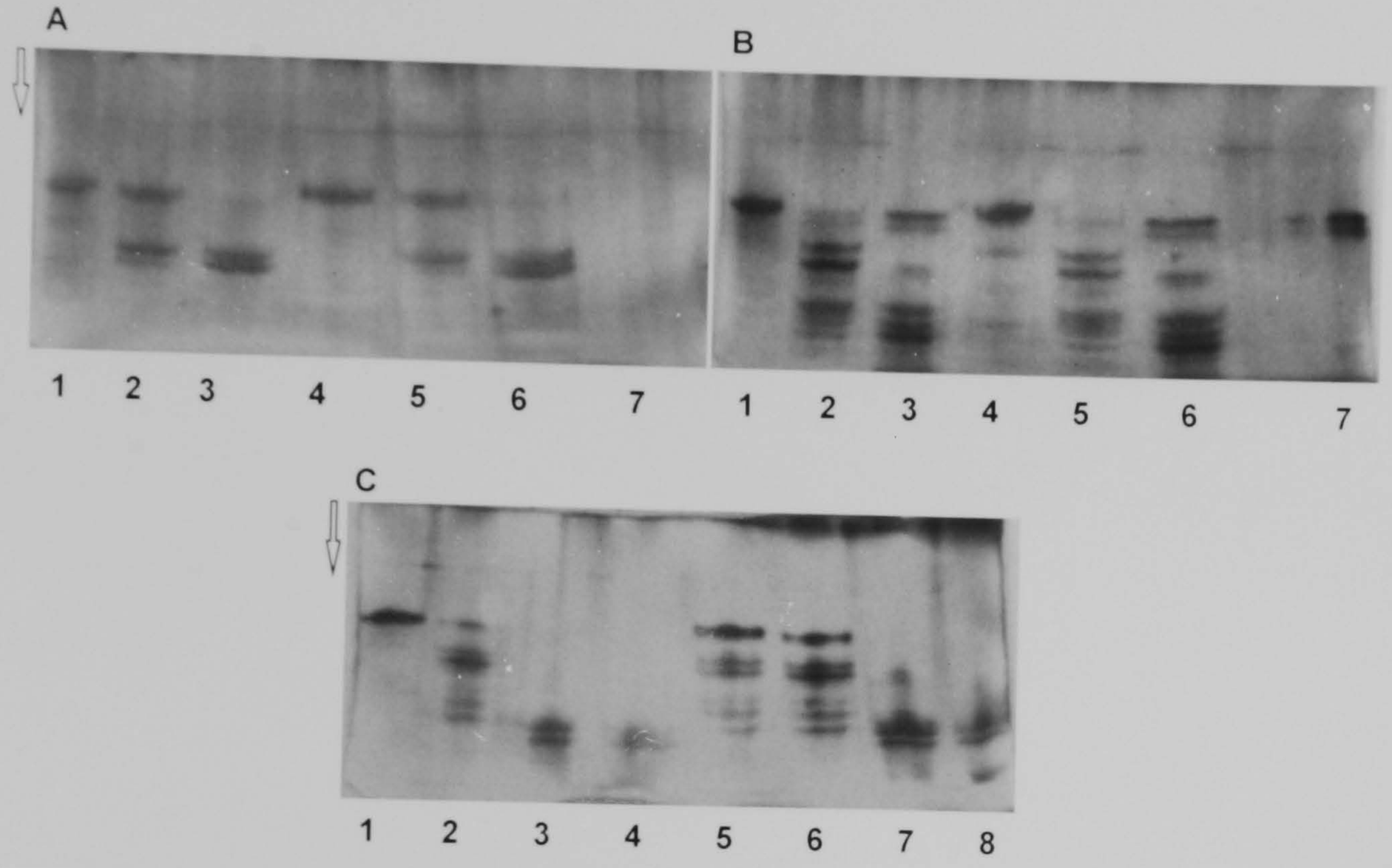


Figure 4.14: SDS polyacrylamide gels showing protease digests of the Deh1 subunits. The peptides were obtained by digesting 2.5 µg of each haloalcohol dehalogenase subunit (see section 2.2.10.3) with (A) lanes 1-3, 30.5 kDa protein, lanes 4-6, 34 kDa protein, lanes 2 and 5 cleavage with Endoproteinase Lys-C (1:100 dilution), lanes 3 and 6 cleavage with Endoproteinase Lys-C (1:10 dilution), lane 7, endoproteinase Lys-C (0.1 µg). (B) as in (A) but endoproteinase Glu-C was used for protein cleavage. (C) lanes 1-4, 30.5 kDa protein, lanes 5-8 34 kDa protein, lanes 2 and 6 cleavage with alkaline protease (1:10,000), lanes 3 and 7 cleavage with alkaline protease (1:1,000), lanes 4 and 8, cleavage with alkaline protease (1:200), lane 9, alkaline protease (0.01 µg). The vertical arrow indicates the direction of protein migration.

Because the enzyme solution used as the antigen (the Octyl-Sepharose eluate) was not sufficiently pure the serum so obtained also showed some unspecific binding on immunoblots. Furthermore, the titre of the serum was very low (1:280). Therefore it was necessary to concentrate and purify the Deh1 polyclonal antibody. An I_gG rich fraction, obtained by precipitation with caprylic acid and ammonium sulphate (see section

2.2.9.3), showed a 5 fold increase in its titre. However, elimination of the unspecific binding was only achieved after immunobloting purification (see section 2.2.9.3).

On Western blot, the purified antibody showed immunocross-reactivity with the 31.5 and 34 kDa proteins of the *Arthrobacter* sp H10a crude extract and the purified fraction of the Deh1 dehalogenase (Figure 4.15 B). Under nondenaturating conditions this antibody reacted with the five protein bands corresponding to the Deh1 dehalogenase (Figure 4.15 E). No immunocross-reactivity was found with the R_f 0.42 band.

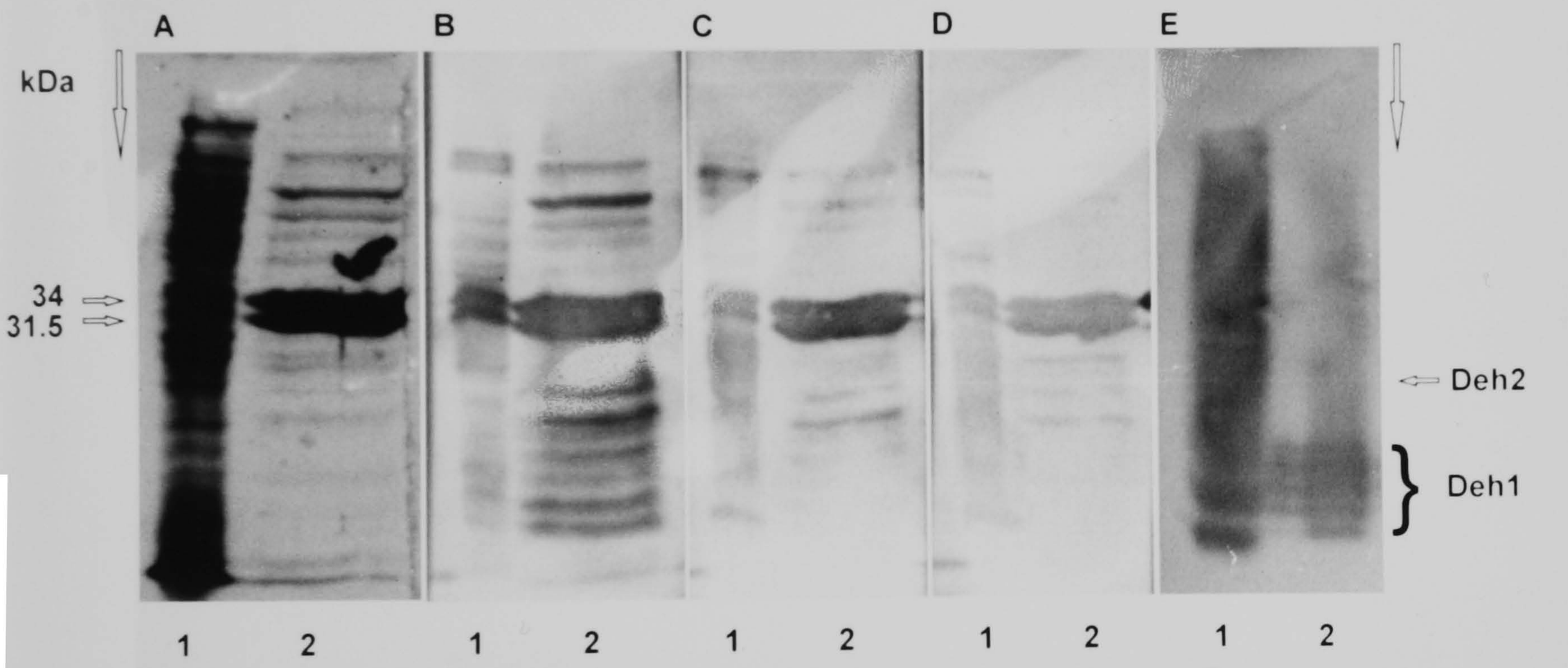


Figure 4.15: SDS polyacrylamide gels and Western blot analysis of the polyclonal antibodies raised against the Deh1 haloalcohol dehalogenase and its subunits. The four panels A-D represent identical gels, and the panel E shows the Western blot of a gel performed under nondenaturating conditions. The specificity of the antibodies raised was tested against crude extract and purified Deh1 dehalogenase of *Arthrobacter* sp H10a (see section 2.2.9.1). In panels B and E the antibody raised against the purified Deh1 enzyme was used. In the panels C and D the used antibodies were raised against the 31.5 and 34 kDa proteins respectively. Lanes: 1, 20 µg *Arthrobacter* sp H10a crude extract; 2 µg purified Deh1 dehalogenase. The vertical arrows indicate the direction of protein migration.

The same results were observed when polyclonal antibodies raised against the purified 31.5 and 34 kDa proteins were tested with the crude extracts and the purified Deh1

enzyme of *Arthrobacter* sp H10a. These antibodies showed immunocross-reactivity with both of subunits of the Deh1 dehalogenase (Figure 4.15 C & D). Furthermore, under nondenaturating conditions these antibodies reacted with the five active bands formed by the Deh1 enzyme. These antibodies showed the same binding properties as the Deh1 antibody.

All the immunobloting experiments shown below were carried out using the purified preparation of the polyclonal antibody raised against the whole Deh1 haloalcohol dehalogenase. The immunochemical relationships between the *Arthrobacter* sp H10a Deh1 enzyme and other bacterial dehalogenases was studied using Western blot techniques. The crude extracts of several haloalcohol degrading bacteria were electrophoresed under native (see section 2.2.8.1) and denaturating conditions (see section 2.2.9.4). The proteins were then transferred on to a nitrocellulose membrane and tested for immunoreaction with the Deh1 antibody (see section 2.2.).

Amongst the haloalcohol degrading bacteria tested only strains H10c and H10f showed immunocross-reactivity with the Deh1 antibody (Figure 4.16 and 4.17). The strain H10c proteins that showed a positive reaction with the antibody co-migrate with the Deh1 enzyme on SDS-PAGE and nondenaturating gels. Of particular interest is the fact that the crude extract of strain H10f has only one protein of 31.5 kDa that showed cross-reactivity with the Deh1 antibody. Furthermore, under nondenaturating conditions the proteins that react with the antibody (R_f 0.68, 0.63 and 0.58) showed no dehalogenase activity (Figure 4.17). The strain H10f possesses a haloalcohol dehalogenase which migrates to the same position as Deh1a, which did not showed immunocross-reactivity with the Deh1 antibody. The Octyl-Sepharose CL-4B fractions 86-108 (Figure 4.8 and 4.9), that displayed higher proportion of the 31.5 kDa protein in relation to the one of 34 kDa, in nondenaturating polyacrylamide gels also showed only three protein bands, with R_f similar to the ones above.

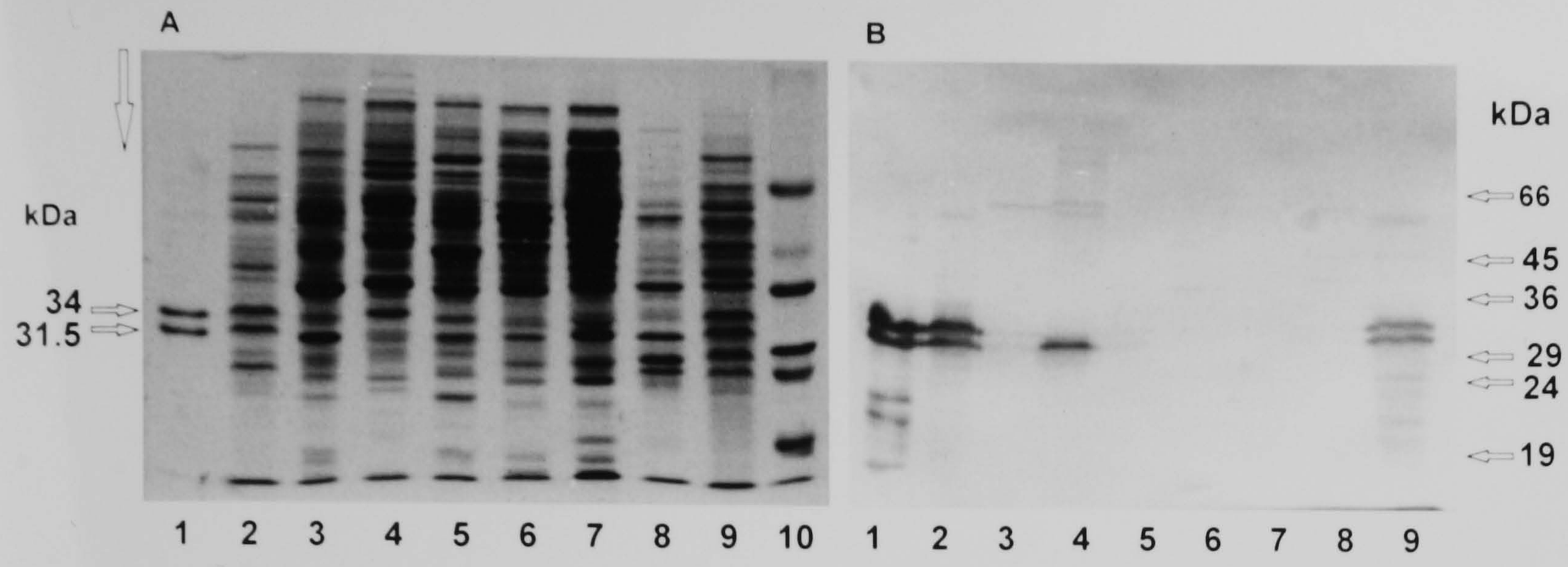


Figure 4.16: SDS polyacrylamide gel and Western blot analysis of the crude extracts of haloalcohol degrading bacteria. The panel A and B represent identical gels that were run in parallel. The immunoreaction of the purified Deh1 antibody against the proteins immobilized on the nitrocellulose membrane was performed as described in section 2.2.9.4. Lanes: 1, purified Deh1 dehalogenase; 2, *Arthrobacter* sp H10a; 3, mixed culture; 4, coryneform rod strain H10f; 5, *Agrobacterium* sp biovar I H10e; 6, *Agrobacterium tumefaciens* HK7; 7, *Pseudomonas cepacia* H10g; 8, *Arthrobacter histidinolovorans* HK1; 9, Coryneform bacteria H10c; 10, molecular weight markers. Lane 1 contained 2.5 µg of protein and lanes 2-9 15 µg of protein. The vertical arrow indicates the direction of protein migration.

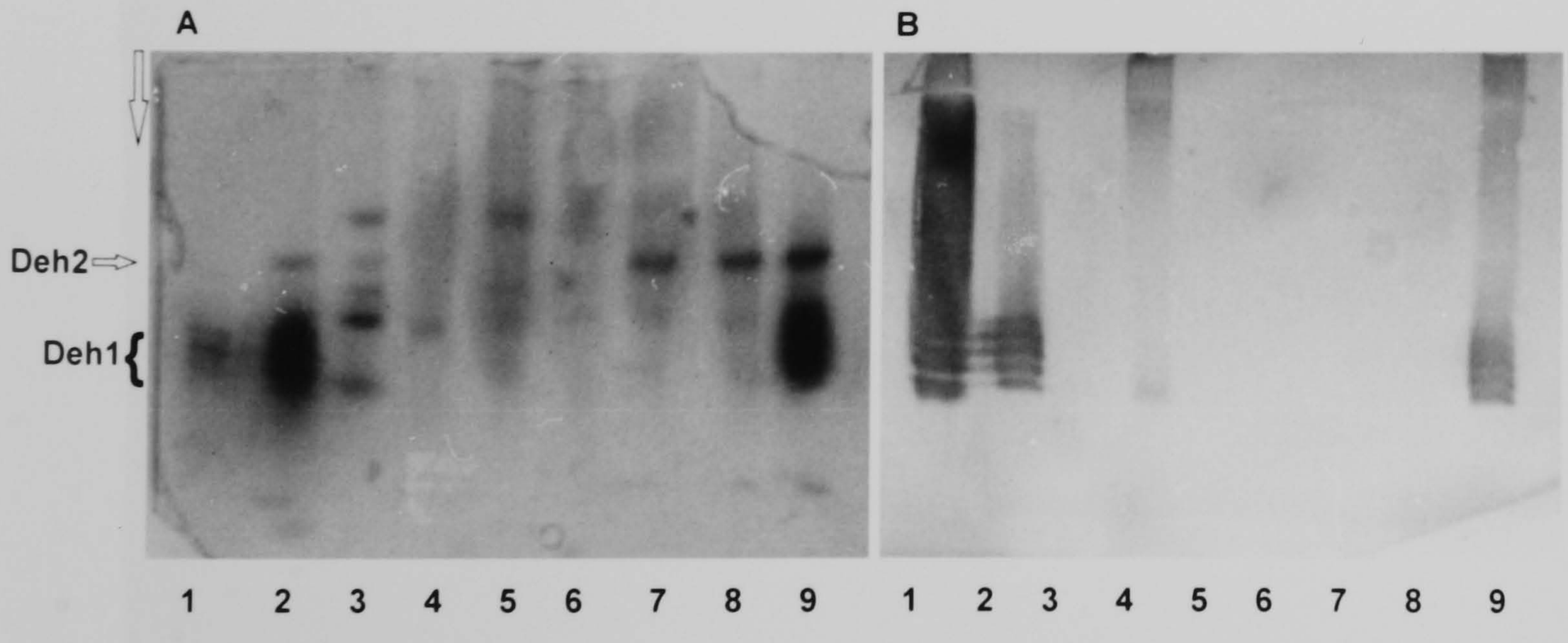


Figure 4.17: Native polyacrylamide gel of the haloalcohol dehalogenases and their Western blot analysis. The panel A and B represent identical gels that were run in parallel. The immunoreaction of the purified Deh1 antibody against the proteins immobilized on the nitrocellulose membrane was performed as described in section 2.2.9.4. Lanes: 1, purified Deh1 dehalogenase; 2, *Arthrobacter* sp H10a; 3, mixed culture; 4, coryneform rod strain H10f; 5, *Agrobacterium* sp biovar I H10e; 6, *Agrobacterium tumefaciens* HK7; 7, *Pseudomonas cepacia* H10g; 8, *Arthrobacter histidinolovorans* HK1; 9, Coryneform bacteria H10c. Lane 1 contained 3 µg of protein and lanes 2-9 20 µg of protein. The vertical arrow indicates the direction of protein migration.

Immunocross reaction of the Deh1 antibody was also investigated with the crude extracts of other bacteria that possess one or more haloacid and haloalkane dehalogenating enzymes. The selection of these isolates was based on the electrophoretic mobility of their dehalogenases on nondenaturating polyacrylamide gels, and included a wide range of different enzymes. As shown in Figures 4.18 and 4.19, no immunological cross-reactivity was found with the crude extracts of haloacid or haloalkane degrading bacteria under denaturating conditions. The same results were obtained under nondenaturating conditions.

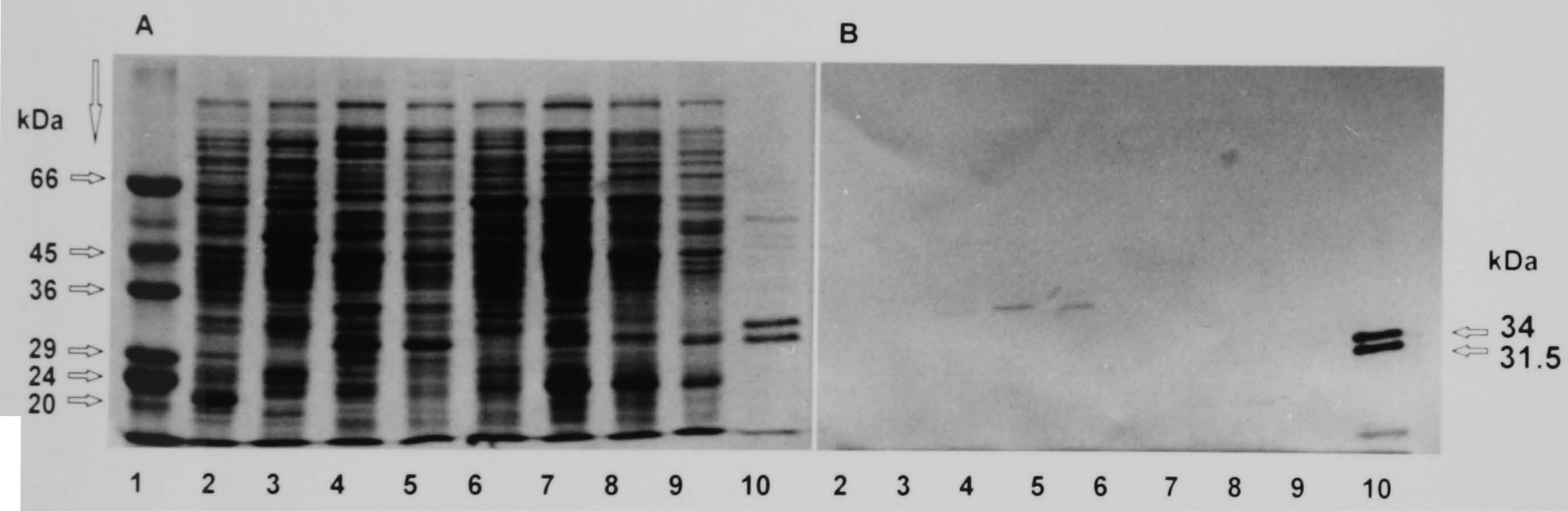


Figure 4.18: SDS polyacrylamide gel and Western blot analysis of the crude extracts of haloacid degrading bacteria. The panel A and B represent identical gels that were run in parallel. The immunoreaction of the purified Deh1 antibody against the proteins immobilized on the nitrocellulose membrane was performed as described in section 2.2.9.4. Lanes: 1, molecular weight standards; 2, strain A5-12; 3, *Pseudomonas cepacia* MBA4; 4, *Pseudomonas* K37; 5, strain I4; 6, *Bacillus* sp R1; 7, strain F10; 8, strain F1; 9, *Pseudomonas putida* PP3; 10, strain H10a Deh1 dehalogenase. Lanes 2-9 contained 15 µg of protein and lane 10, 2.5 µg of protein. The vertical arrow indicates the direction of protein migration.

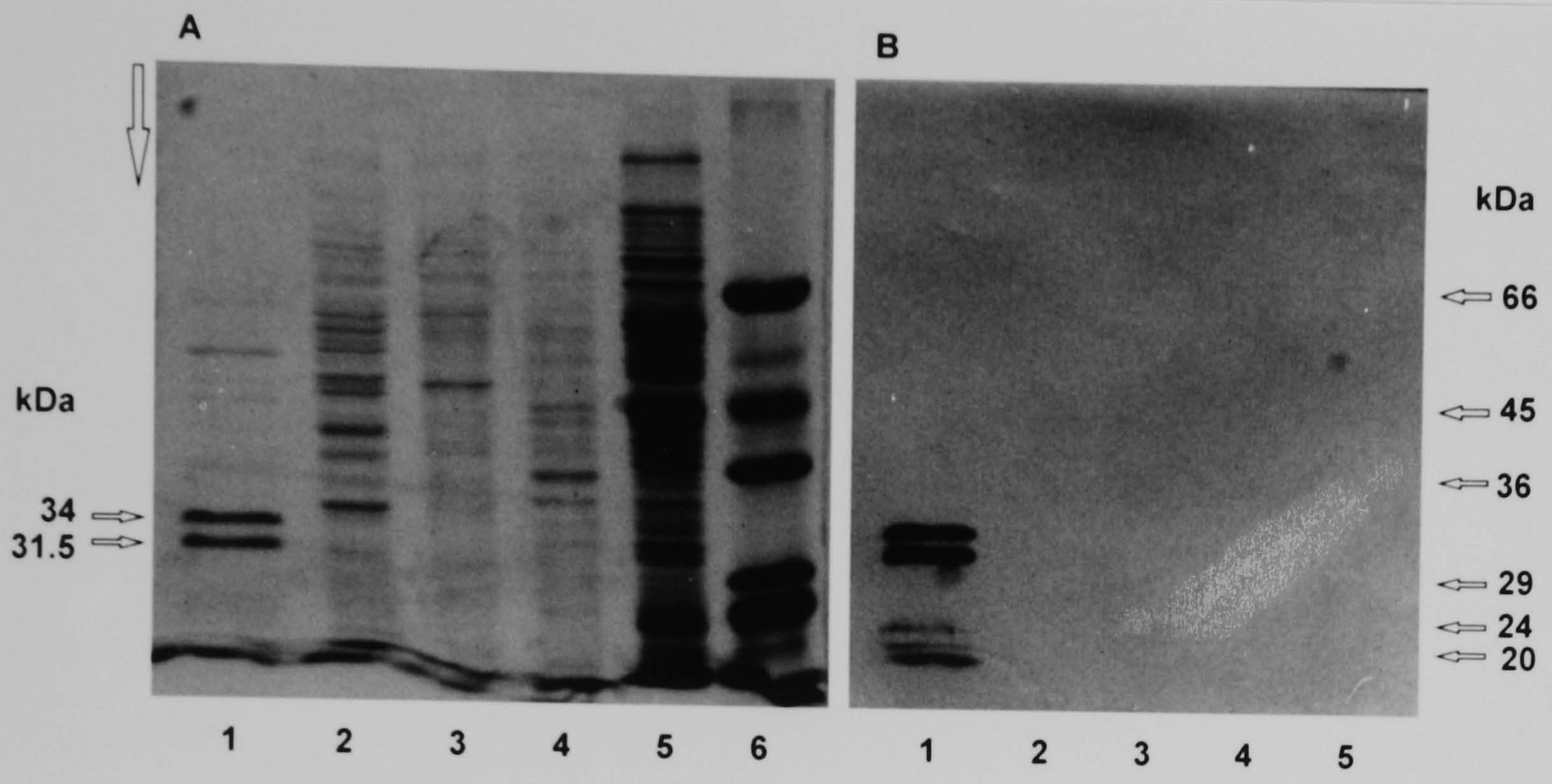


Figure 4.19: SDS polyacrylamide gel and Western blot analysis of the crude extracts of haloalkane degrading bacteria. The panel A and B represent identical gels that were run in parallel. The immunoreaction of the purified Deh1 antibody against the proteins immobilized on the nitrocellulose membrane was performed as described in section 2.2.9.4. Lanes: 1, strain H10a Deh1 dehalogenase; *Arthrobacter* sp HA-1; 3, *Rhodococcus erythropolis* Y2; 4, *Xanthobacter autotrophicus* GJ10; 5, *Pseudomonas* sp E4M; 6, molecular weight markers. Lane 1 contained 2.5 µg of Deh1 dehalogenase and lanes 2-5, 15 µg of protein. The vertical arrow indicates the direction of protein migration.

4.5. Discussion

The purification methods described above proved effective in the partial purification of the *Arthrobacter* sp H10a haloalcohol dehalogenase Deh1, as shown on the SDS-PAGE of the Octyl-Sepharose CL-4B eluate (Figure 4.3). Further purification of the enzyme using gel filtration (G-100) of hydroxypatite columns (Bio-Gel HT) resulted in very little improvement in its purity but resulted in significant loss of activity. The Octyl-Sepharose CL-4B chromatography was the most effective procedure in the purification of the *Arthrobacter* sp H10a haloalcohol dehalogenase. A large fraction of the DEAE-Sephacel

eluated proteins did not bind or were eluted very early in the ammonium sulphate gradient (Figure 4.2) while the dehalogenase was eluted as a single peak at lower ionic strength.

The purified haloalcohol dehalogenase showed two major bands with the molecular weight of approximately 31.5 and 34 kDa on SDS-PAGE. It was shown previously (see section 3.3.) that these two proteins were induced in medium containing glycidol and they were designated as the Deh1 haloalcohol dehalogenase. The molecular mass of the Deh1 enzyme found by gel filtration (200 kDa) suggested that this enzyme is a hexamer composed of two subunits in 1:1 proportions (on the basis of the intensity of the stained protein bands on SDS-PAGE). However, this may not be the case, since it was found that some high molecular weight proteins co-migrated with the 31.5 and 34 kDa subunits on nondenaturating polyacrylamide gels (Figure 4.6). In addition no other protein peaks were found on the Superose 12 column chromatogram (Figure 4.5) probably indicating that these proteins were eluted with the Deh1 dehalogenase and possibly associated with it.

With the exception of the enzyme of *Pseudomonas putida* AJ1/23 which is a tetrameric protein (Smith *et al.*, 1990), all the dehalogenases purified to date have been monomers or dimers of a single subunit. The *Arthrobacter* sp H10a Deh1 and the *Corynebacterium* sp N-1074 (Nakamura *et al.*, 1992) I_b enzymes are the first dehalogenases reported to be composed of two different polypeptides. Although the molecular weight of the subunits of these two dehalogenases was found to be similar, the strain H10a enzyme showed a larger molecular mass than the I_b protein (Table 4.2). The *Arthrobacter* sp AD2 (van den Wijngaard *et al.*, 1991) and the *Corynebacterium* sp N-1074 I_a (Nagasawa *et al.*, 1992) haloalcohol dehalogenases were found to be respectively tetramers or dimers of identical subunits. The molecular weight of the subunits of these two enzymes was very close, 28 and 29 kDa (Table 4.2).

Table 4.2: Molecular weight of the haloalcohol dehalogenases.

	Molecular Weight (kDa)		Reference
	Subunit	Native	
Arthrobacter sp AD2	29	69 - dimer	van den Wijngaard *et al.*, 1991
Corynebacterium sp N-1074 I_a	28	105 - tetramer	Nagasawa *et al.*, 1992
Corynebacterium sp N-1074 I_b	35 & 32	115 -tetramer	Nakamura *et al.*, 1992
Arthrobacter sp H10a	34 & 31.5	200 - hexamer	This work

Peptide mapping experiments have demonstrated a high degree of homology between the two Deh1 subunits. The peptides obtained by protease digestion of the two subunits showed similar profiles on SDS-polyacrylamide gels. The resolution of this type of gel is too low to detect very small differences (less than 1 mm) in the migration of the peptides from different subunits. Unfortunately, the technology available in the laboratory did not allow determination of either the location or the type of modification that caused the different migration profiles in SDS-PAGE. The HPLC profiles of the tryptic digests (Figure 4.12) obtained from the two Deh1 subunits were different, yet some peptides exhibited the same elution volumes. The sequence of the fractions 12 and 12/13 of the 31.5 and 34 kDa proteins respectively, showed 100% of homology in 9 out of 10 amino acids. Further evidence of the homology between the two polypeptides was obtained with the antibodies raised against each one of the purified Deh1 subunits. These antibodies reacted with equal intensity to both subunits on SDS-PAGE immunoblots (Figure 4.15). Separation of the two polypeptides was only achieved after denaturation of the Deh1 enzyme with 0.5% of SDS. The dehalogenase activity was not recovered after removal of the detergent, indicating that irreversible denaturation had occurred. Therefore it was not possible to study the function of each subunit on the overall structure and activity of the enzyme.

The enzyme solution, when electrophoresed under nondenaturating conditions, produced 5 active bands with R_f of 0.51, 0.55, 0.58, 0.62 and 0.65. The dehalogenase band with an R_f of 0.42 found in the H10a crude extracts was lost during the haloalcohol dehalogenase purification. The antibody raised against the Deh1 enzyme showed no immunoreaction, on nondenaturating gels, with the protein band at R_f 0.42. These results suggested that this protein band was not immunologically related with the Deh1 dehalogenase; hence this dechlorination activity seems to result from the activity of a completely different enzyme. The haloalcohol dehalogenase that runs at R_f 0.42 may be related to the enzyme fraction that showed higher affinity towards CPD and was designated as Deh2 (see section 3.3). However, because of the very low levels of haloalcohol dehalogenase activity towards CPD found on strain H10a crude extracts, the fractions obtained during the purification of this enzyme were not tested for CPD dehalogenation. The Deh1 dehalogenase was eluted as a single peak from the Octyl-Sepharose CL-4B column. No other traces of dehalogenase activity towards 1,3-DCP were found on the proteins which did not bind to the hydrophobic matrix or were eluted early in the $(NH_4)_2SO_4$ gradient. The loss of the Deh2 dehalogenase might be due either to its denaturation or because it remained bound to the gel matrix.

The dehalogenase I_b of *Corynebacterium* sp N-1074 has shown similar profiles to the Deh1 enzyme on nondenaturating polyacrylamide gels (Nakamura *et al.*, 1992). The purified I_b dehalogenase migrated as 4 protein bands that, by two dimensional electrophoresis, were shown to be composed of 4 peptides mixed in different ratios of the 32 and 35 kDa subunits. Those subunits were combined on the ratio (35:32) of 4:0, 3:1, 2:2, 1:3 and a fifth band, barely visible in Coomassie stained gels, composed of four 32 kDa polypeptides. Two dimensional gels of the Deh1 dehalogenase performed under conditions similar to the ones described by Nakamura *et al.* (1992) showed similar ratios of the 31.5 and 34 kDa proteins. However, because only a small proportion of the proteins on the first dimension gel was denaturated, these protein spots were too faint to

allow any conclusion to be drawn. It was not possible to determine the composition of each of the protein bands formed on nondenaturing gels. Nevertheless, two factors have been shown to influence their migration: (1) the proportion of the 31.5 and 34 kDa proteins and (2) the size and the relative amount of other proteins (Figure 4.7). The role of the proteins that co-migrate with this two subunits is still unclear. It was not possible to distinguish if this association occurred within the cells or was caused by the change of the environment conditions that occurred during the preparation of the crude extract.

Comparison of the tryptic digest sequences obtained from the 31.5 and 34 kDa subunits did not reveal any significant sequence homology. There is also very little information about other bacterial haloalcohol dehalogenases. Nagasawa *et al.* (1992) and Nakamura *et al.* (1992) claimed that they have cloned both genes encoding the two dehalogenases of *Corynebacterium* sp N-1074, but until now the sequences of these genes have not been published. However, the N-terminal sequence of the enzyme I_a was quite similar to the one of the *Arthrobacter* sp AD2 enzyme (Nagasawa *et al.*, 1992), suggesting once more a high degree of similarity between the two proteins.

Immunobloting experiments were performed in order to test whether the Deh1 haloalcohol dehalogenase of *Arthrobacter* sp H10a was related to other bacterial enzymes that catalyzed the same reaction. Among the bacteria tested only crude extracts of strain H10c and H10f gave positive reaction with the antibodies raised against the strain Deh1 enzyme (Figure 4.16 and 4.17). The haloalcohol dehalogenase of strain H10c showed the same electrophoretic mobility as the Deh1 protein under both native and denaturing conditions. The Deh1 antibody bound the H10c proteins at the same position as the H10a Deh1 enzyme on SDS and native PAGE. Furthermore, the strain H10c is a Coryneform bacterium that showed similar cell morphological features to the *Arthrobacter* sp H10a, suggesting a high degree of similarity between the two bacteria and their dehalogenases.

The strain H10f is also a Coryneform rod that shows poor growth in minimal medium containing halogenated alcohols as the sole carbon and energy source. In addition, the crude extracts of this bacterium have shown very low dehalogenase activity (P.J. Sallis, personal communication). A 31.5 kDa protein from strain H10f showed reaction with the Deh1 antibodies suggesting that this protein might be similar to the low molecular weight subunit of the Deh1 haloalcohol dehalogenase. Under nondenaturating conditions this protein migrated to similar positions as the Deh1 dehalogenase 31.5 kDa enriched fractions that were eluted from the Octyl-Sepharose CL-4B column at low salt concentrations (Figure 4.8 and 4.9). However, no dehalogenase activity was found at these positions on native gels of the strain H10f. These results indicate that the high dehalogenase activity found with the Deh1 enzyme may be associated with either the 34 kDa protein, or, is dependent on the quaternary structure of the enzyme determined by the assembly of the subunits.

The Deh1 antibody was also tested against the crude extracts of dehalogenating bacteria representing a wide number of halidohydrolases, as distinguished by their electrophoretic mobility on nondenaturing gels. The Deh1 antibodies did not show reaction with their crude extracts, indicating that the enzymes probably have no strong homology. These results are in agreement with the lack of homology found between the sequenced Deh1 tryptic digests and the other dehalogenases of known sequence. The same results were obtained with the antibody raised against the *Arthrobacter* sp AD2 haloalcohol dehalogenase (van den Wijngaard *et al.*, 1991). No immunological cross-reactivity was found with the crude extracts of haloacid and haloalkane degrading bacteria, suggesting that the haloalcohol dehalogenating enzymes represent a distinct group of dehalogenases.

CHAPTER 5

BIOCHEMICAL CHARACTERIZATION OF THE *ARTHROBACTER* sp H10a Deh1 HALOALCOHOL DEHALOGENASE

5.1. Introduction

Biochemical and biophysical studies of dehalogenases have been the main source of information used in the characterization and grouping of these enzymes. More recently, the isolation and sequence analysis of genes encoding halidohydrolases has shed more light on the relationships between these enzymes.

One of the first points addressed in the study of the aliphatic dehalogenases was their substrate specificities. This allowed for their subdivision into haloacid, haloalkane and haloalcohol dehalogenases. Among these three groups, the enzymes have also shown different substrate specificities depending on the chain length, position and number of the halogens substituents. Grouping of the halidohydrolases according to their biochemical characteristics proved to be a good system of classification of these enzymes. Molecular biology studies have shown that the 2-haloacid halidohydrolases classified within one mechanism group displayed homologous amino acid sequences (see section 1.3.1). The haloalkane dehalogenating enzymes have been classified into three groups according to the reaction catalyzed and their substrate specificities. Furthermore, the N-terminal amino acid sequence of these dehalogenases was conserved amongst each enzyme group, but different among the three groups (see section 1.3.2).

Only recently, the haloalcohol degrading enzymes have received some attention, and only three enzymes have so far been purified. The haloalcohol dehalogenases from the Coryneform strain AD3, *Arthrobacter* sp AD2 (van den Wijngaard *et al*, 1991) and enzyme I_a of *Corynebacterium* sp N-1074 (Nagasawa *et al*., 1992) have shown similar substrate profiles, with great activity towards the brominated analogues and relatively high activity towards CPD. Biophysical characterization of these enzymes revealed that: (1) the AD3 and the AD2 dehalogenases were immunologically related (van den

Wijngaard *et al.*, 1991); (2) and the *Corynebacterium* sp N-1074 I_a enzyme possessed an N-terminal amino acid sequence quite similar to the one of strain AD2 (Nagasawa *et al.*, 1992).

The haloalcohol dehalogenase I_b of *Corynebacterium* sp N-1074, which demonstrated very low activity towards CPD and only a slight increase in activity towards the brominated analogues, was immunologically distinct from the enzyme I_a (Nakamura *et al.*, 1992). The former enzyme may represent a distinct group of haloalcohol dehalogenases. The comparison of the substrate profiles of crude extracts of other halohydrin degrading bacteria indicated that the haloalcohol dehalogenases might be a relatively heterogeneous group of enzymes (see section 1.3.3). However, a detailed study on the biochemistry of these enzymes is necessary to enable definition of their groupings.

Using activity stained polyacrylamide gel electrophoresis (Tsang *et al.*, 1988) it was possible to distinguish 10 different 2-haloacid halidohydrolases on the basis of the electrophoretic mobilities. Although this method is useful in identifying different isoenzymes it is limited in distinguishing homologuos enzymes. For example the 2-haloacid halidohydrolases of *Pseudomonas cepacia* MBA4, *Xanthobacter autotrophicus* GJ10 and *Pseudomonas* sp CBS3 showed different electrophoretic mobilities (Tsang *et al.*, 1988). However, they belong to the same group of 2-haloacid halidohydrolases (type 1) and have also shown homologous amino acid sequences (van der Ploeg *et al.*, 1991; Murdiyatmo *et al.*, 1992).

The biochemical characteristics of the purified *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase will be described in this chapter. Their features will be compared with the other dehalogenases which catalyze the same type of reaction.

5.2. Degradation of halohydrins and epoxides by *Arthrobacter* sp H10a

5.2.1. Dehalogenation of haloalcohols

The products formed during incubation of haloalcohols and epoxides with crude extracts and the purified Deh1 haloalcohol dehalogenase from *Arthrobacter* sp H10a were qualitatively analyzed by gas chromatography-mass spectrometry (see section 2.2.3.5). The H10a crude extracts catalyzed the complete degradation of 1,3-DCP to CPD (Table 5.1). When the Deh1 enzyme was the biocatalyst used, 1,3-DCP was dehalogenated with simultaneous production of ECH. The Deh1 dehalogenase showed no activity towards ECH, while this compound was quickly hydrolyzed to CPD when incubated with the crude extract of *Arthrobacter* sp H10a (Table 5.1). Degradation of CPD by the crude extract and the Deh1 dehalogenase was a slow process, and trace amounts of glycerol or GDL were produced, respectively (Table 5.1). The Deh1 enzyme showed no activity towards GDL. This compound was slowly hydrolyzed to glycerol by the crude extract of strain H10a (Table 5.1). No significant degradation of these substrates was found with the controls that contained all the assay components except the enzyme preparations.

Table 5.1: Degradation of halogenated alcohols and epoxides by *Arthrobacter* sp H10a. The indicated substrates were incubated with the strain H10a crude extracts (5 U ml^{-1}) and the purified Deh1 haloalcohol dehalogenase (5 U ml^{-1}), for 2 h at 30°C (see section 2.2.6.1). 500 µl samples were removed and extracted with 1.0 ml of ethyl acetate. The reaction products were determined by gas chromatography-mass spectrometry as described in section 2.2.3.5 and 2.2.3.6.

Substrates	Products		
	No enzyme	Crude extract	Deh1 haloalcohol dehalogenase
1,3-DCP	1.3-DCP	CPD	1.3-DCP, ECH
CPD	CPD	CPD, Glycerol	CPD, GDL
ECH	ECH	CPD	ECH
GDL	GDL	GDL, Glycerol	GDL

5.2.2. Transhalogenation

The reverse reaction, halogenation of epihalohydrins, was tested with ECH and EBH in the presence of 0.1 M halide. At timed intervals samples were removed and the product formation qualitatively monitored on a gas chromatograph-mass spectrometer as described in section 2.2.3.6. When ECH was used as the substrate in the presence of 0.1 M KBr, the epoxide was rapidly consumed and EBH, BCP and 1,3-DBP were produced almost simultaneously (Table 5.2). When the starting substrates were EBH and KCl, the epoxide was rapidly converted to BCP (Table 5.2). Only small amounts of ECH were produced during the first 30 min, while the EBH concentration continued to decrease and the BCP concentration increased. At the end of the reaction the compounds detected were ECH, EBH and BCP (Table 5.2). The rate of chlorination of EBH was slower than the rate found for bromination of ECH. The blank, containing all the reaction mixture components except the Deh1 dehalogenase, showed no convertion of the substrates.

Table 5.2: Transhalogenation of epoxides catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. The reaction vials (1.0 ml) contained 100 mM TRIS-SO_4, pH 8.0, 100 mM of the indicated salt and 10 mM of the epoxide. The reaction mixtures were incubated at 30°C until the reaction stooped (ca. 2 h). A 100 µl sample was removed and analysed on a GC-MS (see section 2.2.3.6).

	Substrate / Salt	Products
Reaction 1	ECH / KBr	ECH, BCP, EBH, 1,3-DBP (1:3:4:0.8)
Reaction 2	EBH / KCl	ECH, BCP, EBH (1:4:1)

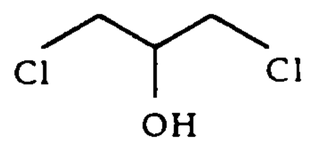
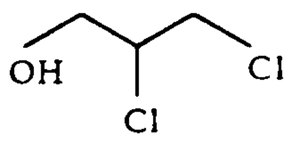
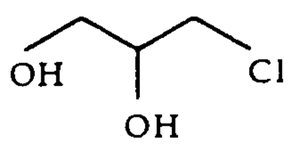
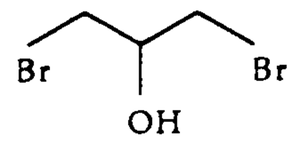
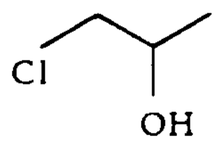
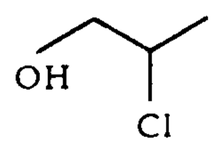
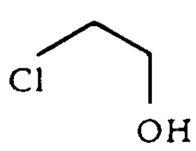
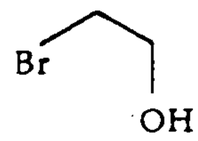
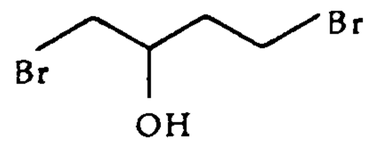
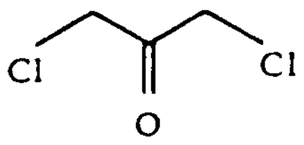
5.2.3. Substrate specificity

The *Arthrobacter* sp H10a dehalogenase activity was studied towards a range of halogenated hydrocarbons using crude extracts, which contained the two dehalogenases, and purified Deh1 enzyme, (Table 5.3). It was found that haloacids, haloalkanes and halophenols were not dehalogenated by either the Deh1 enzyme or the H10a crude extracts. The enzyme preparations were not capable of dehalogenating 2-chloroethylether or 1,1,1-trichloro-2-methyl-2-propanol. The Deh1 dehalogenase showed no activity towards epichlorohydrin. However, some chloride release (0.034 U mg^{-1}, 1.7%) was observed when this compound was incubated with the H10a crude extracts. Both enzyme preparations dehalogenated mono- and di-substituted chloro- and bromo- alcohols with hydroxy and halogen substituents on vicinal carbons (Table 5.3). Neither the H10a crude extract nor the purified Deh1 dehalogenase showed activity towards 3-chloro- or 3-bromo-propanol. Furthermore, the activity towards ß-substituted hydroxy hydrocarbons was higher than the α-substituted, as found with 2,3-DCP and 2-chloropropanol (Table 5.3). Of the chlorinated alcohols tested, 1,3-DCP was the best substrate.

The H10a crude extracts and the purified Deh1 dehalogenase demonstrated different substrate specificities. The most notable difference was found in their activities towards 1,3-DBP, 1,4-DBB and CPD (Table 5.3). The relative activity of the Deh1 enzyme towards the brominated alcohols was low when compared to the values obtained with the H10a crude extracts. The activity towards CPD of the Deh1 dehalogenase was less than 0.1%, while activity towards CPD of the H10a crude extract accounted for 8% of the activity towards 1,3-DCP. The purified Deh1 enzyme showed no activity towards haloalcohols with the hydroxy group positioned in the α-carbon, as found with 2,3-DCP and 2-chloropropanol (Table 5.3). The relative activities of the H10a crude extract towards 2-bromoethanol and 1-chloro-2-propanol were twice the values found for the

Deh1 enzyme (Table 5.3). The Deh1 enzyme also catalyzed the dehalogenation of 1,3-dichloroacetone (Table 5.3).

Table 5.3: Relative activity of the crude extract and the purified Deh1 enzyme of *Arthrobacter* sp H10a towards halogenated hydrocarbons. All the assays were performed at 30°C and contained 6.5 ml 100 mM TRIS-SO_4, pH 8.0, 6.5 µl of the indicated substrate, and 5-200 µl of enzyme solution (section 2.2.6.1). A rate of 100% corresponds to 1.95 U mg^{-1} (crude extracts) or 168 U mg^{-1} (Deh1 haloalcohol dehalogenase), which was found with 1,3-DCP as substrate. All the other values were standardized with respect to either of these values. No halide release was found with epichlorohydrin, chloroacetic acid, dichloroacetic acid, 2-chloropropionic acid, 1,3-dichloropropane, 1,2-dibromopropane, 1-chloropropane, 1-chlorobutane, 1,2-dibromobutane, 1,2-dibromoethane, 2-chloroethylether, 2,4-dichlorophenol, 2,4-dibromophenol. ND, not determined.

Compound	Structure	Relative activity (%)	
		Crude extract	Deh1 dehalogenase
1,3-dichloro-2-propanol (1,3-DCP)		100	100
2,3-dichloro-1-propanol (2,3-DCP)		ND	0
3-chloro-1,2-propanodiol (CPD)		8	>0.1
1,3-dibromo-2-propanol (1,3-DBP)		12400	60
1-chloro-2-propanol (1-MCP)		64	27
2-chloro-1-propanol (2-MCP)		1	0
2-chloroethanol (MCE)		<0.1	1.2
2-bromoethanol (MBE)		56	28
1,4-dibromo-2-butanol (1,4-DBB)		1157	40
1,3-dichloroacetone (DCA)		ND	72

5.3. Kinetic studies on the Deh1 haloalcohol dehalogenase

5.3.1. Kinetics of degradation of 1,3-DCP and CPD

The initial rates of 1,3-DCP and CPD degradation, catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase, were determined at different substrate concentrations (see section 2.2.6.5). The dehalogenation of 1,3-DCP and CPD by the Deh1 haloalcohol dehalogenase followed the Michaelis-Menten kinetics (equation 5.1). The apparent K_m and V_{max} values (Table 5.4) were determined using the Lineweaver-Burk equation (equation 5.2, Figure 5.1)

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad (5.1)$$

$$\frac{1}{v} = \frac{K_m}{[S]} * \frac{1}{V_{max}} + \frac{1}{V_{max}} \qquad (5.2)$$

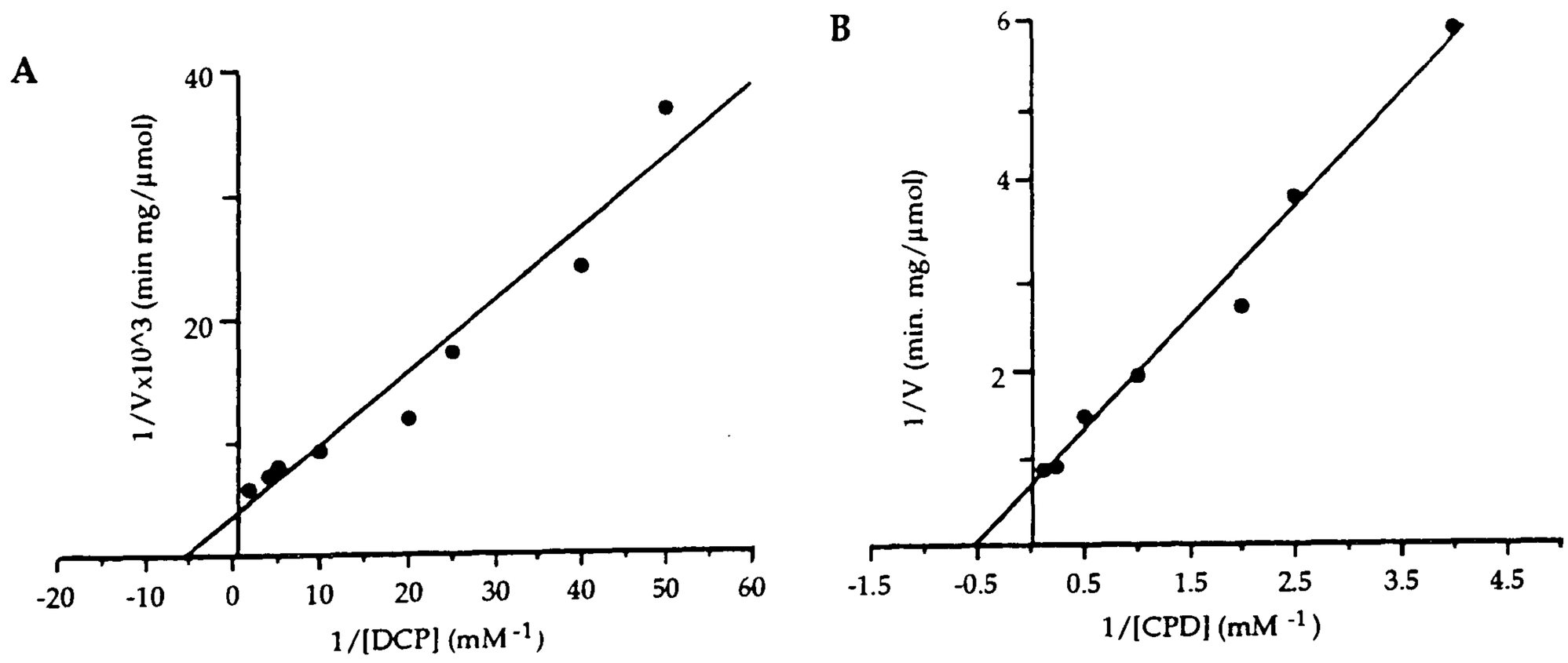


Figure 5.1: Lineweaver-Burk plot of the initial rates of degradation of (A) 1,3-DCP and (B) CPD obtained at different substrate concentrations. The K_m was determined using the value obtained where the line intercepts the X axis ($-1/K_m$) and V_{max} using the Y axis intercept value ($1/V_{max}$).

Table 5.4: Michaelis-Menten constants of the Deh1 haloalcohol dehalogenase.

The values of the constants were determined as described in Figure 5.1.

Substrate	V_{max} (µmol min^{-1} mg^{-1})	K_m (mM)
1,3-dichloro-2-propanol	236	0.11
3-chloro-1,2-propanodiol	1.55	2.02

5.3.2. Inhibition of the Deh1 haloalcohol dehalogenase

5.3.2.1. Effect of substrate analogues

In order to better understand the dehalogenation mechanism of the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase, several halogenated compounds were tested for possible effects on enzyme activity. No inhibition was found with the following compounds at 10.0 mM concentration: 3-chloro-1-propanol, 4-chloro-1-butanol, 2-chloroethylether and 1-chloropropane. Dichloroacetic acid (DCA) and chloroacetic acid (MCA) were found to inhibit the Deh1 dehalogenase. The effect of the haloacids on the initial rates of dehalogenation of 1,3-DCP at different substrate concentration was determined (see section 2.2.6.5). Lineweaver-Burk plots of these data (Figure 5.2) showed that both K_m and V_{max} apparent values were affected by MCA and DCA, giving rise to almost parallel lines. The kinetic constants obtained in this conditions are listed in Table 5.5.

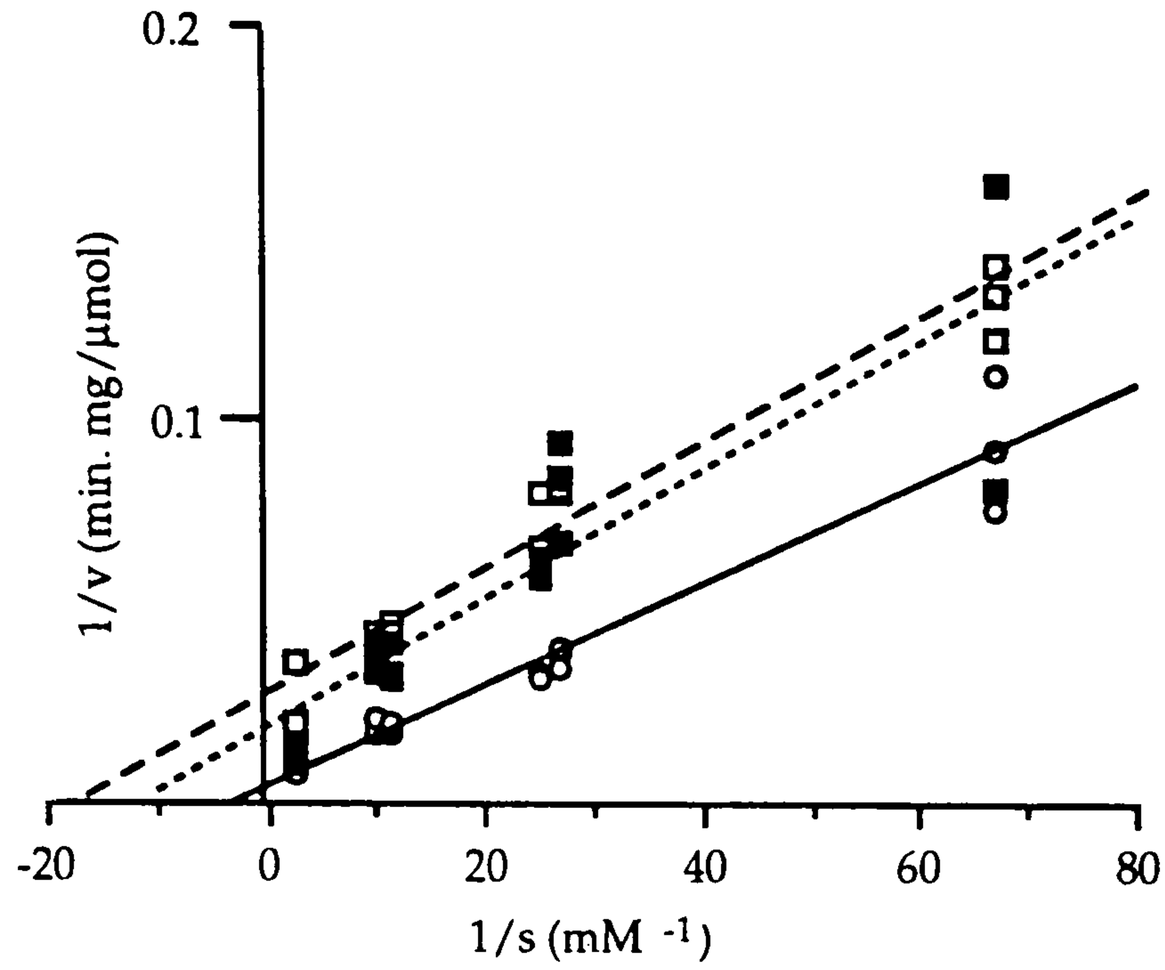


Figure 5.2: Lineweaver-Burk plot of the initial rates of 1,3-DCP dehalogenation by the Deh1 haloalcohol dehalogenase in the presence of MCA and DCA. The initial rates of 1,3-DCP dehalogenation in the presence of the halogenated acids were determined as described in section 2.2.6.5. The Michaelis-Menten constants were calculated using the Lineweaver-Burk transformation of the data whereas the intercepts of the line with the x and the y axis corresponded to $-1/K_m$ and $1/V_{max}$, respectively. The K_I was determined using the intercept of the line with the y axis ($1/V_{max} * [I]/K_I$). (—), no haloacids; (···), 3 mM MCA; (– –), 0.5 mM DCA.

Table 5.5: The effect of MCA and DCA on the Michaelis-Menten constants of the Deh1 haloalcohol dehalogenase towards 1,3-DCP. The values of the constants were determined as described in Figure 5.2.

	No haloacids	3 mM MCA	0.5 mM DCA
V_{max} (μmol min^{-1} mg^{-1})	232 ± 12	52 ± 12	39 ± 2
K_m (mM)	0.25 ± 0.01	0.087 ± 0.02	0.063 ± 0.003
V_{max}/K_m	927 ± 46	594 ± 138	612 ± 31
K_I (mM)		0.57 ± 0.13	0.07 ± 0.004

5.3.2.2. Effect of cations and chelators

The effect of several cations and chelating agents on the dehalogenation of 1,3-DCP catalyzed by the Deh1 haloalcohol dehalogenase was studied. Under the reaction conditions (see section 2.2.6.6), the cations Ca^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} and K^{+}, at concentrations of 1 mM, had no effect on Deh1 activity (Table 5.6). The Deh1 enzyme was completely inactivated in the presence of 1 mM Zn^{2+} and 0.1 mM Hg^{2+}. The chelating agents tested, EDTA and 1,10-phenantroline, also had no effect on the dehalogenation of 1,3-DCP (Table 5.6). The blanks containing all the compounds except the Deh1 dehalogenase showed no chloride release.

Table 5.6: The effect of cations and chelating agents on the Deh1 haloalcohol dehalogenase of *Arthrobacter* sp H10a. The pure Deh1 enzyme was incubated for 10 minutes at 30°C with the compound indicated, before the activity was determined with 1,3-DCP as substrate. All the assays were performed at 30°C in a reaction mixture containing 6.5 ml 100 mM TRIS-SO_4, pH 8.0, 10 mM 1,3-DCP and 5 µl of the Deh1 dehalogenase (see section 2.2.6.6).

Reagent	Concentration (mM)	% of inhibition
$CaCl_2$	1.0	0
$CuSO_4$	1.0	0
$FeSO_4$	1.0	0
$NiSO_4$	1.0	0
$ZnCl_2$	1.0	100
$HgCl_2$	0.1	100
K_2SO_4	1.0	0
KCl	1.0	0
EDTA	1.0	0
1,10-phenantroline	0.1	0

5.3.3. The effect of the pH

The effect of the pH on the dehalogenation rate of 1,3-DCP catalysis by the purified Deh1 haloalcohol dehalogenase was also studied. The Deh1 dehalogenase precipitated at pH values below 6.0. Above this pH, the activity increased concomitantly with pH up to pH values of 8.25 (Figure 5.3). The Deh1 haloalcohol dehalogenase showed more than 95% of the maximum activity at pH values between 8.25 and 10.5. Above pH 10.5 the enzyme activity decreased very rapidly (Figure 5.3). Spontaneous dehalogenation of 1,3-DCP was found above pH 8.5 on the blanks, which contained all the compounds except the Deh1 enzyme. Where spontaneous dehalogenation occurred, the rates of dehalogenation were calculated by subtracting the degradation rates determined in the blanks.

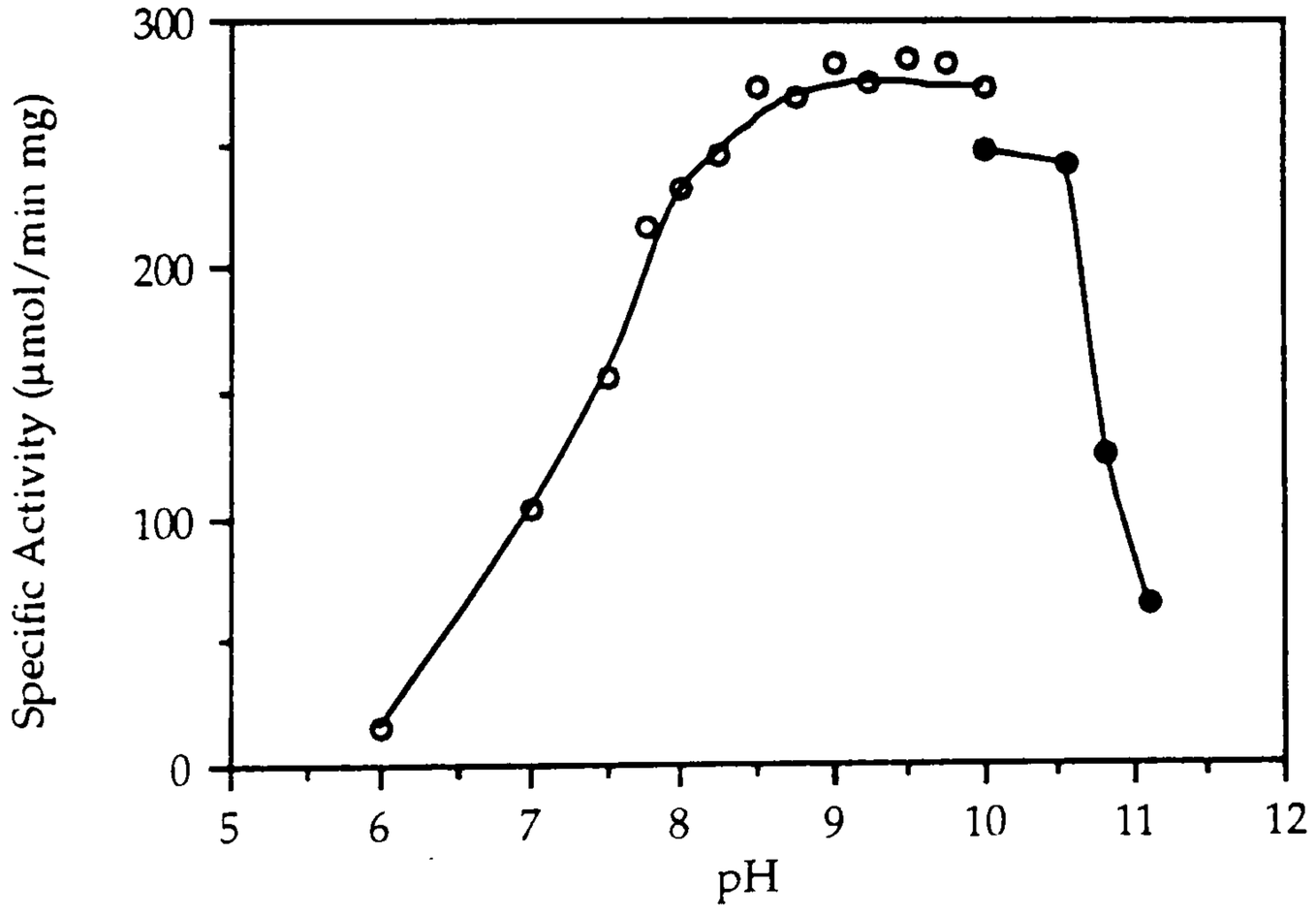


Figure 5.3: The effect of the pH on the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. The activity towards 1,3-DCP was determined over a pH range of 6.0 to 10.0 in 200 mM TRIS-glycine-sodium maleate buffer (○) and pH 10.0 to 11.1 in 200 mM CAPS buffer (●) (see section 2.2.6.4).

5.3.4. Effect of the temperature

The dehalogenation of 1,3-DCP catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase was studied at different temperatures, as described in section 2.2.6.3. The rates of dehalogenation of 1,3-DCP increased simultaneously with temperature to a maximum at 50°C (Figure 5.4 A). A rapid loss of activity was observed when the temperature increased above 50°C and no activity was observed at temperatures above 58°C (Figure 5.4 A). Whenever spontaneous dehalogenation of 1,3-DCP occurred (above 37°C), the rates of dehalogenation were calculated by subtracting the degradation rates determined in the blanks (all the components except the Deh1 enzyme were present). From the Arrhenius equation (Equation 5.3) it was possible to estimate an activation energy of 55 KJ mol^{-1} for the dehalogenation of 1,3-DCP (Figure 5.4 B).

$$k = A\, e^{-Ea/RT} \qquad (5.3)$$

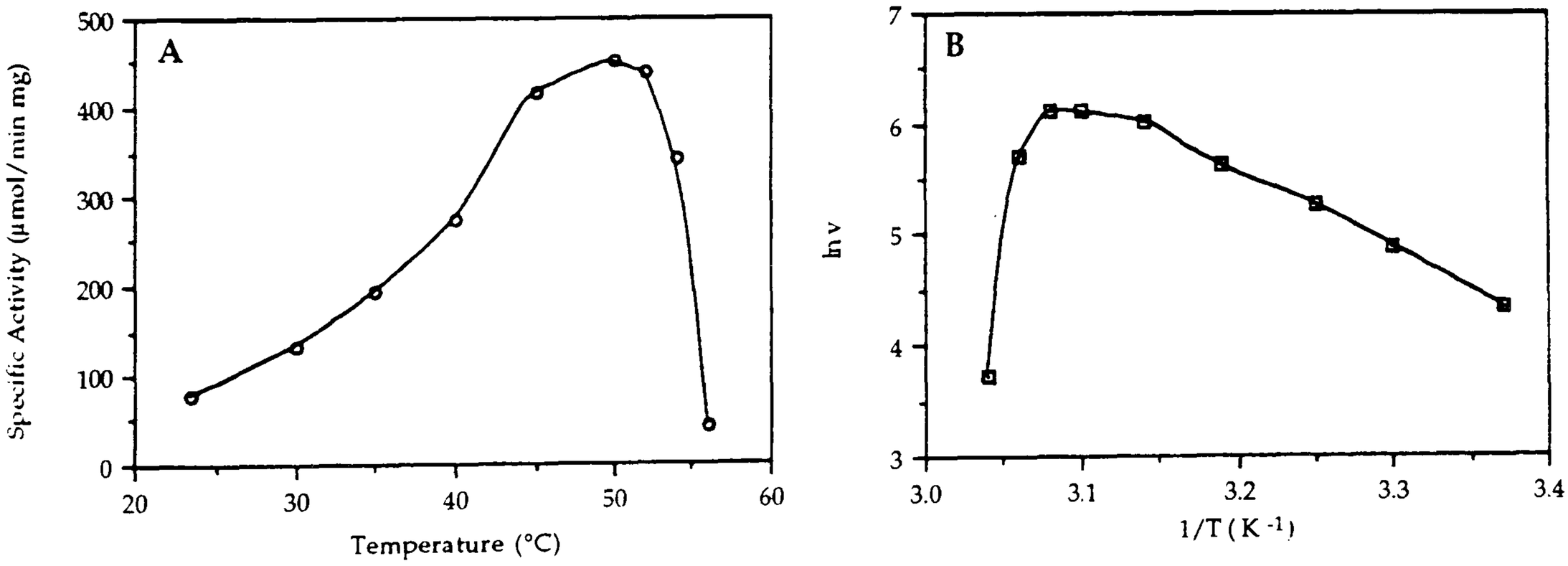


Figure 5.4: The effect of the temperature on the rates of dehalogenation catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. (A) The activity towards 1,3-DCP was determined at different temperatures as described in section 2.2.6.3. (B) According to the Arrhenius equation (equation 5.3), the plot of ln(velocity) against 1/T gives a straight line with a slope of -Ea/R. The Ea value was determined from the slope obtained, considering R equal to 8.31 J $mol^{-1}K^{-1}$.

5.4. Modification of the amino acid residues of the Deh1 haloalcohol dehalogenase

5.4.1. Modification of histidine residues

Histidine (His) residues are relatively highly reactive molecules, often participating in the stabilization of protein structure or in catalysis. At pH values below 6.0 His residues are predominantly ionized. In these circumstances, His can be a relatively strong nucleophile. However, in the non-ionized form (pH > 6.0) one N atom is an electrophile while the other is a nucleophile (Creighton, 1983). The most frequently used reagent for the modification of histidine residues in proteins is diethylpyrocarbonate (DEP) (Lundbald, 1991). This reagent is reasonably specific for histidine in the pH range of 5.5 to 7.5. Under alkaline conditions, DEP can also react with lysine, methionine and tyrosine residues. Under the conditions used (see section 2.2.), the reaction of DEP with the Deh1 haloalcohol dehalogenase had no effect on the enzyme activity towards 1,3-DCP (Table 5.7).

Table 5.7: Effect of histidine modifying reagent on the activity of the Deh1 haloalcohol dehalogenase towards 1,3-DCP. The specific activity of the enzyme, which was previously incubated with the indicated reagent (see section 2.2.7.2), was determined as described in section 2.2.6.1.

Reagent (concentration)	Diethylpyrocarbonate (1.0 mM)
Reaction conditions	100 mM borate buffer, pH 8.0, 30°C, 10 min
Reaction	Appendix A1
% of inhibition	0 %

5.4.2. Modification of cysteine residues

The thiol group of cysteine (Cys) is the most reactive of all amino acid side chains. This residue often plays an important role in stabilizing protein structure (disulphide or

hydrogen bonds) and/or at the active site. The modification of Cys depends on the charge and size of the reagent used, but also on the ionic state of the -SH group. Because of the importance of Cys residues in proteins, a large number of chemicals have been developed that specifically modify this amino acid. These reagents can be classified according to the type of reaction they promote: (1) oxidation of the SH group, (2) formation of a mercaptide (e.g. $HgCl_2$, p-chloromercuric benzoate), (3) alkylation of SH groups (iodoacetamide) and (4) reaction of the SH with a double bond (N-ethylmaleimide). The reaction of haloalcohol dehalogenase Deh1 with three thiol binding reagents produced different effects on the catalytic activity of the enzyme. Addition of N-ethylmaleimide (NEM) and p-chloromercuric benzoate (PMB) resulted in the loss of 85 to 100% of the dehalogenase activity (Table 5.8). When the substrates 1,3-DCP and MCE were present in the reaction mixture, the levels of inactivation decreased (8 to 24%). Under the reaction conditions used (see section 2.2.7.1.2), iodoacetamide had no effect on the Deh1 activity towards 1,3-DCP.

Table 5.8: Effect of cysteine modifying reagents on the activity of the Deh1 haloalcohol dehalogenase towards 1,3-DCP. The specific activity of the enzyme, which was previously incubated with the indicated reagent (see section 2.2.7.1), was determined as described in section 2.2.6.1. ND, not determined

Reagent (concentration)	Iodoacetamide (1 mM)	N-Ethylmaleimide (2 mM)	p-chloromercuric benzoate (0.1 mM)
Reaction condition	60 mM CAPS, pH 8.5, 30°C, 20 min	100 mM TRIS-SO_4, pH 8.0, 30°C, 10 min	100 mM TRIS-SO_4, pH 8.0, 30°C, 10 min
Reaction	Appendix A2.1	Appendix A2.2	Appendix A2.3
% inhibition	0	85	100
+ 1,3-DCP	ND	8	12
+ MCE	ND	13	24

5.4.3. Modification of lysine residues

Lysine (Lys) residues come second, after cysteine, amongst the strongest nucleophilic groups in a protein, often being involved in the stabilization of the protein structure and/or catalytic activity of the enzyme. Because chemical modification of lysine residues in proteins is based on the nucleophilic character of the ε-amino group, it is difficult to selectively modify this amino acid. A number of reagents which are used to modify Lys residues also have the potential to react with the N-terminal amino group, tyrosyl and cysteinyl residues. The role of lysine residues on the Deh1 haloalcohol dehalogenase activity was tested by its modification with three different compounds, ethylacetamidate, pyridoxal-5'-phosphate and formaldehyde. These compounds react with α and ε-amino groups under very mild conditions. Under the reaction conditions used (see section 2.2.7.4), ethylacetamidate had no effect on the Deh1 activity (Table 5.9). Pyridoxal-5'-phosphate and formaldehyde also had no effect on the activity of the Deh1 haloalcohol dehalogenase. However, addition of sodium borohydride, which is necessary to reduce the Schiff base formed during reaction of the above reagents with the amino group, resulted in the loss of 30% of enzyme activity. The loss of activity was not prevented upon addition of 1,3-DCP and MCE to the reaction mixture (Table 5.9).

Table 5.9: Effect of lysine modifying reagents on the activity of the Deh1 haloalcohol dehalogenase towards 1,3-DCP. The specific activity of the enzyme, which was previously incubated with the indicated reagent (see section 2.2.7.4), was determined as described in section 2.2.6.1. ND, not determined

Reagents (concentration)	Ethyl acetamide (10 mM)	Pyridoxal phosphate (1 mM) + $NaBH_4$ (8 mM)	Formaldehyde (4 mM $NaBH_4$ (10 mM)	$NaBH_4$ (8 mM)
Reaction conditions	100 mM Borate buffer, pH 7.0, 35°C, 30 min	100 mM borate buffer, pH 8.0, 28°C, 30 min	0.25 M borate buffer, pH 9.0, 0°C, 20 min	100 mM borate buffer, pH 8.0, 28°C, 30 min
Reaction	Appendix A3.1	Appendix A3.2	Appendix A3.3	
% inhibition	0	30	60	30
+ 1,3-DCP	ND	ND	ND	35
+ MCE	ND	ND	ND	30

5.4.4. Modification of arginine residues

The strong basic guanidine group of the arginyl residue (Arg) is virtually unreactive, but often acts as an anion recognition site in proteins (Lundbald, 1991). Modification of arginyl residues with phenylglyoxal and 2,3-butadione, occurs in very mild conditions and both reagents have been shown to be highly specific for this amino acid side chain (Lundbald, 1991). Under the reaction conditions used (see section 2.2.7.3.1), phenylglyoxal had no effect on the Deh1 haloalcohol dehalogenase activity towards 1,3-DCP (Table 5.10). The modification of the Deh1 enzyme with 2,3-butadione resulted in a 60% decrease in dehalogenase activity towards 1,3-DCP (Table 5.10). When 1,3-DCP was added to the reaction mixture, the inactivation of the enzyme by 2,3-butadione was reduced to 20%. However, MCE had no protection effect on the modification of argynyl residues by 2,3-butadione (Table 5.10).

Table 5.10: Effect of arginine modifying reagents on the activity of the Deh1 haloalcohol dehalogenase towards 1,3-DCP. The specific activity of the enzyme, which was previously incubated with the indicated reagent (see section 2.2.7.3), was determined as described in section 2.2.7.3. ND, not determined

Reagent (concentration)	Phenylglyoxal (5.0 mM)	2,3-butadione (10 mM)
Reaction conditions	40 mM borate buffer, pH 7.0, 35°C, 30 min	100 mM borate buffer, pH 8.0, 35°C, 30 min
Reaction	Appendix A4.1	Appendix A4.2
% inhibition	0	60
+ 1,3-DCP	ND	20
+ MCE	ND	60

5.4.5. Modification of carboxylic acid residues

A wide variety of methods and compounds have been developed to selectively modify carboxylic acid residues in proteins (Lundbald, 1991). However, most of these reactions occur at acidic pH, which caused the precipitation of the Deh1 haloalcohol dehalogenase. The modification of the Deh1 carboxylic acids was studied with two carbodiimides, one water soluble (CMC) and one relatively hydrophobic (DCCD) and with a isoxazolium salt (Woodward's reagent). The selection of these reagents was based on the fact that modification could be performed at pH 7.4 to 8.0. Under the conditions used, reaction of any of the above carboxyl reagents with the Deh1 enzyme resulted in no loss of the activity towards 1,3-DCP (Table 5.11).

Table 5.11: Effect of carboxyl modifying reagents on the activity of the Deh1 haloalcohol dehalogenase towards 1,3-DCP. The specific activity of the enzyme, which was pre-incubated with the indicated reagent (see section 2.2.7.5), was determined as described in section 2.2.6.1. CMC, (1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-p-toluenesulfonate; DCCD, dicyclohexylcarbodiimide; Woodward's reagent, N-ethyl-5-phenylisoxazolium-3'-sulphonate.

Reagent (concentration)	CMC (10 mM)	DCCD (0.16 mM)	Woodward's reagent (1 mM)
Reaction conditions	70 mM TRIS-SO_4, pH 8. 25°C, 20 min	40 mM MOPS, pH 7.4, 30°C, 60 min	40 mM TRICINE, pH 7.9, 25°C, 20 min
Reaction	Appendix A5.1	Appendix A5.1	Appendix A5.2
% inhibition	0	2.5	0

5.5. Discussion

The degradation of haloalcohols when catalyzed by the H10a crude extracts resulted in the accumulation of the corresponding alcohols. However, the haloalcohols were dehalogenated to the corresponding epoxides when the purified Deh1 enzyme was used as the biocatalyst. Enzymes that catalyze the conversion of vicinal halohydrins into epoxides have been designated as halohydrin epoxidases by Castro and Bartnicki (1968), haloalcohol dehalogenases and haloalcohol halogen-halide lyases (EC 4.5.1) by van den Wijngaard *et al.* (1989; 1991) or as halohydrin hydrogen-lyases by Nagasawa *et al.* (1992). The Deh1 showed no activity towards the epoxides, while they were rapidly hydrolyzed to the corresponding alcohols by the H10a crude extracts, indicating the presence of an epoxide hydrolase activity. These results suggest that degradation of haloalcohols catalyzed by the *Arthrobacter* sp H10a was a two step process. In the first step a haloalcohol dehalogenase catalyzed the removal of the chloride with simultaneous formation of the epoxide ring. The epoxide ring formed was subsequently hydrolyzed by an epoxide hydrolase present in the H10a crude extracts (Figure 5.5). If a second halogen was present it was removed in the same manner.

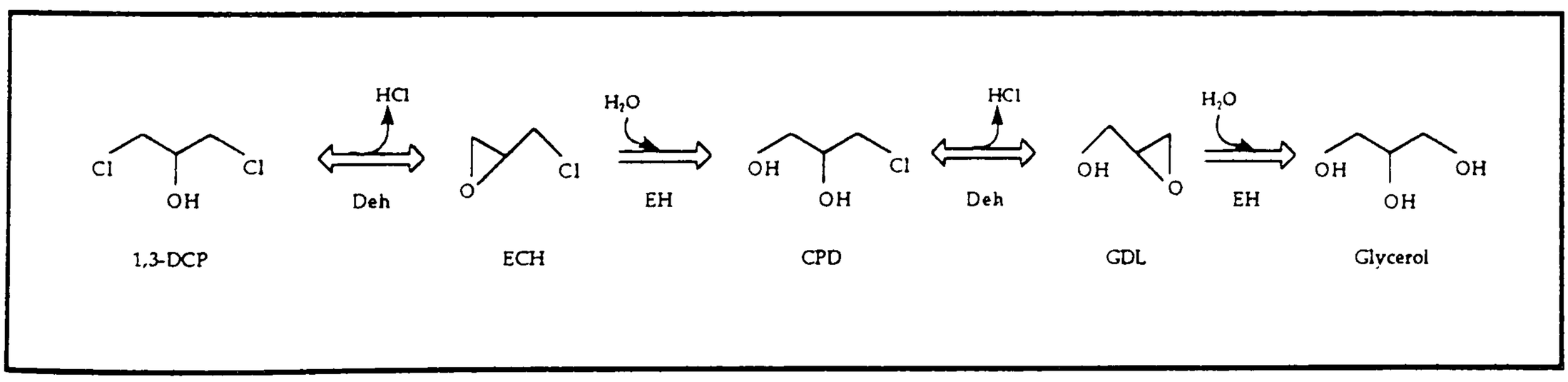


Figure 5.5: Proposed route for the degradation of 1,3-DCP by *Arthrobacter* sp H10a. Deh, haloalcohol dehalogenase; EH, epoxide hydrolase; 1,3-DCP, 1,3-dichloro-2-propanol; ECH, epichlorohydrin; CPD, 3-chloro-1,2-propanodiol; GDL, glycidol.

Similar microbial degradation routes were reported for the dehalogenation of several haloalcohols (Castro & Bartnicki, 1968; Kasai *et al.*, 1990; Nakamura *et al.*, 1991; Suzuki & Kasai, 1991; Kasai *et al.*, 1992a; Suzuki *et al.*, 1992) and epichlorohydrin (van den Wijngaard *et al.*, 1989). Alternative routes have been reported for the microbial degradation of haloalcohols such as 2-chloroethanol (Stucki & Leisinger, 1983; Strotman *et al.*, 1990) and 3-chloropropanol (Castro & Bartnicki, 1965). In this case, dehalogenation takes place after oxidation of the alcohol to the corresponding acids. The haloalkane dehalogenase of the 1,2-dichloroethane utilizing bacteria, *Xanthobacter autotrophicus* GJ10, was capable of dehalogenating 2-bromoethanol and epichlorohydrin to the corresponding alcohols (van den Wijngaard *et al.*, 1992), indicating another possible mechanism of dehalogenation of haloalcohols.

Although the haloalcohol degradation pathway found in *Arthrobacter* sp H10a was common to a number of halohydrin utilizing bacteria, some differences were observed in the enzymes that catalyse these reactions. In H10a, as well as for most of the other microorganisms, the epoxide ring opening step was qualitatively more rapid than epoxide formation as shown by the fast degradation of 1,3-DCP to CPD and CPD to glycerol by crude extracts from strain H10a, thus preventing the accumulation of toxic epoxides in the cell. The bacteria that showed faster epoxide forming steps than the hydrolyzing ones could only use a limited number of carbon sources and growth was restricted to the substrates where it was possible to maintain the concentration of epoxides in the cell at low levels. The *Alcaligenes* sp DS-S-7G could only utilize 1-halo-1,2-propanediols despite the fact that its cell-free extract was capable of catalyzing the degradation of a number of halohydrins and epoxides (Suzuki *et al.*, 1992). The *Flavobacterium* sp, which has been isolated by enrichment on 2,3-DBP, was shown to possess more effective halohydrin epoxidases than the ones that catalyze the epoxide opening steps (Castro & Bartnicki, 1968). Although the haloalcohol dehalogenase revealed higher dehalogenation rates with 1,3-DBP (2222 U mg^{-1}) than with 2,3-DBP (123 U mg^{-1}), this strain showed very poor growth on the former compound while it

grew very well on 2,3-DBP. The *Arthrobacter* sp AD2 grew very slowly on halogenated haloalcohols and epoxides, because this bacterium did not possess an enzyme which catalyzed the hydrolysis of the epoxide ring. Hence growth by this organism on the above compounds depends on the spontaneous hydrolysis of the epoxides (van den Wijngaard *et al.*, 1989).

The Deh1 haloalcohol dehalogenase catalyzed the reverse reaction, which was the halogenation of epihalohydrins (Table 5.2). The main product detected, from transhalogenation of EBH and ECH, was 1-bromo-3-chloro-2-propanol. In both reactions the two epoxides were also found. These results suggest that the transhalogenation of epibromohydrin into epichlorohydrin (and vice versa) was a result of the combination of halogenation and dehalogenation reactions (Figure 5.6). Whenever detailed studies of the haloalcohol dehalogenases have been published, these enzymes have been found to also catalyze the reverse reaction (Castro & Bartnicki, 1968; van den Wijngaard *et al.*, 1991; Nagasawa *et al.*, 1992; Nakamura *et al.*, 1992). Nakamura *et al.* (1991a) used this catalytic feature of the *Corynebacterium* sp N-1074 I_a haloalcohol dehalogenase, to produce ß-hydroxynitriles from the corresponding epoxides and cyanide.

The *Arthrobacter* sp H10a crude extracts and the purified Deh1 dehalogenase showed no activity towards halogenated acids, alkanes and phenols. These enzyme preparations have only shown activity towards haloalcohols with a halogen positioned on a carbon vicinal to the hydroxy group (Table 5.3). Similar dehalogenases which showed high specificity towards vicinal halohydrins have been found in microorganisms that degrade halogenated alcohols and epoxides (Castro & Bartnicki, 1968; van den Wijngaard *et al.*, 1989; Kasai *et al.*, 1990; Nakamura *et al.*, 1991; Suzuki & Kasai, 1991; Kasai *et al.*, 1992a, Suzuki *et al.*, 1992).

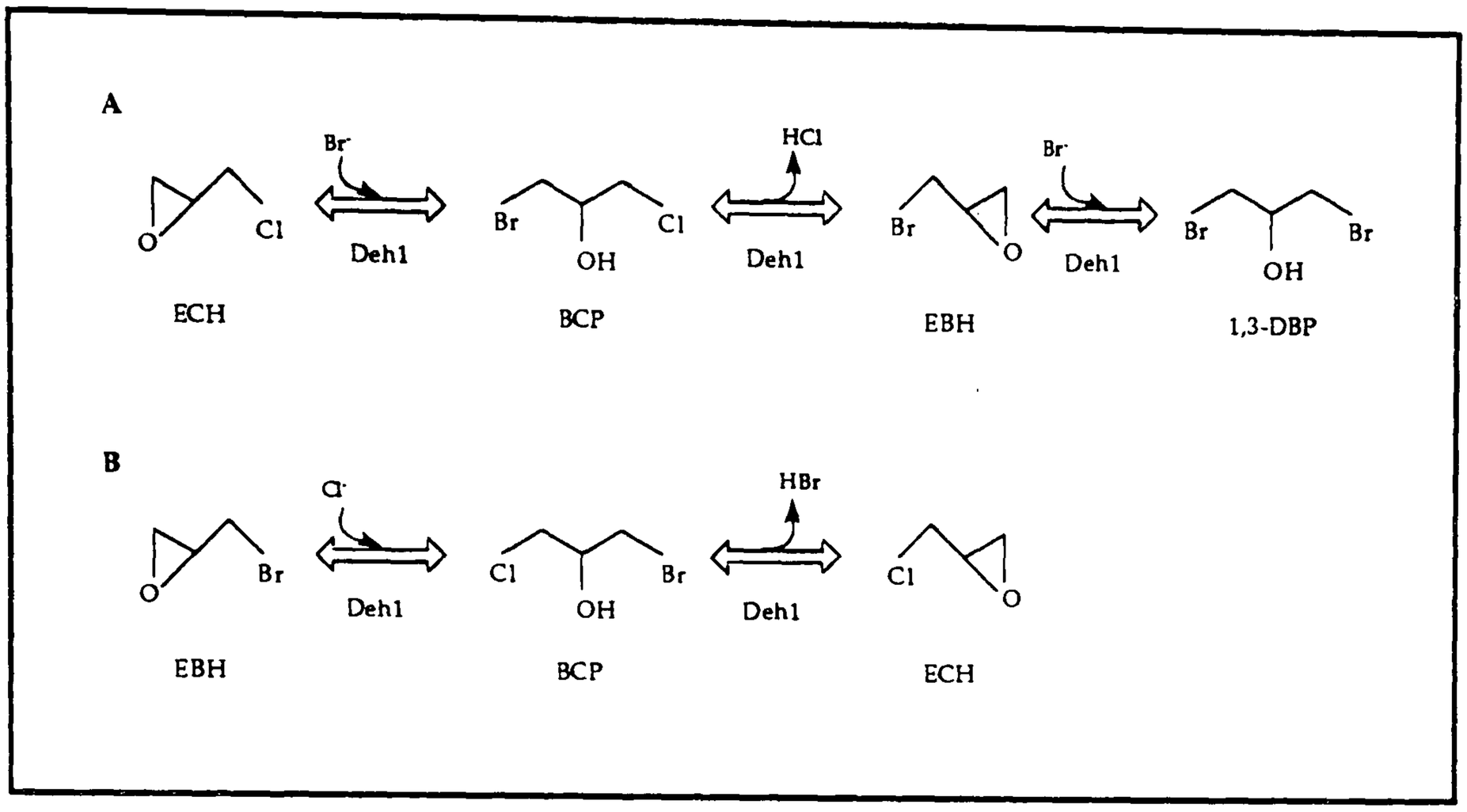


Figure 5.6: Possible reactions for the transhalogenation of epoxides catalysed by the *Arthrobacter* sp Deh1 haloalcohol dehalogenase. (A), transhalogenation of epichlorohydrin and (B) epibromohydrin. ECH, epichlorohydrin; EBH, epibromohydrin, BCP, 1-bromo-3-chloro-2-propanol, 1,3-DBP, 1,3-dibromo-2-propanol.

The H10a crude extracts and the purified Deh1 dehalogenase have shown different substrate specificities, representing a further indication that *Arthrobacte*r sp H10a possesses more than one haloalcohol dehalogenase. The Deh1 showed high specific activity towards 1,3-DCP and 1-MCP, but low towards CPD and MCE (Table 5.3). With exception of halogenated ethanols, the activity of this enzyme towards brominated alcohols was lower than with the chlorinated analogues. Similar results were found with the crude extracts of *Alcaligenes* sp DS-S-7G (Suzuki *et al.*, 1992). The dehalogenase(s) of this strain showed higher activity towards CPD than BPD. However, no significant difference was found in the activity towards the 2,3-dihalo-1-propanol analogues. No activity towards 1,3-DCP was detected on the strain DS-S-7G crude extracts, suggesting its dehalogenase(s) was different from the Deh1 enzyme. As found with the *Arthrobacter* sp AD2 and the *Pseudomonas* sp AD1 (van den Wijngaard

et al., 1989), the Deh1 enzyme was capable of dehalogenating 1,3-dichloroacetone. The substrate profile demonstrated for the *Arthrobacter* sp H10a Deh1 dehalogenase showed no significant similarity to the published data found for other haloalcohol degrading enzymes.

The specific activity of the H10a crude extracts showed 10 times higher activity towards the brominated than the corresponding chlorinated alcohols. The activity towards CPD was also higher with the crude extracts than with the purified Deh1 enzyme, suggesting that the Deh2 dehalogenase might have a similar substrate profile to the ones found in *Pseudomonas* sp AD1, the *Arthrobacter* sp AD2 (van den Wijngaard *et al.*, 1989), and the *Corynebacterium* sp N-1074 I_a (Nagasawa *et al.*, 1992).

The *Arthrobacter* sp H10a haloalcohol dehalogenase Deh1 showed a maximum activity at 50°C (Figure 5.4 A) and a broad pH optimum from 8.5 to 10.5 (Figure 5.3), for the dehalogenation of 1,3-DCP. In order to minimize non-enzymatic degradation of the substrate the assays were routinely performed at 30°C and pH 8.0. The other haloalcohol dehalogenases, that have been purified (van den Wijngaard *et al.*, 1991; Nagasawa *et al.*, 1992), also showed a broad pH optimum but at lower pH values (8.0 to 9.0). The three haloalcohol dehalogenases showed similar optimum temperatures (50 to 55°C) and, where determined, similar activation energies (49.4 to 55 KJ mol^{-1}).

The K_m values found for the Deh1 haloalcohol dehalogenase for the dehalogenation of 1,3-DCP and CPD were 10 times lower than the ones found with the other two enzymes (Table 5.12). Despite the fact that the reaction conditions were different, these results suggest that the Deh1 dehalogenase had higher affinity towards halogenated alcohols. The apparent V_{max} found for 1,3-DCP dehalogenation was 100 times higher than the values found for the other two enzymes (Table 5.12).

Table 5.12: The apparent Michaelis-Menten constants of haloalcohol dehalogenating enzymes. ND, not determined. (1) this work; (2) van den Wijngaard *et al.*, 1991; (3) Nagasawa *et al.*, 1992.

Microorganism / enzyme	Reaction conditions	1,3-DCP		CPD		Ref.
		K_m (mM)	V_{max} (µmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (µmol min^{-1} mg^{-1})	
Arthrobacter sp H10a Deh1	pH 8.0, 30°C	0.11	236	2.02	1.55	1
Arthrobacter sp AD2	pH 7.5, 30°C	8.5	9.0	48	3.1	2
Corynebacterium sp N-1074 / I_a	pH 8.0, 20°C	2.44	3.13	16.7	ND	3

The activity of the Deh1 haloalcohol dehalogenase was inhibited by haloalkanoic acids (Figure 5.2). When dehalogenation of 1,3-DCP occurred in the presence of MCA and DCA, the apparent V_{max} values decreased by a factor of 5 to 6, while the K_m values decreased 3 to 4 fold (Table 5.5). The results obtained suggest that the haloacids had a mixed type of inhibition effect on the Deh1 dehalogenase. Since the effect on the apparent value of V_{max} was greater than the observed decrease on the ratio V_{max}/K_m, the inhibition was considered to be as mixed type, predominantly uncompetitive. Chloroacetic acid behaved as a competitive inhibitor of the *Arthrobacter* sp AD2 haloalcohol dehalogenase (van den Wijngaard *et al.*, 1991).

The activity of the *Arthrobacter* sp H10a Deh1 dehalogenase was completely inhibited by the presence of Hg^{2+} and Zn^{2+}. The latter ion can coordinate amino acid residues that contain N or OH groups (Dugas, 1989). While Hg^{2+} is known to be a strong chelating agent for thiol groups (Dugas, 1989). Other thiol reagents, such as PMB and NEM also caused the inactivation of the dehalogenase activity (Table 5.8). In addition, inactivation of the enzyme was prevented by addition of 1,3-DCP and 2-MCE to the reaction mixture. These results suggest that one or more cysteine residues may be involved in the dehalogenation mechanism or in maintaining the quaternary structure of

the enzyme. Hg^{2+} caused partial inhibition of the *Arthrobacter* sp AD2 (van den Wijngaard *et al.*, 1991) and the *Corynebacterium* sp N-1074 haloalcohol dehalogenase I_a (Nagasawa *et al.*, 1992). However, no significant inactivation was caused by other thiol reagents (NEM and PMB) indicating that cysteine residue(s) did not play a critical role in the catalytic activity and structural stability of these enzymes. This is in agreement with the study of the amino acid composition of the strain AD2 dehalogenase which did not reveal the presence of cysteine or cystine residues (van den Wijngaard *et al.*, 1991).

Modification of lysine residues of the Deh1 dehalogenase with three different reagents (ethyl acetamidate, pyridoxal-5'-phosphate and formaldehyde), had no effect on the dehalogenase activity towards 1,3-DCP. However, addition of sodium borohydride to the protein solution resulted in a decrease of 35% in enzyme activity (Table 5.9). Sodium borohydride is known to reduce aldehydes and disulfide bonds (Lunbald, 1991), supporting the previous results that suggested that cysteine residues were important for Deh1 dehalogenase activity. Modification of the Deh1 dehalogenase arginine residues by phenylglyoxal resulted in no alteration of the dehalogenase activity (Table 5.10). When a smaller reagent was used, 2,3-butadione, a decrease of 60% on the enzyme activity towards 1,3-DCP was observed. Substrate protection experiments showed that 1,3-DCP can protect the enzyme from the inhibitory effect of 2,3-butadione while the two carbon haloalcohol, chloroethanol, had no protection effect suggesting that an arginine residue may be located in or near to the active site. The amino acid modification experiments on the Deh1 haloalcohol dehalogenase suggested that one or more cysteine and arginine residues are probably important for the activity and/or maintenance of the three dimensional structure of the enzyme.

Modification of histidine residues with DEP (Table 5.7) and carboxylic amino acids with CMC (1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl] carbodiimide), DCCD (dicyclohexyl carbodiimide) and Woodward's reagent, resulted in no loss of enzymatic activity. These

results indicate that these amino acids may not play an important role on the structure and catalytic activity of the Deh1 haloalcohol dehalogenase. Carboxylic modifying reagents also showed no effect on the activity of the haloalcohol dehalogenase I_a of *Corynebacterium* sp N-1074 (Nagasawa *et al.*, 1992).

CHAPTER 6

SYNTHESIS OF CHIRAL EPIHALOHYDRINS USING THE Deh1 HALOALCOHOL DEHALOGENASE

6.1. Introduction

Recently there has been an increasing interest in the synthesis of small chiral molecules which can be used in the synthesis of compounds for medical applications. Optically active epichlorohydrin is a versatile C3 chiral building block for the synthesis of chiral pharmaceutics such as ß-adernegic blockers, vitamins, pheromones, natural products and new materials such as ferro electric crystals (Kasai *et al.*, 1992). Chemical synthesis of optically active ECH from mannitol (Baldwing *et al.*, 1978) and glycidol using the Sharpless epoxidation (Katsukit & Sharpless, 1980), requires expensive starting materials, can be technically difficult and the enantiomeric excess (e.e.) of the isomer formed is often low (GDL 91% e.e. synthesized using the Sharpless method). Iriuchijime *et al.* (1982) synthesized (R)-ECH via (S)-1-acetoxy-2,3- dichloropropane obtained by asymmetric hydrolysis of the racemate using pancreatin and stepsin from hog pancreas. However, the yield and optical purity of the recovered epoxide were low (yield, 15.4%, >90% e.e.) for an effective production method. Optically pure (R)-ECH could be obtained using an alkene degrading strain, *Norcadia* sp H8, that was capable of stereoselectively degrading ECH (Weijers & deBont, 1991). Habets-Crutzen *et al.* (1985) studied the synthesis of optically active epichlorohydrin from allylchloride by alkene assimilating bacteria. However, due to its toxic effect on the microorganism, S-ECH was produced in low concentrations and with low enantiomeric excess (80-98% e.e.).

Most of the methods reported for the production of chiral epoxides using dehalogenating bacteria have been based on the enantioselective degradation of racemic mixtures of either 2,3-DCP (Kasai *et al.*, 1991, Kasai *et al.*, 1992a) or CPD (Suzuki & Kasai, 1991; Suzuki *et al.*, 1992). The unassimilated haloalcohol, that accumulated in the growth medium, was then chemically converted to the epoxide by adding hydroxide. Using this approach both isomers of ECH and GDL were obtained in high enantiomeric excess (>99%). A disadvantage of such optical resolution method was that the resulting yield of

the desired enantiomer was less than 35%. However, halogenated alcohols can be produced economically in the petrochemical industry, thus this method could be commercially attractive for the manufacture of valuable chiral epoxides. Optically pure R-epichlorohydrin has been produced at a large scale (19 batches of 80 l each) using a R-2,3-DCP with *Pseudomonas* sp OS-K-29 cells immobilized in alginate beads (Kasai *et al.*, 1992b).

From an industrial point of view, the production of optically active compounds by enantioselective microbial transformation of prochiral starting materials is more attractive since a quantitative yield of the desired enantiomer can be obtained. Nakamura *et al.* (1993) studied the enantioselective transformation of 1,3-DCP into R-CPD by *Corynebacterium* sp N-1074. Using this method, they obtained a molar conversion yield of 97.3%. However, the enantiomeric excess of the R-CPD produced was too low (87.3%) to be useful as a chiral building reagent.

The relative stability of dehalogenases in solution and the non-requirement of cofactor for maintenance of activity makes these enzymes suitable industrial biocatalysts. An L-2-haloacid halidohydrolase from *Pseudomonas putida* AJ1 has been used in the synthesis of D-lactate (Hasan *et al.*, 1991a). The L-2-MCPA was converted to D-lactate by the L-2-haloacid halidohydrolase, while the unreacted D-2-MCPA was then chemically dehalogenated to produce D-lactic acid. By combining the enzymatic and chemical dehalogenation, 2-MCPA could be totally converted into D-lactate (87% yield) with an enantiomeric excess of more than 94%. The *Pseudomonas cepacia* MBA4, L-2-haloacid dehalogenase IVa has been successfully immobilized into hollow fibers and used for dehalogenation of MCA (Diaz *et al.*, 1989). Although no studies were made on the stereoselective capabilities of the immobilized enzyme, the results suggest that the bioreactor may be useful for the resolution of racemic mixtures of 2-haloalkanoic acids. The *Pseudomonas putida* AJ1 L-2-haloacid halidohydrolase was also used to produce

long chain D-2-hydroxy acids from the corresponding halogenated analogues, in organic solvents (Hasan *et al.*, 1991b).

The potential of the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase for the production of optically active epichlorohydrin was tested as this enzyme possesses a number of characteristics of a good biocatalyst (stability, no requirement of co-factors and high activity). This chapter describes the study of the stereospecificity of 1,3-DCP dehalogenation and halogenation of epihalohydrins catalyzed by the Deh1 dehalogenase. The production of optically active epihalohydrins using dehalogenases from haloalcohol degrading bacteria requires an enzyme preparation that does not contain epoxide hydrolase activity. Therefore the cost associated with protein purification makes the bioreaction costly. Immobilization of the Deh1 haloalcohol dehalogenase looked a more effective process for the production of epichlorohydrin, since in this way the enzyme could be recovered and reused in a continuous mode. Studies on the immobilization of the Deh1 haloalcohol dehalogenase are also described.

6.2. Preparation of the biocatalyst

The aim of preparing the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase for the production of chiral epoxides was to obtain an epoxide hydrolase free fraction while favouring the recovery of large amounts of the dehalogenase activity. Therefore, the ammonium sulphate precipitation step was eliminated from the purification procedure since it resulted on the loss of a large proportion of the haloalcohol dehalogenase activity (see section 4.2). The crude extract (8.3 g of protein, 8.5 U mg^{-1}) was first fractionated on a DEAE-Sephacel column (see section 2.2.5) and the fractions monitored for dehalogenation of 1,3-DCP (see section 2.2.6.1) and degradation of epichlorohydrin (see section 2.2.6.2). The epoxide hydrolase was eluted as two separated peaks, designated as EH1 (fractions 67-79) and EH2 (fractions 80-104)

(Figure 6.1). The haloalcohol dehalogenase was co-eluted with the two peaks. Fraction II, which contained only one of the epoxide hydrolase enzymes (EH2) and most of the haloalcohol dehalogenase, was further fractionated on an Octyl-Sepharose CL-4B column (see section 2.2.5). The haloalcohol dehalogenase eluted as a broad peak at low ionic strength and no epoxide hydrolase activity was detected in these fractions (Figure 6.2). Degradation of epichlorohydrin was observed in fractions 31 to 33. The fractions obtained from this column, which showed dehalogenase activity, were pooled (volume, 315 ml; specific activity, 137 U mg^{-1}; protein concentration, 0.175 mg ml^{-1}) and dialyzed against 2 l of 100 mM TRIS-SO_4, pH 8.0, 1 mM DTT. On SDS-PAGE this enzyme preparation showed two major bands, corresponding to the 31.5 and 34 kDa subunits of the Deh1 haloalcohol dehalogenase, and several minor bands (Figure 6.3 A). On nondenaturing gels the enzyme preparation showed only the 5 active protein bands corresponding to the above enzyme (Figure 6.3 B). This enzyme preparation was used in the immobilization experiments and to study the synthesis of chiral epoxides.

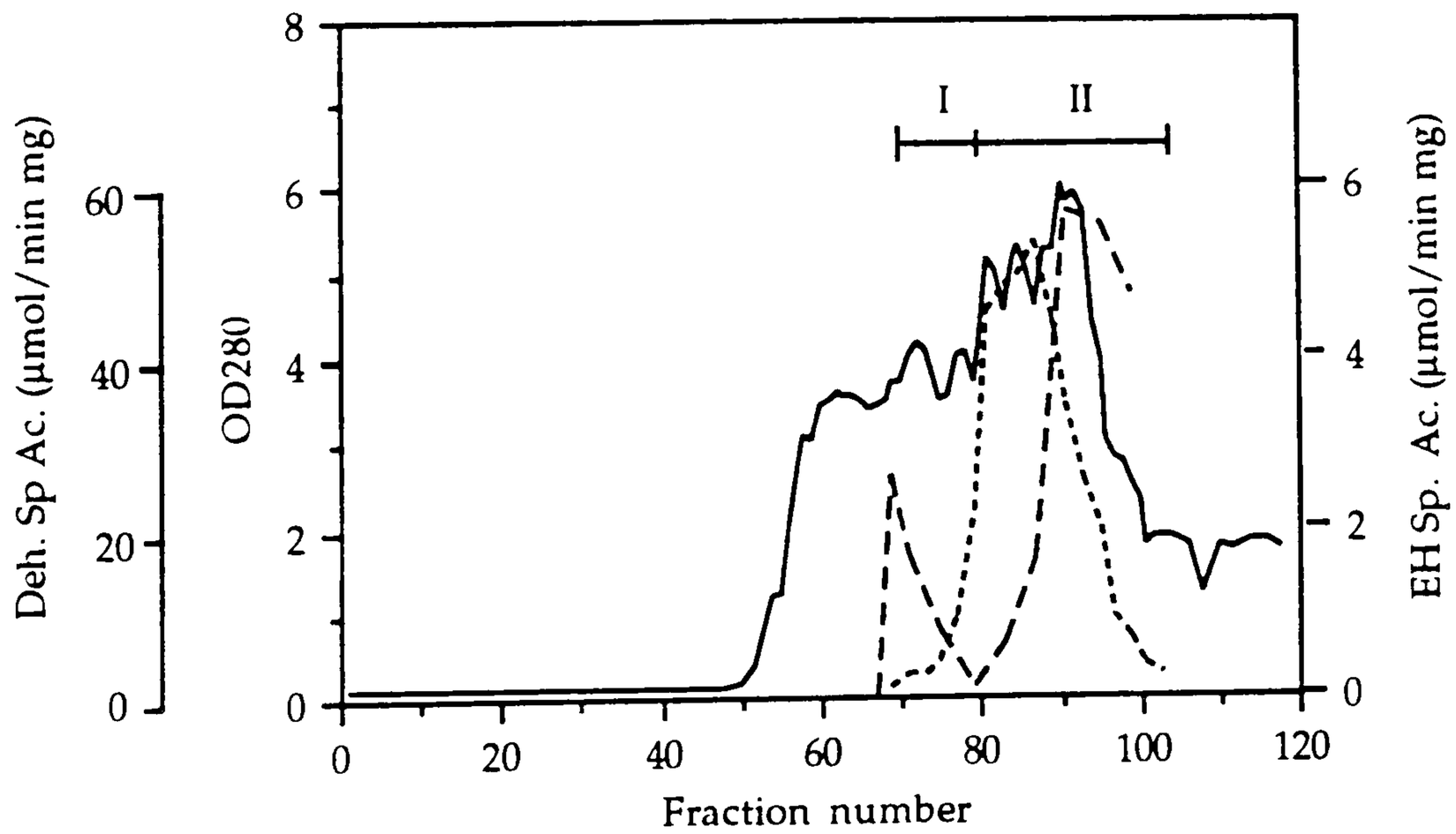


Figure 6.1: Elution profile of the *Arthrobacter* sp H10a crude extract on a DEAE-Sephacel column. The cell free extract (8.3 g of protein) was loaded onto a DEAE-Sephacel column (5 x 42 cm) equilibrated with 10 mM TRIS-SO_4, pH 7.0, 1 mM DTT. The column was washed with 1 l of equilibration buffer and eluted with a linear gradient of 0-1 M $(NH_4)_2SO_4$. Fractions were monitored for protein (—), haloalcohol dehalogenase (···) and epoxide hydrolase activity (—).

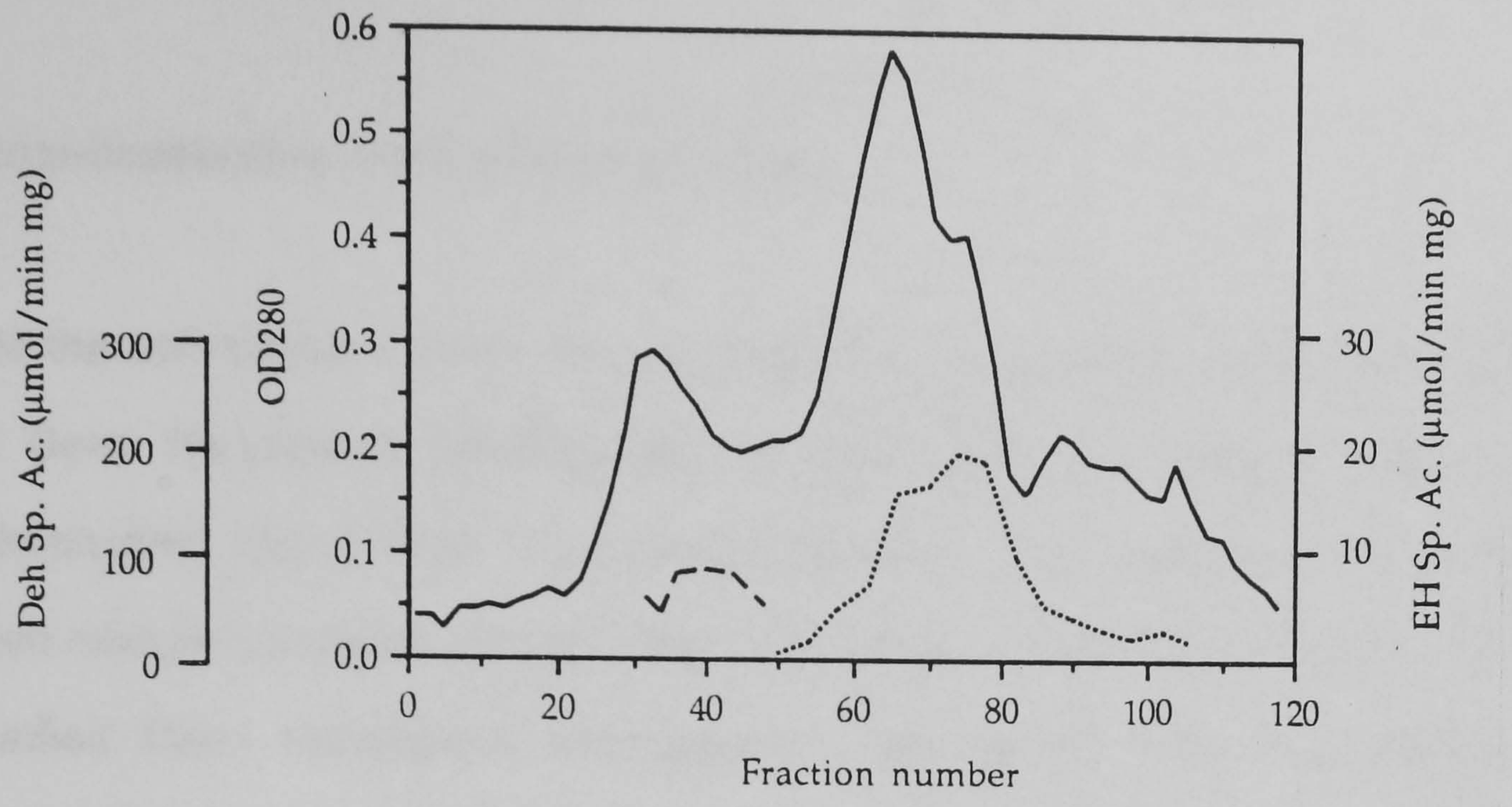


Figure 6.2: Elution profile of fraction II from the DEAE-Sephacel column on a Octyl-Sepharose CL-4B column. The DEAE-Sephacel eluate was loaded onto a Octyl-Sepharose CL-4B column (2.5 x 35 cm) equilibrated with 50 mM TRIS-SO_4, pH 7.0, 1.17 M $(NH_4)_2SO_4$, 1 mM DTT. The column was washed with 500 ml of the equilibration buffer and eluted with a linear gradient of 1.17-0 M $(NH_4)_2SO_4$. The fractions were monitored for protein (—), haloalcohol dehalogenase (···) and epoxide hydrolase activity (—).

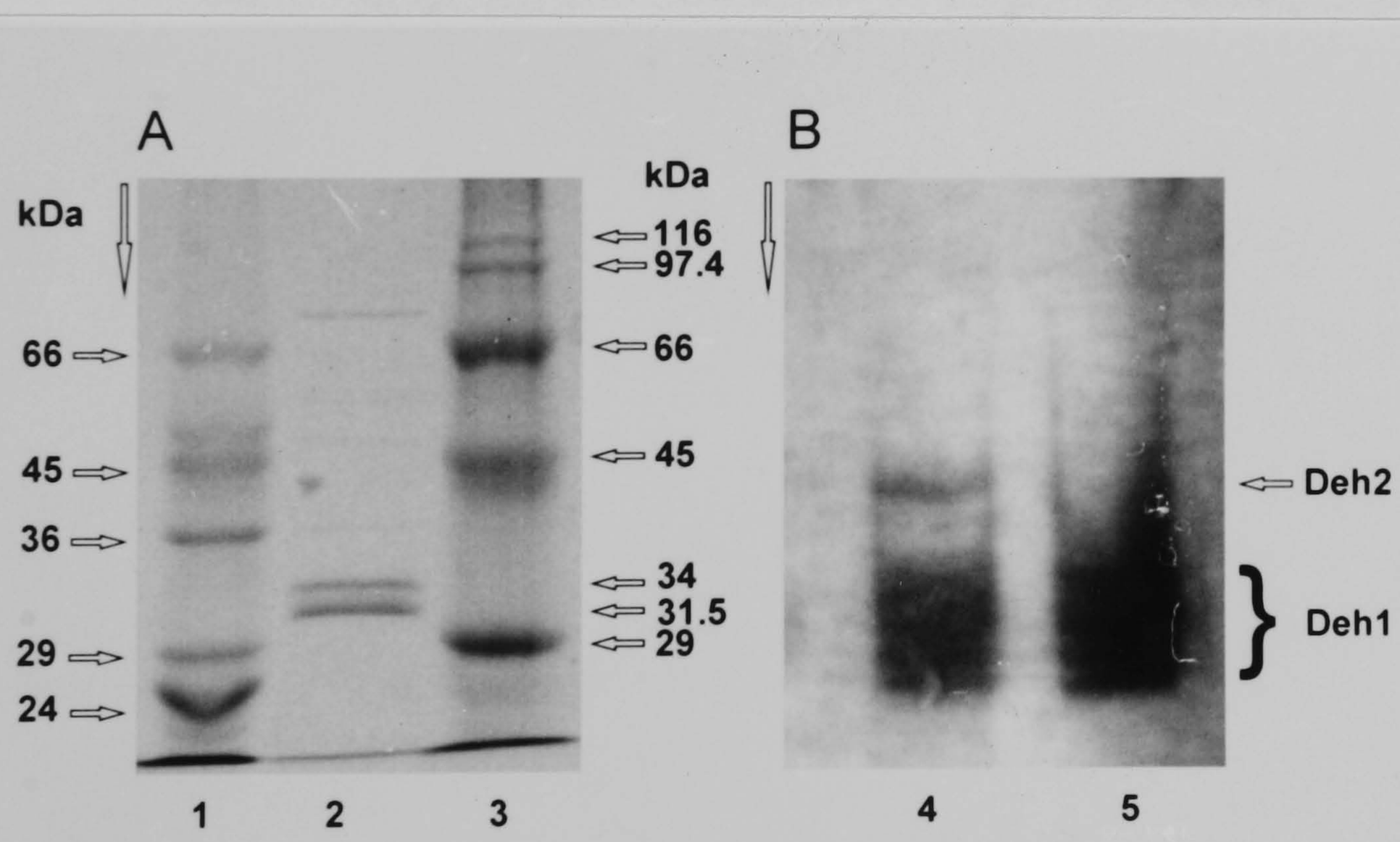


Figure 6.3: Polyacrylamide gels of the *Arthrobacter* sp H10a, epoxide hydrolase free enzyme preparation. A protein sample of the protein solution obtained after the Octyl-Sepharose CL-4B column, was electrophoresed on a (A) 12.5% polyacrylamide SDS gel (see section 2.2.8.2) and a (B) 8% polyacrylamide nondenaturing gel (see section 2.2.8.1). Lanes: 1, MW markers; 2 and 5, Octyl-Sepharose CL-4B eluate (5 µg protein); 3, high molecular weight markers; 4, *Arthrobacter* sp H10a crude extract (20 µg of protein). The vertical arrow indicates the direction of protein migration.

6.3. Enantioselective production of epoxides

Due to the commercial interest of chiral epoxides, the capacity of the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase to stereospecifically produce optically pure epichlorohydrin (ECH) from 1,3-dichloro-2-propanol, was evaluated. A preliminary reaction mixture contained 100 mM TRIS-SO_4, pH 8.0 10 mM 1,3-DCP and 0.01 U ml^{-1} of purified Deh1 haloalcohol dehalogenase (see section 4.2). The reaction was performed at room temperature for 5 h. The epichlorohydrin produced, was extracted with ethyl acetate and concentrated (see section 2.2.3.7). The optical purity of the epoxide obtained was determined by gas chromatograph fitted with a FS-Cyclodex alpha II/P (0.23 mm x 50 m) as described in section 2.2.3.7. As shown in Figure 6.4, the Deh1 dehalogenase produced (R)-ECH with an enantiomeric excess ((R-S)/(R+S) in which R is the major enantiomer) of 75%. Further studies on the optimization of the optical purity of (R)-ECH as well as on the yields of the epoxide obtained are described bellow.

6.3.2. Dehalogenation

The effect of the reaction conditions on the optical purity of (R)-ECH obtained by dehalogenation of 1,3-DCP catalyzed using the partial purified Deh1 haloalcohol dehalogenase (see section 6.2) were studied. Unless otherwise stated the reaction mixture (100 ml) contained 10 mM 1,3-DCP, 200 mM TRIS-glycine-acetate buffer, pH 8.0 and 0.175 U ml^{-1} of Deh1 dehalogenase (see section 2.2.6.7). The reaction was carried out in a water bath at 30°C. Samples, removed at 60 min reaction time, were analyzed for 1,3-DCP and ECH concentration (see section 2.2.3.6) and the optical purity of the ECH enantiomers produced was determined (see section 2.2.3.7).

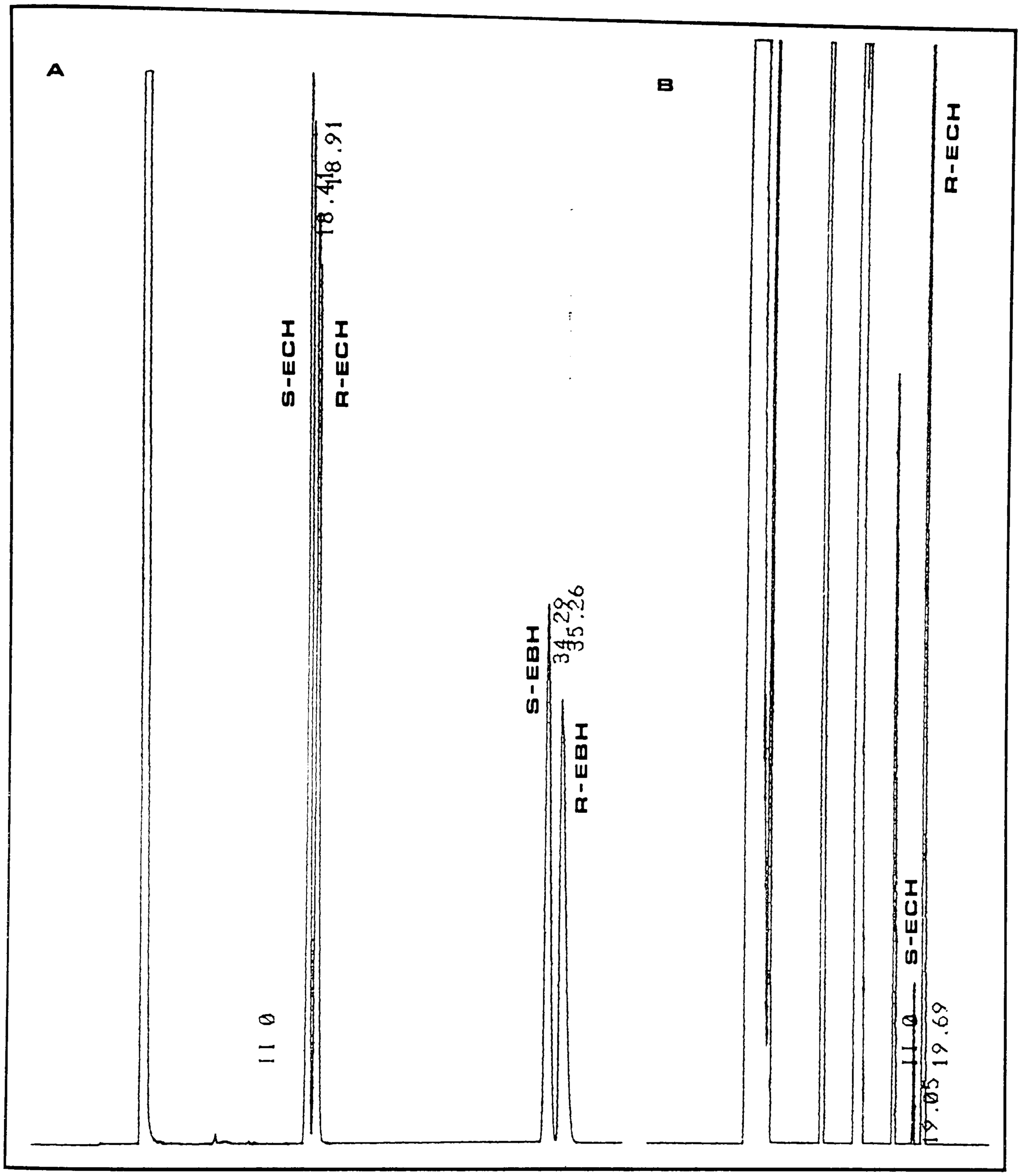


Figure 6.4: Estimation of epichlorohydrin optical purity produced by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase by gas chromatography. Samples: (A), 10% (RS)-ECH, 10% (RS)-EBH; (B), ECH obtained by dehalogenation of 1,3-DCP (10 mM) catalyzed by the Deh1 dehalogenase (0.01 U ml^{-1}) in TRIS-SO_4, pH 8.0, for 5 h at room temperature. Analysis conditions: column, 0.23 mm x 50 m FS-Cyclodex alpha II/P; column temperature, 40°C; injector and detector (FID) temperature, 200°C; carrier gas, helium (11 Psi); split ratio 1:50, sample volume 0.1 µl (A) and 0.5 µl (B).

The effect of the composition of the reaction buffer on the optical purity of (R)-ECH was studied. The highest optical purity of (R)-ECH (89.3% e.e.) was obtained using TRIS-glycine-acetate buffer (200 mM, pH 8.0). When the bioreactions were performed in only TRIS buffer (R)-ECH was produced to a lower enantiomeric excess (79.4% in 100 mM TRIS-SO_4 and 100 mM TRIS-Cl 44.7% e.e.). The effect of 1,3-DCP concentration on the optical purity of the epoxide produced was also investigated. At low concentrations of the substrate the enantiomeric excess of (R)-ECH decreased (Figure 6.5). The optical purity of (R)-ECH showed only a small increase at 1,3-DCP concentrations above 10 mM, in the reaction mixture. An increase in the bioreaction temperature resulted on a slight decrease in the optical purity of (R)-ECH produced (91.3%, 88.9% and 85.1% e.e. at 20, 30 and 37°C, respectively). However, the rates of dehalogenation decreased with the temperature (see section 5.3.3).

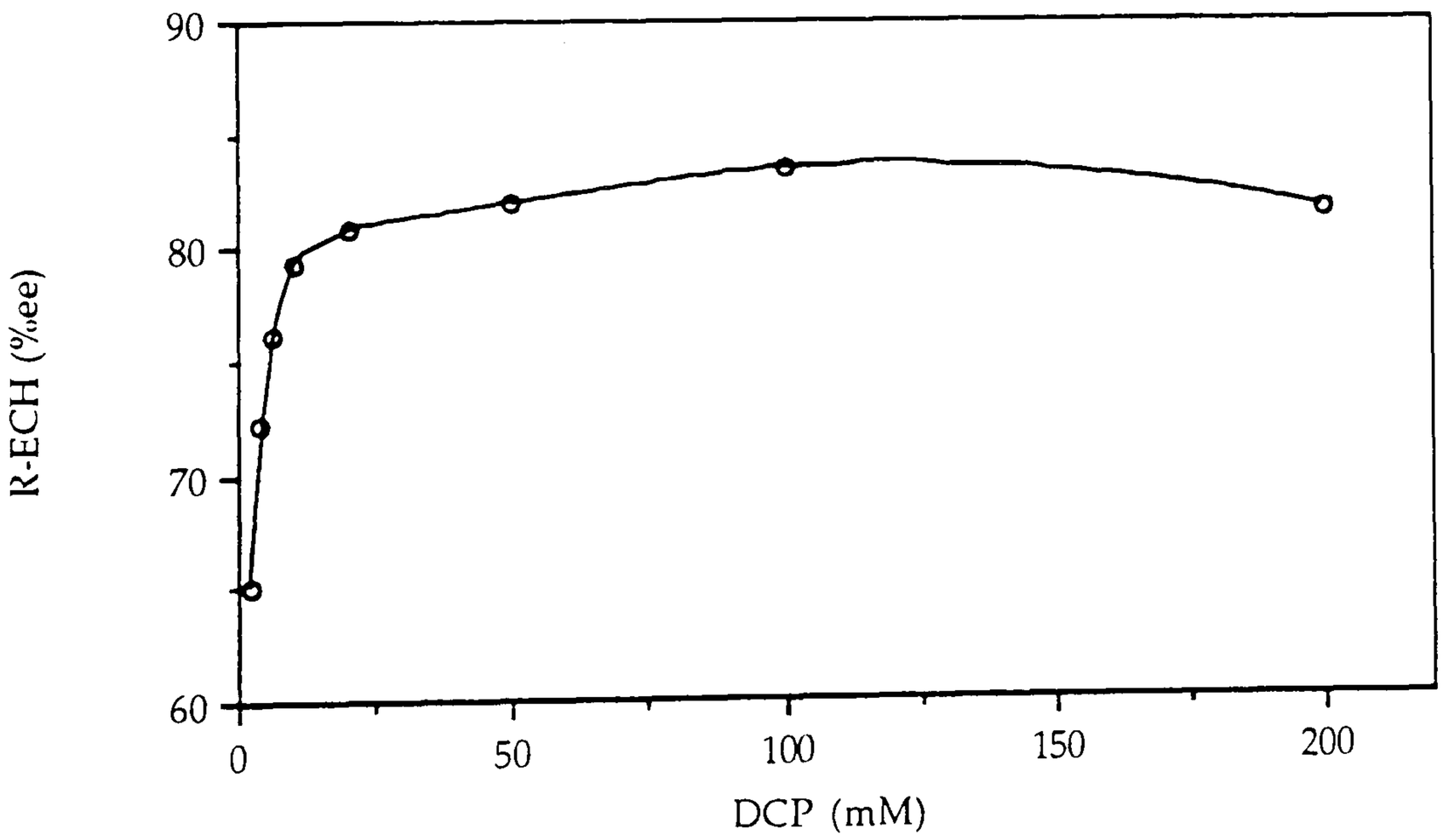


Figure 6.5: The effect of the concentration of 1,3-DCP on the optical purity of (R)-epichlorohydrin produced by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. The reactions were performed at 30°C in a reaction mixture containing 1,3-DCP, 100 mM TRIS-SO_4, pH 8.0 and 0.175 U ml^{-1} of Deh1 haloalcohol dehalogenase (see section 2.2.6.7). A sample was removed at 60 min reaction time and the optical purity of (R)-ECH analyzed (see section 2.2.3.7).

Transformation of 1,3-DCP into (R)-ECH, catalyzed by the Deh1 haloalcohol dehalogenase, at different pH (7.5, 8.0 and 8.5) was monitored with time. As seen in Figure 6.6, the rates of dehalogenation decreased with decrease in the pH of the reaction mixture. The yield of (R)-ECH produced at pH 8.0 (39%) was lower than when the bioreaction occurred at pH 7.5 and 8.5 (61-63% yield). The production profiles of (R)-ECH were similar, independent of the pH of the bioreaction. A maximum of (R)-ECH was obtained after 30 minutes and was maintained during 90 min, after which its concentration decreased eventual reaching zero after prolonged incubation. Variation of bioreaction pH showed no effect on the rates of (R)-ECH production and also on its optical purity (89-89.5% e.e.). However, the rates of (R)-ECH degradation increased with the pH. Formation and degradation of (S)-ECH was not affected by the pH of the bioreaction (Figure 6.6). Using the chromatographic conditions described in section 2.2.3.6, it was not possible to identify the degradation products of 1,3-DCP and ECH. Since ECH degradation was stereospecific ((R)-ECH degradation occurs while (S)-ECH was still being produced) it must be enzyme catalyzed. No significant degradation of 1,3-DCP was observed in the controls, which contained all the reaction components except the enzyme preparation.

The effect of high halogen concentrations (200 mM KBr), on the dehalogenation of 1,3-DCP, was also studied (Figure 6.7). Under these conditions, dehalogenation proceeded very slowly (5.02 µmol min^{-1} mg^{-1}in comparison with 163.3 µmol min^{-1} mg^{-1}, obtained for the reaction preformed without KBr). The yield of (R)-ECH production was very low even at the maximum level of production (yield 10.7%). However, (R)-ECH was obtained in very high enantiomeric excess (>95%). As found in bioreactions that occurred without added halogen, ECH was degraded after prolonged incubation. No degradation of 1,3-DCP occurred in the controls containing all the reaction components except the enzyme preparation.

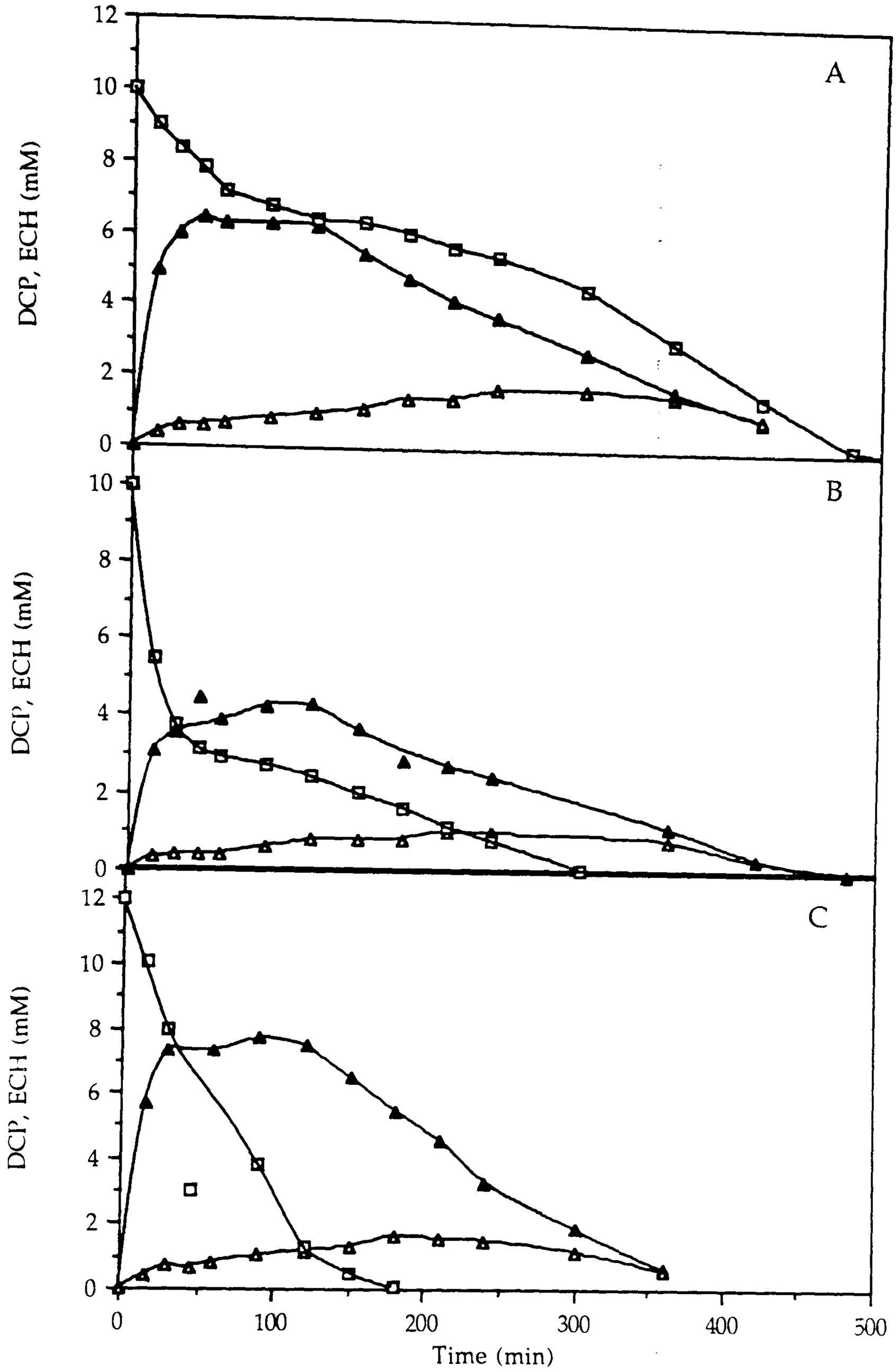


Figure 6.6: Effect of the pH on the formation and optical purity (R)-epichlorohydrin catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. The reaction was carried out at 30°C in 200 mM TRIS-glycine-acetate buffer, pH 7.5 (A), 8.0 (B) and 8.5 (C), 10 mM 1,3-DCP and 0.175 U ml^{-1} Deh1 haloalcohol dehalogenase (see section 2.2.6.7). A sample was removed at time intervals and 1,3-DCP, and ECH concentration (see section 2.2.3.6) and the optical purity of the obtained ECH enantiomers determined (see section 2.2.3.7). Symbols: (□) 1,3-DCP, (▲) (R)-ECH, (Δ) (S)-ECH.

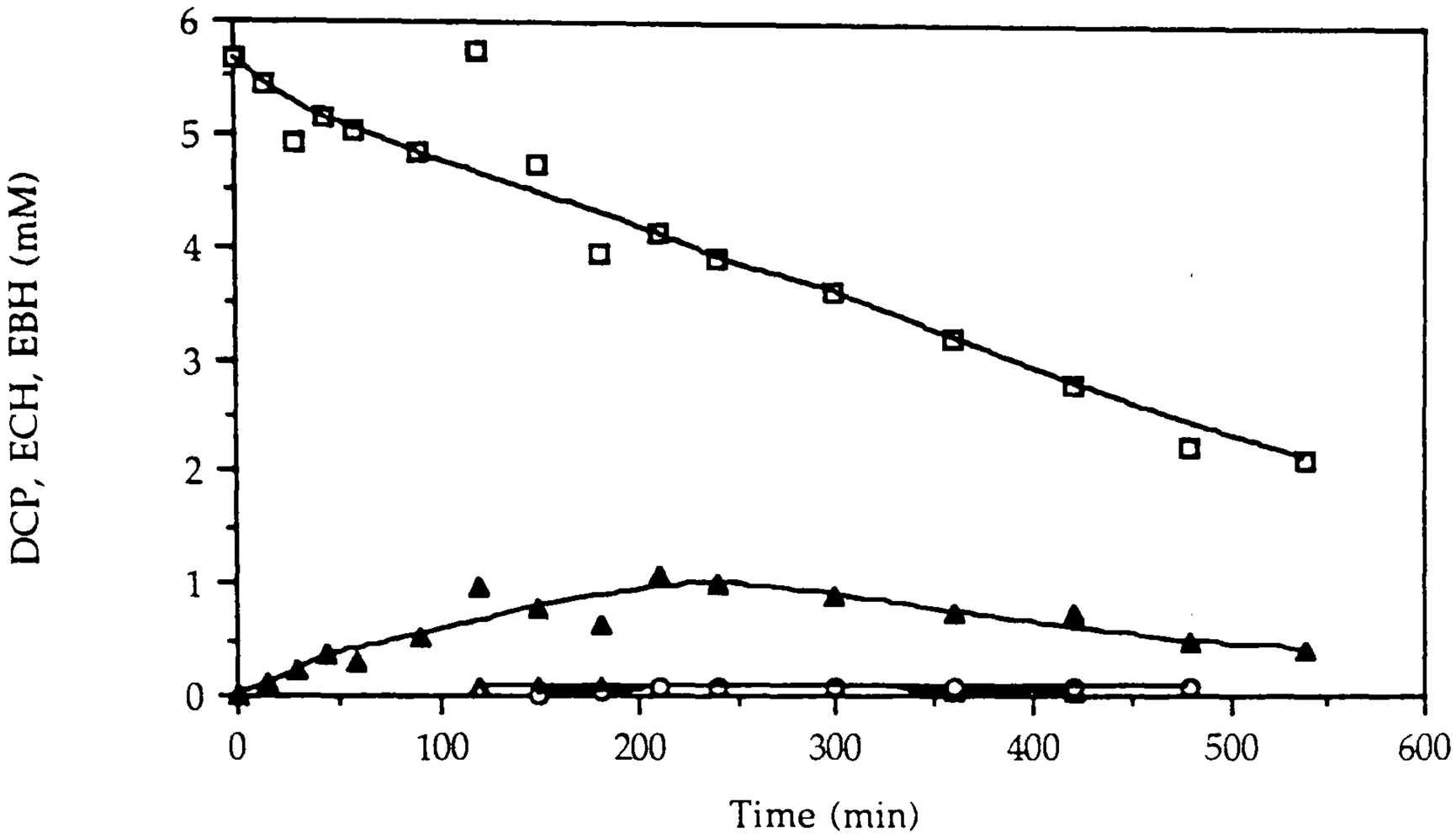


Figure 6.7: Effect of high halogen concentration on the formation and optical purity of (R)-epichlorohydrin catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. The reaction was carried out at 30°C in 200 mM TRIS-glycine-acetate buffer pH 8.0, 200 mM KBr 10 mM 1,3-DCP and 0.175 U ml^{-1} Deh1 haloalcohol dehalogenase (see section 2.2.6.7). A sample was removed at time intervals and 1,3-DCP and ECH concentration (see section 2.2.3.6) and the optical purity of the obtained ECH enantiomers determined (see section 2.2.3.7). Symbols: (□) 1,3-DCP, (Δ) (R)-ECH, (Δ) (S)-ECH, (●) (R)-EBH, (○) (S)-EBH.

6.3.3. Halogenation

The fact that the Deh1 haloalcohol dehalogenase is able to stereoselectively dehalogenate 1,3-DCP to (R)-epichlorohydrin, led us to investigate the selectivity of epoxide halogenation that was found to be catalyzed by the same enzyme in the presence of halogen ions (see section 5.2.2). The effect of the chloride concentration on

the stereoselective halogenation of the epoxide was studied. The bioreactions were carried out at 30°C and the reaction mixture contained 200 mM TRIS-glycine-acetate buffer, pH 8.0, 10 mM ECH, 0.175 U ml^{-1} of Deh1 haloalcohol dehalogenase and the indicated concentration of the halogen. The reaction samples were analyzed for 1,3-DCP and ECH concentration (see section 2.2.3.6) and the optical purity of ECH on the reaction mixture determined. The enantiomeric excess of the (S)-ECH that accumulated in the reaction mixture increased significantly with the concentration of chloride up to 40 mM (Figure 6.8). Further increase on the halogen concentration showed little effect on the optical purity of (S)-ECH.

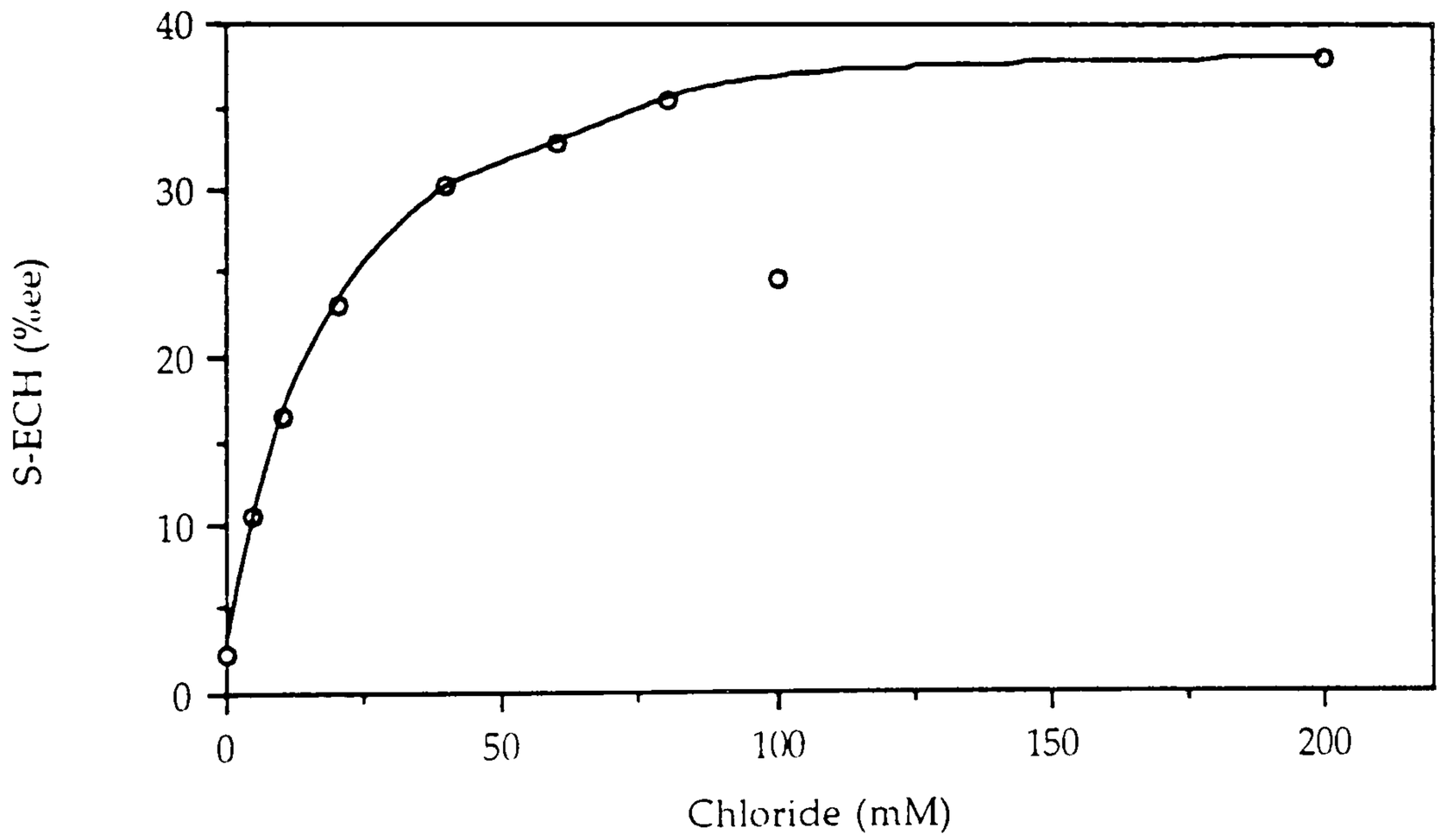


Figure 6.8: The effect of the concentration of chloride on the optical purity of (S)-epichlorohydrin produced by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. The bioreactions were performed at 30°C in a reaction mixture containing KCl, 10 mM ECH, 100 mM TRIS-SO_4, pH 8.0 and 0.175 U ml^{-1} of Deh1 haloalcohol dehalogenase (see section 2.2.6.7). A sample was removed at 60 min reaction time and the optical purity of (S)-ECH analyzed (see section 2.2.3.7).

The effect of chloride concentration on the halogenation of ECH, catalyzed by the Deh1 haloalcohol dehalogenase, was studied with time. The three chloride concentrations selected for detailed study (10, 50 and 200 mM) were chosen on the basis of the differences of the (S)-ECH optical purity values previously obtained (Figure 6.8). At low salt concentrations ECH halogenation proceeded slowly, and stereoselective degradation of (R)-ECH was only observed in the first hour of reaction (Figure 6.9 A). The maximum of enantiomeric purity of (S)-ECH was obtained at the 45 min reaction time (20% e.e.), at which point 32% of ECH has been degraded. Formation of 1,3-DCP was observed on the first 45 min of reaction (yield 12.4%), after which its degradation started and it was completely degraded during prolonged incubation (Figure 6.9 A).

When the chloride concentration in the reaction mixture was increased to 50 mM the rate of ECH consumption increased and (R)-ECH was dehalogenated preferentially (Figure. 6.9 B). Under these conditions, the maximum enantiomeric excess of (S)-ECH obtained was 63.6% for 44% of ECH degraded. 1,3-DCP was produced while ECH was present in the reaction mixture (28% yield at maximum of production), after which the concentration of the haloalcohol started to drop (Figure 6.9 B). When the concentration of chloride was increased to 200 mM, a maximum of 1,3-DCP was obtained after 30 min of reaction and the haloalcohol concentration was maintained for more than 500 min (Figure 6.9 C). The Deh1 enzyme halogenated (R)-ECH preferentially, although the rates of ECH degradation were lower than ones obtained with 50 mM KCl. The maximum of (S)-ECH optical purity (51.2% e.e.) was obtained at 90 min reaction time, at this point 60% of the epoxide was degraded (Figure 6.9 C). No significant degradation of ECH was found in the blanks, which contained all the components except the Deh1 haloalcohol dehalogenase. As found for the dehalogenation reaction, both substrate and product, disappeared after prolonged incubation (Figure 6.9). Using the conditions described (see section 2.2.3.6) products could not be detected by GC-MS.

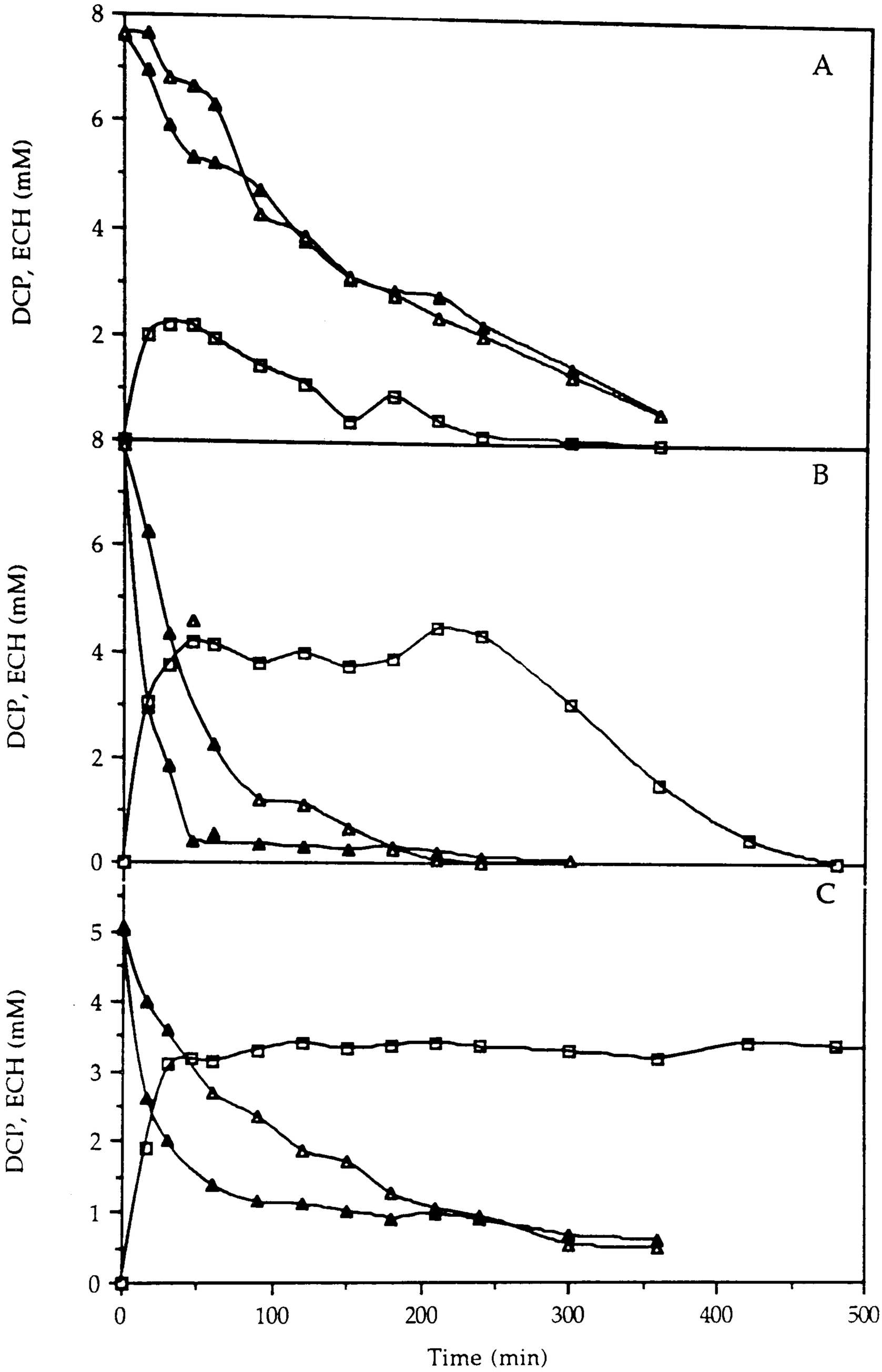


Figure 6.9: Effect of chloride concentration on the halogenation and optical purity of epichlorohydrin catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. The reaction was carried out at 30°C in 200 mM TRIS-glycine-acetate buffer pH 8.0, 10 mM ECH, 0.175 U ml^{-1} Deh1 haloalcohol dehalogenase and 10 (A), 50 (B) or 200 (C) mM of KCl (see section 2.2.6.7). A sample was removed at time intervals and 1,3-DCP and ECH concentration (see section 2.2.3.6) and the optical purity of the obtained ECH enantiomers determined (see section 2.2.3.7). Symbols: (□) 1,3-DCP, (▲) (R)-ECH, (Δ) (S)-ECH.

6.3.4. Transhalogenation

In order to distinguish between the disappearance of ECH due to halogenation of the epoxide and the reverse reaction of dehalogenation of the haloalcohol formed, halogenation of ECH was carried out with bromide and vice versa. The transhalogenation reactions were performed at 30°C, in a reaction mixture containing 200 mM TRIS-glycine-acetate, pH 8.0, 10 mM of epihalohydrin and 0.175 U ml^{-1} of the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase (see section 2.2.6.7). Halogen was added to the reaction mixture to a final concentration of 50 or 200 mM. Samples, removed during reaction, were analyzed for 1,3-DCP, 1,3-DBP, ECH and EBH concentrations (see section 2.2.3.6). The optical purity of the epoxides was also determined (see section 2.2.3.7).

When ECH and bromide were used as substrates, (S)-ECH was preferentially halogenated while (R)-ECH accumulated in the reaction medium during the first 90 min of bioreaction, after this time the concentration of (R)-ECH started to decrease eventually reaching zero after prolonged incubation (Figure 6.10). Increasing the halogen concentration had little effect on the halogenation profiles of ECH. The maximum optical purity for (R)-ECH was observed at the end of the bioreaction when most of the epoxide had been degraded (87.4% and 75.2% e.e., when the KBr concentration was 200 and 50 mM, respectively). An increase in the bromide concentration had no effect on the optical purity or the percentage of epibromohydrin formed (Figure 6.10). (R)-EBH was preferentially formed, but its enantiomeric excess never exceeded the 70.5% e.e. value, obtained at 15 min reaction time.

Most of the ECH was halogenated to produce 1,3-DBP independent of the bromide concentration in the reaction mixture (Figure 6.10). During the course of the reaction, 1,3-DCP was transiently formed and disappeared after prolonged incubation. In both bioreactions 1-bromo-3-chloro-2-propanol was detected, but, as this compound was not

commercially available, its quantification using GC-MS was not possible. Bromide showed no effect on epichlorohydrin stability as found in the blanks that contained all the bioreaction components except the Deh1 haloalcohol dehalogenase.

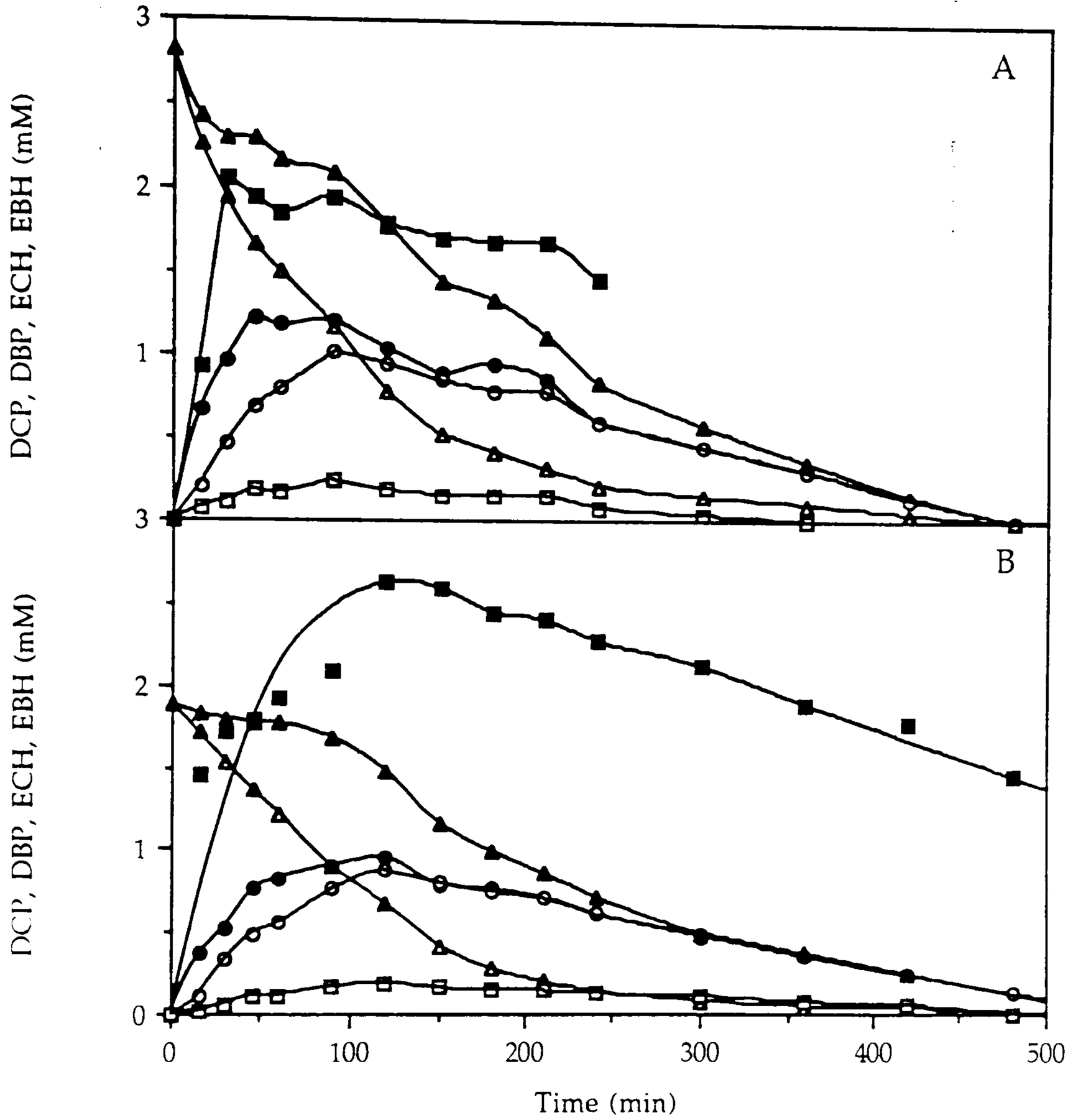


Figure 6.10: Effect of bromide concentration on the halogenation of epichlorohydrin and optical purity of the obtained epoxides catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. The reaction was carried out at 30°C in 200 mM TRIS-glycine-acetate buffer pH 8.0, 10 mM ECH and 0.175 U ml^{-1} Deh1 haloalcohol dehalogenase and 50 mM (A) or 200 mM (B) KBr (see section 2.2.6.7). A sample was removed at time intervals and 1,3-DCP, 1,3-DBP, ECH and EBH concentration determined (see section 2.2.3.6). The optical purity of the obtained ECH and EBH enantiomers was determined as described in section 2.2.3.7. Symbols: (□) 1,3-DCP, (■), 1,3-DBP, (▲) (R)-ECH, (Δ) (S)-ECH, (●) (R)-EBH, (○) (S)-EBH.

Halogenation of epibromohydrin, catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase in the presence of either 50 or 200 mM KCl, occurred preferentially at the (R)-isomer. Optically pure (S)-EBH (>95% e.e.) could be detected, after 90 min of reaction when the bioreaction was carried out in the presence of 200 mM KCl, with a yield of 14.5% (Figure 6.11 B). At lower chloride concentrations, the enantiomeric excess of (S)-EBH never exceeded 78.9% with a corresponding yield of 22.5%, that was obtained after reaction time of 45 min (Figure 6.11 A). (S)-ECH was stereoselectively formed, with a maximum enantiomeric excess of 91.5% (yield 30%) and 87.9% e.e. (yield 37%) at 50 and 200 mM KCl, respectively. The rates of (S)-EBH halogenation and (R)-ECH formation were not significantly affected by the chloride concentration. However, degradation of (R)-EBH and (S)-ECH was faster at higher salt concentrations (Figure 6.11). During the course of the reaction containing 50 mM KCl, 1,3-DCP was transiently formed at low concentrations (Figure 6.11 A). When the bioreaction was carried out in the presence of 200 mM KCl, 20% of EBH was converted to 1,3-DCP (Figure 6.11 B). 1-Bromo-3-chloro-2-propanol was detected under both conditions. However, its quantification was not possible, due to the fact that this compound was not commercially available. Epibromohydrin was stable in the controls, that contained all the components except the enzyme preparation. As found in all the bioreactions, in the presence of the enzyme preparation, the reaction components were degraded after prolonged incubation. Using the analytical procedure described in section 2.2.3.6. it was not possible to determine the degradation products.

6.4. Immobilization of the Deh1 haloalcohol dehalogenase

Enzymes offer important advantages over microorganisms since they can yield greater amounts of the desired product with less contamination. The reaction cost increment caused by the use of enzymes can be compensated for by the immobilization of the biocatalyst thereby prolonging the life of the biocatalyst by recycling it. 1,3-DCP can be

obtained at low cost from the petrochemical industry, hence purification of the Deh1 dehalogenase represents a significant additional process cost. Therefore, the stability and the performance of the Deh1 enzyme immobilized onto hollow fibers and alginate beads was studied.

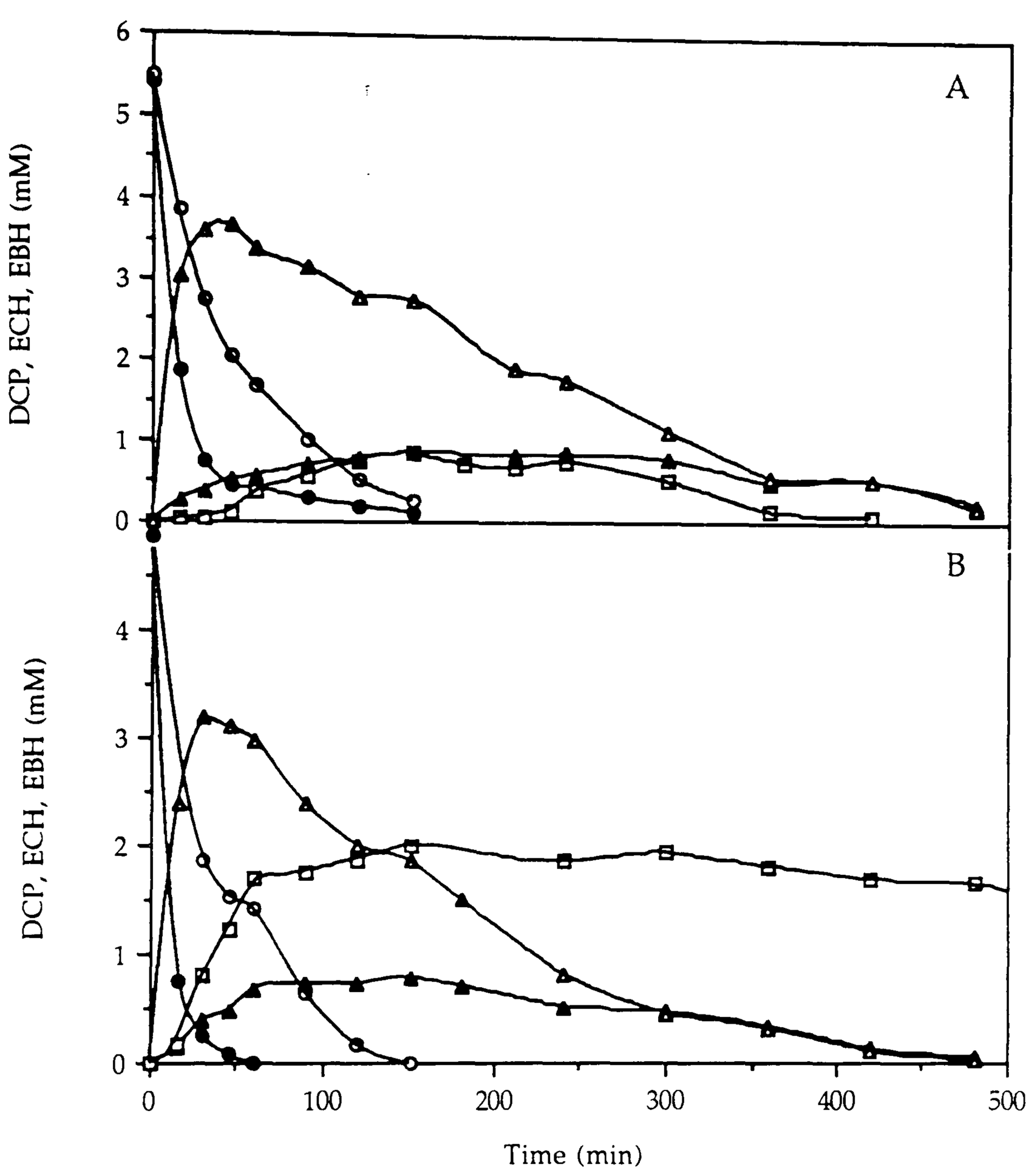


Figure 6.11: Effect of chloride concentration on the halogenation of epibromohydrin and optical purity of the obtained epoxides, catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. The reaction was carried out at 30°C in 200 mM TRIS-glycine-acetate buffer pH 8.0, 10 mM EBH and 0.175 U ml^{-1} Deh1 haloalcohol dehalogenase and 50 mM (A) or 200 mM (B) KCl (see section 2.2.6.7). A sample was removed at time intervals and 1,3-DCP, 1,3-DBP, ECH and EBH concentration determined (see section 2.2.3.6). The optical purity of the obtained ECH and EBH enantiomers was determined as described in section 2.2.3.7. Symbols: (□) 1,3-DCP, (Δ) (R)-ECH, (Δ) (S)-ECH, (●) (R)-EBH, (○) (S)-EBH

6.4.1. Immobilization in hollow fibers

A similar enzyme reactor to the one described by Diaz *et al.* (1989) was used in these experiments (Figure 6.12). The reactors contained nine Romicon P10 hollow fibers (Romicon, Woburn, Massachusetts) with a nominal molecular weight cut-off of 10,000. The fibers, 9 cm in length with a diameter of 0.11 cm, were inserted into the reactor after being conditioned (see section 2.2.11.1). The enzyme preparation (1.57 mg epoxide hydrolase free solution plus 2.5 mg of BSA in 8 ml of 100 mM TRIS-SO_4, pH 8.0) was loaded into the shell-side space surrounding the fibers and allowed to associate with them during incubation overnight at 4°C (see section 2.2.11.1). The following experiments were carried out at 30°C by submerging the reactor in a thermostatically controlled water bath. The hollow fiber reactor was operated in a single-pass mode, which is, a 100 mM TRIS-SO_4, pH 8.0, 10 mM 1,3-DCP was pumped (0.48 ml min^{-1}) into the shell side of the reactor, allowed to pass thought the fiber and collected from its lumen. The chloride in the effluent stream was periodically determined as described in section 2.2.3.2.

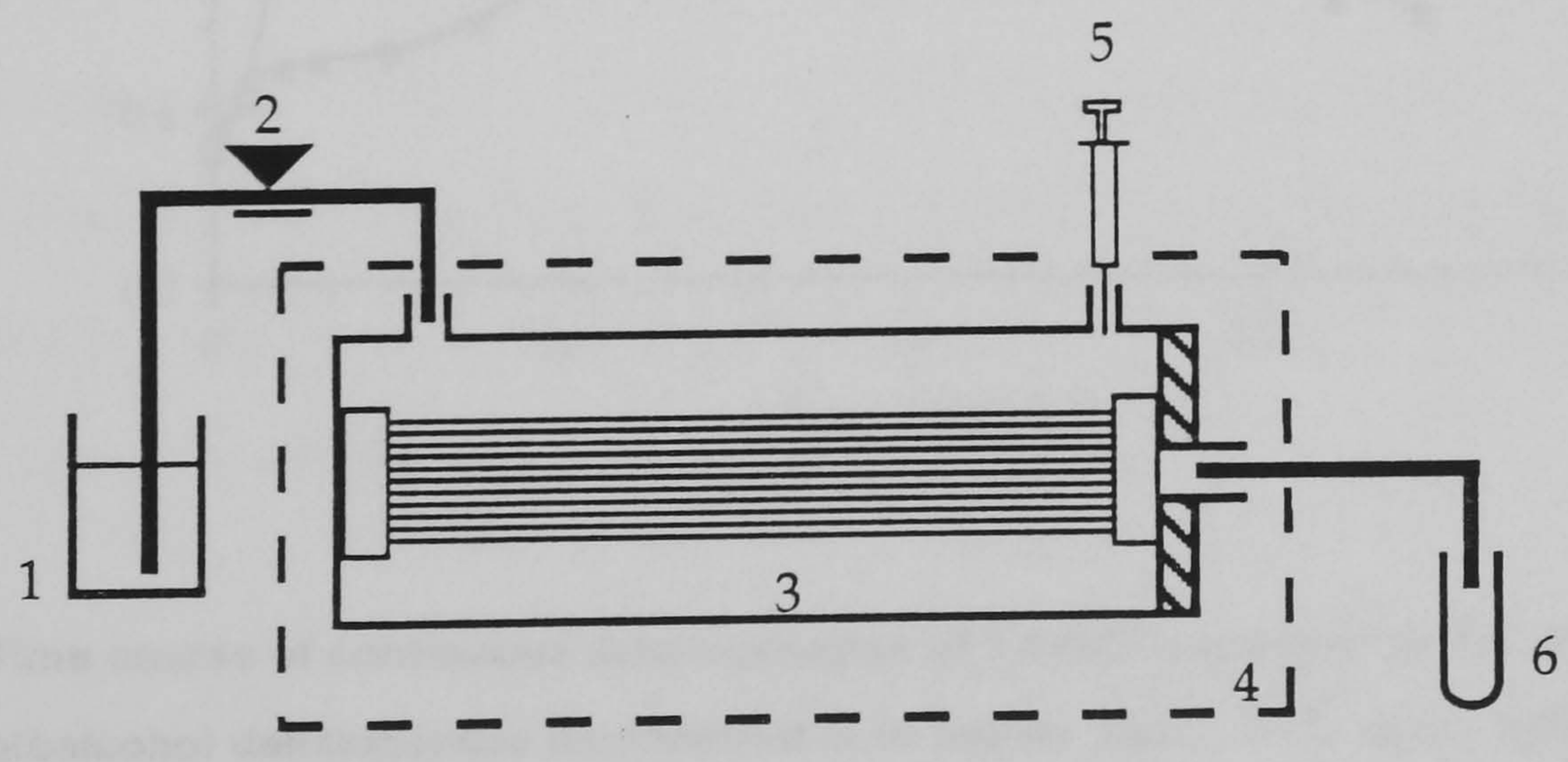


Figure 6.12: Schematic representation of the hollow fiber reactor. 1, Substrate reservoir; 2, peristaltic pump; 3, reactor vessel containing nine Romicon P10 hollow fibers; 4, thermostatically controlled water bath; 5, enzyme loading port; 6, sample collection (after Diaz *et al.*, 1989).

The performance of the immobilized Deh1 haloalcohol dehalogenase was studied in two consecutive assays. As shown in Figure 6.13, the chloride concentration increased with time, up to a maximum of 3.3 µmol ml^{-1} (20 min reaction time), after which it started to decrease. The substrate was removed by washing the shell-side of the reactor with 5 volumes (60 ml) of 100 mM TRIS-SO_4, pH 8.0, to remove all traces of substrate and reaction products. A second reaction was started by adding fresh 10 mM 1,3-DCP in buffer, and the reaction was carried out under the same conditions. Little chloride was release observed on the second bioreaction (Figure 6.13). Protein release was monitored (see section 2.2.3.1) on the washing solutions and effluents, and shown to be negligible.

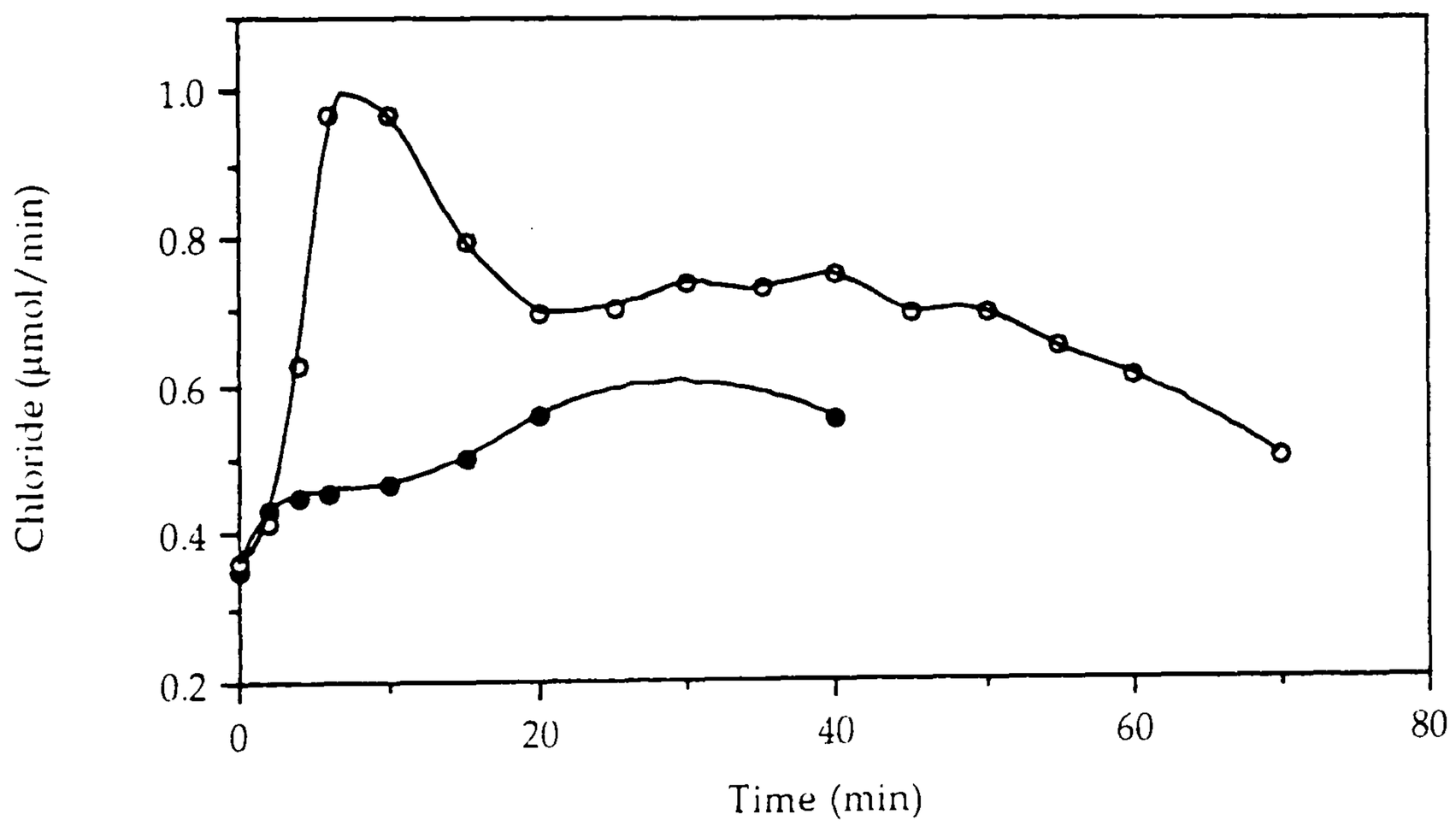


Figure 6.13: Time course of continuous dehalogenation of 1,3-DCP catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase immobilized onto hollow fibers. The reactor contained 1.57 mg of immobilized epoxide hydrolase free solution and was operated in a transverse mode (see section 2.2.11.1). The bioreaction was performed at 30°C in 100 mM TRIS-SO_4, pH 8.0 containing 10 mM 1,3-DCP. Samples were periodically taken from the effluent stream and the chloride concentration determined (see section 2.2.3.2). (○), first reaction; (●)second reaction.

6.4.2. Immobilization in alginate beads

The performance of the Deh1 haloalcohol dehalogenase in alginate beads was studied in a semi-continuous mode (after reaction the liquor was removed and the system refilled with fresh substrate solution). The epoxide hydrolase free enzyme preparation (2.0 mg in 5 ml of 100 mM TRIS-SO_4, pH 8.0) was mixed with the same volume of 4% alginate (sodium alginate from *Laminaria hyperborea*) and the beads formed as described in section 2.2.11.2. The Deh1 dehalogenase immobilized in the alginate beads was transferred to a open water-jacked vessel heated up to 30°C. The bioreaction was started by adding 30 ml of 10 mM 1,3-DCP in 100 mM TRIS-SO_4, pH 8.0 saturated with $CaSO_4$. Samples were removed from the reactor at different time intervals and the chloride determined (see section 2.2.3.2).

Chloride concentration increased in the reactor to a maximum of 3.9 µmol ml^{-1} corresponding to 30% degradation of the 1,3-DCP (Figure 6.14). The bioreaction continued for another 2.5 h without any increase in the halogen concentration. The reaction mixture was removed and the beads washed, as described above, to remove the chloride and a new batch of 1,3-DCP in TRIS-SO_4, pH 8.0 saturated with $NaSO_4$, was added. The reaction was performed under the same conditions (see above) and the chloride release determined. No dehalogenase activity was observed when the immobilized Deh1 dehalogenase was reused (Figure 6.14).

As found with the Deh1 immobilized onto hollow fibers, when the enzyme was used for long periods its activity disappeared. No significant decrease in dehalogenase activity was found when the immobilized enzyme was stored for 24 h at room temperature. The reaction mixtures and the washing buffers were monitored for protein release (see section 2.2.3.1), and this was seen to be negligible.

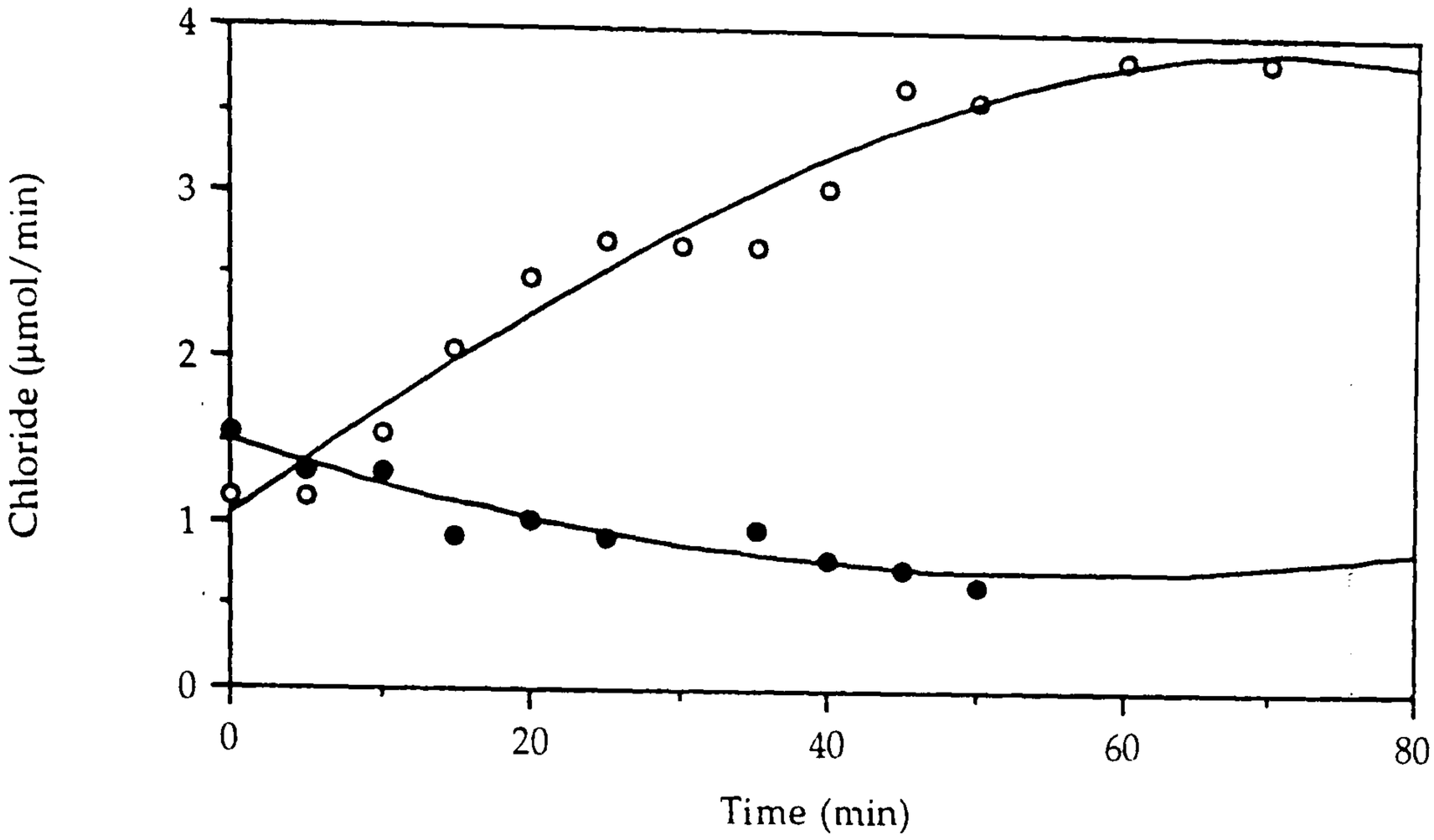


Figure 6.14: Time course of the dehalogenation of 1,3-DCP catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase immobilized on alginate beads. The reactor was operated on a semi-continuous method and contained 2.0 mg of epoxide hydrolase free enzyme solution immobilized by entrapment on 2% alginate beads (see section 2.2.11.2). The reaction was performed on a water-jacked vessel heated to 30°C. The reaction mixture contained 10 mM 1,3-DCP, 100 mM TRIS-SO_4, pH 8.0 saturated with $CaSO_4$. Samples were periodically taken and the chloride release determined. (○), first reaction; (●)second reaction.

6.5. Discussion

In spite of the availability of numerous methods for the synthesis of enantiomerically pure epichlorohydrin, a short and direct route that produces pure isomer with high yields would be commercially viable. Up to now, the approaches developed consist of the production of the chiral halohydrin by stereospecific assimilation of one of the isomers (Kasai *et al.*, 1991; Kasai *et al.*, 1992a). The halohydrin isomer is then chemically converted to epichlorohydrin resulting on a slight decrease in its enantiomeric purity (Kasai *et al.*, 1992b) with yields lower than 74% (obtained from the pure isomer) (Iriuchijima *et al.*, 1982; Kasai *et al.*, 1992b).

Direct production of optically active ECH from halohydrins, using either whole cells or enzyme preparations, has not yet been attempted. Production of ECH optical isomers using the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase was investigated. It was found that this enzyme dehalogenated 1,3-DCP and stereoselectively formed (R)-ECH with an enantiomeric excess of 75% (Figure 6.4). The enzyme I_b of *Corynebacterium* sp N-1074 also produced enantioselectively (R)-ECH (68.2% e.e.) from 1,3-DCP (Nakamura *et al.*, 1992). However, the resulting ECH from 1,3-DCP, catalyzed by the fraction I_a, was almost a racemate (11.7% e.e.) suggesting that enantioselective dehalogenation of halohydrins is not a general property of all the enzymes that catalyze this reaction.

The optical purity of (R)-ECH produced by the Deh1 haloalcohol dehalogenase could be improved by altering the reaction conditions. The buffer composition was seen to have an effect on the optical purity of the isomer formed. The best results were obtained when a TRIS-glycine-acetate buffer was used. The enantiomeric excess of (R)-ECH produced was lower when TRIS buffer pH was adjusted with an inorganic acid (HCl or H_2SO_4). Nakamura *et al.* (1991) have suggested that the dehalogenase I_a of *Corynebacterium* sp N-1074 was capable of catalyzing the addition of nucleophiles (e.g. CN^-) to epoxides. As such, high concentrations of chloride and, most probably, sulphate ions may stimulate the reverse reaction in which the (R)-isomer was consumed preferentially resulting on lower purity. The pH at which the bioreaction occurred, had little effect on the optical purity of (R)-ECH (Figure 6.6).

As expected, in the reverse reaction of ECH chlorination, the (R)-isomer was stereoselectively halogenated to 1,3-DCP (Figure 6.9). The maximum optical purity of (S)-ECH (63.6% e.e.) obtained was found when the concentration of KCl was 50 mM. This maximum occurred very early in the reaction and only a small percentage of ECH was halogenated, as such the yield was low (41%, relative to the total amount of ECH added). The low e.e. value obtained may be due to the fact that dehalogenation of the

halohydrin so formed might be occurring simultaneously and (R)-ECH was formed preferentially as observed on the above reaction. This effect was most notable at lower chloride concentrations (10 mM) in which the optical purity of (S)-ECH accumulated in the reaction mixture was low (20% e.e.).

The most interesting results were obtained from the transhalogenation experiments. When ECH and KBr were used as the substrates the Deh1 haloalcohol dehalogenase brominated preferentially the (S)-isomer and the epibromohydrin produced showed the (R)-configuration (Figure 6.10). However, if EBH and KCl were the starting reaction substrates, the enzyme preferentially chlorinated the (R)-isomer (Figure 6.11), as found in the halogenation experiments. Under these conditions, the transhalogenation product had the (S)-configuration. These results showed that the Deh1 haloalcohol dehalogenase dechlorinates halohydrins and preferentially forms the (R)-isomer. Under conditions that favour the reverse reaction, this enzyme chlorinates stereoselectively the (R)-epoxides. However, debromination of halohydrins catalyzed by the Deh1 enzyme results in the formation of the (S)-epoxide, and during the reverse reaction the (S)-isomer was selectively used. These observations suggest that either (1), the reactions involving the bromide and the chloride ions occurred at different active sites; or (2), due to stearic constraints imposed by the larger bromide ion, the substrate is positioned differently in the active site and hence, reaction occurring on, or forming the (S)-isomer is favoured.

By changing the reaction conditions it was possible to use the Deh1 haloalcohol dehalogenase to enantioselectively produce both isomers of ECH and EBH (Table 6.1). The best results were obtained when EBH plus 200 mM KCl were used as starting substrates. In these reaction conditions, enantiomericaly pure (S)-ECH could be obtained (yield, 14.5%) while the enantiomeric excess of (S)-ECH formed was 87.9% e.e. (yield, 36.6%). The enantiomeric excess and the yields of the ECH transhalogenation products, (R)-ECH (yield 7.1%; 87.4% e.e.) and (R)-EBH (yield

70.3%; 70.5%) were lower. The Deh1 haloalcohol dehalogenase showed higher enantioselectivity when halogen concentration was 200 mM.

Table 6.1: Production of optically active chiral epoxides by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. The configuration and the enantiomeric excess (% e.e.) of the epoxide formed by the Deh1 haloalcohol dehalogenase under different reaction conditions (see section 2.2.) and the yields obtained at its maxima production were determined.

Reaction conditions	Maximum optical purity of the epoxide obtained by stereoselective					
	Dehalogenation			Halogenation		
	Isomer	% e.e.	Yield (%)	Isomer	% e.e.	Yield (%)
Substrate: DCP						
⇨pH 7.5	(R)-ECH	89.0	61.0			
⇨pH 8.0	(R)-ECH	89.0	39.0			
⇨pH 8.5	(R)-ECH	89.5	63.0			
⇨pH 8.0, 200 mM KBr	(R)-ECH	>95	10.7			
Substrate: ECH						
⇨10 mM KCl				(S)-ECH	20.0	42.0
⇨50 mM KCl				(S)-ECH	63.6	41.0
⇨200 mM KCl				(S)-ECH	51.2	23.0
Substrate: EBH						
⇨50 mM KBr	(R)-EBH	70.5	11.6	(R)-ECH	75.2	18.7
⇨200 mM KBr	(R)-EBH	70.3	9.6	(R)-ECH	87.4	7.1
Substrate: EBH						
⇨50 mM KCl	(S)-ECH	97.5	30.0	(S)-EBH	78.9	22.5
⇨200 mM KCl	(S)-ECH	87.9	36.6	(S)-EBH	>95	14.5

An enantioselective biotransformation can be considered successful if the enantiomeric excess (e.e.) is above 95% (Santaniello *et al.*, 1992). Optically pure (R)-ECH was obtained when dehalogenation of 1,3-DCP, catalyzed by the Deh1 enzyme, occurred in the presence of 200 mM KBr (Figure 6.7). Under these conditions, dehalogenation was slow and the yields obtained were very low (10.7%). The high optical purity obtained

under these conditions may be due to the fact that the (S)-isomer formed would be selectively brominated (Figure 6.11) while the (R)-isomer accumulated in the reaction mixture. Further experiments with lower concentrations of bromide might result in faster dehalogenation rates, but it is possible that the optical purity of (R)-ECH could decrease as seen in the transhalogenation experiment (Table 6.1).

Obtaining an epoxide free hydrolase enzyme preparation was necessary in order to avoid subsequent degradation of epihalohydrin formed by dehalogenation of 1,3-DCP. It was possible to separate the epoxide hydrolase from the haloalcohol dehalogenase activity using a two step purification process, DEAE-Sephacel and Octyl-Sepharose column chromatography. The enzyme preparation obtained contained only the Deh1 haloalcohol dehalogenase (Figure 6.3) with only small amounts of other contaminants.

Immobilization of the Deh1 dehalogenase appeared to be an attractive alternative to a batch process since the biocatalyst could be recovered and re-used making the bioreaction economically feasible. However, using the two methods tested the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase could not be successfully immobilized. The reactors were operated in a continuous and a semi-continuous mode, respectively. Enzyme immobilization in hollow fibers resulted in a progressive decrease of the dehalogenase activity, suggesting inactivation of the Deh1 enzyme (Figure 6.13). Similar results were obtained when the dehalogenase was immobilized in alginate beads. The loss of activity was not due to denaturation during the prolonged incubation at temperatures above 4°C, since activity was maintained when the immobilized enzyme was stored at room temperature for 24 h. The loss of activity might be related to the requirement of an unknown co-factor for the enzyme activity.

Whole cells have advantages over purified dehalogenases, in that the enzyme activity would be restored either by recycling cofactors. However, due to the fact that the *Arthrobacter* sp H10a possess a highly active epoxide hydrolase enzymes, the ECH

formed would be rapidly degraded, which would result in lower yields. The possibility of using a microorganism that lacks epoxide degrading enzymes, such as the *Arthrobacter* sp AD2 (van den Wijngaard *et al.*, 1989) or a genetic engineered microorganism that contains the Deh1 dehalogenase gene (or other gene with similar features), would be of interest.

These results constitute only a preliminary study of the potential of the Deh1 haloalcohol dehalogenase on the production of the chiral epoxides. Further optimization of the reaction conditions may lead to the production of optically pure epoxides with better yields. In addition studies on the enantioselectivity features of this enzyme may reveal very interesting results, specially if other substrates and ions (e.g. CN^-) are used as substrates.

CHAPTER 7

GENERAL DISCUSSION

7.1. Introduction

The study of the degradation of many man-made chemicals by microorganisms has revealed a large number of different enzymes and catabolic pathways that were previously unknown. Toxic substances create a selective environmental pressure that favours the microorganisms that are able to completely degrade these chemicals or to transform them into intermediary molecules not harmful for the cell. These new activities may be acquired in two ways, mutation of either the regulatory or structural genes, or by horizontal transference of genetic information through plasmids or transposons. Evidence for evolutionary relationships has been obtained from comparative studies of the biochemical and biophysical features of the enzymes involved in the metabolic pathways. More recently, molecular biological techniques have allowed a more detailed approach to the study of these relationships. A better understanding of the biochemistry and genetics of degradation of xenobiotic compounds will allow for the manipulation of the genes that encode these enzymes and ultimately to construct strains with enhanced degradation abilities. With exception of 2-haloacid and 1,2-DCE dehalogenation, detailed biochemical and genetic studies of biodegradation of chlorinated hydrocarbons is lacking.

Although halogenated alcohols are important environmental pollutants, there is little information on their bacterial degradation. Much of the insight on dehalogenation of halohydrins is due to the ever growing interest on the synthesis of chiral halohydrins and epoxides for production of pharmaceuticals. Due to the commercial interest of these biotechnological products most of the studies have focused on the stereoselectivity of the enzymes involved in their synthesis. Little effort has been made towards the understanding of the mechanisms of biodegradation and the catalytic properties of the enzymes involved in such reactions.

The purification and biochemical characterization of a dehalogenase from a haloalcohol degrading bacteria, *Arthrobacter* sp H10a, have been described in the previous chapters. Here an overall image of the results obtained during this study as well as the comparison of the biochemical characteristics of this enzyme with others previously studied will be discussed. Based on the biochemical features of this enzyme a putative model for the mechanism of dehalogenation is also proposed. A comparison of the biochemical data available suggests that haloalcohol dehalogenases are a diverse family of enzymes. The classification of these enzymes on the basis of substrate specificity is also suggested.

7.2. The *Arthrobacter* sp H10a haloalcohol dehalogenases

The *Arthrobacter* sp H10a synthesizes constitutively, at least, two dehalogenases. Deh1 that is induced to higher levels by epoxides and shows greatest activity towards 1,3-DCP and Deh2, which has a higher affinity towards CPD and is induced to greater protein levels by haloalcohols (Table 3.3). These enzymes catalyze the dehalogenation of vicinal halohydrins (Table 5.3). Other studies on the Deh1, have shown that the dehalogenation products were the corresponding epoxides (Table 5.1), indicating that the enzymes belong to the haloalcohol dehalogenase family. Van den Wijngaard *et al.* (1991) proposed the systematic name of these enzymes as haloalcohol hydrogen-halide lyase (EC 4.5.1).

During the last four years a number of studies have focused on haloalcohol degrading bacteria. However, only a few of these studies concern the purification and the characterization of enzymes involved on the degradation of these compounds. Enzymes which catalyze the conversion of halohydrins to the corresponding epoxides, have been purified from a *Flavobacterium* sp (Castro & Bartnicki, 1968) *Arthrobacter* sp AD2 (van den Wijngaard *et al.*, 1991) and *Corynebacterium* sp N-1074 (Nagasawa *et*

al., 1992; Nakamura *et al.*, 1992). Purification and characterization of the *Arthrobacter* sp H10a dehalogenases involved in the degradation of 1,3-DCP was the main aim of the work presented in this thesis. The Deh1 haloalcohol dehalogenase has been purified and characterized. These studies have shown that the dehalogenase was constituted by two nonidentical subunits (relative molecular weight of 31.5 and 34 kDa), which associate with other proteins to form a large active enzyme of 200 kDa.

The *Arthrobacter* sp H10a and the *Corynebacterium* sp N-1074 (Nakamura *et al.*, 1992) haloalcohol dehalogenases showed on native gels similar activity profiles, suggesting a high degree of homology between the dehalogenating systems of the two bacteria. These bacteria possess two enzymes which catalyze the dehalogenation of 1,3-DCP. The enzymes designated Deh1 and I_b migrated as five protein bands, while the enzymes Deh2 and I_a migrated as a single protein band, localized just above the other dehalogenase (Figure 4.4). As suggested by the substrate profile of *Arthrobacter* sp H10a crude extracts (Table 5.3), the Deh2 and the I_a dehalogenases (Nagasawa *et al.*, 1992) also showed similar affinities for some substrates (very high activity towards the brominated alcohols and high activity towards CPD). Furthermore, the haloalcohol dehalogenase found in *Arthrobacter* sp AD2 exhibited the same catalytic profile (van den Wijngaard *et al.*, 1991). This enzyme also shared similar molecular weight subunits (28 and 29 kDa), N-terminal amino acid sequence, susceptibility to SH reagents, pH (8.0-9.0) and temperature optimum (50-55°C) with the dehalogenase I_a of *Corynebacterium* sp N-1074 (Nagasawa *et al.*, 1992).

The *Arthrobacter* sp H10a Deh1 dehalogenase is composed of two subunits which share similar molecular weights with dehalogenase I_b of the *Corynebacterium* sp N-1074 (Nakamura *et al.*, 1992). These dehalogenases were the first reported to be composed by two different subunits. However the molecular weight of the native Deh1 (200 kDa) was seen to be greater than that of enzyme I_b (115 kDa). Nakamura *et al.* (1992) attributed the formation of multiple bands on native gels by the I_b enzyme, to the

ratio of the two subunits on the composition of each protein band. Migration of the protein bands of Deh1 dehalogenase was determined not only by the ratio of the two subunits but also on the ratio and the molecular weight of other proteins that were found to comigrate with the two subunits (Figure 4.6 & 4.7).

Differences between the Deh1 and the *Corynebacterium* sp N-1074 I_b (Nakamura *et al.*, 1992) dehalogenases were also found on studies on their affinities towards the some substrates. The enzyme I_b showed high specificity for 1,3-dihalo-2-propanols with less than 1% of activity towards the other chlorinated alcohols tested (2,3-DCP, CPD, 1-MCP and 2-MCE). The Deh1 dehalogenase showed lower specificity and activity towards 1,3-DBP than with its chlorinated analogue. Further biochemical characterization of the *Corynebacterium* sp N-1074 I_b enzyme is necessary to determine how closely related are these two dehalogenases.

The biochemical studies have shown that the Deh1 dehalogenase has high affinity for 1,3-DCP with K_m values of 0.11 mM, which was significantly lower than the values obtained with other dehalogenases (2.44 and 8.5 mM, van den Wijngaard *et al.*, 1991; Nagasawa *et al.*, 1992, respectively). This enzyme also showed higher affinity towards CPD than the other two as shown by the lower apparent K_m (2.2 mM, Table 5.12). Deh1 enzyme has an optimum activity at higher pH values than the ones determined for the strain AD2 and N-1074 I_a dehalogenases. Sulphydryl reagents caused only slight inhibition of the dehalogenase activity of the latter enzymes whilst they caused total loss of the Deh1 activity.

The Deh1 and the *Corynebacterium* sp N-1074 I_b dehalogenases showed higher specificity towards 1,3-DCP than the *Arthrobacter* sp AD2 (van den Wijngaard *et al.*, 1991) and *Corynebacterium* sp N-1074 I_a (Nagasawa *et al.*, 1992) haloalcohol dehalogenases. In addition the Deh1 and I_b enzymes showed also higher specificity towards 1,3-DCP than the other two dehalogenases, suggesting that they are highly

specific and efficient biocatalysts. However, because both microorganisms possess the two types of dehalogenases, it might constitute a metabolic advantage for the bacteria since it allows a wider range of compounds to be used as carbon sources and with a greater efficiency.

7.3. Model for the dehalogenation mechanism of 1,3-DCP

Bartnicki and Castro (1969) studied the stereospecificity of the conversion of halohydrins into epoxides by the *Flavobacterium* sp haloalcohol dehalogenase. They have observed that transhalogenation of ECH and EBH occurred via 1-bromo-3-chloro-2-propanol and proposed that halogenation and dehalogenation proceed by attack on the terminal epoxide carbon (C_1). The study of the dehalogenation of 3-bromo-2-butanol also revealed that the epoxide formation step was a stereospecific *trans* elimination of HBr. Both reactions were explained by the same pattern of bond movements at the active site, in which negatively and positively charged loci were involved (Figure 7.1). The same transhalogenation products were found when the *Arthrobacter* sp AD2 (van den Wijngaard *et al.*, 1991) and *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenases were used as biocatalysts. This suggests that the reaction occurred also at the C_1. These studies indicate that enzymatic dehalogenation and halogenation reactions catalyzed by the haloalcohol dehalogenases follow a common pattern of bond cleavage and formation. The different substrate specificities found for the dehalogenases might be related to the amino acids involved in the catalysis that are located at the active site.

Studies on the Deh1 haloalcohol dehalogenase relative activity towards different halohydrins (Table 5.3) revealed some interesting features. This enzyme showed low activity towards 1,3-DCP analogues which do not possess halogens at the C_3 (e.g. CPD and 1-MCP). Furthermore, it was found that the Deh1 dehalogenase

stereoselectively degraded or formed epoxides, indicating that the position of the C_3 of either the halohydrins or the epoxides is probably restricted to a certain area in the active site. This might be explained by the existence of a third locus in the active site of the enzyme which binds to the C_3 or to the halogen bound to it. The low activity towards CPD could be due to steric effects or to the fact that the hydrogen of the hydroxy group is an electrophile as opposed to the chlorine which is a nucleophile. In the case of 1-MCP the hydrogen might be too far away from the third locus to interact with it.

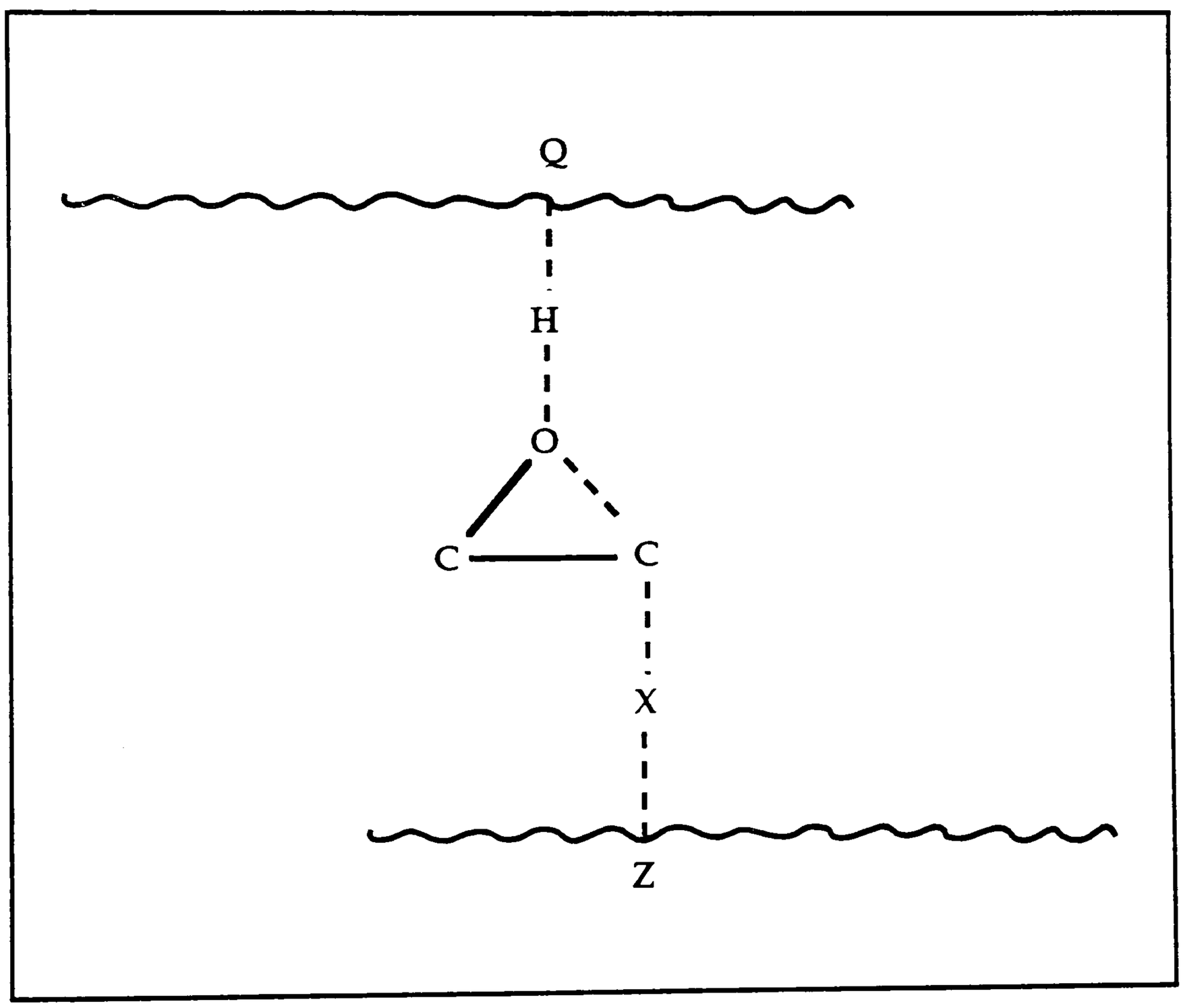


Figure 7.1: Putative dehalogenation mechanism of the haloalcohol dehalogenase of Flavobacterium sp. (After Bartnicki & Castro, 1969).

Although chemical modification of protein amino acids are not definitive indicators of the residues involved in the catalytic activity of an enzyme, it has been used as a

preliminary study to identify amino acids that might be located at the active site. The limitations of these methods are of three main types. For example, loss of activity during chemical modification can be caused by conformational alteration of the enzyme. Conservation of enzyme activity may also indicate lack of reaction either due to steric effects or to the ionic state of the amino acid or the reagent may not be the one that promotes the reaction. Protection of the enzymes by substrates during chemical modification of specific amino acids can be due to conformational alterations induced by the binding of the substrate to the enzyme making the targeted amino acid no longer available to the modifying reagent.

Having these considerations in mind, chemical modification studies were performed on the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase in order to compare its modification pattern with other dehalogenating enzymes and to elucidate the possible amino acid composition of the active site. Modification of cysteine and lysine residues resulted, respectively, in the total or partial loss of enzyme activity. In the case of Arg modification with 2,3-butadione, 1,3-DCP showed a protection effect but 2-MCE could not protect the enzyme activity (Table 5.10). These results suggest that an arginine residue might be located at, or very near to, the third locus, because the two carbon halohydrin (2-MCE) could not protect the Deh1 activity. Riordan *et al.* (1977) have suggested that arginyl residues function as general anion recognition sites in proteins. As such, an arginine residue that is possibly located at the third locus, may be involved in the stabilization of the C_3 position by interacting with the electronegative halogen bound to it. Substrate protection experiments using 1,3-DCP and 2-MCE have shown that these compounds could protect the Deh1 activity from inactivation by cysteinyl binding reagents. It is possible that a cysteine is either involved in catalysis or is positioned near to the amino acids that participate in the reaction. Cysteines and halogens are strong nucleophiles and so, they should not be positioned close to each other at the active site. Therefore, the reactive thiol group may be located near the C_2 hydroxy group.

Although it may be premature to suggest a detailed reaction mechanism of dehalogenation catalyzed by the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase, the susceptibility to amino acid modifying reagents, the substrate specificity and enantioselectivity of the enzyme indicate that the active site may have at least three loci that are involved in the reaction (Figure 7.2). According to this model, the binding of 1,3-DCP to the active site occurs at three points: the C_1 chlorine substituent interacts with the electrophile(s) of locus 1 (L_1), while the C_3 halogen interacts most probably with an Arg positioned at locus 3 (L_3). In this position the hydroxy group (at carbon 2) is located at locus 2 (L_2). Reaction should start with the nucleophilic attack, involving possibly a Cys, to the hydrogen of the hydroxy group (Figure 7.2 A). Probably, the negatively charged oxygen, formed in a transition state (Figure 7.2 B), might react with the C_1 resulting in the formation of the epoxide ring and cleavage of the C_1-Cl bond (Figure 7.2 C). Hydrolysis of the S-H bond would be necessary to restore the ionic state of the active site (Figure 7.2 D). It is possible that the reverse reaction occurs at the same site involving the same movement of bonds as proposed for the *Flavobacterium* sp halohydrin epoxidase (Bartnicki & Castro, 1969).

This model is consistent with the pH profile found for the Deh1 dehalogenase. The enzyme showed a maximum activity at alkaline pH, which would facilitate the hydrolysis of the S-H bond. The decrease in activity observed at pH values above 10.5 indicates that a strongly basic amino acid, such as arginine or lysine, that might be involved in the reaction, becomes uncharged. Furthermore the rapid increase on the activity between pH 6 and 8.5, indicates a residue with a pK_a of 7-8 is probably involved in catalysis (Price & Stevens, 1989, see pp 154-157). These results may relate to the cysteine which promotes the nucleophilic attack to the alcohol, as hypothesized above (pK_a 7.2 to 8.5, near a positive charged group; Webb, 1959) .

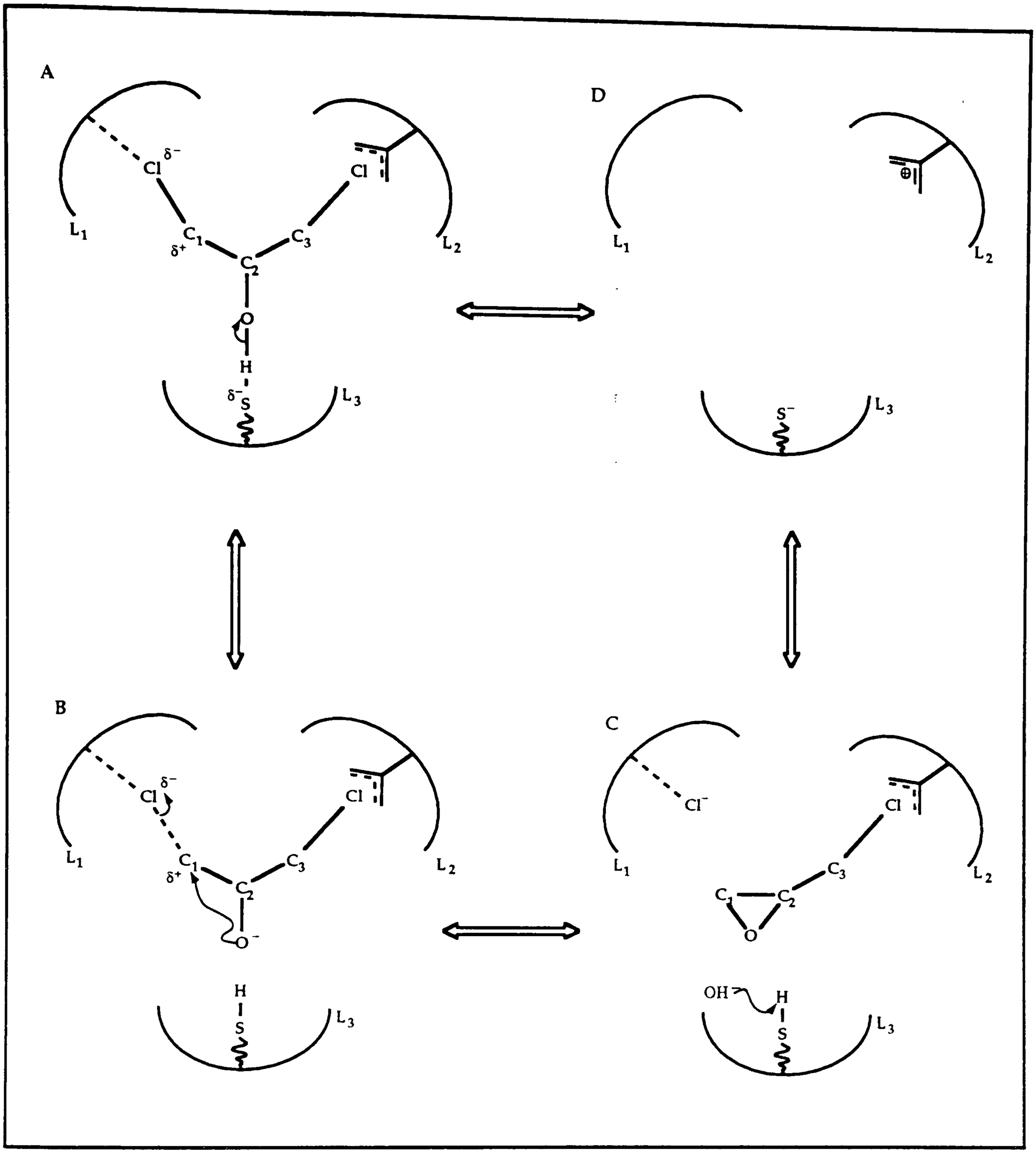


Figure 7.2: Proposed model of dehalogenation and halogenation for the Arthrobacter sp H10a Deh1 haloalcohol dehalogenase.

The importance and requirement of the third locus (L_3) for catalysis can be observed in the Deh1 haloalcohol dehalogenase stereospecificity when it catalyzed the bromination or chlorination of epoxides and also during the reverse reaction. The Deh1 dehalogenase dechlorinates 1,3-DCP and selectively formed (R)-ECH (Table 6.1). In

contrast, the dehalogenase acted on and formed selectively (S)-ECH, when bromide ions were involved in the reaction (Table 6.1). As opposed to what was observed with other dehalogenases (Hardman, 1991), the Deh1 enzyme showed higher activity towards 1,3-DCP than with the brominated analogue. This could be the result of a stearic effect due to the size of the halide ion. Since bromide is a larger atom, when positioned at the L_1 site, it might push the C_1 away from the L_1, and as a consequence the (R)-ECH molecule might turn clockwise. In this position the halogen at the C_3 also rotates and moves away from the L_3, and reaction rates would be slower due to stearic constrains. However, when (S)-ECH is at the active site, that is, with the chloride of C_3 and the hydroxy group placed at the L_3 and L_2, respectively, the C_1 might be placed away from the original position occupied by the same carbon of the (R)-isomer. This can be attributed to the fact that the length of C_1-C_2 bond is shorter (1.47 Å) than the one of C_2-C_3 (1.57 Å; Parker & Isaacs, 1959) (Figure 7.3). This would explain why bromination of ECH is slower than its chlorination and also why its bromination occurs selectively at the (S)-isomer. The same stearic effects should determine the configuration of the product during debromination of haloalcohols. Further experiments on the Deh1 stereoselectivity of dechlorination of CPD and 1-MCP and halogenation of the corresponding epoxides might expose other restrictions imposed on the substrates by the three dimensional structure of the active site.

In the case of the two carbon halohydrins (2-MHE), the movement of the substrate, induced by the presence of the bromide, would not be restricted by the position of L3, and so, the enzyme activity should be higher towards 2-MBE than with its chlorinated analogue. This hypothesis is consistent with the results obtained for the Deh1 haloalcohol dehalogenase (Table 5.3).

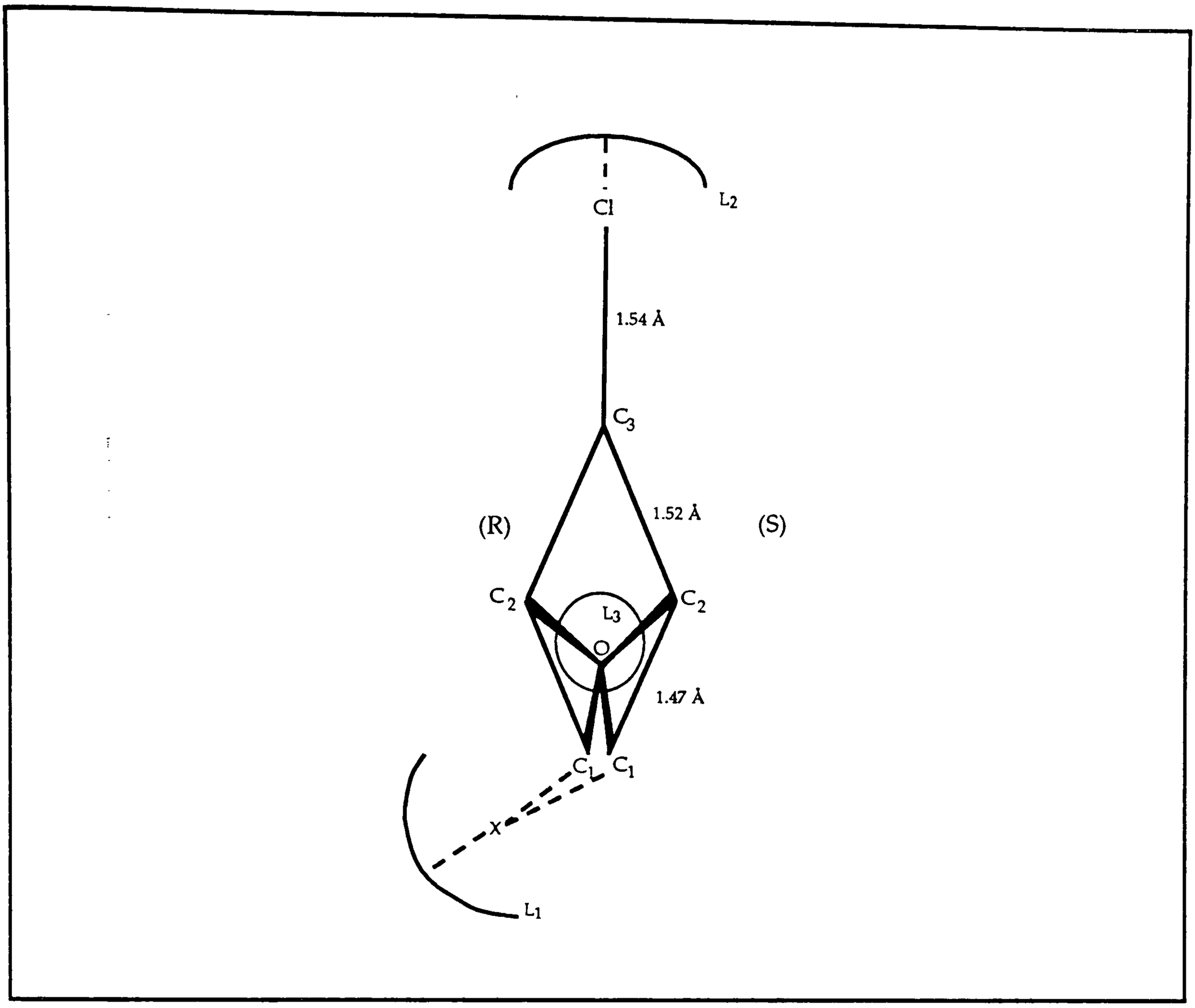


Figure 7.3: Proposed model for the position of both enantiomers of epichlorohydrin at the Deh1 active site.

The haloalcohol dehalogenase I_a of *Corynebacterium* sp N-1074 revealed very low enantioselectivity, producing ECH in an almost racemic form (Nakamura *et al.*, 1992). This enzyme also showed low specificity and higher activity towards 1,3-DBP than to its chlorinated analogue. These observations suggest that a possible third locus would have little influence on the enzymatic catalysis of this dehalogenase. Furthermore, this enzyme was not significantly inhibited by S-binding reagents, suggesting that the amino acids involved in the catalysis are different from those in the Deh1 haloalcohol dehalogenase.

7.4. Grouping of the haloalcohol dehalogenases

Grouping of enzymes into related families can be useful in respect that biochemical knowledge obtained from one enzyme may be applicable to another one of the same family. Furthermore, highly conserved regions in gene sequences in an enzyme group may indicate an amino acid or sequences of amino acids that are essential for the enzyme's activity (Asmara *et al.*, 1993). Besides the work done on the Coryneform bacteria (van den Wijngaard *et al.*, 1991; Nagasawa *et al.*, 1992; Nakamura *et al.*, 1992) and *Flavobacterium* sp (Castro & Bartnicki, 1968; Bartnicki & Castro, 1969; Geigert *et al.*, 1983) there is little information on the enzymatic features of haloalcohol degrading bacteria (e.g. the presence of isoenzymes and characterization) that would enable their grouping. In addition, most of these microorganisms have been isolated in screening programs aimed at the resolution of commercially important chiral glycerol derivatives, hence there has been little emphasis on the study of their enzymes. Despite these facts it is possible to group different dehalogenases on the basis of their substrate specificity which proved to be a good indicator of enzyme similarity (see section 5.1).

Comparison of the activity profiles found with the crude extracts of haloalcohol degrading bacteria suggests some diversity among their dehalogenating enzymes. Nondenaturating polyacrylamide gels performed with our laboratory isolates (Figure 4.17 A), revealed that a large number of haloalcohol degrading microorganisms possess more than one dehalogenase. As such, the activity profiles of haloalcohol degrading bacteria that were studied using the crude extracts, might have resulted from the action of more than one enzyme. However, it is still possible to distinguish five different types of haloalcohol dehalogenases according to their substrate specificity (Table 7.1, Figure 7.4). The group 1 enzymes, represented by the *Alcaligenes* sp DS-S-7G dehalogenase(s) (Suzuki *et al.*, 1992), were not capable of dehalogenating 1,3-dihalo-2-propanol (1,3-DHP).

The haloalcohol dehalogenases found in Coryneform bacteria showed very low or no activity towards 2,3-DHP. These enzymes can be further subdivided into three different groups according to their activity towards brominated alcohols and CPD. As such, group 2 enzymes, like the ones of *Arthrobacter* sp AD2 (van den Wijngaard *et al.*, 1991), the I_a of *Corynebacterium* sp N-1074 (Nagasawa *et al.*, 1992) and the Deh2 of *Arthrobacter* sp H10a, were 100 fold more active towards 1,3-DBP than towards the chlorinated analogue. These dehalogenases also shared a number of biochemical and biophysical features (see section 7.2). The *Corynebacterium* sp N1074 I_b (Nakamura *et al.*, 1992) and the *Arthrobacter* sp H10a Deh1 dehalogenases showed lower activity towards CPD (≤ 0.01%) than the group 2 enzymes. Although, these two enzymes share some biochemical and biophysical characteristics they have shown different substrate profiles and native molecular weight (see section 7.2), suggesting that they are different. The activity of the Deh1 dehalogenase towards the brominated alcohols was lower than with the chlorinated ones (Group 3). The *Corynebacterium* sp N-1074 haloalcohol dehalogenase I_b revealed high specificity towards 1,3-DCP and similar but higher activity towards the brominated alcohols (Group 4). More information on the biochemical features of the I_b dehalogenase are required in order to better establish the degree of similarity between the group 3 and 4 enzymes. Nakamura *et al.* (1992) claimed that they have cloned the I_b gene, but its sequence was not been published yet. Consequently, comparison of the dehalogenases amino acid sequences was not possible.

A fifth but non-homogeneous group of enzymes is constituted by the dehalogenases exhibiting broad substrate specificity (1,3-DHP, 2,3-DHP, HPD and 1-MHP). The dehalogenases of *Alcaligenes* sp DS-K-S-38 (Kasai *et al.*, 1992a) and *Pseudomonas* sp OS-K-29 (Kasai *et al* 1990) have shown opposite stereospecificity on the degradation of 2,3-DCP. The *Pseudomonas* AD1 dehalogenase(s) can be distinguished from the others by its relatively high activity towards the brominated

alcohols (van den Wijngaard *et al.*, 1989). While the *Flavobacterium* sp showed high specificity towards 1,3-DBP (Castro & Bartnicki, 1968). Although, major differences were found among these enzymes, it was not possible to accurately group these enzymes due to the lack of information on the enzymatic features of these bacteria.

Table 7.1: Haloalcohol dehalogenases. When possible the relative activity was standardized with respect to 1,3-DCP. 1, This work; 2, van den Wijngaard *et al.*, 1991; 3, Nagasawa *et al.*, 1992; 4, Nakamura *et al.*, 1992; 5, Castro & Bartnicki, 1968 and Geigert *et al.*, 1983; 6, van den Wijngaard *et al.*, 1989; 7, Kasai *et al.*, 1990; 8, Kasai *et al.*, 1992a; 9, Suzuki *et al.*, 1992.

Microorganism		1,3-DHP		2,3-DHP		HPD		1-MHP		2-MHE		Ref.
		Cl	Br	Cl	Br	Cl	Br	Cl	Br	Cl	Br	
Arthrobacter sp H10a	CFE	1.00	124.00			0.08		0.64		>0.01	0.56	1
	Deh1	1.00	0.60	0.00		>0.01		0.27		0.01	0.28	1
Arthrobacter sp AD2		1.00	311.00	0.00		0.10		0.11	37.8	0.00	1.02	2
Corynebacterium sp N-1074	I_a	1.00	125.00	>0.01		0.38		0.23		>0.01	0.57	3
	I_b	1.00	1.66	>0.01		0.01				>0.01	0.09	4
Flavobacterium sp			1.00		0.06		0.09	0.02	0.08	0.01	0.05	5
Pseudomonas sp AD1		1.00	17.5			0.07		0.90	3.86		0.38	6
Pseudomonas sp OS-K-29		1.00		0.10	0.19	0.33		0.08		0.12		7
Alcaligenes sp DS-K-S-38		1.00		0.50	0.60	1.10		0.50				8
Alcaligenes sp DS-S-7G		0.00		0.36	0.39	1.00	0.73	0.12		0.00		9

Dehalogenase enzymes have been grouped according to their relative electrophoretic mobility on polyacrylamide gel electrophoresis under non-denaturating conditions (Tsang *et al*, 1988). When this technique was applied to haloalcohol dehalogenases it shown that the enzymes of Coryneform strains migrated in positions different to those of others belonging to Gram negative isolates (Figure 4.17A). The model proposed for the classification of the haloalcohol dehalogenases based on their activity profiles have shown also that the Coryneform enzymes formed a distinct group of enzymes (enzymes which revealed very low or no activity towards 2,3-DCP). The antibody raised

against the Deh1 haloalcohol dehalogenase exhibited only immunocross-reactivity with two coryneform bacteria proteins. The haloalcohol dehalogenase of one of them (strain H10c) had the same electrophoretic mobilities as the ones of the *Arthrobacter* sp H10a Deh1 enzyme. The *Arthrobacter* sp HK1, which has shown no immunocross-reactivity with the Deh1 antibody, possesses only one haloalcohol dehalogenase that has the same electrophoretic mobility as the Deh2 enzyme.

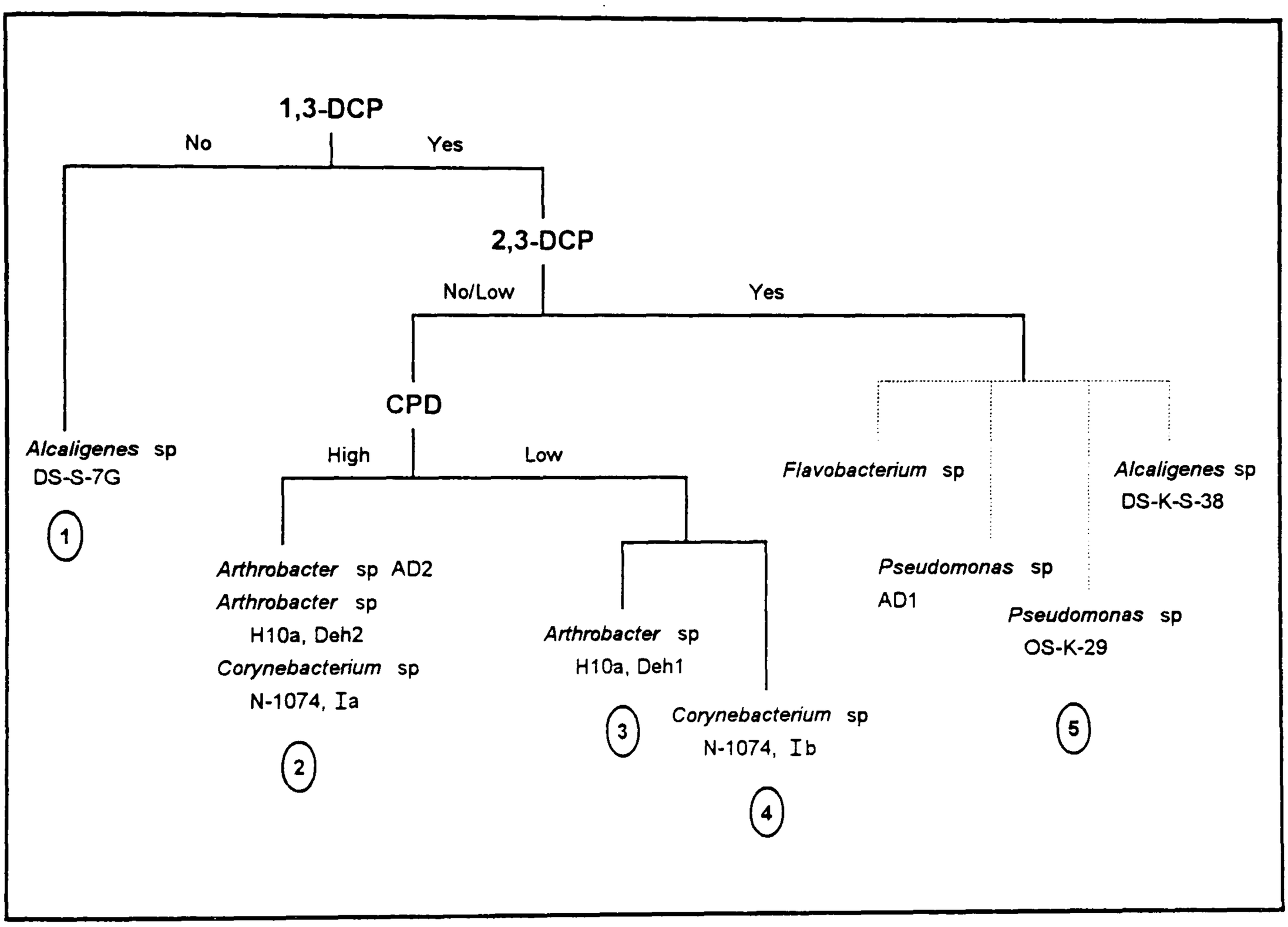


Figure 7.4: Diagram showing the proposed classification of the haloalcohol dehalogenases based on the substrate specificity of the enzymes.

As such, based on the electrophoretic mobility on native gels, the haloalcohol dehalogenases of Coryneform bacteria of this laboratory collection can be subdivided into two immunologically distincts groups. One represented by the Deh2 and the *Arthrobacter* sp HK1 dehalogenases, that correspond to the group 2 enzymes in the

above classification. The second group, which contains the Deh1 dehalogenase and the immunologically related strain H10c dehalogenase, would be representatives of the group 3. Unfortunately, there is no information on the biochemical features of the strain H10c and HK1 dehalogenases that would allow to establishment of a more reliable link between the two classification systems.

7.5. Regulation of the enzymes involved on degradation of haloalcohols

Degradation of haloalcohols by the *Arthrobacter* sp H10a occurs via the formation of the corresponding epoxides (Table 5.1). The epoxides are then hydrolyzed by an epoxide hydrolase(s) to the corresponding alcohols. Accumulation of the toxic epoxides inside the cell is avoided by a faster epoxide degrading system (Table 5.1). This can be achieved either by expression of high levels of an epoxide hydrolase or an enzyme with higher specific activity. Regulation can also take place at the dehalogenation step either by controlling the expression of the enzyme or its activity. The two dehalogenases of *Arthrobacter* sp H10a were produced constitutively. However the Deh1 activity of the crude extracts was increased by 2-3 fold if glycidol was present on the growth medium (Table 3.3). However, the increase of the dehalogenase activity had not significant effect on the chloride release. These results suggest that besides regulation of gene expression, other factor(s) control the dehalogenase activity in the cell. This might be related to the capacity of the dehalogenase to catalyze the reverse reaction. The excess of epoxide in the growth medium would be converted to the less harmful alcohol, which could later be used for growth. In addition, if halogenation and dehalogenation occurs at the same site as proposed above (see section 7.3), epoxides would behave as competitive inhibitors for the dehalogenation reaction. In this case the bacterium has several ways of controlling the concentration of epoxide in the cell: gene expression of both (1) dehalogenase and (2) epoxide hydrolase and (3) by switch the haloalcohol dehalogenase to catalyze the reverse reaction.

Regulation of the *Arthrobacter* sp AD2 haloalcohol dehalogenase is also similar to that found with the strain H10a. The specific activity of the dehalogenase of AD2 cells grown on epichlorohydrin was 3 to 4 times higher than the CPD grown cells. Most of haloalcohol degrading bacteria found to date have a faster epoxide hydrolyzing system than their dehalogenase(s), transforming dehalogenation into the limiting step of the degradation of haloalcohols. Where microorganisms have faster dehalogenating enzymes, their growth is restricted to substrates for which the dehalogenase had a poor substrate specificity (Castro & Bartnicki, 1968; Suzuki *et al.*, 1992), further stressing the importance of an efficient epoxide degrading system for the degradation of halohydrins. As such, engineering of a better catalyst should be directed to the expression of higher levels of epoxide hydrolase.

7.6. Future directions

Interesting questions were brought to light during the characterization of the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase. Firstly the high degree of homology found between the two subunits raises the question if the small differences in molecular weight between the two subunits are determined at the DNA level or are due to post transcriptional and/or post-translational events? Secondly, why do these two polypeptides associate to form a high molecular weight enzyme? Could this be advantageous in terms of higher enzyme activity or stability? To our knowledge this is the first time that a dehalogenase constituted by two homologous subunits has been isolated. The answer to the above questions might reveal some of the mechanisms that might take place in the cell when it is submitted to an environmental pressure to degrade unusual substances.

Isolation and sequencing of the gene(s) that encode(s) the Deh1 dehalogenase and characterization of mutants deficient in either of the subunits (possibly strain H10f) might shed more light on the function of these polypeptides. In addition, cloning and characterization of the Deh1 dehalogenase gene(s) will also clarify the relation between the two subunits and point out amino acids, or sequence of amino acids that might have a relevant role on the enzyme activity. Gene duplication seems a probable evolutionary mechanism, since independent of the growth conditions the proportion of the two subunits is 1:1 (determined by the band intensity in SDS-PAGE and Western blot analysis). Gene duplication renders the organism with a metabolic advantage since this often leads to increased enzymatic activity (Hardman, 1991).

Although the *Arthrobacter* sp H10a Deh1 and the *Corynebacterium* sp N-1074 I_b showed some differences, they share a distinct fingerprint on native gels (Nakamura *et al.*, 1992) indicating some degree of homology between them. As suggested above (see section 7.4) the Deh2 dehalogenase belongs to the same enzyme group as the *Arthrobacter* sp AD2 (van den Wijngaard *et al.*, 1991) and the *Corynebacterium* sp N-1074 (Nakamura *et al.*, 1992) dehalogenases. The fact that these coryneform bacteria have been isolated from soil samples collected in different parts of the world (UK, Netherlands and Japan), raises also the interesting question about the evolution of these dehalogenases.

Although the results that were obtained from biochemical studies performed on the *Arthrobacter* sp H10a Deh1 haloalcohol dehalogenase do not allow a precise formulation of a mechanism of dehalogenation, they point to the proposed mechanism with three binding sites being involved. Further studies on the reaction mechanism of this enzyme require cloning and sequence of the gene(s) and crystallographic analyses of the protein structure. A better understanding of the dehalogenase structure and function will ultimately lead to engineering of an enzyme with higher stereoselectivity and activity.

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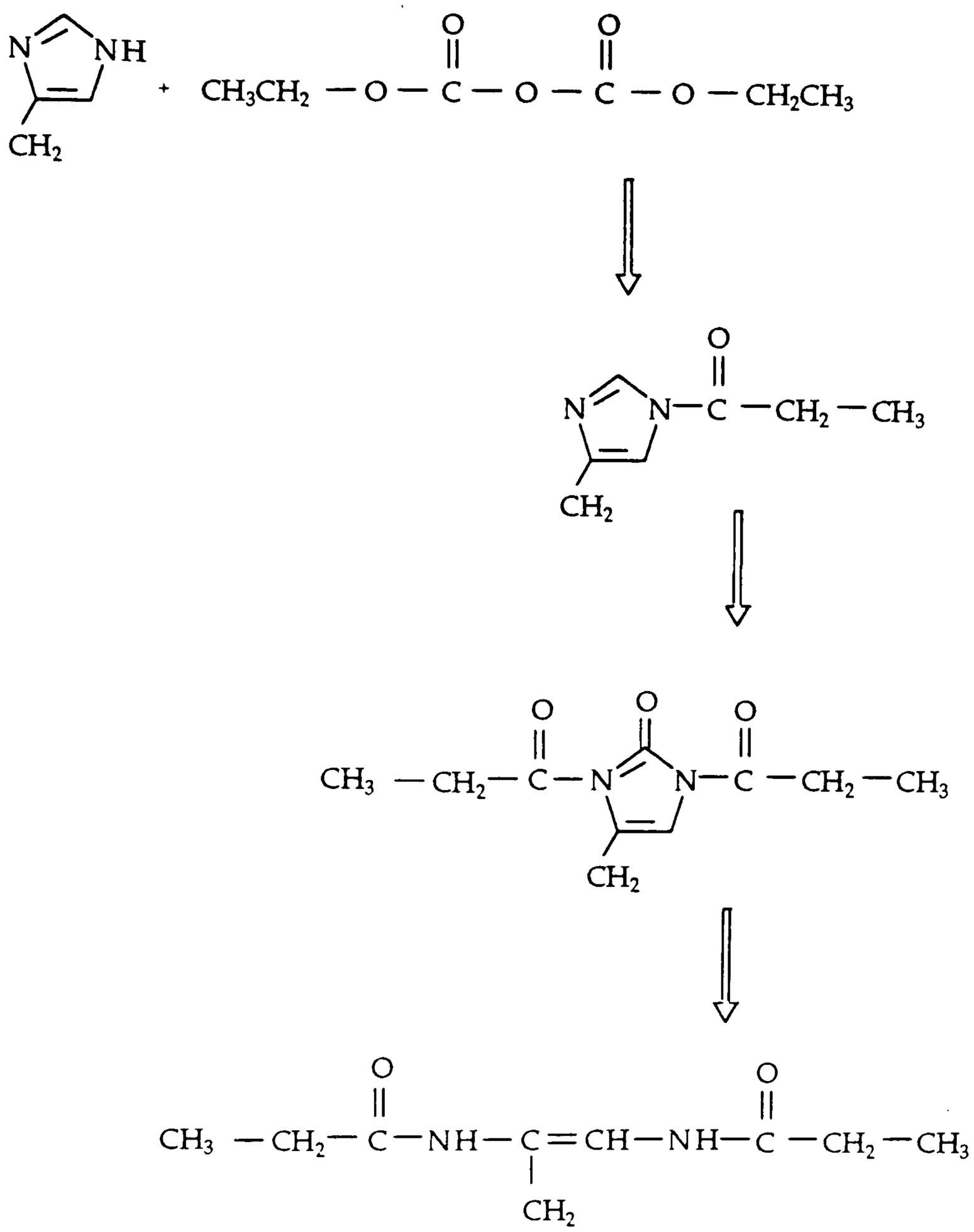
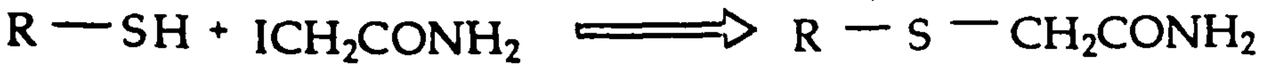
APPENDIX A

A1 Modification of histidine with diethylpyrocarbonate

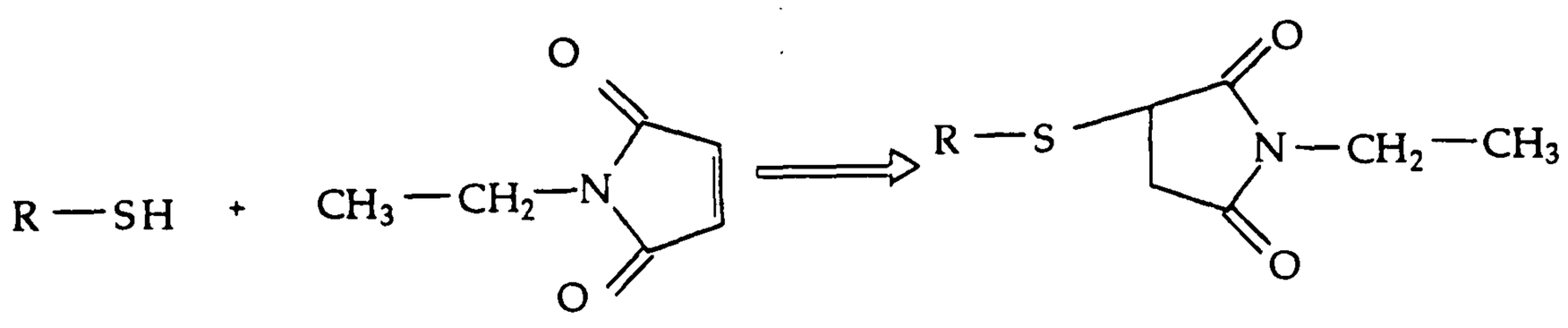


A2 Modification of cysteine

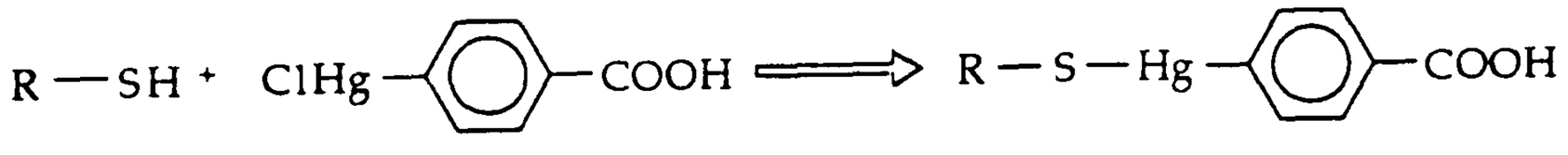
A2.1 With iodoacetamide



A2.2 With N-ethylmaleimide

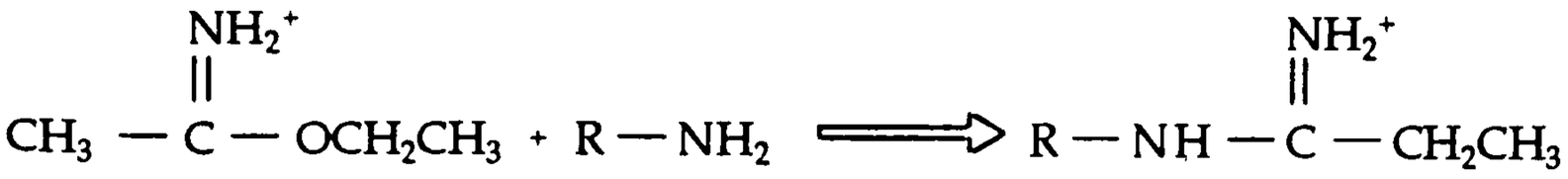


A2.3 With p-chloromercuric benzoate

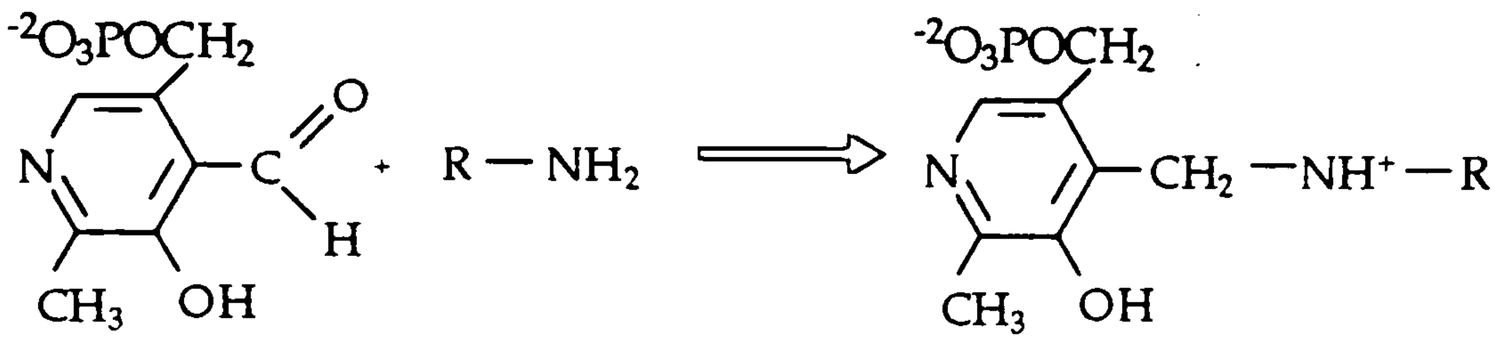


A3 Modification of lysine

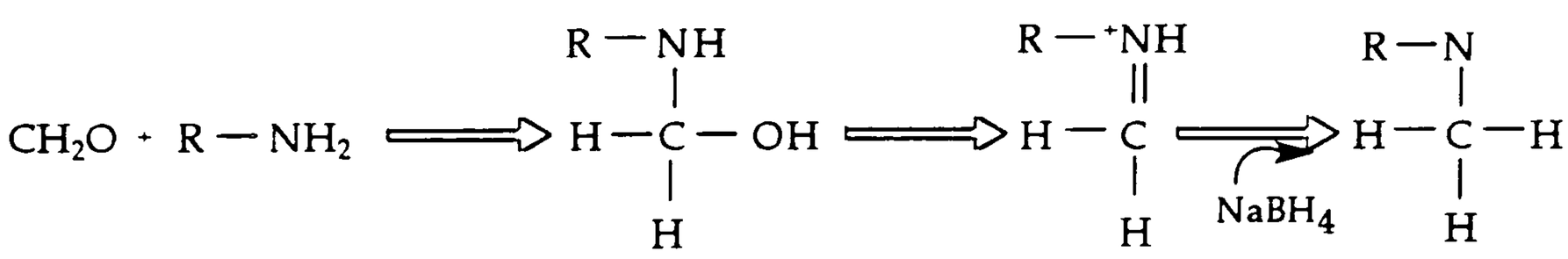
A3.1 With ethyl acetamidate



A.3.2 With pyridoxal phosphate

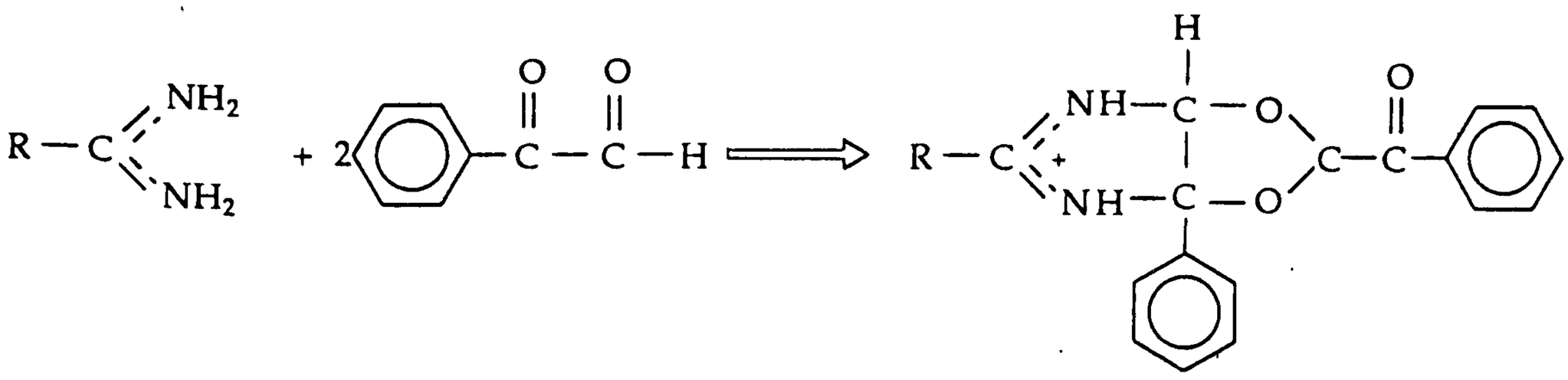


A3.3 With formaldehyde

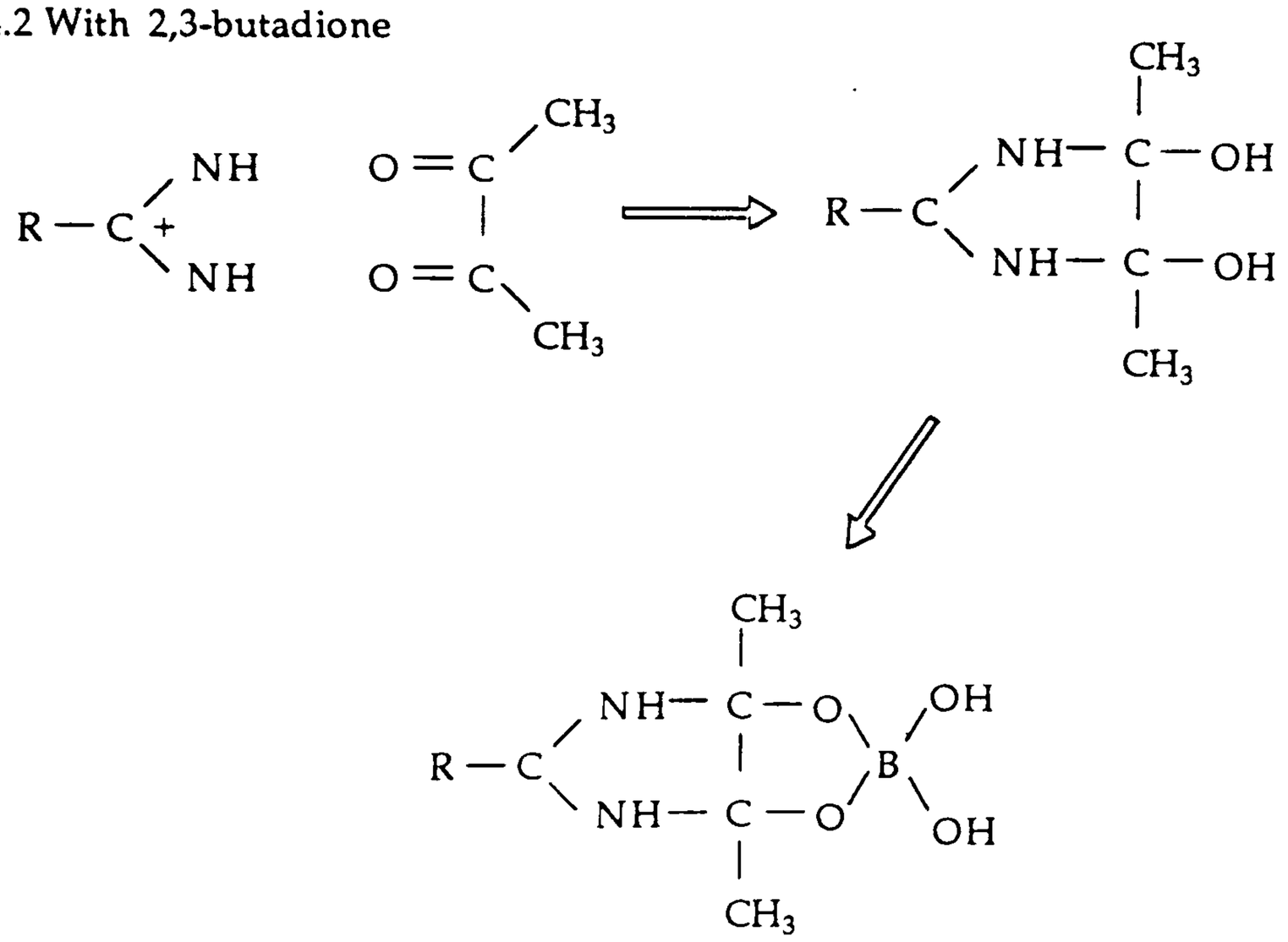


A4 Modification of arginine

A4.1With phenylglyoxal

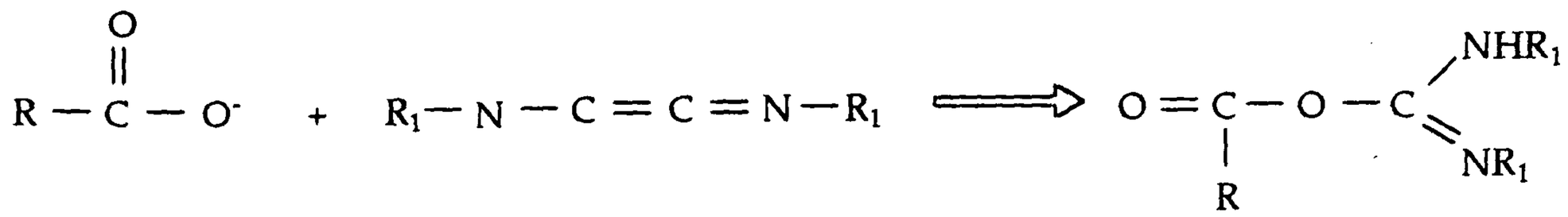
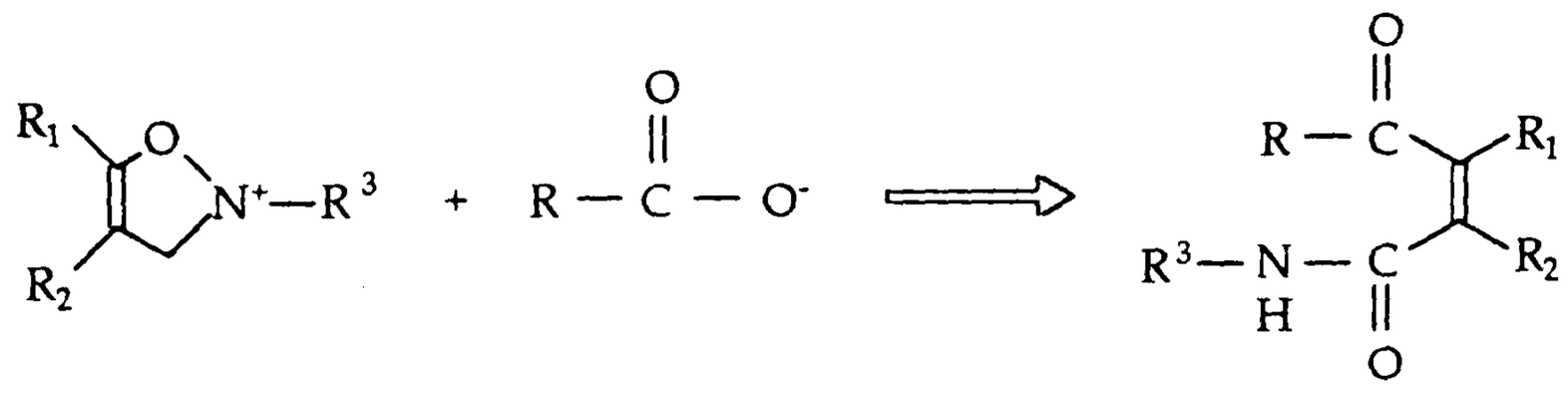


A4.2 With 2,3-butadione



A5. Modification of carboxyl groups

A5.1 With a carbodiimide



A5.2 With a isoxazolium sat