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# Biodegradation of the chlorophenoxy herbicide (*R*)-(+)-mecoprop by *Alcaligenes denitrificans*

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**Abstract**

An *Alcaligenes denitrificans* strain capable of utilizing the herbicide (*R*)-(+)-2(2-methyl-4-chlorophenoxy)propionic acid (mecoprop) as a sole carbon source was isolated from soil and cultured in liquid medium. Crude cell extracts of the bacterium were utilized in spectrophotometric assays to elucidate a biochemical pathway for degradation of mecoprop. Results indicated a reaction sequence analogous to the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D). GC-MS analysis provided direct evidence for the biotransformation of mecoprop to the transient metabolite 4-chloro-2-methylphenol (MCP). No NADPH-dependent activity was observed during this reaction. Pyruvate was verified as the second product derived from the aliphatic side chain of mecoprop. MCP was subsequently transformed to a substituted catechol by an NADPH-dependent monooxygenase. When grown on mecoprop, *A. denitrificans* was adapted to oxidize catechol and its 4- and 3-methylated derivatives indicating the broad substrate specificity of catechol dioxygenase. The microorganism was demonstrated to adopt the *ortho* mechanism of aromatic cleavage which resulted in the formation of 2-methyl-4-carboxymethylene but-2-en-4-olide, a reaction intermediate of the $\beta$-ketoadipate pathway.

## Introduction

The structurally related chlorinated phenoxy alkanoics, including 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl 4-chlorophenoxyacetic acid (MCPA) and 2(2-methyl 4-chlorophenoxy) propionic acid (mecoprop) are among the most widely used herbicides to control broad leaved weeds in cereal crops throughout the world (Anonymous 1992; Worthing & Walker 1991). Many soil bacteria have been found to be able to grow on 2,4-D and MCPA as the sole source of organic carbon, and this has contributed to the successful elucidation of biodegradation pathways for the complete mineralization of these xenobiotics (Bollag et al. 1968a, b; Gamar & Gaunt 1971; Gaunt & Evans 1971a, b; Loos et al. 1967a, b; Tiedje & Alexander 1969).

It is widely accepted that the microbiological decomposition of herbicides in top soils occurs largely via aerobic metabolism (Sinton et al. 1986). Enzyme activities of an *Arthrobacter* species suggested that 2,4-D degradation was initiated by side chain cleavage of 2,4-dichlorophenol (Loos et al. 1967a, b). The phenol was hydroxylated to 3,5-dichlorocatechol by the *Arthrobacter* species and the catechol was oxidized to *cis, cis*-2,4-dichloromuconic acid, a step that effects an *ortho* cleavage of the aromatic ring. *Cis, cis*-2,4-dichloromuconic acid then underwent lactonization to 2-chloro-4-carboxymethylene but-2-en-4-olide with release of the second chloride ion and further conversion to chloromaleylacetate. Finally, chloromaleylacetate was transformed into succinyl-CoA enabling the ring carbon to be assimilated for microbial growth. Essentially the same pathway was proposed for MCPA degradation by a *Pseudomonas* species (Evans et al. 1971a).

On account of its chemical structure, mecoprop is considered likely to be a more recalcitrant compound than 2,4-D or MCPA, since the propionic acid

side chain is linked to the oxygen atom of the phenoxyacid moiety via the $\alpha$-carbon atom adjacent to the carboxyl group (Kilpi 1980). Several mechanisms have been proposed for the degradation of mecoprop based on the precedents of 2,4-D and MCPA degradation but no pathway has yet been elucidated (Oh & Tuovinen 1991a, b, 1994). Lindholm and coworkers (1982) suggested decarboxylation of the propionic acid side chain of mecoprop to form MCPA which could then be degraded by the previously proposed pathway for the phenoxyacetic herbicide (Lindholm et al. 1982). Confirmation that phenoxyalkanoic acids with side chains longer than two carbon atoms can be degraded by a pathway involving some element of side-chain cleavage was provided by the observation that a *Flavobacterium* species isolated from soil degraded 4-(2,4-dichlorophenoxy)butyric acid with formation of 2,4-dichlorophenol (MacRae et al. 1963).

Research into the biodegradative pathway of mecoprop has been hindered by the difficulty of obtaining pure cultures of mecoprop-degrading bacteria. We have recently reported the stereoselective degradation of (*R*)-(+)-mecoprop by *Alcaligenes denitrificans* (Tett et al. 1994). The bacterium is capable of growth on (+)- and racemic mecoprop as the sole carbon and energy source, and can also degrade 2,4-D and MCPA. In this paper we report initial investigations to elucidate the biodegradative pathway of mecoprop.

## Materials and methods

### *Bacteria and culture conditions*

*Alcaligenes denitrificans*, able to utilize mecoprop as the sole carbon and energy source, was isolated from soil as described by Tett et al. 1994. The bacterium was grown in minimal medium containing (*R*)-(+)-mecoprop as the organic carbon source (Tett et al. 1994) unless indicated otherwise.

### *Chemicals*

Gibbs reagent (2,6-dichloroquinone-4-chloroimide) and the reduced form of $\beta$-nicotinamide adenine dinucleotide were obtained from Sigma Chemical Company Ltd., (Dorset, UK), MCP was obtained from Aldrich Chemicals (Gillingham, Dorset, UK). Catechol, 4-methylcatechol, 3-methylcatechol and 4-carboxymethylbut-2-en-4-olide were kindly donated by Prof. D.W. Ribbons; 5-chloro-3-methylcatechol and 2-methyl-4-carboxymethylene but-2-en-4-olide were donated by Prof. H. Knackmuss. *Cis, cis*-muconic acid was synthesized using the amended method of Boeseken and Engelberts (Pandell 1976) and the identity of the product confirmed by $[H]^1$-NMR. *Cis, cis*-muconic acid was also produced through the peracetic acid oxidation of catechol. The (*R*)-(+)-isomer of mecoprop was used throughout this study and was obtained as a donation from Pennine Chemical Services Ltd. (Huddersfield, West Yorkshire, UK).

### *Measurement of gross mecoprop degradation*

The degradation of mecoprop was measured directly by monitoring the change in the maximum absorption peak of mecoprop ($A_{279}$) in a scanning UV spectrophotometer. The culture supernatant was scanned from 200–500 nm to determine both the breakdown of mecoprop and the accumulation of biotransformation products.

### *Enzyme assays*

A 24 h-culture of *A. denitrificans* was used to inoculate mecoprop medium at a concentration of 1 g $l^{-1}$. The batch culture was incubated overnight at 30 °C and sparged with air (4 l $min^{-1}$). When the microbial population had reached mid log phase (16–21 h), the cells were harvested by centrifugation at 5000 g for 15 min. The cell pellet was washed once with 50 mM sodium phosphate buffer pH 7.0 and respun under the same conditions as above. The cells were resuspended in a minimal amount of buffer to produce a concentrated cell suspension of at least 250 mg cells $ml^{-1}$. Cell lysis was achieved by sonication on ice at $22 \times 10^{-1}$ nm for $4 \times 30$ seconds. The cell debris was removed by centrifugation at 12,000 *g* at 4 °C for 15 min, then further centrifuged at 300,000 *g* at 4 °C for 90 min. The supernatant which was used immediately for measurements of enzyme activities had a protein concentration of 11 mg $ml^{-1}$. All enzymes were assayed spectrophotometrically at 25 °C using an equivalent no-substrate control.

The activity of mecoprop monooxygenase in the cell extract was measured by the decrease in absorbance at 340 nm due to oxidation of the added cofactor NAD(P)H. The reaction mixture contained: 800 $\mu$l of 50 mM phosphate buffer pH 7.0, 20 $\mu$l of 10 mM NAD(P)H, 50–200 $\mu$l of enzyme extract, 20 $\mu$l of 50 mM mecoprop and distilled water to a total volume of 1 ml. The activity of MCP monooxygenase

was determined in an identical manner but substituting mecoprop with 20 $\mu$l of 50 mM MCP.

Catechol dioxygenase was assayed by a modification of the method described by Hegeman (Hegeman 1966). Each cuvette contained: 20 $\mu$l of 25 mM an aromatic cis-diol (catechol, 4-methylcatechol or 3-methylcatechol), 20 $\mu$l of crude cell extract, 900 $\mu$l of 50 mM phosphate buffer pH 7.0 and distilled water to a total volume of 1 ml.

The activity of muconate lactonizing enzyme was followed by the rate of decrease in muconic acid as assayed at its $\lambda$max. The assay mixture contained: 60 $\mu$l of 2.5 $\mu$M *cis, cis*-muconic acid, 10 $\mu$l of cell extract, 20 $\mu$l of 3 mM $MnCl_2$ solution and 50 mM Tris-Cl buffer pH 8.0 to a total volume of 1 ml.

The delactonizing enzyme that is responsible for the metabolism of 2-methyl-4-carboxymethylene but-2-en-4-olide was assayed by following the decrease in the maximum absorbance of the analogue 4-carboxymethylbut-2-en-4-olide (230 nm). The reaction mixture contained: 60 $\mu$l of 25 mM 4-carboxymethylbut-2-en-4-olide, 10 $\mu$l crude cell extract and Tris-Cl buffer pH 8.0 to a total volume of 1 ml.

*Detection of phenols using Gibbs reagent*

The putative production of phenolic compounds as early intermediates in the metabolism of mecoprop by *A. denitrificans* was monitored using the Gibbs reagent. The reaction mixture (100 ml) contained basal salts medium and 5 g $l^{-1}$ mecoprop. In order to promote phenol accumulation and hence visualization, 2, 2′-bipyridyl (1 mM) was added to the medium of some cultures to inhibit ring cleavage enzymes requiring $Fe^{2+}$. The reaction was initiated with a 10% inoculum of *A. denitrificans* grown on mecoprop. At regular time intervals, a 5 ml sample was removed from the reaction and the cells were precipitated by centrifugation. The supernatant was acidified to pH 3.0 with 1 M HCl and extracted into ethyl acetate. The extracts were then separated using TLC (dichloromethane : methanol : acetic acid; 9:1:0.1). Phenolic compounds were visualized using Gibbs reagent (2% (w/v) 2,6 dichloroquinone chloroimide in absolute ethanol).

*Diagnostic test for aromatic ring cleavage enzymes*

*A. denitrificans* was spread onto protocatechuate agar of the following composition (g $l^{-1}$ distilled water): protocatechuate, 0.2; $KH_2PO_4$, 0.5; $K_2HPO_4$, 0.5; $(NH_4)_2SO_4$, 0.2; $MgSO_4$. $7H_20$, 0.5; yeast extract, 0.01; agar, 15.0. The plates were incubated at 30 °C for 7 days. The cleavage pathway in the resulting colonies was determined colorimetrically (Stanier et al. 1966; Kilby 1948). The surfaces of the protocatechuate agar plates were flooded with 2 $cm^3$ 10% (w/v) aqueous $FeCl_3$ which forms a black precipitate with protocatechuate and allowed to stand for 30 min. After this time interval, excess reagent was tipped off and the plates examined for clear haloes surrounding the colonies indicating utilization of protocatechuate.

*Respirometric assays*

The ability of the bacterium to oxidize mecoprop was studied by the measurement of oxygen uptake by whole cells using a standardized oxygen electrode. Cells were grown overnight in 500 ml of minimal medium containing 1 g $l^{-1}$ mecoprop, harvested by centrifugation at 4 °C, washed once with phosphate buffer (50 mM, pH 7.0), and suspended in the buffer to a protein concentration of 440 $\mu$g$ml^{-1}$. For assay of oxygen-dependent mecoprop metabolizing activity, the culture was suspended in phosphate buffer to achieve an oxygen uptake of 1–5 mg $O_2$ $l^{-1}$ $min^{-1}$. The reaction was started by the addition of substrate at a final concentration of 1 mM.

*Gas liquid chromatography (GLC) analysis*

Samples were withdrawn from mecoprop-grown cultures (5 g $l^{-1}$) and centrifuged for 15 min at 3000 *g*. Aliquots of medium (20 ml) were acidified to pH 3.0 with 0.1 M HCl and extracted into ethyl acetate. Samples were analysed isothermally by gas liquid chromatography (Schimadzu GC-14A with FID; column: cyanopropylphenyldimethyl siloxane, 25m, i.d. = 0.2 mm, helium as carrier gas). Mecoprop and intermediates in the biodegradation pathway were identified by comparing their retention times with those of equivalent chemically-synthesized reference standards using naphthalene (RT 4.44 min) as the internal standard.

*Volatile fatty acid analysis*

At 2 h intervals throughout growth of *A. denitrificans* on mecoprop (1 g $l^{-1}$), three separate 1 ml aliquots were aseptically removed and to each sample, was added 100 $\mu$g $ml^{-1}$ lysozyme. The tubes were incubated on ice for 10 min and then placed in a boiling

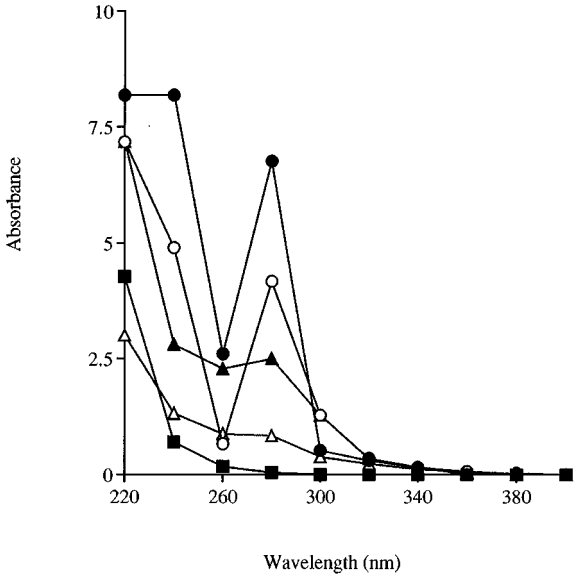


*Figure 1.* Change in UV absorption spectrum with time during batch culture growth of *A. denitrificans* on the (*R*)-(+)-enantiomer of mecoprop. At intervals during growth of the culture, samples of the medium were centrifuged to remove bacterial cells, diluted fivefold with water and the UV spectrum of the supernatant recorded between 220 and 400 nm. Symbols: ●, starting spectrum at inoculation; ○, spectrum 24 h after inoculation; ▲, spectrum after 32 h; △, spectrum after 46 h and ■, spectrum after 48 h.

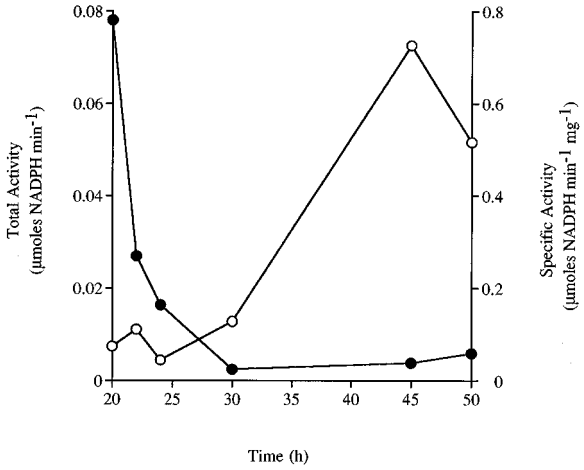


*Figure 2.* Change in MCP monooxygenase activity during growth of *A. denitrificans* on (*R*)-(+)-mecoprop. At various intervals during growth of the culture, bacterial cells were harvested, sonicated and the resulting enzyme activity of the crude cell extracts was determined as described in the text. The concentration of protein increased in crude cell extracts from 2.0 mg $ml^{-1}$ at 20 h to 18.6 mg $ml^{-1}$ at 50 h. Symbols: ●, specific activity of MCP monooxygenase and ○, total activity of MCP monooxygenase.

water bath for 60–90 seconds. The samples were diluted 1:100 in deionised water prior to injection.

Samples were analysed for volatile fatty acid concentrations by ion-exclusion HPLC. An ICE (Ion Chromatography Exclusion, type AS1, Dionex Ltd.) column was used, with eluent (5 mM heptafluorobutyric acid) flow rate 0.8 ml $min^{-1}$; regenerant (5 mM tertabutylammoniumhydroxyde) flow rate 2 ml $min^{-1}$. Peaks were identified by a comparison of their retention times to those of authentic standards of putative reaction products as follows: mecoprop (6.76 min), pyruvate (7.11 min), propionate (16.99 min), acetate (13.99 min), lactate (11.62 min).

## Results

The changes in the UV spectra of culture supernatants of *A. denitrificans* while growing on (*R*)-(+)-mecoprop is shown in Figure 1. The sequence of changes with time may be summarized as follows:

– in the first 24 h of growth there was a decrease in $A_{279}$ indicating the disappearance of mecoprop;
– at 32 h, there was a further decrease in $A_{279}$ accompanied by a shift in maximum absorption ($\lambda$max) towards 260 nm, probably representing the accumulation of an intermediate;
– at 46 h, there was a decline in absorbance at 260 nm, the $\lambda$max occurring at 230 nm, this is more evident at 48 h.

In an attempt to elucidate a biodegradative pathway for (*R*)-(+)-mecoprop by *A. denitrificans* we postulated a pathway then undertook enzyme assays to test the hypothetical route. Crude enzyme extracts were produced from (*R*)-(+)-mecoprop-grown *A. denitrificans* to assay for a range of enzymes. The first step in the biodegradation may involve a mecoprop monooxygenase. However, spectrophotometric tests for this enzyme based on the oxydation of NAD(P)H failed to detect its presence, and evidence for this step was drawn from whole-cell methods using GLC analysis and respirometry.

Assays for activity of MCP were performed by monitoring the consumption of NADPH by mecoprop-grown cells. The specific and total activities for MCP monooxygenase are plotted in Figure 2, the maximum specific activity of the monooxygenase was 0.78 $\mu$moles NADPH $min^{-1}$ $mg^{-1}$ protein. The reaction was specific for NADPH and there was no conversion of MCP to 5-chloro-3-methylcatechol in the

*Table 1.* Specific activities of catechol dioxygenase on three catechol analogues of 5-chloro-3-methylcatechol. The extinction coefficients were measured at the wavelengths cited in 50 mM phosphate buffer (pH 7.0). The protein concentration of the crude cell extract was 12.0 mg $ml^{-1}$. One enzyme unit is defined as that amount of enzyme that catalyses the formation of 1 $\mu$mol of the product or the disappearance of 1 $\mu$mol of the substrate per minute under the assay conditions

| Substrate | Wavelength $\lambda$ max (nm) | Molar extinction coefficient ($\times$ $10^3$ M) | Specific activity (units $min^{-1}$ $mg^{-1}$ protein) |
|---|---|---|---|
| Catechol | 274 | 2.59 | 0.07 |
| 3-methylcatechol | 272 | 1.73 | 0.23 |
| 4-methylcatechol | 278 | 2.91 | 0.26 |

*Table 2.* Spectrophotometric investigation of the effect of substrate concentration on the specific activity of the muconate lactonizing and delactonizing enzymes of (*R*)-(+)-mecoprop-grown *A. denitrificans*. The assays were conducted at 260 nm and 230 nm respectively with crude cell extracts containing 12.4 mg $ml^{-1}$ protein and started by the addition of substrate. The data represent the change in specific activity as a function of concentration of *cis, cis*-muconic acid and 4-carboxymethylbut-2-en-4-olide, structural analogues of 4-chloro-2-methyl muconate and 2-methyl-4-carboxymethylene but-2-en-4-olide

| [*cis, cis*-muconic acid] ($\mu$M) | Specific activity ($\mu$mols *cis, cis*-muconic acid utilized $min^{-1}$ $mg^{-1}$ protein) | [4-carboxymethyl-but-2-en-4-olide] ($\mu$M) | Specific activity ($\mu$mols lactone (utilized $min^{-1}$ $mg^{-1}$ protein) |
|---|---|---|---|
| 0.0 | 0 | 0.0 | 0 |
| 0.03 | 0.016 | 0.13 | 0.08 |
| 0.09 | 0.017 | 0.25 | 0.15 |
| 0.18 | 0.029 | 0.38 | 0.27 |
| 0.27 | 0.033 | 0.63 | 0.40 |
| 0.36 | 0.034 | 1.38 | 0.55 |

presence of NADH. Similarly, there was no detectable monooxygenase activity in extracts obtained from cells grown on nutrient broth. The monooxygenase was found to be intracellular and not membrane-bound as the activity was almost exclusively present in the supernatant and not in the pellet of cellular debris.

Utilization of catechol was monitored by the production of the corresponding muconic acid which absorbs maximally at 265 nm. The rate of change of optical density is directly proportional to the enzyme concentration within a 10-fold range of enzyme dilutions. Catechol dioxygenase activity toward a number of catechols was induced in cells of *A. denitrificans* grown on mecoprop, but not on cells grown on nutrient broth. The bacterium possessed an enzyme capable of utilizing catechol, 3-methyl and 4-methylcatechol. The specific activity of catechol dioxygenase was calculated using the molar extinction coefficients of the tested substrates (Table 1).

The spectrum of *cis, cis*-muconic acid has a $\lambda$max at 257 nm. Muconate cycloisomerase activity was assayed by following the decrease in absorbance at 260 nm without interference from the lactone at this wavelength. Crude cell extracts prepared from cells grown on both (*R*)-(+)-mecoprop and benzoic acid utilized muconic acid in the presence of Mn (II). In order to demonstrate lactonizing enzyme activity, the assay required very specific reaction conditions. The dependence of the rate of lactonizing enzyme activity on substrate concentration with *cis, cis*-muconic acid is demonstrated in Table 2. Maximum absorption of 4-carboxymethylbut-2-en-4-olide occurs at 220 nm. Disappearance of the lactone was followed at 230 nm since absorbance of $\beta$-ketoadipate is neglible at this wavelength. 4-carboxymethyl but-2-en-4-olide utilization was observed in cell extracts produced separately from cells grown on benzoate and mecoprop. The rate of delactonizing enzyme activity as a function of 4-carboxymethylbut-2-en-4-olide concentration is shown in Table 2.

The Gibbs reaction was used to detect the presence of phenol-containing compounds within the biodegra-

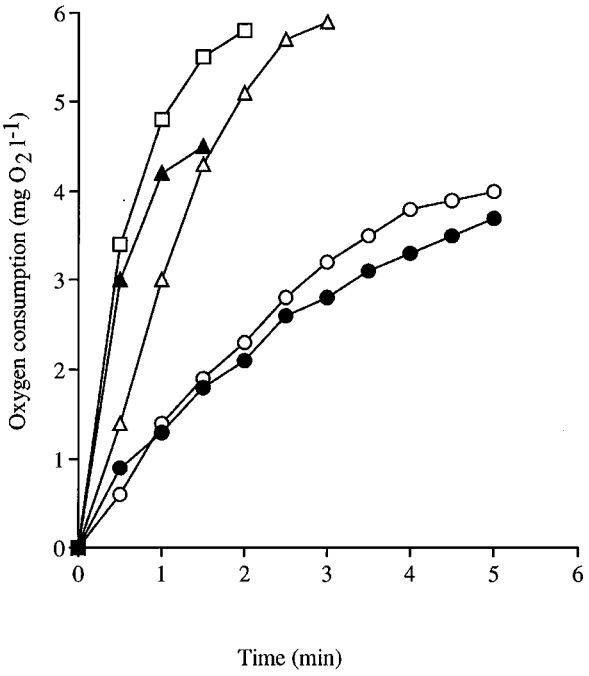


*Figure 3.* Oxygen uptake by (*R*)-(+)-mecoprop grown *A. denitrificans*. Substrate stimulated oxygen consumption was determined by subtracting the endogenous rate of oxygen uptake in the absence of substrate from the total oxygen consumption. The data represent the mean of triplicate values. The substrates indicated were added at zero time. Symbols: ●, mecoprop stimulated oxydation; ○, MCP fed reaction; ▲, oxygen consumption from oxidation of catechol; △, addition of 3-methylcatechol and ■, addition of 4-methylcatechol.

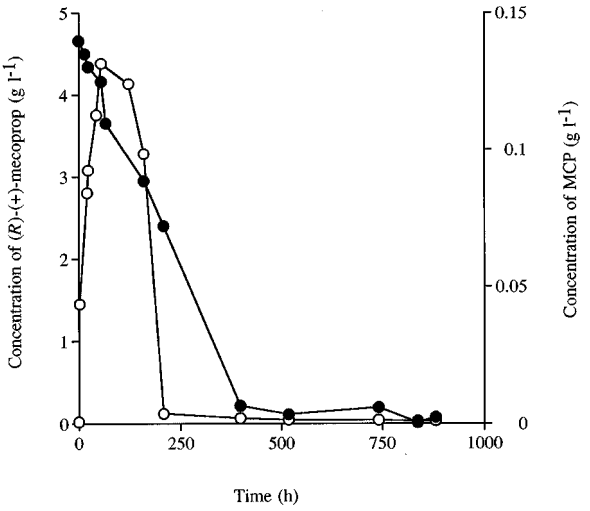


*Figure 4.* Changes in concentration of (*R*)-(+)-mecoprop and MCP during batch culture growth of *A. denitrificans* using gas liquid chromatography. Culture supernatants were analysed for the presence of mecoprop and the hypothetical metabolite MCP in media containing an initial substrate concentration of 5 g $l^{-1}$ (*R*)-(+)-mecoprop. Symbols: ●, (*R*)-(+)-mecoprop and ○, MCP.

dation pathway. Although reported to be specific for 4-alkoxy-substituted phenolic compounds, mecoprop, 4-chloro-2-methylphenol and muconate were all detected using the reaction giving respective Rf values of 0.47, 0.71 and 0.08. There was no positive Gibbs response of phenol formation observed when mecoprop biodegradation was monitored in the presence of 2,2′-bipyridyl.

Using diagnostic tests to determine *ortho* and *meta* cleavage, mecoprop-grown *A. denitrificans* gave a negative test for *meta* cleavage but a positive Rothera reaction demonstrating that the degradation of mecoprop by *A. denitrificans* proceeds via *ortho* cleavage.

Oxygen uptake was measured and compared when (*R*)-(+)-mecoprop grown cells were supplied with mecoprop, MCP, catechol, 3-methyl and 4-methylcatechol. All substrates were rapidly oxidized without a lag phase (Figure 3). The rate of oxygen consumption during oxidation of each catechol was greater than that observed for oxidation of mecoprop or MCP.

GLC analysis was used to follow the breakdown of (*R*)-(+)-mecoprop and to detect and identify specific intermediates of the mecoprop biodegradation pathway (Figure 4). Results from GC-MS confirmed that MCP was an intermediate soon after the cells were exposed to mecoprop. Other intermediates were identified within the culture supernatants, occurring in minor quantities throughout growth of the bacterium and identified by their retention times against equivalent chemically-synthesized reference standards as follows: 5-chloro-3-methylcatechol RT 11.54 min, 2-methyl-4-carboxymethylene but-2-en-4-olide (lactone) RT 9.76 min. Upon disappearance of MCP after approximately 200 h growth on mecoprop, the formation of a more hydrophobic peak with a retention time of 5.8–5.9 min was observed. The compound increased steadily in the supernatants and was still present after 900 h. This peak could not be identified by GLC or GC-MS. After 900 h, a total amount of 1 mg of mecoprop remained at a 93% extraction efficiency (Figure 4). The muconic acid was not detected in the extracts from *A. denitrificans* and the chemically-synthesized standard was not able to dissolve in the ethyl acetate extraction solvent. Muconic acid was considered too polar for the extraction and partitioning characteristics required in the gas chromatographic procedure to isolate mecoprop.

**Discussion**

The 2,4-dichlorophenoxyacetic acid (2,4-D) biodegradation pathway is a paradigm for microbial metabolism of chloroaromatic compounds (Chaudhry & Chapalamadugu 1991; Häggblom 1992). The scheme for mineralization of 2,4-D was first established in an *Arthrobacter* species but variations of this pathway have been reported (Bollag et al. 1968a, b; Evans et al. 1971b; Gaunt & Evans 1971a; Loos et al. 1967a; Loos 1975; Tiedje et al. 1969). Unlike 2,4-D, mecoprop possesses an alkyl substituent on the aromatic ring in addition to a chlorine atom. As indicated previously, this has a radical influence on how the compound is degraded (Dagley 1987).

On the basis of the reaction sequence for 2,4-D degradation, the first metabolite in the metabolism of mecoprop would be MCP (Figure 5). This compound is also formed in conjunction with glyoxylate as the primary intermediates of MCPA metabolism (Gamar & Gaunt 1971; Gaunt & Evans 1971a). The biotransformation reaction may be initiated by the action of a monooxygenase(s) in the presence of a hydrogen donor (NADH or NADPH) and molecular oxygen. However, it was not possible to demonstrate mecoprop stimulated oxidation of NAD(P)H in crude cell extracts of *A. denitrificans*. Conversely, the enzyme responsible for the initial biotransformation may not be a monooxygenase with NAD(P)H-dependent activity but rather an $\alpha$-ketoglutarate-coupled dioxygenase which catalyses an entirely different reaction mechanism for cleavage of the aliphatic side chain (Abbott & Udenfriend 1974). Dioxygenase activity is dependent on the presence of $\alpha$-ketoglutarate, $Fe^{2+}$ and a reducing agent (Fukumori & Hausinger 1993). The inability of the microorganism to transform mecoprop in the presence of 2,2′-bipyridyl suggests that the initial reaction step itself was inhibited which is consistent with the concept that the enzyme responsible for phenol formation is iron dependent.

As the initial enzyme proved to be unstable in cell extracts, experiments were carried out with whole cells. Disappearance of mecoprop was monitored on GLC and GC-MS. Analysis of supernatants revealed that cells produced MCP when incubated with mecoprop.

Oxygen consumption data supported the view that MCP is an early metabolite in the degradative pathway of (*R*)-(+)-mecoprop. MCP was rapidly oxidized without a lag by *A. denitrificans* implicating side-chain loss at the first metabolic step and demonstrating the oxygen dependency of the reaction. Although it was expected that mecoprop would consume twice the amount of oxygen of MCP and three times the amount of catechol, it is possible that the amount of oxygen consumption observed was due to different rates of transport and/or biooxidation of the substrates at comparable protein concentrations.

In addition to the production of MCP, a second primary metabolite was generated as the product of the three carbon aliphatic side chain. After 4 h batch culture growth, pyruvate (3 ppm) was formed as a result of cleavage of the ether link and could be fed directly into the TCA cycle.

MCP is subsequently converted to the corresponding catechol, a common central intermediary metabolite of aromatic compounds (Sandmann & Loos 1988). The introduction of an additional polar group into the ring is achieved by the employment of a monooxygenase and enables the compound to be further degraded by the $\beta$-keto adipate pathway. Trace amounts of 5-chloro-3-methylcatechol were detected in mecoprop grown cultures. Experiments with cell extracts showed that molecular oxygen and NADPH were required for MCP utilization. This implicates a monooxygenase catalysed reaction and is consistent with work by Bollag (1967) who partially purified an enzyme that could hydroxylate 2,4-dichlorophenol to 3,5-dichlorocatechol with NADPH (Bollag et al. 1967).

In respirometric experiments, catechol, 4-methylcatechol and 3-methylcatechol were oxidized without a lag by mecoprop grown cells. This further suggests that a catechol is formed as an intermediate when cells grow on mecoprop. The phenomenom illustrates a degree of nonspecificity of the mecoprop degrading enzyme systems of *A. denitrificans* towards methylated catechols and catechol intermediates.

There are two different metabolic pathways which can be used by microorganisms to metabolize gem-diols such as catechol, the *ortho* cleavage pathway (initiated by catechol 1, 2-oxygenase or protocatechuate 3,4-oxygenase) and the *meta* cleavage pathway (initiated by catechol 2, 3-oxygenase or protocatechuate 4, 5-oxygenase). The mechanism of aromatic cleavage is of great significance because it determines a long series of subsequent biochemical steps differing in many enzymic respects. Most microorganisms able to metabolize aromatic substrates possess either the *ortho* or the *meta* pathway however, there are microorganisms which exhibit both mechanisms of ring cleavage (Worsey & Williams 1975). Many investigations of the ring cleavage mechanism have been conducted with structures simpler than those of

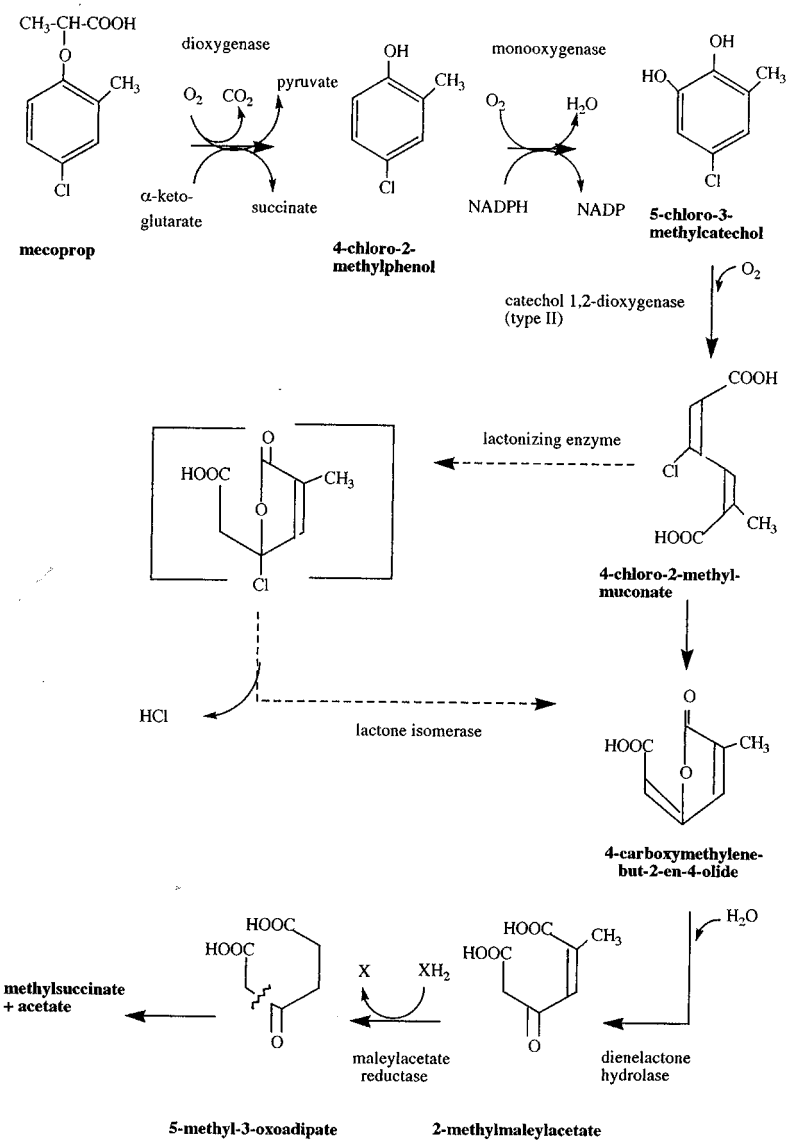


*Figure 5.* Proposed pathway for the degradation of (*R*)-(+)-mecoprop by *A. denitrificans*. Direct evidence was obtained for transformation of the herbicide as far as 2-methyl-4-carboxymethylene but-2-en-4-olide (—>), by detection of the metabolites using GLC, but the remaining conversion of the lactone to the putative products acetate and methyl succinate remains speculative (- - - >).

most pesticides (Ornston & Stanier 1966). In general, *ortho*- and *meta*-substituted methyl phenols seem to be preferentially metabolized by a *meta*-cleavage pathway starting with hydroxylation of methyl phenol to form 3-methylcatechol. In contrast, halogenated aromatics are generally degraded by *ortho* cleavage. It is not possible to predetermine the mechanism of aromatic cleavage of compounds possessing both methyl and halogen ring substituents. *Pseudomonas cepacia* MB2, for example, adopted the *meta* cleavage pathway for utilisation of 3-chloro-2-methylbenzoic acid via 4-chloro-3-methylcatechol (Higson & Focht 1992). In contrast, Pieper and coworkers described degradation of 2-chloro-4-methylphenoxyacetic acid via 5-chloro-3-methylcatechol (Pieper et al. 1993).

Results from the *ortho* and *meta* cleavage diagnostic tests indicated that mecoprop-grown *A. denitrificans* adopts the *ortho* cleavage mechanism of aromatic ring

cleavage for growth on protocatechuate. Further evidence for the metabolism of this aromatic substrate was obtained by the observation of clear haloes surrounding colonies of *A. denitrificans* indicating utilization of protocatechuate.

If mecoprop is degraded by *A. denitrificans* by *ortho* cleavage, catechol 1, 2-dioxygenase attack on 5-chloro-3-methylcatechol would produce 4-chloro-2-methylmuconate. In contrast to MCP monooxygenase, the ring cleavage enzyme is unstable in the presence of oxygen. The increase in absorbance at 265 nm in spectrophotometric assays of cell extracts fed with analogues of 5-chloro-3-methylcatechol is consistent with the production of a muconic acid. The ability of the crude cell extract to transform both catechol and the two methylated derivatives provides further evidence for the relative non-specificity of catechol dioxygenase. This is consistent with halobenzoate degradation in which both *Pseudomonas* and *Alcaligenes* species have been reported to possess catechol-1, 2-dioxygenases with high activities towards substituted catechols (Commandeur & Parsons 1990; Haigler et al. 1988; Tiedje et al. 1969). The relaxed substrate specificity of catechol 1,2-dioxygenase is characteristic of the modified *ortho* cleavage pathway for degradation of chloroaromatic compounds (Schlömann 1994). The formation of this ring cleavage product at the expense of 4-methylcatechol can be followed by monitoring the change in maximum absorbance with time over a range of wavelengths. The maximum absorption peak initially occurred at 278 nm which is consistent with that of 4-methylcatechol. With time, the peak became broader and there was a gradual shift in absorbance towards 250 nm indicative of formation of a muconic acid. There was no increase in absorbance at 375 nm corresponding to the production 2-hydroxymuconic acid semialdehyde of the *meta* cleavage pathway. The absence of catechol dioxygenase activity in uninduced cells indicates that mecoprop or intermediates formed from it are required for induction of catechol dioxygense and subsequent enzymes in the pathway. Expression of the aromatic degradation has been repressed in the presence of a non-aromatic growth substrate.

Results from the scanning spectrophotometric study following the transformation of 4-methylcatechol, reveal that after 50 min incubation of the crude cell extract with the substrate, the maximum absorption peak shifts back to a higher wavelength of 270–272 nm. This maybe due to the production of the derivatised butenolide ($\lambda$max 278 nm) occurring either biologically or through the spontaneous lactonization of 4-chloro-2-methylmuconate if it is unstable (Gaunt & Evans 1971b). Spectrophotometric assays revealed that cell extracts harvested after independent growth on both mecoprop and benzoic acid were indeed able to utilize 4-carboxymethylbut-2-en-4-olide, the lactone derivative formed during metabolism of benzoate. This indicates the broad substrate specificity of the dienelactone hydrolase induced by growth on mecoprop being able to metabolize an intermediate other than that derived from the mecoprop substrate. This is in contrast to the modified *ortho* cleavage pathway of chlorocatechols where the dienelactone hydrolases are more specific (Schlömann 1994). Both the lactonizing and delactonizing enzymes are stable under the above extraction conditions for several weeks. GLC also revealed the presence of a compound with a retention time equivalent to that of 2-methyl-4-carboxymethylene but-2-en-4-olide in culture supernatants during growth of *A. denitrificans* on (*R*)-(+)-mecoprop. Although it is considered that muconic acids and muconlactones are likely intermediates in the oxidative biotransformation of catechols, it is yet to be established whether this occurs via the *ortho* or modified *ortho* cleavage pathways. As far as it is known, this is the most comprehensive series of investigations to determine the biodegradative pathway of mecoprop.

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