Metabolism of 4-Chloro-2-methylphenoxyacetate by a Soil Pseudomonad

RING-FISSION, LACTONIZING AND DELACTONIZING ENZYMES

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1. A cell-free system, prepared from *Pseudomonas* N.C.I.B. 9340 grown on 4-chloro-2-methylphenoxyacetate (MCPA) was shown to catalyse the reaction sequence: 5-chloro-3-methylcatechol $\rightarrow$ *cis-cis*- γ -chloro- α -methylmuconate $\rightarrow$ γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide $\rightarrow$ γ -hydroxy- α -methylmuconate. 2. The activity of the three enzymes involved in these reactions was completely resolved and the lactonizing and delactonizing enzymes were separated. 3. This part of the metabolic pathway of 4-chloro-2-methylphenoxyacetate is thus confirmed for this bacterium. 4. The ring-fission oxygenase required Fe^{2+} or Fe^{3+} and reduced glutathione for activity; the lactonizing enzyme is stimulated by Mn^{2+} , Mg^{2+} , Co^{2+} and Fe^{2+} ; no cofactor requirement could be demonstrated for the delactonizing enzyme. 5. *cis-cis*- γ -Chloro- α -methylmuconic acid was isolated and found to be somewhat unstable, readily lactonizing to γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide. 6. Enzymically the lactonization appears to be a single-step dehydrochlorinase reaction.

Evidence for the metabolic pathway involved in the bacterial degradation of MCPA* has been presented by Gaunt & Evans (1971). In view of the fact that crude cell-free extracts of the pseudomonad used in this work were found to be able to catalyse many of the steps involved, this organism appeared eminently suitable for study of MCPA metabolism at an enzymic level. This paper describes the identification and separation of the ring-fission, lactonizing and delactonizing enzymes present in this cell-free extract and confirms this part of the proposed route of dissimilation of the herbicide employed by *Pseudomonas* N.C.I.B. 9340.

MATERIALS AND METHODS

Materials. 5-Chloro-3-methylcatechol and γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide were prepared as described by Gaunt (1962). Crude γ -hydroxy- α -methylmuconic acid was obtained as the alkaline hydrolysis product of γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide (Gaunt & Evans, 1971).

Determinations. Spectrophotometric and manometric determinations and the determination of chloride were carried out as described in the previous paper (Gaunt & Evans, 1971).

* Abbreviation: MCPA, 4-chloro-2-methylphenoxyacetate.

Titration and the measurement of acid production during enzyme reactions were performed in an automatic titrator (Radiometer Ltd., Copenhagen, Denmark).

Protein was determined by the method of Warburg & Christian (1942).

Chromatography. Solvent systems and detection agents are as described by Gaunt & Evans (1971).

Culture of organism. Cells were grown and harvested as described in the previous paper (Gaunt & Evans, 1971).

Preparation of cell-free extracts. Frozen or freshly harvested cells were suspended in 10 ml of buffer/g fresh wt. of cell paste. Either 50 mM- Na_2HPO_4 - KH_2PO_4 or tris- H_2SO_4 buffer, pH 8.0, was used. The bacteria were disrupted by exposure for 10 min at 0-5°C to the output of an MSE-Mullard ultrasonic disintegrator at an average frequency of 20 kHz. After treatment, suspensions were centrifuged at 20000g for 30 min at 0°C. The supernatant (referred to below as the crude cell-free extract) was used in all enzyme studies.

Before any protein fractionation of this extract was attempted, the nucleic acid content was decreased by the protamine sulphate procedure used by Stanier (1955). All operations were carried out at 0-5°C. Crude cell-free extracts were first saturated with solid $(\text{NH}_4)_2\text{SO}_4$ and, after being left for 15 min to complete precipitation, the protein was spun down. The precipitate was dissolved in 50 mM buffer (phosphate or tris- H_2SO_4 as described in the text), pH 8.0, and dialysed for 4 h against 10 litres of the same buffer, with 1 cm-diameter dialysis tubing and constant stirring. The protein concentration of the enzyme solution was adjusted to 10-15 mg/ml and the pH lowered to 6.0.

with *m*-acetic acid. A freshly prepared solution of protamine sulphate (20 mg/ml in water) was slowly added to the well-stirred solution. The precipitate was spun down at intervals and treatment continued until the E_{280}/E_{260} ratio of the supernatant reached a value of 0.75. In crude cell-free extracts this ratio generally started at about 0.6 and the addition of 1.0–1.5 ml of protamine solution/30 ml of enzyme solution was required to raise it to 0.75. Attempts to increase the ratio above 0.75 resulted in the loss of considerable amounts of protein. The pH of the supernatant was readjusted to 8.0 with *m*-NaOH before further fractionation.

Assay of 5-chloro-3-methylcatechol 1,2-oxygenase. The rate of oxidation of 5-chloro-3-methylcatechol was measured by either O_2 uptake or by acid production.

O_2 uptake was followed in the Warburg apparatus at 30°C with enzyme solution diluted to 2.7 ml with 50 mM buffer. Substrate (5 μ mol) in 0.3 ml of water was added to initiate the reaction. Enzyme activity was measured in terms of μ mol of O_2 uptake/min. The unit of activity is defined as that amount of enzyme required to catalyse the uptake of 1 μ mol of O_2 /min.

Acid production was followed in the automatic titrator with enzyme solution (prepared in 20 mM buffer) diluted to 5 ml with water. The stirrer in the reaction chamber was adjusted to beat air into the incubation mixture to ensure adequate aeration. The enzyme solution was titrated to the required pH and the reaction initiated by addition of 1 ml of substrate solution (containing 1–5 μ mol), previously adjusted to the same pH. Acid production was titrated automatically with 20 mM-NaOH. Enzyme activity was expressed as μ equiv. of acid produced/min.

At the end of either type of assay, release of Cl^- ion could be measured by diluting a measured amount of the reaction mixture (containing 1–4 μ equiv. of Cl^- ion) with 15 ml of water and 2 ml of *m*-HNO₃ in a 50 ml beaker, and titrating immediately with 1 mM-AgNO₃.

Assay of lactonizing enzyme. Substrate for this enzyme was prepared in the following way. 5-Chloro-3-methylcatechol (220 mg) was incubated with the ring-fission enzyme (see the Results section) and the reaction product extracted into ether as described below. The ether was washed with an equal volume of water and was then extracted with a solution of NaOH (140 mg) in water (110 ml). *cis-cis*- γ -Chloro- α -methylmuconate was thus obtained in aqueous solution without decomposition into the lactone. This stock solution contained 8 μ mol of γ -chloro- α -methylmuconate/ml as determined by Cl^- release on acidification. Before use this solution was diluted with an equal volume of 0.1 M buffer and carefully adjusted to pH 8.0 with 0.5 M-H₂SO₄.

Two methods of assay were used, both performed at room temperature. (1) The enzyme solution was diluted to 4 ml with 50 mM-phosphate buffer, pH 8.0, containing 6 μ mol of MnSO₄. Substrate solution (8 μ mol of γ -chloro- α -methylmuconic acid in 2 ml of 50 mM-phosphate buffer, pH 8.0) was added to initiate the reaction. Release of Cl^- ion was followed at 2 min intervals by diluting 1 ml of the reaction mixture with 17 ml of water and 2 ml of *m*-HNO₃ and titrating immediately with 1 mM-AgNO₃. Activity was expressed as μ equiv. of Cl^- ion released/min per ml of enzyme solution. The unit of activity is defined as that amount of enzyme catalysing the release of 1 μ equiv. of Cl^- ion/min.

(2) In some assays release of Cl^- ion could not be used to measure activity, e.g. owing to addition of Ag⁺ or Cl^- to the system. When the purified lactonizing enzyme was used for such assays, activity was measured as the change in the E_{278}/E_{265} ratio (the u.v. maxima of the lactone and ring-fission compound respectively) with the same reaction mixture as above. For the ring-fission compound this ratio is 0.72 and for the lactone 1.12 (in water and in ether), and there is a linear relationship between this ratio and the proportion of each component present. The reaction was followed at 2 min intervals by reading samples diluted 1:50 with water at 278 and 265 nm against a control cell containing the same system with the exclusion of substrate.

In view of the instability of the ring-fission compound, care was taken to determine the rate of lactone formation under the conditions of each particular enzyme assay but in the absence of enzyme. This was particularly important during investigation of the pH optimum of the enzyme. Values obtained for non-enzymic conversion were subtracted from those obtained during enzyme assay to give the value for enzyme activity.

Assay for the delactonizing enzyme. The u.v. spectrum of γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide has λ_{max} at 278 nm whereas the hydrolysis product, γ -hydroxy- α -methylmuconate has negligible absorption at this wavelength (see Fig. 1). The decrease in extinction at 278 nm can therefore be used to measure delactonizing activity. The assay was performed in 1 cm light-path quartz spectrophotometer cuvettes at room temperature. Each cell contained enzyme in 1.0 ml of 67 mM-tris-H₂SO₄ buffer, pH 8.0, and 2 ml of water; 0.14 μ mol of lactone was added to one cuvette to initiate the reaction. The extinction at 278 nm was recorded at 1 min intervals; for complete reaction the ΔE observed was 0.81. During the reaction the change of pH was inconsequential. Activity was expressed as μ mol of lactone hydrolysed/min per ml of enzyme solution. The unit of activity is defined as the amount of enzyme catalysing the hydrolysis of 1 μ mol of lactone/min under the conditions described.

RESULTS

All the initial efforts to obtain cell-free extracts capable of metabolizing 5-chloro-3-methylcatechol were unsuccessful. Most batches of cells were able to oxidize this substrate rapidly, but little or no activity was noted in cell-free extracts. With later batches of cells, variable success was achieved, but it was only after about 18 months' culture of the organism that ring-splitting activity was obtained regularly in cell-free extracts. The reason for preliminary failures remains unexplained.

Reaction of crude cell-free extracts

5-Chloro-3-methylcatechol was rapidly oxidized by active crude cell-free extracts prepared in phosphate buffer, up to a concentration of 5 μ mol of catechol/ml. During the reaction 1 mol of oxygen was taken up, 3 equiv. of acid produced and 1 equiv. of Cl^- ion released/mol of substrate oxi-

dized. No carbon dioxide was evolved. The u.v. spectrum of the reaction mixture after completion of oxidation showed $\lambda_{\max}$ at 250 nm (alkaline) and no spectrum (acid).

Cell-free extracts also rapidly metabolized the lactone, γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide, without oxygen uptake, but with the release of 1 equiv. of acid/mol of substrate, to give a product with the same u.v. characteristics as are described above.

These results are consistent with the view that the end product of reaction of crude cell-free extracts with 5-chloro-3-methylcatechol is γ -hydroxy- α -methylmuconate. This requires the presence of at least three enzymes, ring-splitting, lactonizing and delactonizing, according to the proposed metabolic sequence. It was necessary to separate these to confirm this supposition. Isolation of the ring-fission compound, the postulated *cis-cis*- γ -chloro- α -methylmuconate, was the first requirement in this work, both to confirm its existence and to enable a study of the lactonizing enzyme.

Ring-fission enzyme (5-chloro-3-methylcatechol 1,2-oxygenase)

The addition of Fe^{2+} or Fe^{3+} to crude cell-free extracts was found to inhibit release of Cl^- ion during oxidation of 5-chloro-3-methylcatechol. A concentration of 5 mM- Fe^{2+} or 1 mM- Fe^{3+} resulted in complete inhibition, as shown in Table 1.

This behaviour is similar to the observations of Suda, Matsuoka & Kamahora (1952) that Fe^{2+} (1 mM) partially inhibited the conversion of *cis-cis*-muconate into β -oxoadipate, and of Sistrom & Stanier (1954) who found inhibition of lactonization of *cis-cis*-muconate by both Fe^{2+} and Fe^{3+} .

It was further found that cell-free extracts prepared in tris buffer also failed to catalyse dehalogenation, even in the absence of iron.

Oxidation of 5-chloro-3-methylcatechol without release of Cl^- ion clearly implies accumulation of the ring-fission compound. In agreement with this, the use of iron or tris buffer to prevent dehalogenation resulted in the release of only 2 equiv. of acid/mol of catechol oxidized. The u.v. spectrum of the reaction product when dehalogenation was inhibited showed $\lambda_{\max}$ at 265 nm. This was stable to alkaline pH values, but on acidification the spectrum changed to give $\lambda_{\max}$ at 278 nm (see Fig. 1). The new spectrum was identical in all respects with that of γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide. Quantitative release of Cl^- ion accompanied this change in spectrum at low pH.

Confirmation of identity of the product of spontaneous dehalogenation of the ring-fission compound. 5-Chloro-3-methylcatechol (25 mg) was incubated with 12 ml of crude cell-free extract (prepared in phosphate buffer), 90 ml of 50 mM-phosphate buffer, pH 8.0, and ferrous sulphate to give a final concentration of 5 mM, at 30°C in a 1-litre baffled flask on a rotary shaker. Oxidation was complete in 30 min, as shown by the disappearance of the blue colour of the iron-catechol complex. At this stage no release of Cl^- ion occurred and the u.v. spectrum showed $\lambda_{\max}$ at 265 nm. On adjustment of the pH to 2.8 rapid dehalogenation occurred. The dehalogenated product was extracted into ether and the extract dried over anhydrous sodium sulphate. Evaporation of the solvent gave a white solid, which recrystallized from water as colourless crystals (6 mg), m.p. 180–181°C. The mixed melting point with γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide was undepressed. This confirms that the ring-fission compound decomposes under acid conditions to yield the lactone.

Isolation of the ring-fission product. This proved difficult because of the rapid lactonization of the ring-fission compound under acid conditions. Throughout isolation attempts the proportion of

Table 1. *Effect of iron concentration on dehalogenation of 5-chloro-3-methylcatechol by crude cell-free extracts of Pseudomonas N.C.I.B. 9340*

The system contained 0.5 ml of crude cell-free extract prepared in phosphate buffer, 1.7 ml of 50 mM-phosphate buffer, pH 8.0, 0.5 ml of water containing FeSO_4 or FeCl_3 and 5 μmol of 5-chloro-3-methylcatechol in 0.3 ml of water. The temperature was 30°C.

Iron concentration	Total O_2 uptake (μmol)	Total Cl^- release ($\mu\text{equiv.}$)	Cl^- release (% of that calculated from O_2 uptake)
No addition	4.4	3.7	85
0.1 mM- Fe^{2+}	4.1	1.2	30
0.5 mM- Fe^{2+}	4.4	1.3	30
1 mM- Fe^{2+}	4.4	1.0	23
2 mM- Fe^{2+}	4.4	0.7	16
5 mM- Fe^{2+}	4.4	0.0	0
1 mM- Fe^{3+}	4.0	0.9	22

Fig. 1. Effect of acidity on the u.v. spectrum of the ring-fission product of 5-chloro-3-methylcatechol 1,2-oxygenase. 5-Chloro-3-methylcatechol ($5\mu\text{mol}$) was incubated with a crude cell-free extract at pH 7.0 in the presence of $5\text{mM}\cdot\text{Fe}^{2+}$ in a Warburg flask. When O_2 uptake was complete the incubation mixture was diluted and the u.v. spectrum recorded against a blank cell containing the same system with the exception of substrate. ●, Spectrum of ring-fission product, pH 7; ○, spectrum 1 h after acidification to pH 1 (i.e. lactone); △, spectrum after adjusting acid solution to pH 13 (lactone hydrolysis product).

lactonization was followed by measuring the E_{278}/E_{265} ratio. Two problems required consideration in this isolation: first, extraction of the ring-fission product into a suitable organic solvent and secondly the removal of this solvent under anhydrous conditions. The presence of water when all of the organic solvent had been evaporated was found to result in immediate lactonization. It was observed that the rate of dehalogenation was largely independent of the degree of acidity below pH 3, but appeared to vary with the concentration of the ring-fission compound. At a concentration of $0.1\text{--}0.2\mu\text{mol/ml}$ about 1 h was required for complete conversion into lactone, whereas at a concentration of $1\text{--}2\mu\text{mol/ml}$ it was almost immediate. The problem of extraction was thus overcome by first diluting the reaction mixture containing the compound to a concentration of about $0.2\mu\text{mol/ml}$. This slowed up dehalogenation, and rapid extraction with large volumes of ether successfully extracted most of the ring-fission compound before extensive lactonization had occurred. The use of anhydrous sodium sulphate to dry the ether extract proved inefficient and an appreciable amount of water remained in solution. This was overcome, however, by evaporation of the ether below 0°C . Water was thus frozen and prevented from catalysing lactonization.

5-Chloro-3-methylcatechol (150mg) was incubated with 40ml of a crude cell-free extract prepared in tris buffer, 360ml of $67\text{mM}\cdot\text{tris}\cdot\text{H}_2\text{SO}_4$ buffer, pH 8.0, 150mg of ferrous sulphate heptahydrate and 150mg of GSH at 30°C . The reaction was performed in two batches, each in a 1-litre baffled flask with vigorous shaking. After completion of the reaction (15min) the mixture was diluted ten times with water and was then extracted in 500ml portions. Each portion was extracted twice with ether at neutral pH and was then acidified to below pH 3 with dilute sulphuric acid and rapidly extracted twice with 300ml volumes of ether. This treatment enabled extraction of most of the ring-fission compound in $4\text{--}5\text{min}$ after addition of acid. The u.v. spectrum of the acid-ether extract showed λ_{max} at 265nm and an E_{278}/E_{265} ratio 0.75, indicative of only $5\text{--}10\%$ lactonization. After standing over anhydrous sodium sulphate for 24h , the ethereal solution was filtered and evaporated under high vacuum at -10 to -20°C . When all of the solvent had been removed, residual ice was removed by freeze-drying, leaving a pale-brownish solid (130mg). Recrystallization with charcoal treatment from 1,2-dichloroethane gave cream crystals (75mg), m.p. $123\text{--}124^\circ\text{C}$ (decomp.). Further recrystallization from benzene gave small white needles (61mg), m.p. 133°C (decomp.). The E_{278}/E_{265} ratio of an ether solution of these crystals was 0.72, showing the absence of lactone. On melting, hydrogen chloride was evolved and lactonization occurred. This resulted in resolidification followed by remelting at $160\text{--}170^\circ\text{C}$. The crystalline material was unstable when exposed to the atmosphere, being slowly converted into the lactone.

The isolated acid was readily soluble in water, but spontaneous lactonization took place. However, since 1mol (2equiv.) of ring-fission compound gave 1equiv. of lactone plus 1equiv. of hydrogen chloride, titration results were unaffected. Moreover, this decomposition enabled measurement of chlorine as Cl^- ion. Titration gave equiv.wt. 94 and chloride analysis gave 18.5% (Calc. for $\text{C}_7\text{H}_7\text{ClO}_4$: equiv.wt. 95; Cl, 18.6%).

This evidence strongly supports the previous suggestion that the ring-fission compound is γ -chloro- α -methylmuconate.

Properties of the ring-fission enzyme

This enzyme converts 5-chloro-3-methylcatechol into *cis-cis*- γ -chloro- α -methylmuconate. It was stable to storage at -20°C for at least 6 weeks without loss of activity.

Effect of pH. As assayed by the acid production method the optimum pH for the enzyme was about 7.5.

Cofactor requirement. During attempts at enzyme

Table 2. Requirement for iron by the ring-fission enzyme

The system contained enzyme preparations in phosphate buffer, pH 8.0, diluted to 2.2 ml with the same buffer, 0.5 ml of water plus or minus 3 μ mol of FeSO₄ and 5 μ mol of 5-chloro-3-methylcatechol in 0.3 ml of water.

Fraction	Vol. (ml)	Activity (μ mol of O ₂ /min per ml of enzyme soln.)		Specific activity in the presence of Fe ²⁺ (μ mol of O ₂ / min per mg of protein)
		No Fe ²⁺	1 mM-Fe ²⁺	
Crude cell-free extract	32	0.52	0.62	
100%-saturated-(NH ₄) ₂ SO ₄ precipitate	35	0.28	0.51	
After dialysis	35	0.22	0.57	0.071
First isoelectric precipitate at pH 5.0 with dialysed (NH ₄) ₂ SO ₄ precipitate as starting material	12	0.0	0.46	0.045
Second isoelectric precipitate obtained by decreasing from pH 5.0 to pH 4.5	12	0.0	0.50	0.050
Supernatant after isoelectric precipitation	40	0.0	0.0	0.0

Table 3. Effect of thiol compounds on the ring-fission activity of isoelectric precipitate

The system contained 0.5 ml of the pH 5.0 isoelectric precipitate dissolved in 50 mM-phosphate buffer, pH 8.0, 2.2 ml of 50 mM-phosphate buffer, pH 8.0, containing the cofactors and 5 μ mol of 5-chloro-3-methylcatechol in 0.3 ml of water. Cysteine hydrochloride was neutralized before use. Fe²⁺ was added as the sulphate; Fe³⁺ was added as the chloride.

Cofactor added (1 mM)	Specific activity (μ mol of O ₂ /min per mg of protein)
None	0.0
GSH	0.0
Cysteine hydrochloride	0.0
Fe ²⁺ alone	0.045
Fe ³⁺ alone	0.041
Fe ²⁺ + cysteine hydrochloride	0.037
Fe ²⁺ + GSH	0.075
Fe ³⁺ + GSH	0.082

purification it was found that protamine treatment resulted in complete loss of activity. However, the enzyme appeared stable to ammonium sulphate and isoelectric precipitation. During these procedures a requirement for iron became apparent, and isoelectric precipitation by addition of M-acetic acid to the dialysed ammonium sulphate precipitate to pH 5.0 followed by further addition of acetic acid to pH 4.5, gave enzyme preparations that were completely resolved with respect to this element. The activities of enzyme fractions after successive purifications, in the presence and absence of iron are shown in Table 2. Fe²⁺ and Fe³⁺ were equally effective in restoring activity to such preparations (see Table 3), whereas Mn²⁺, Mg²⁺, Co²⁺, Ni²⁺ and Zn²⁺ had no effect. The activity of isoelectric precipitates was considerably increased by

addition of GSH. Cysteine hydrochloride could not replace GSH in this respect (see Table 3).

Effect of enzyme concentration. Oxidation of 5-chloro-3-methylcatechol by cell-free preparations was proportional to the amount of enzyme present up to the limit at which the Warburg technique fails from insufficient aeration.

Effect of tris buffer. Cell-free extracts prepared in tris buffer showed a greater response to added iron than did those prepared in phosphate buffer. They also aged more rapidly on storage at -20°C. Activity of stored preparations was completely revived by addition of iron and GSH, as shown in Table 4.

Separation of lactonizing and delactonizing enzymes

A 1:2 (w/v, wet weight) suspension of cells in 50 mM-phosphate buffer, pH 8.0, was sonically disintegrated. The crude cell-free extract thus obtained was used as starting material for the enzyme preparation. Activity of both enzymes was still high after precipitation by saturation with ammonium sulphate, dialysis and protamine treatment. The protamine supernatant was fractionated into four protein precipitates by careful addition of a saturated solution of ammonium sulphate at 0°C: 0-35%, 35-40%, 40-45% and 45-66%-saturated ammonium sulphate. Each fraction was dissolved in 50 mM-phosphate buffer, pH 8.0, and assayed for activity. A virtually complete separation of the lactonizing and delactonizing enzymes was obtained in this way, the 0-35%-saturated-ammonium sulphate fraction containing most of the lactonizing enzyme and the 45-66%-saturated-ammonium sulphate fraction containing most of the delactonizing activity. This finding is similar to that of Sistrom & Stanier (1954) in an investigation of

Table 4. *Effect of storage at -20°C on the ring-fission activity of enzyme preparations in tris buffer*

The system contained 0.3 ml of crude cell-free extract prepared in tris buffer, 2.4 ml of 67 mM-tris buffer, pH 8.0, containing cofactors as indicated and 5 μ mol of 5-chloro-3-methylcatechol in 0.3 ml of water.

Length of storage of enzyme preparation	Cofactors added (1 mM)	Activity (μ mol of O ₂ /min per ml of enzyme soln.)
Freshly prepared enzyme	None	0.90
	GSH + Fe ²⁺	1.26
After 2 days storage	None	0.57
	Fe ²⁺	0.73
	GSH	0.79
	GSH + Fe ²⁺	1.26

the conversion of *cis-cis*-muconate into β -oxo-adipate. The results of a typical enzyme separation are presented in Table 5.

Properties of the lactonizing enzyme (cis-cis- γ -chloro- α -methylmuconate dehydrochlorinase)

During reaction of this enzyme, quantitative release of Cl⁻ ions occurred but there was no detectable increase in acidity. The end product showed $\lambda_{\max}$ at 278 nm identical with that of the lactone. Solutions of γ -chloro- α -methylmuconate in the absence of enzyme were examined for Cl⁻ ion release or change in extinction during all experiments.

Confirmation of the identity of the reaction product. A sample (10 units) of purified enzyme (0–35% saturated-ammonium sulphate fraction) was diluted with 10 ml of 50 mM-phosphate buffer, pH 8.0, containing 30 μ mol of manganous sulphate and incubated with 80 μ mol of ring-fission compound. The release of Cl⁻ ion was complete in 30 min. The incubation mixture was then acidified and extracted with ether. The extract was washed with water, dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The solid residue was recrystallized from water to give white crystals (6 mg), m.p. 180–181°C, undepressed by admixture with authentic γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide.

Effect of pH. The optimum pH found for the enzyme was 7.0.

Effects of cations and inhibitors. The activity of crude cell-free extracts was drastically decreased by dilution. At high dilutions (100-fold) all lactonizing activity was lost, whereas under the same conditions the delactonizing enzyme was unaffected. Lactonizing activity was fully restored, however, by addition of Mn²⁺ at 1 mM. Apart from the effect of dilution, complete resolution of the lactonizing enzyme with respect to this cation was never achieved. Activity was stimulated by Mn²⁺, Mg²⁺, Co²⁺ and Fe²⁺ and inhibited by Zn²⁺, Ni²⁺,

Cu²⁺, Ag⁺, potassium cyanide, EDTA and *p*-chloro-mercuribenzoate. Quantitative results of this survey are shown in Table 6.

Effects of tris buffer and iron. Neither tris buffer nor iron had any inhibitory effect on the purified dehydrochlorinase. In fact iron markedly stimulated activity, as shown in Table 6. This was a most unexpected result, bearing in mind the inhibition of dehalogenation by these substances during the incubation of crude cell-free extracts with 5-chloro-3-methylcatechol. No explanation for this observation is evident and no further study was undertaken.

Properties of the delactonizing enzyme (γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide hydrolase)

Reaction of this enzyme results in the release of 1 equiv. of acid/mol of lactone. The u.v. spectrum of the reaction product showed $\lambda_{\max}$ at 250 nm (alkaline) and no spectrum (acid).

Isolation of reaction product. A sample (4 units) of purified delactonizing enzyme (45–66% saturated-ammonium sulphate fraction) was incubated with 20 mg of lactone in 40 ml of 50 mM-phosphate buffer, pH 8.0. When the hydrolysis was complete (40 min), the reaction mixture was acidified, extracted once with ether and then subjected to continuous ether extraction for 24 h. The continuous ether extract was dried over anhydrous sodium sulphate, filtered and the solvent evaporated to yield a light-brown oil. The u.v. spectrum showed no extinction under acid conditions, $\lambda_{\max}$ at 250 nm (alkaline), and was almost identical with that of γ -hydroxy- α -methylmuconic acid. Positive 2,4-dinitrophenylhydrazine and Rothera reactions confirmed the presence of a β -oxo group. The spectrum of the 2,4-dinitrophenylhydrazone ($\lambda_{\max}$ at 375 nm) was very similar to that of the 2,4-dinitrophenylhydrazone of γ -hydroxy- α -methylmuconic acid ($\lambda_{\max}$ at 375 nm). Chromatography of the crude oil showed several acidic spots, the major two of which corresponded to γ -hydroxy- α -methylmuconic acid (R_F 0.35 and 0.65). With regard to previous failures (see Gaunt &

Table 5. Results of the separation of lactonizing and delactonizing enzymes

Fraction	Vol. (ml)	Protein (mg/ml)	Lactonizing enzyme			Delactonizing enzyme		
			Activity (units/ml)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)	Activity (units/ml)	Total activity (units)
Crude cell-free extract	48		2.75	132		100	0.71	34
100%-saturated- (NH ₄) ₂ SO ₄ precipitate	50		2.15	107		81	0.30	15
After dialysis	60	24	1.5	90	0.0625	68	0.255	15.3
After protamine treatment	110	16	0.835	91.5	0.052	69	0.0985	10.9
0-35%-saturated- (NH ₄) ₂ SO ₄ precipitate	26	11	2.00	52	0.182	38	0.022	0.57
35-40%-saturated- (NH ₄) ₂ SO ₄ precipitate	22	10	0.60	13.2	0.06	10	0.026	0.57
40-45%-saturated- (NH ₄) ₂ SO ₄ precipitate	12	6	0.05	0.6	0.008	0.5	0.078	0.94
45-66%-saturated- (NH ₄) ₂ SO ₄ precipitate	19	11	0.0	0.0	0.0	0	0.39	7.4
								0.0355
								22

Evans, 1971), no attempt was made to isolate this product further. The evidence is, however, fully consistent with the view that the delactonizing enzyme catalyses a simple hydrolysis of the lactone to γ -hydroxy- α -methylmuconate.

Effect of pH. Activity of the delactonizing enzyme was at a uniform maximum amount from pH 7.5 to pH 10.0. Above this pH enzyme activity decreased rapidly, and spontaneous hydrolysis of the lactone was appreciable.

Effects of cations and inhibitors. No cofactor requirement could be found for the enzyme. Cu²⁺, EDTA and *p*-chloromercuribenzoate had no effect on activity, but Ag⁺ and potassium cyanide markedly inhibited the enzyme. Full details of these results are shown in Table 7.

DISCUSSION

These results unequivocally established the proposed route for metabolism of 5-chloro-3-methylcatechol. Since γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide has now been identified as an intermediate in metabolism of both MCPA and 5-chloro-3-methylcatechol, the intermediary role proposed by Gaunt & Evans (1971) for the latter compound in MCPA oxidation is supported.

The ring-fission enzyme resembles many other catechol oxygenases in the general cofactor requirement for iron and thiol groups (Suda, Hashimoto, Matsuoka & Kamahora, 1951; Ottey & Tatum, 1956; Hayaishi, Katagiri & Rothberg, 1955; Ribbons & Evans, 1960), although their resolution has not been achieved for all such enzymes (Stanier & Ingraham, 1954; Cain & Cartwright, 1960; Ribbons & Evans, 1960; Cain, 1961). Ring fission is accompanied by the uptake of 1 mol of oxygen and the release of 2 equiv. of acid/mol of substrate, indicating cleavage between the hydroxyl groups with the formation of a muconic acid and identifying the enzyme as a catechol 1,2-oxygenase. The product of this reaction should be γ -chloro- α -methylmuconate, a presumption that is endorsed by titration results, chlorine analysis and conversion into the lactone γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide.

Although chemical isolation was not successful, the end product of this reaction sequence, i.e. the product after reaction of the delactonizing enzyme, is undoubtedly γ -hydroxy- α -methylmuconate. Certainly no further enzyme reaction involving saturation or cleavage of the molecule takes place with the fractionated enzyme, since this would destroy the conjugated-bond system necessary for u.v. absorption.

Without an extensive chemical survey of the compounds under discussion, the isomeric forms involved cannot be rigidly defined. However,

Table 6. *Effects of various cations and inhibitors on activity of the lactonizing enzyme*

The system contained 0.8 ml of 0–35% saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction, 3.2 ml of 50 mM-phosphate buffer, pH 8.0, containing the cofactor or inhibitor and 2.0 ml of γ -chloro- α -methylmuconate solution (8 μmol). All cations were added as the sulphate except AgNO_3 .

Cation or inhibitor	Activity ($\mu\text{equiv. of Cl}^-/\text{min per ml of enzyme soln.}$)	Activity (% of control)
None	0.67	100
Mn^{2+} (1 mM)	1.95	290
Mg^{2+} (1 mM)	1.01	150
Co^{2+} (1 mM)	1.01	150
Fe^{2+} (1 mM)	0.90	134
Fe^{2+} (5 mM)	1.50	223
Zn^{2+} (1 mM)	0.25	36
Ni^{2+} (1 mM)	0.135	20
Cu^{2+} (1 mM)	0.0	0
Ag^{+} (1 mM)	0.0	0
KCN (1 mM)	0.29	44
EDTA (1 mM)	0.195	29
<i>p</i> -Chloromercuribenzoate (1 mM)	0.0	0

* Assayed by the spectrophotometric method.

Table 7. *Effects of cations and inhibitors on the delactonizing enzyme*

The system contained 0.03 ml of 50–66% saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction, 1.0 ml of 67 mM-tris buffer, pH 8.0, 2.0 ml of water and 0.14 μmol of lactone. All cations were added as the sulphate except AgNO_3 .

Inhibitor	Activity ($\mu\text{mol of lactone hydrolysed/min per ml of enzyme soln.}$)	Activity (% of control)
None	0.545	100
Cu^{2+} (1 mM)	0.545	100
Mn^{2+} (1 mM)	0.545	100
Fe^{2+} (1 mM)	0.545	100
Mg^{2+} (1 mM)	0.545	100
Ag^{+} (1 mM)	0.078	14
EDTA (1 mM)	0.545	100
<i>p</i> -Chloromercuribenzoate (0.1 mM)	0.545	100
KCN (1 mM)	0.286	52
KCN (2 mM)	0.235	43

enzymic ring cleavage by catechol 1,2-oxygenases yields *cis-cis*-muconates (Evans, Smith, Linstead & Elvidge, 1951); *cis-trans*-isomers are rarely oxidized by bacterial systems and, if so, only at a very low rate (Sistrom & Stanier, 1954). Thus the isolated γ -chloro- α -methylmuconate is almost certainly the *cis-cis* form. Preparations of γ -chloro- α -methylmuconate were always completely metabolized by cell-free systems. Thus no evidence of isomerization to a biologically inactive form was observed.

However, a small amount of a biologically inactive compound tentatively identified as a γ -chloro- α -methylmuconate was isolated from MCPA cultures (Gaunt & Evans, 1971). This behaviour of γ -chloro- α -methylmuconate thus differs from that of β -chloromuconate. The latter compound has only been isolated as a biologically inactive

isomer from cultures of cells metabolizing 4-chlorophenoxyacetate or 4-chlorocatechol (Evans, Smith, Moss & Fernley, 1971). This leads to the deduction that *cis-cis*- β -chloromuconate tends to isomerize rather than lactonize, whereas with *cis-cis*- γ -chloro- α -methylmuconate the reverse seems to be true.

Chemical lactonization of *cis-cis*- γ -chloro- α -methylmuconate should give a lactone with an exocyclic double bond in the *cis* configuration. Consideration of an atomic model showed no stress within this structure that might lead to a tendency for spontaneous isomerization to the *trans* form. Since the synthesized lactone is identical with the lactone isolated from bacterial and enzymic incubations, it is probable that the metabolic product is also in the *cis* form.

Mechanism of lactone formation. The mechanism

Scheme 1. Possible two-step reactions for the lactonization of γ -chloro- α -methylmuconate.

so far considered for lactone formation is a single-step reaction, i.e. the enzyme lactonizes by dehydrochlorination. The reaction is unlike typical bacterial lactonizations in this series, since the product contains two double bonds. In view of this, the similarity of this enzyme with the lactonizing enzyme described by Sistrom & Stanier (1954) was somewhat unexpected; both enzymes appeared in the same ammonium sulphate fraction, and both required Mn^{2+} or Mg^{2+} for activity (see also Cain, 1961). However, whereas γ -chloro- α -methylmuconate dehydrochlorinase was stimulated by Fe^{2+} and Co^{2+} , the enzyme studied by Sistrom & Stanier (1954) was inhibited. Again this dehydrochlorinase bears no resemblance to 1,1,1-trichloro-2,2-di-(*p*-chlorophenyl)ethane dehydrochlorinase, the only other dehydrochlorinating system so far described. No cofactor requirement could be found for the latter enzyme, despite an exhaustive study by Lipke & Kearns (1959), and *p*-chloromercuribenzoate was the only inhibitor found. Compared with this, apart from the cation requirement already discussed, γ -chloro- α -methylmuconate dehydrochlorinase was inhibited by Cu^{2+} , Zn^{2+} , Ag^+ , potassium cyanide and EDTA as well as by *p*-chloromercuribenzoate.

It is conceivable that lactonization could occur by a two-step reaction, i.e. dehalogenation followed by lactonization, or vice versa. Possible mechanisms are suggested in Scheme 1. The only mechanism of dehalogenation so far enzymically

demonstrated in bacteria is replacement of halogen by a hydroxyl group (Davies & Evans, 1962). However, it seems unlikely that this mechanism operates, i.e. the first route proposed in Scheme 1, since if γ -hydroxy- α -methylmuconate is formed directly from γ -chloro- α -methylmuconate the lactone becomes a quite unnecessary intermediate in 5-chloro-3-methylcatechol metabolism.

In the second possibility dehydrochlorination would yield a triple-bonded compound and lactonization of this would necessitate the same molecular rearrangement as occurs in other bacterial lactonizations of muconates (see Dagley, Evans & Ribbons, 1960); the similarity in enzyme properties could then be understood. However, no evidence for the existence of such an intermediate has been found. In both chemical and enzymic reactions the rates of dehalogenation and lactone formation are identical. Further, if two enzymes are required for this reaction and it is assumed that the dehydrochlorinase has no requirement for Mn^{2+} whereas the lactonizing enzyme needs this cation, then in the absence of Mn^{2+} an accumulation of the triple-bonded intermediate would be expected. No such dissociation of enzyme action was observed.

The third possible mechanism is also credible, but unlikely on the grounds that no halogenated lactone was ever detected and in any case the most likely chemical reaction of such a compound would be hydrolytic dehalogenation rather than the required dehydrochlorination; e.g. Boeseken & Metz (1935)

and Fernley (1959) both found unsaturated chloro-lactones to be unstable, halogen being readily replaced by a hydroxyl group in aqueous solution.

It thus seems probable that lactonization is the mechanism of chlorine elimination from the molecule, and that the reaction is the one-step process originally proposed.

No demonstrable cofactor requirement was found for γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide hydrolase. In this respect it resembles the de-lactonizing enzyme described by Sistrom & Stanier (1954), although Cain (1961) found a Ca^{2+} requirement for another enzyme catalysing the hydrolysis of muconolactone. The reaction mechanism appears to be a simple addition of water to the molecule, without any double-bond migration (cf. hydrolysis of muconolactone), giving γ -hydroxy- α -methyl-muconate.

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