Bacterial Metabolism of 4-Chlorophenoxyacetate

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1. A pseudomonad capable of utilizing 4-chlorophenoxyacetate (CPA) as sole source of organic carbon was isolated from soil. 2. The organism was grown in liquid culture and the following compounds were isolated and identified in culture extracts: 4-chloro-2-hydroxyphenoxyacetate, 4-chlorocatechol, β -chloromuconate probably the *cis-trans* isomer and γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide. 3. Cells grown on 4-chlorophenoxyacetate were able to metabolize 4-chloro-2-hydroxyphenoxyacetate, 4-chlorocatechol and γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide without a lag period. They were not adapted to 4-chlorophenol, or to either culture isolated or synthetic β -chloromuconate, possibly because of stereospecificity towards the *cis-cis* isomer. 4. On the basis of isolation and induction evidence, the following metabolic pathway is proposed for the breakdown of 4-chlorophenoxyacetate by this organism: 4-chlorophenoxyacetate $\rightarrow$ 4-chloro-2-hydroxyphenoxyacetate $\rightarrow$ 4-chlorocatechol $\rightarrow$ *cis-cis*- β -chloromuconate $\rightarrow$ γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide $\rightarrow$ maleylacetate and fumarylacetate $\rightarrow$ fumarate and acetate.

Pesticides, herbicides, synthetic detergents and waste products of chemical industries constitute a formidable addition of foreign chemicals to the two major sites of biological dissimilation, the soil and natural waters. Some of these are very poisonous substances, and could be a hazard to life if they accumulated in the environment. Gale (1947) has expressed the view that 'it is probably not unscientific to suggest that somewhere or other, some organisms may exist which can, under suitable conditions, oxidize any substance which is theoretically capable of being oxidized'. This doctrine of biochemical omnipotency has been questioned (Carson, 1963; Alexander, 1965); instances of apparent microbial fallibility have assumed prominence, because of the long persistence of a variety of pesticides with allegedly unfortunate consequences to wild life.

If a herbicide is to be of wide application in farming practice, it should not affect soil biochemical processes adversely, nor persist for such a length of time that it will interfere with the growth of the succeeding crop. Inactivation by adsorption to the soil colloids cannot go on indefinitely; leaching only means a transfer to some other locality. Short of their physicochemical instability in the environment, ultimate destruction and removal from the biosphere is mainly dependent on microbial action.

The hormone herbicides, CPA*, 2,4-D, MCPA and

* Abbreviations: CPA, 4-chlorophenoxyacetate; 2,4-D, 2,4-dichlorophenoxyacetate; MCPA, 4-chloro-2-methylphenoxyacetate.

their derivatives have been outstandingly successful in crop protection, and are therefore of world-wide importance. Generally speaking, they are toxic to dicotyledonous plants, often referred to as broad-leaved weeds, but relatively harmless, at least in the normal dosages used, to monocotyledonous plants such as the Gramineae.

The detoxication of 2,4-D in soils was attributed to the activities of the microflora by Nutman, Thornton & Quastel (1945). Subsequently several workers have described the isolation of microbial species from enrichment cultures, alleged to be the biological agents of inactivation of these herbicides, e.g. unidentified bacteria Newman & Thomas (1950), *Bacterium globiforme* Audus (1950), *Flavobacterium aquatile* and a *Corynebacterium*-like organism Jensen & Petersen (1952a,b), *Flavobacterium peregrinum* n. sp. Stapp & Spicher (1954), *Pseudomonas* sp. Evans & Smith (1954), *Corynebacterium* sp. Rogoff & Reid (1956), *Mycoplana* sp. Walker & Newman (1956), *Achromobacter* sp. Steenson & Walker (1956, 1957) and Bell (1957), *Nocardia coeliaca* Jensen & Petersen (1952a,b), *Nocardia* sp. Symonds (1958), *Rhizobium meliloti* Nilsson (1957), *Pseudomonas* N.C.I.B. 9340 Gaunt & Evans (1961) and *Arthrobacter* sp. Loos, Roberts & Alexander (1967). Studies on the metabolism of aryloxyacetic acids by fungi, e.g. *Aspergillus niger* strains, have also appeared (Faulkner & Woodcock, 1964; Bocks, Lindsay-Smith & Norman, 1964). Previous work on this topic has been well summarized by Audus (1964) and Kearney & Kaufman (1969).

It is therefore evident that a great variety of soil micro-organisms are capable of attacking these herbicides, although apparently to varying degrees. A study of their biochemical pathways of metabolism, however, necessitates not only the isolation of a capable organism, but its growth in pure culture, preferably with the herbicide as the sole source of organic carbon. Some of the above isolates grew very poorly, or not at all, in liquid medium under these circumstances. These desirable experimental conditions were first realized by using CPA as substrate. Preliminary reports of this work have already appeared (Evans & Smith, 1954; Evans & Moss, 1957; Evans, Gaunt & Davies, 1961); the bacterial metabolism of CPA is now presented in greater detail.

MATERIALS AND METHODS

Organism. Soil (5g) from the A₀ horizon of a conifer site was continuously perfused in a glass perfusor (Collins & Sims, 1956) with a solution of CPA (0.01%, w/v) in a mineral salts medium (see below) at room temperature (15–18°C). The medium was periodically readjusted to pH 6.5 with dilute NH₃. After 2 weeks subcultures were made on to CPA (0.05%, w/v)–mineral salts medium solidified with agar (2%, w/v) and a pure strain was isolated of an organism capable of utilizing the herbicide as sole carbon source for growth, both on solid and in liquid media. It was placed in the family *Pseudomonadaceae* (Bergey's *Manual of Determinative Bacteriology*, 7th ed., 1957). The organism was maintained on CPA–mineral salts–agar slopes, with subculturing every 2 weeks.

Culture media. The basal inorganic salts medium had the following composition (g/l): (NH₄)₂SO₄, 1; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.5; NaCl, 0.1; CaCl₂, 0.1; FeCl₃·6H₂O, 0.01; MnSO₄·4H₂O, 0.001; CuSO₄·5H₂O, 0.001; ZnSO₄·7H₂O, 0.001; adjusted to pH 7. CPA (sodium salt) (0.05–0.1%, w/v) was added, and the medium sterilized in Pyrex bottles (10 litres) by autoclaving at 15 lb/in² for 30 min. Starter cultures (250 ml) were grown in baffled Erlenmeyer flasks (1 litre) on a rotary shaker at 25°C and used as inocula for the larger-scale cultures (10–50 litres) grown under forced aeration. They were maintained at 25°C either in a thermostatically controlled water bath or in a large incubator cabinet.

Harvesting of cells. Cells were usually harvested towards the end of the exponential phase of growth, but before the complete disappearance of substrate (2–4 days), by centrifugation either at 5000g in polythene bottles (150 ml) in the six-place conical head of an MSE refrigerated centrifuge at 0°C or at 13,000g in the Sharples centrifuge with a cellophan lining to the rotor. The bacterial cream was scraped off and washed with 10 vol. of Na₂HPO₄–KH₂PO₄ buffer, pH 7 (50 mM), and either used immediately or stored at –25°C until required.

Extraction of cultures. Cultures or the supernatants obtained after centrifuging cultures were passed through a multiplate Seitz filter (Ford EK pads) under pressure (10 lb/in²) and concentrated to a convenient bulk in a climbing film evaporator at 25–30°C under reduced pressure. Concentrates were acidified to below pH 3 and

extracted three times with diethyl ether (equal vol.). Combined ether extracts were shaken with 5% (w/v) NaHCO₃ to remove acidic components, leaving the neutral-ether fraction. The bicarbonate fraction was carefully reacidified to below pH 3 and extracted with ether three times. Both the acid-ether and neutral-ether extracts were dried over anhydrous Na₂SO₄ and the solvent was removed under reduced pressure before examination by chromatography and spectrophotometry.

Materials. CPA, m.p. 155–156°C, prepared by the method of Minton & Stephens (1922), was obtained as a gift from Monsanto Chemicals Ltd., Ruabon, Denbighshire, U.K. It was recrystallized from water, with charcoal treatment, until free of phenolic impurities.

4-Chloro-2-hydroxyphenoxyacetic acid, m.p. 124–130°C (depending on the rate of heating), was synthesized for us by the method of Brown & McCall (1955).

4-Chlorocatechol, m.p. 90–91°C, was prepared by the method of Dakin (1909).

4-Chloroguaiacol, m.p. 36–37°C, was prepared from 4-nitroguaiacol (Paul, 1906) by the method of Brown & McCall (1955).

β -Chloromuconic acid was prepared by the peracetic acid oxidation of *p*-chlorophenol under conditions similar to those employed by Elvidge *et al.* (1950a) and Parke & Williams (1952) for the preparation of *cis-cis*-muconic acid from phenol. The peracetic acid reagent used was that of Greenspan (1947). The β -chloromuconic acid so obtained was recrystallized from ethanol or glacial acetic acid to m.p. 235–238°C. It thus differs from the product obtained by Boeseken & Metz (1935), which had m.p. 223°C. It is uncertain which isomer of β -chloromuconic acid was synthesized (see below).

β -Chloromuconolactone, m.p. 123°C, was prepared by the method of Neunhoeffer (1935), through the action of sulphuric acid (oleum) on 4-chloro-2-nitrophenol at 110–125°C.

β -Hydroxymuconolactone, m.p. 198°C, was prepared by hydrolysis of β -chloromuconolactone, as described by Neunhoeffer (1935).

γ -Carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide, m.p. 156°C, was prepared by the method of Eisner, Elvidge & Linstead (1951).

Determinations. Total phenols were determined by the method of Folin & Ciocalteu (1927). *o*-Dihydroxyphenols were determined by the method of Evans (1947).

Chloride ion was determined by a modification of the electrometric 'bottled end-point' method described by Strouts, Gilfillan & Wilson (1955) and developed by Moss (1956). The apparatus consisted of a standard Ag–AgCl half-cell connected by a saturated KNO₃–agar bridge with a second half-cell containing the unknown solution. This solution was titrated with 1 mM-AgNO₃ until the Ag⁺ ion concentration equalled that in the reference half-cell. A spot galvanometer was used to determine the end point, which could be measured to 0.02 μ equiv. of Cl[–]. The standard half-cell contained the following solutions: 5.01 ml of 0.1 M-AgNO₃, 5.00 ml of 0.1 M-NaCl, 2 ml of M-HNO₃ and water to give a final volume of 20 ml. The test half-cell contained 2 ml of M-HNO₃ plus test solution in a volume of 20 ml. A silver electrode, coated with AgCl, was immersed in each half-cell and connected through the galvanometer. This method proved very reproducible and gave a linear calibration from 0 to 6 μ equiv. of Cl[–].

Microanalyses were performed by Dr Weiler and Dr Strauss, Oxford, U.K.

Spectrophotometry. U.v. spectra were obtained with a Unicam SP 500 spectrophotometer; i.r. spectra were determined on a double-beam Grubb-Parsons instrument with KCl disc or Nujol, by Dr A. J. Neale, Monsanto Chemicals Ltd.

Chromatography. Both ascending and descending techniques were used. The most commonly used solvent systems were: (a) water-saturated benzene-acetic acid (4:1, v/v); (b) ethanol-water-aq. ammonia (sp.gr. 0.88) (16:3:1, by vol.).

Phenolic compounds were detected by spraying chromatograms either with Folin-Ciocalteu reagent (British Drug Houses Ltd., Poole, Dorset, U.K.) diluted with water to give a 20% (v/v) solution, followed by exposure to ammonia vapour, or with diazotized *p*-nitroaniline or sulphaphilic acid followed by 10% (w/v) Na_2CO_3 .

o-Dihydroxyphenols were located by either the Evans (1947) reagent or the Tollens reagent, i.e. ammoniacal 0.1M- AgNO_3 solution.

Acidic substances were detected with a mixture of 0.04% (w/v) Bromocresol Green and 0.04% (w/v) Bromophenol Blue in 96% (v/v) ethanol containing 5 ml of *m*-NaOH/l.

Unsaturated aliphatic compounds were detected by the decolorization of 10 mM- KMnO_4 in 0.5% (w/v) metaphosphoric acid (Dickman, 1952).

U.v.-absorbing compounds were detected by using a mercury-vapour lamp emitting at 254 nm and contact printing on Ilford document paper. Fluorescent spots were located visually, and certain absorbing compounds could also be detected by inspection under u.v. light.

Manometric experiments. These were performed in a conventional Warburg constant-volume apparatus at 30°C. A 1 ml portion of cell suspension (containing approx. 5 mg dry wt. of cells) and 1.8 ml of 50 mM-sodium phosphate-potassium phosphate buffer, pH 7.0, were placed in the main flask, 0.1 ml of 20% (w/v) KOH was put in the centre well and substrate in 0.3 ml of water was added from the side arm. Generally 6–12 μmol of substrate was used. However, these concentrations of catechol derivatives were found to be definitely inhibitory, and so the amount for these substrates was decreased to 1–2 μmol .

RESULTS

Changes in u.v.-absorption spectra of CPA cultures during growth

The u.v. spectra of liquid cultures utilizing 0.05% (w/v) CPA were followed at intervals; culture samples were centrifuged, the clear supernatants suitably diluted and the u.v. absorptions determined. Results for a typical culture are shown in Fig. 1. The sequence of changes may be summarized as follows: (I) during the early phase of growth (6–12 h), there is a decrease in E_{277} , indicating the disappearance of CPA; (II) between 12 and 24 h there occurs a definite increase in E_{275} ; (III) a further increase in absorption, accompanied by a movement in λ_{max} towards 270 nm takes place

Fig. 1. Changes in u.v. spectrum of CPA cultures during growth. CPA cultures (10 litres, containing 0.05% CPA in mineral salts medium) were grown under forced aeration at 25°C. Samples (10 ml) were periodically withdrawn aseptically, centrifuged at 10000g for 10 min, the clear supernatant fluid was diluted fivefold with water and the absorption spectrum scanned between 220 and 300 nm. $\blacktriangle$, Starting spectrum at inoculation, *t*, 0 h; $\square$, stage I, *t*, 6–12 h; $\blacksquare$, stage II, *t*, 12–24 h; $\bullet$, stage III, *t*, 24–48 h; $\circ$, stage IV, *t*, 48–72 h; $\triangle$, stage V, *t*, 72–96 h.

between 24 and 48 h; (IV) there follows a decrease in absorption, but with a λ_{max} clearly defined at 264 nm, probably representing the accumulation of an intermediate, which persists for a few hours between 48 and 72 h; (V) during the fourth day, a rapid decrease in extinction at all wavelengths between 220 and 330 nm takes place.

Formation of phenolic substances during growth

Even when growth was barely visible to the naked eye, CPA cultures gave a positive Folin-Ciocalteu test. This increased markedly during the early exponential phase of growth. Towards the end of this phase the Evans test also became positive, indicating the presence of an *o*-dihydroxyphenol; this latter could be easily missed, since it persisted only for a short time in the life of the culture. Subsequently the Folin-Ciocalteu test diminished, but did not entirely disappear.

Release of Cl^- ions during growth

A Cl^- -free culture medium was prepared by replacing sodium chloride, calcium chloride and ferric chloride with sodium nitrate, calcium nitrate

and ferrous sulphate respectively. This medium, after inoculation, allowed growth of the pseudomonad with the utilization of the substrate (CPA), without any apparent abnormality in cultural characteristics or morphology of the organism, as judged by staining techniques. The release of Cl^- ions was followed in such a culture. When the u.v. spectrum of the culture fluid showed that all the CPA had disappeared, 82% of the organically bound chlorine was found as inorganic chloride in the cell-free medium.

4-Chloro-2-hydroxyphenoxyacetic acid

A Folin-positive Evans-negative compound was often found to accumulate during the early stages of culture growth, although its concentration never exceeded 0.5 mg/l of culture medium. Culture medium (100 litres) was filtered, concentrated and extracted when the accumulation of this compound was at a maximum. The phenolic substance was found in the acid-ether extract, contaminated with large amounts of residual CPA. The two components were separated by chromatography on Whatman no. 3MM paper with solvent (a). The band containing the phenolic component was eluted into 0.1 M-sodium hydroxide. The eluate was acidified and exhaustively extracted with diethyl ether. The extract was dried over anhydrous sodium sulphate and the ether was then removed under vacuum to give a light-brown solid. This was chromatographed by repeating the above procedure and the final product recrystallized from hot water with charcoal treatment to give fine white needles (20 mg), m.p. 129°C; λ_{max} in water at 284 nm, equiv.wt. 202 (Found: C, 47.5; H, 3.4; Cl, 17.6. Calc. for $\text{C}_8\text{H}_7\text{ClO}_4$: C, 47.4; H, 3.5; Cl, 17.5%; equiv.wt. 202.5). This was found to be identical with an authentic sample of 4-chloro-2-hydroxyphenoxyacetic acid (m.p. 130°C) by mixed m.p. 130°C and by u.v. and i.r. spectrophotometry.

4-Chlorocatechol

Cultures showing maximum accumulation of an Evans-positive substance were harvested in 10-litre batches, the supernatants were made alkaline by the addition of sodium hydrogen carbonate and extracted three times with 1 litre of ether. The ether extract was found by chromatography to contain only one phenolic component, which had the same R_F value and colour reactions as 4-chlorocatechol. The extract was evaporated to dryness, leaving a brown oil (10 mg) that solidified on standing. This was recrystallized with charcoal treatment from chloroform, to give white needles, m.p. 88°C, unchanged by admixture with authentic 4-chlorocatechol. The culture isolate gave a dibenzoyl

derivative, m.p. 96°C, undepressed by authentic 4-chlorodibenzoylcatechol.

β -Chloromuconic acid

CPA cultures exhibiting λ_{max} at 264 nm were centrifuged and the supernatants acidified to pH 3 and exhaustively extracted with ether. Chromatography of the acid-ether extract in solvent (a) revealed a strongly absorbing unsaturated acidic compound which on elution had λ_{max} at 264 nm in both ethanol and 0.1 M-sodium hydroxide solution. About 30 mg of crude crystalline material showing these properties was isolated from a number of cultures. Recrystallization from ethanol or glacial acetic acid with charcoal treatment gave a white microcrystalline solid, m.p. 235–238°C, undepressed by admixture with a sample of synthetic β -chloromuconic acid (Found: C, 40.9; H, 3.0; Cl, 20.2; equiv.wt. 88.4. Calc. for $\text{C}_6\text{H}_5\text{ClO}_4$: C, 40.8; H, 2.8; Cl, 20.1%; equiv.wt. 88.3).

γ -Carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide

Crude isolates of β -chloromuconic acid from CPA cultures were shown by i.r. spectroscopy to be contaminated occasionally with a lactone. One such sample on microanalysis gave a chlorine content corresponding to 37% β -chloromuconic acid and λ_{max} at 272 nm (cf. β -chloromuconic acid, 264 nm). The mixture (24 mg) was triturated with hot 1,2-dichloroethane (1 ml) and the supernatant cooled to -20°C , when crystals were deposited

Fig. 2. U.v. spectra of γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide and its hydrolysis product. - · - ·, Spectrum of lactone in water; - · - ·, spectrum after addition of NaOH; —, spectrum after reacidification.

(13mg). Recrystallization from the same solvent gave white needles, m.p. 164°C, devoid of halogen with $\lambda_{\max}$ in water at 277nm (ϵ 15000). Micro-analysis of this lactone gave: C, 51.6; H, 3.0%. $C_6H_4O_4$ requires C, 51.4; H, 2.86%. Synthetic γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide had m.p. 156°C and $\lambda_{\max}$ in water at 277nm (ϵ 14500).

The u.v. spectra of the natural and synthetic lactones showed identical behaviour on treatment with alkali and subsequent acidification (Fig. 2). On addition of alkali the peak at 277nm disappeared, being replaced by a smaller peak at 243nm; on acidification the latter peak vanished, but was restored on addition of excess of alkali. This behaviour is explained by a spontaneous hydrolysis of the γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide to β -hydroxymuconic acid, which then undergoes a pH-dependent keto-enol tautomerism, with loss of the conjugated double bond in acid solution (Scheme 2). Chromatography of both lactones in solvent (a) gave one spot with R_F 0.8, which showed $\lambda_{\max}$ at 243nm on elution, indicating that hydrolysis had occurred.

The i.r. spectra of these two lactones both showed a peak at 1800cm⁻¹, but in other respects there were slight discrepancies, supporting the inference from melting-point data that they were not identical. Geometric isomerism is possible about the exocyclic double bond, and it is suggested that the natural lactone is the *cis* and that the synthetic lactone the *trans* form of γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide.

Metabolic studies with CPA-grown cells

Adaptation data. Useful criteria in determining whether substances isolated from cultures actually participate in a metabolic pathway may be obtained from adaptation evidence. CPA-grown cells, harvested at mid-exponential phase of growth, were examined in the Warburg respirometer for their ability to oxidize a number of compounds, including those isolated from CPA cultures. These results show that 4-chloro-2-hydroxyphenoxyacetate, 4-chloroguaiacol and 4-chlorocatechol were immediately metabolized without a lag-phase; *p*-chlorophenol and *p*-chloroanisole were not attacked. Surprisingly, neither the synthetic β -chloromuconate nor that isolated from CPA cultures were metabolized by CPA-grown cells, indicating that the biologically active form is a different isomer of this acid. Further evidence bearing on this behaviour is discussed below. Both isolated and synthetic samples of γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide were readily oxidized by CPA-grown cells. Two hypothetical intermediates, namely β -chloromucrolactone and β -hydroxymuconolactone, were not attacked.

Fig. 3. Metabolism of 4-chlorocatechol by adapted *Pseudomonas* cells. Cells previously adapted to 4-chlorocatechol were suspended in 10 ml of 50 mM-phosphate buffer, pH 6.5, and added to 190 ml of 1.4 mM-4-chlorocatechol in the same buffer; a control flask, omitting the cells, was also included. The flasks were incubated with aeration at 25°C. Samples were periodically removed aseptically for analysis. ●, 4-Chlorocatechol; ○, β -chloromuconic acid; ▲, Cl⁻ ions; △, further metabolites (see the text).

In the above experiments, results obtained with CPA-grown cells were compared with the behaviour of organisms grown in nutrient broth. Such organisms failed in all cases to oxidize the substrates mentioned.

Metabolism of 4-chlorocatechol. CPA-grown cells were suspended in 10 ml of 50 mM-phosphate buffer, pH 6.5, and added to 190 ml of 1.4 mM-4-chlorocatechol and incubated with aeration at 25°C. Samples were aseptically withdrawn periodically for the following determinations, the results of which are shown in Fig. 3.

(i) U.v. absorption. Samples (1 ml) were diluted to 10 ml with water and centrifuged at 0°C (10000g for 10 min). The absorption spectra of the supernatants were determined between 220 and 300 nm. The extinction values at 284 nm (4-chlorocatechol) and 264 nm (β -chloromuconic acid) showed a gradual shift in the absorption maximum from the former peak to the latter during the first 8 h of the experiment, with a decrease in extinction at 264 nm thereafter. By the use of Vierordt's method (Gillam & Stern, 1954), the concentrations of 4-chlorocatechol and β -chloromuconate in each sample were calculated; the assumption was made that no other u.v.-absorbing components were present.

(ii) Chromatographic examination. Samples (5 ml) of the supernatants were acidified to below pH 3, ether extracted and the solvent was removed. The aqueous residues showed no absorption between 220 and 300 nm. The ether extracts were subjected to chromatography in solvent mixture (b). They

showed the disappearance of 4-chlorocatechol, accompanied by the production of a substance (R_F 0.45) identical with synthetic β -chloromuconic acid and giving an extinction maximum at 264 nm on elution with ethanol containing a drop of 0.1 M-sodium hydroxide; no other components absorbing between 220 and 300 nm were present on the chromatograms. After 12 h the supernatant was devoid of 4-chlorocatechol but it took 16 h for the β -chloromuconate to disappear.

(iii) 4-Chlorocatechol was determined independently by the method of Folin & Ciocalteu (1927); there was close agreement with the values obtained by Vierordt's method.

(iv) Cl^- ion determination. The production of Cl^- ions closely follows the curve for 'further metabolites' obtained by subtracting the contributions of 4-chlorocatechol and β -chloromuconate at any given time from the total material present.

In a repeat experiment 0.1 μmol of synthetic β -chloromuconate/ml was added to the cell suspension at 13 h. The concentration of the extracellular substance absorbing at 264 nm formed from 4-chlorocatechol (which we contend is a β -chloromuconic acid) was known before the addition and in this case was 0.02 $\mu\text{mol}/\text{ml}$. By following the extinction and Cl^- ion release over the next 10 h (13–23 h) it was shown that the culture-formed β -chloromuconate was completely metabolized, whereas none of the synthetic acid disappeared.

Subsequent metabolism of β -chloromuconate. Evans, Smith, Linstead & Elvidge (1951) had already shown that the bacterial metabolism of *cis-cis*-muconate to β -oxoadipate was mediated through a (+)-muconolactone (γ -carboxymethyl- $\Delta^{\alpha\beta}$ -butenolide). They also suggested that this lactone underwent a tautomeric change to β -oxoadipate enol-lactone before yielding oxoadipate; this latter step has since been demonstrated by Ornston & Stanier (1964). *Neurospora crassa* metabolized protocatechuate through β -carboxymuconolactone (Gross, Gafford & Tatum, 1956), whereas *Pseudomonas* sp. form the γ -carboxymuconolactone (Ornston & Stanier, 1966) before β -oxoadipate production.

Cis-cis-(syn-*cis*)- β -Chloromuconate (the isomer expected from the *ortho*-fission of 4-chlorocatechol) could lactonize in a variety of ways. Whether the γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide isolated from CPA cultures is produced directly from β -chloromuconate or is mediated by another chloro compound is difficult to establish at present, since neither the β -chloromuconic acid isolated from culture nor a synthetic sample was attacked by CPA-grown cells or a cell-free extract. Whether β -chloromuconolactone participated could, however, be tested.

Synthetic ($\pm$)- β -chloromuconolactone was unstable in aqueous solution, being very easily hydro-

β -Chloromuconolactone

β -Hydroxymuconolactone

Scheme 1. Hydrolysis of β -chloromuconolactone to give β -hydroxymuconolactone in aqueous solutions.

Fig. 4. Effect of pH on the spontaneous dechlorination of β -chloromuconolactone in aqueous solution. β -Chloromuconolactone (1.5 $\mu\text{equiv.}/\text{ml}$) was incubated at 20°C in buffers consisting of various proportions of disodium phosphate (0.2 M) and citric acid (0.1 M) and samples were removed periodically for Cl^- ion determination. $\times$, pH 7.0; $\circ$, pH 4.0; $\bullet$, pH 2.2.

lysed to β -hydroxymuconolactone (Scheme 1) with the liberation of Cl^- ions. The product formed, β -hydroxymuconolactone, gives λ_{max} at 251 nm in neutral aqueous solution, changing on acidification to a peak at 225 nm of much lower intensity. The hydrolysis of β -chloromuconolactone is pH-dependent, as shown by following quantitatively the u.v. spectrum and Cl^- ion release at different pH values (Fig. 4). Titration of β -chloromuconolactone showed that 3 equiv. of alkali had been taken up. The ready release of Cl^- ions from β -chloromuconolactone in aqueous solution made this compound an attractive possibility as an intermediate after β -chloromuconate. Adaptation data for CPA-grown cells in buffered neutral solution of β -chloromucono-

DISCUSSION

The organism used here performed an *ortho*-cleavage of 4-chlorocatechol to give a β -chloromuconate. CPA-grown cells and cell-free extracts,

Scheme 2. Proposed pathway of CPA degradation by a soil pseudomonad.

Table 1. *Extent of steric hindrance between various groups as revealed by scale models of β -chloromuconic acid isomers*

$\alpha\beta$	$\gamma\delta$	$\beta\gamma$
<i>cis</i>	<i>cis</i>	<i>syn-cis</i>
<i>cis</i>	<i>cis</i>	<i>syn-trans</i>
<i>trans</i>	<i>cis</i>	<i>syn-cis</i>
<i>trans</i>	<i>cis</i>	<i>syn-trans</i>
<i>cis</i>	<i>trans</i>	<i>syn-cis</i>
<i>cis</i>	<i>trans</i>	<i>syn-trans</i>
<i>trans</i>	<i>trans</i>	<i>syn-cis</i>
<i>trans</i>	<i>trans</i>	<i>syn-trans</i>

Atoms or groups that interfere with one another
(possible minor effects shown in parentheses)

α -CO₂H and δ -CO₂H
 β -Cl and δ -CO₂H
 (α -H and δ -CO₂H)
 β -Cl and δ -CO₂H (β -Cl and α -CO₂H)
 (α -CO₂H and δ -H)
 None
 None
 (α -CO₂H and β -Cl)

however, failed to attack the sample of this acid isolated from culture, or that prepared synthetically; yet in culture an intermediate is transiently produced with a u.v.-absorption spectrum identical with that of β -chloromuconate, and is readily metabolized as is shown by the loss of its u.v. spectrum after initial development and by Cl⁻ ion release.

Scale models (Courtauld) of β -chloromuconic acid isomers based on covalent atomic radii, and, in accordance with current theory, with conjugated systems given a planar arrangement (where possible), show the steric interference between various groups to be according to Table 1. The *cis-cis* isomer, produced by *ortho*-cleavage of 4-chlorocatechol, shows steric hindrance in both the *syn-cis* and *syn-trans* forms, and this unstable arrangement would tend to invert to the more stable *cis-trans* isomer. This phenomenon is well known in the muconic acid series (cf. Elvidge, Linstead, Sims & Orkin, 1950b); peracetic acid oxidation of *p*-cresol yields the *cis-trans* isomer of β -methylmuconic acid (Elvidge, Linstead & Sims, 1951). Since the sizes of the methyl and chlorine groups are almost the same, we are of the opinion that the β -chloromuconic acid isolated from culture, and synthesized, may be the *cis-trans* isomer, whereas the enzyme acting on the culture intermediate is stereospecific towards the *cis-cis* form of β -chloromuconate.

Direct enzymic evidence of the subsequent fate of *cis-cis*- β -chloromuconate could not be obtained, because of the failure to isolate this unstable isomer. Chlorine is lost from the molecule at some stage, but the precise mechanism remains uncertain. There exists, however, circumstantial evidence about its further metabolism.

At the time this work was undertaken, the only analogous situation was the metabolism of *cis-cis*-muconate and of *cis-cis*- β -carboxymuconate to

β -oxoadipate (Evans, 1956). In both cases a lactonization step is involved. If analogous lactonization occurs in the metabolism of β -chloromuconate, then β -chloromuconolactone could be an intermediate. This compound was found to be very unstable, undergoing ready hydrolysis to β -hydroxymuconolactone with the release of Cl⁻ ions. Such a step could account for the loss of organically bound chlorine during metabolism of CPA. However, the failure of CPA-grown cells to metabolize either β -chloromuconolactone or β -hydroxymuconolactone suggests that neither is involved in the metabolic pathway.

A more likely step for the further metabolism of β -chloromuconate is a completely different lactonization mechanism: lactone formation by the elimination of HCl to give γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide. This might be enzymically feasible with *cis-cis*- β -chloromuconate, since the chlorine and carboxyl groups will be adjacent in the molecule. In support of this are two observations: (a) the isolation of this lactone from culture extracts; (b) the ability of CPA-grown cells to oxidize the lactone. It is possible, however, that the lactone could arise by other means from *cis-cis*- β -chloromuconate, and thus the finding of the compound in extracts is insufficient evidence of a direct metabolic role.

Thus from the present data it is not possible to say categorically that γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide is the next intermediate to β -chloromuconate. However, subsequent studies on the metabolism of MCPA by a pseudomonad affords additional evidence that an analogous step is involved in the breakdown of this closely related phenoxyacetic acid (Gaunt & Evans, 1961). The ring-fission product in this case is γ -chloro- α -methylmuconate, and this is readily converted by elimination of HCl either chemically at low pH or by an enzyme preparation (from MCPA-grown

cells) into γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide. It thus seems probable that the γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide isolated from CPA cultures is in fact a true intermediate in the metabolic pathway, and that the pathway presented in Scheme 2 is accurate. γ -Carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide is readily attacked by CPA-grown cells and crude cell-free extracts possibly through maleylacetate to give substrates for the tricarboxylic acid cycle.

Some aspects of the degradation of the chlorophenoxyacetates and the chlorocatechols have also been investigated by Loos, Bollag & Alexander (1967), Bollag, Helling & Alexander (1968) and Tiedje, Duxbury, Alexander & Dawson (1969), with an *Arthrobacter* sp.; their results confirm our previous work in this field (Evans & Smith, 1954; Evans & Moss, 1957; Evans *et al.* 1961). It is also noteworthy that some fungi (e.g. *A. niger*) are capable of a non-specific hydroxylation of CPA, but the 4-chloro-2-hydroxy- and 4-chloro-3-hydroxy-phenoxyacetate produced were not further metabolized (Faulkner & Woodcock, 1964).

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