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

PRELIMINARY EVIDENCE FOR THE METABOLIC PATHWAY

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1. A pseudomonad capable of utilizing the herbicide 4-chloro-2-methylphenoxyacetate as a sole carbon source was isolated from soil and cultured in liquid medium. 2. Analysis of induction patterns of 4-chloro-2-methylphenoxyacetate-grown cells suggests that 5-chloro-*o*-cresol and 5-chloro-3-methylcatechol are early intermediates in the oxidation of 4-chloro-2-methylphenoxyacetate. Cells were not adapted to oxidize 4-chloro-6-hydroxy-2-methylphenoxyacetate. 3. In culture, 4-chloro-2-methylphenoxyacetate rapidly disappeared and the chlorine in the molecule was quantitatively released as Cl^- ion. 4. A lactone (γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide) was isolated from cultures and established as an intermediate. 5. The following metabolic pathway is suggested: 4-chloro-2-methylphenoxyacetate $\rightarrow$ 5-chloro-*o*-cresol $\rightarrow$ 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. 6. The tentative identification of 5-chloro-*o*-cresol, a γ -chloro- α -methylmuconate and γ -hydroxy- α -methylmuconate in culture extracts supports this scheme. However, the catechol was never observed to accumulate in cultures. 7. The detection of 4-chloro-6-hydroxy-2-methylphenoxyacetate, 2-methylphenoxyacetate, a dehalogenated cresol and oxalate in culture extracts is discussed in relation to the proposed metabolic pathway.

MCPA* is one of the most effective hormone herbicides; it is usually more persistent than 2,4-D in soil at similar dosages (Audus, 1964), taking approximately twice the length of time to disappear (i.e. 6 weeks or more). Several investigators have isolated, by elective culture, micro-organisms capable of metabolizing this group of herbicides. Thus Steenson & Walker (1957) applied the sequential induction technique to *Achromobacter* sp. growing on MCPA and concluded that 5-chloro-*o*-cresol was an intermediate in its metabolism. Bollag, Helling & Alexander (1967) showed the production of this phenol by *Flavobacterium peregrium* growing on MCPA; further, cell-free extracts catalysed the conversion of MCPA into 5-chloro-*o*-cresol, thus confirming this compound as an intermediate.

The object of the present work was to investigate, in more detail, the metabolic pathway of MCPA by a *Pseudomonad* sp. (Gaunt, 1962), N.C.I.B. 9340, that grew very well on this herbicide. To establish the pathway involved, induction patterns were investigated for this organism, and

MCPA cultures examined for the transient accumulation of possible intermediates. Some of these results were briefly reported by Gaunt & Evans (1961) and Evans, Gaunt & Davies (1961).

MATERIALS AND METHODS

Isolation of organism. A Gram-negative flagellated rod-shaped bacterium was isolated from a soil perfusor in which MCPA degradation was in active progress. It was grown in a mineral salts medium with MCPA as sole carbon source. This organism was characterized as a Group III *Pseudomonas* sp. (Shewan, Hobbs & Hodgkiss, 1960) by Dr J. M. Shewan of the Torry Research Station, Aberdeen, U.K. and has been placed in the National Collection of Industrial Bacteria at Torry Research Station under the reference number N.C.I.B. 9340.

Culture of organism. Cells were grown under forced aeration in 10-litre or 50-litre batches by using the following medium (g/l): KNO_3 , 1; K_2HPO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; NaCl , 0.05; CaCl_2 , 0.05; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.01; 0.1% MCPA added as the sodium salt. The pH was about 7.5 and required no adjustment during growth. Unadapted cells were grown in a medium in which MCPA was replaced by 0.1% glucose. For adaptation experiments, cultures were harvested during the exponential phase in a Sharples compressed-air-driven centrifuge. Cells were washed once

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

by suspension in 50 mM- Na_2HPO_4 - KH_2PO_4 buffer, pH 7.0, and then centrifuged and stored at -20°C until required.

Manometric experiments. These were performed as described in the previous paper (Evans, Smith, Moss & Fernley, 1971).

Preparation of culture extracts. The supernatant culture medium after harvesting of the cells was concentrated in a climbing-film evaporator at 30°C . The concentrate was acidified to below pH 3.0 and extracted four times with ether. This extract was shaken twice with 5% (w/v) NaHCO_3 to separate neutral and acidic components and the bicarbonate phase then acidified and extracted with ether. Finally the ether-extracted culture concentrate was continuously extracted with ether for 24 h. The acid, neutral and continuous ether extracts so obtained were dried over anhydrous Na_2SO_4 , filtered and concentrated to a small volume before chromatographic examination. U.v. spectra of extracts were followed at each stage during this procedure.

Chromatography. Descending chromatograms were run on Whatman no. 54 paper at room temperature with ethanol-water-aq. ammonia (sp.gr. 0.88) (16:3:1, by vol.) (Elliott, 1954). Neutral phenols were coupled with diazotized sulphanilic acid and spotted on paper previously sprayed with 4% (w/v) Na_2CO_3 . The resulting sodium salts of the azo dyes were then run in butan-2-one saturated with water (Hosfield, 1951). Phenolic compounds were detected with (a) aq. 20% (v/v) Folin-Ciocalteu reagent (British Drug Houses Ltd., Poole, Dorset, U.K.), followed by exposure to ammonia fumes (Mitchell & Davies, 1954), (b) diazotized *p*-nitroaniline or diazotized sulphanilic acid followed by 10% (w/v) Na_2CO_3 (Bray, Thorpe & White, 1950).

Acids were detected with (a) 0.1% (w/v) Bromocresol Green in 99.5% (v/v) ethanol (Lugg & Overell, 1948) and (b) aniline-xylose reagent (Nordmann & Nordmann, 1960).

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

Carbonyl compounds were detected by a saturated solution of *o*-dianisidine in acetic acid followed by heating at 100°C for 10 min (Feigl, 1954a).

Compounds with a marked u.v. absorption were detected by contact printing on Ilford document paper, by using a mercury-vapour lamp source emitting at 253.7 nm.

Reactions applied to crystalline isolates. Aldehydes and ketones were detected by the formation of insoluble hydrazones when treated with 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2 M-HCl.

β -Oxo acids were detected by the Rothera (1908) reaction.

Lactones were detected by a modification of the technique of Feigl (1954b). One drop of an ethereal solution of the lactone was warmed with 1 drop of a saturated ethanolic solution of hydroxylamine hydrochloride, saturated ethanolic KOH was added to make the mixture alkaline, and the mixture was cooled and acidified with 0.5 M-HCl. On addition of 1 drop of aq. 1% (w/v) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ a purple colour indicated the presence of a lactone ring.

Chlorine in an organic compound was detected by the Beilstein test (Shriner, Fuson & Curtin, 1956).

Determinations. Spectrophotometric determinations

were carried out in the Unicam SP.500 spectrophotometer in 1 cm-light-path cuvettes.

MCPA was determined by a modification of the chromotropic acid procedure described by LeTourneau & Krog (1952). The test solution was acidified to below pH 3 and extracted into ether to give an extract containing 10–60 μg of MCPA/ml. A sample (1 ml) of the ether layer was evaporated to dryness in a reductase tube and 5 ml of chromotropic acid reagent (200 mg of 1,8-dihydroxynaphthalene-3,6-disulphonic acid dissolved in 100 ml of conc. H_2SO_4) was added. The mixture was heated on a boiling-water bath for 5 min. After cooling, the mauve colour was measured in the Unicam SP.600 spectrophotometer, reading at 580 nm against a reagent blank. This procedure gave a linear response over the range 5–80 μg of MCPA.

Chlorine was determined by a micropotentiometric method based on that of Strouts, Gilfillan & Wilson (1955) as developed by Moss (1956). It enabled determination of 2–200 μg of chloride to be made to an accuracy within 1 μg .

Phenols were determined with Folin-Ciocalteu (1927) reagent.

o-Dihydroxy compounds were determined by the method of Evans (1947).

Catalytic hydrogenation was carried out in the Warburg apparatus as described by Milton & Waters (1955).

Titration was performed on a micro scale by using an automatic titrator (Radiometer Ltd., Copenhagen, Denmark).

I.r. spectra were kindly recorded by Dr A. J. Neale of Monsanto Chemicals Ltd., Ruabon, Denbighshire, U.K.

Materials. MCPA was obtained from the Mirvale Chemical Co. Ltd., Mirvale, Yorks., U.K. and was recrystallized to m.p. 119–121 $^\circ\text{C}$. 5-Chloro-*o*-cresol was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. 4-Chloro-6-hydroxy-2-methylphenoxyacetic acid, 4-chloro-2-formylphenoxyacetic acid, 4-chloro-2-formyl-6-hydroxyphenoxyacetic acid, 2-carboxy-4-chlorophenoxyacetic acid, 5-chloro-3-methylcatechol, 5-chloro-*m*-cresol and α -methylmuconic acid were synthesized (Gaunt, 1962). Other compounds were commercially available. Samples of 3-methylcatechol, 5-chloro-3-methylcatechol and 2,3-dihydroxybenzoic acid were generously given by Dr J. P. Brown of Monsanto Chemicals Ltd.

RESULTS

Adaptation experiments

MCPA-grown cells were examined for their ability to oxidize a large variety of possible aromatic intermediates. In the compounds tested, the following parts of the MCPA molecule were altered: the 2-methyl group at different oxidation levels ($-\text{CH}_3$, $-\text{CO}_2\text{H}$ and $-\text{CHO}$), with and without the 4-chlorine atom, the ether-linked side chain and a hydroxyl group in the 6-position.

There was no evidence for oxidation of the methyl group before ring fission. Cells showed no adaptation to expected oxidation products such as 4-chloro-2-formylphenoxyacetate, 4-chloro-2-formyl-6-hydroxyphenoxyacetate, 2-carboxy-4-

Fig. 1. Adaptation experiments with MCPA-grown *Pseudomonas* N.C.I.B. 9340 cells. MCPA-grown cells (1 ml of a suspension containing 5 mg dry wt. of cells) were incubated in 50 mM-sodium phosphate-potassium phosphate buffer, pH 7, with or without 2 μ mol of the substrates indicated below in a conventional Warburg constant volume apparatus at 30°C. ●, MCPA, 5-chloro-*o*-cresol and 5-chloro-3-methylcatechol; ▲, 3-methylcatechol; ○, 2-methylphenoxyacetate; ■, 4-chloro-2-formylphenoxyacetate and 2-carboxy-4-chlorophenoxyacetate; △, 4-chloro-2-formyl-6-hydroxyphenoxyacetate, 4-chloro-6-hydroxy-2-methylphenoxyacetate, 5-chlorosalicylaldehyde, 5-chlorosalicylate, 2,3-dihydroxybenzoate, *o*-cresol, salicylate, salicylaldehyde, gentisate, 4-chloro-2-methylanisole and without substrate.

chlorophenoxyacetate, 5-chlorosalicylaldehyde, 5-chlorosalicylate, 2,3-dihydroxybenzoate, salicylate, salicylaldehyde and gentisate, whereas 5-chloro-*o*-cresol and 5-chloro-3-methylcatechol were immediately metabolized (Fig. 1).

Cells were not adapted to compounds lacking the 4(*p*)-chlorine atom: neither 2-methylphenoxyacetate nor *o*-cresol was attacked and oxidation of 3-methylcatechol was slight compared with the 5-chloro derivative (Fig. 1). It thus appears unlikely that the chlorine atom is lost before ring fission.

Cells did not attack 4-chloro-6-hydroxy-2-methylphenoxyacetate, whereas 5-chloro-*o*-cresol was rapidly oxidized, implicating side-chain loss at the first metabolic step. It has been suggested that side-chain loss involves initial decarboxylation to an

Fig. 2. Changes in u.v. spectra of MCPA cultures during growth of *Pseudomonas* N.C.I.B. 9340. An MCPA culture was inoculated and the u.v. spectrum read after 15 times dilution. At intervals during the growth of the culture, samples of the medium were centrifuged to remove bacterial cells, diluted and the u.v. spectrum recorded. ○, Starting spectrum; ●, spectrum 82 h after inoculation; ▲, spectrum after 96 h; △, spectrum after 120 h; ■, spectrum after 144 h.

anisole derivative (Fernley, 1959; Bell, 1960). However, MCPA-grown cells did not oxidize 4-chloro-2-methylanisole, which does not support this theory.

Glucose-grown cells showed no ability to oxidize any of the above compounds.

Examination of cultures for accumulation of intermediates

During growth of cultures complete breakdown of MCPA and quantitative release of the chlorine atom as inorganic chloride was observed. The u.v. spectrum, Folin reaction, MCPA and Cl^- ion concentrations in the medium were followed during culture development in attempts to detect the accumulation of possible intermediates. In early cultures of the organism a striking series of u.v.-spectrum changes were observed (Fig. 2). MCPA has a spectrum with a λ_{max} at 279 nm. There was an initial tendency for intensification of absorption in the region 250–300 nm, the extinction at 279 nm increasing by as much as 50%. The 275–280 nm peak then tended to diminish, broaden and move in position towards 250 nm; eventually a slight 250–255 nm peak remained, which gradually decreased to zero. It was further observed that in cultures still showing a high λ_{max} at 278 nm (82 h position in Fig. 2) MCPA disappearance and Cl^- ion release were complete. The spectrum clearly represented

Fig. 3. U.v. spectra of isolated lactone from MCPA cultures of *Pseudomonas* N.C.I.B. 9340. O, Aqueous solution; ●, solution at pH 13; ■, alkaline solution after acidification.

a dehalogenated metabolite, the isolation of which was then attempted.

Isolation of γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide. Five 10-litre cultures were harvested and extracted at a point when MCPA disappearance was just complete and a u.v. spectrum with $\lambda_{\max}$ at 278 nm was still present. The unknown intermediate was readily extracted into the acid-ether fraction. On concentration of the ether extract to about 50 ml white crystals were deposited. The supernatant was decanted and the crystals were washed with a small volume of dry ether. On further concentration of the extract more crystals were deposited and these were collected as before. This process was repeated until the volume reached about 2 ml. The crystalline product was combined and recrystallized from aqueous ethanol to give white plates (1 g), m.p. 181–183°C (Found: C, 54.9; H, 3.9. Calc. for $C_7H_7O_4$: C, 54.5; H, 3.9%). Titration indicated a monobasic acid, equiv.wt. 151; u.v. spectrum had $\lambda_{\max}$ at 278 nm (ϵ 19 200) (Fig. 3). The material decolorized potassium permanganate and catalytic hydrogenation showed uptake of 1.5 mol of H_2 /mol, indicative of two double bonds, although the theoretical uptake of 2 mol of H_2 /mol was never attained. The crystals gave a positive lactone test and negative 2,4-dinitrophenylhydrazine, Rothera and Beilstein reactions. In neutral aqueous solution a slow hydrolysis occurred (an instantaneous reaction at pH 13), accompanied by release of a second carboxyl group. The hydrolysis product gave positive 2,4-dinitrophenylhydrazine and Rothera reactions, showing the presence of a β -oxo group. Its u.v. spectrum showed $\lambda_{\max}$ at 250 nm (alkaline) (ϵ 9000), no spectrum in acid (Fig. 3).

These properties are very similar to those of γ -carboxymethylene- $\Delta^{\alpha\beta}$ -butenolide (Evans *et al.* 1971) and are consistent with the view that the

isolated compound was the lactonic acid, γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide. The peracetic acid oxidation of 5-chloro-*o*-cresol (Gaunt, 1962) gave a product identical in every respect with this lactone; mixed m.p. was undepressed and u.v. and i.r. spectra were identical. Although this synthesis is not entirely unambiguous it adds further support to the structure proposed. Under alkaline conditions irreversible hydrolysis occurred to yield γ -hydroxy- α -methylmuconate, which underwent pH dependent keto-enol tautomerism, explaining the acid-alkali shift (see Scheme 1). MCPA-grown cells were fully adapted to metabolize the lactone.

Attempts were made to prepare γ -hydroxy- α -methylmuconic acid, since this seemed a likely metabolite of the lactone. Lactone (20 mg) was hydrolysed by dissolving it in 0.5 ml of 2 M-sodium hydroxide; after acidification the solution was subjected to continuous ether extraction for 24 h. The hydrolysis product was obtained from the extract as a pale-yellow oil that did not crystallize on standing for several days at -20°C . Although this gave a crystalline 2,4-dinitrophenylhydrazone, m.p. 225°C (decomp.), the analysis of the product was very poor (Found: C, 48.4; H, 4.4; N, 17.7. Calc. for $C_{13}H_{12}O_8N_4$: C, 44.3; H, 3.4; N, 15.9%). A further attempt to isolate the acid as the sodium salt, by the procedure used by MacDonald, Stanier & Ingraham (1954) for the isolation of β -carboxymuconic acid, also failed to yield a pure product. These results doubtless reflect the known instability of β -oxo acids.

γ -Hydroxy- α -methylmuconate as a culture component. Many cultures developed the slight 250–255 nm u.v. peak shown in Fig. 2 after 120 h. This spectrum showed the characteristic acid-base change noted above for the lactone hydrolysis product. Since the spectrum was only noted in cultures after the disappearance of the lactone, it seemed likely that γ -hydroxy- α -methylmuconate was a normal metabolite of the lactone. The 250 nm peak disappeared from the culture medium after continuous ether extraction for 24 h. The u.v. spectrum of the extract was identical with that of γ -hydroxy- α -methylmuconic acid prepared from the lactone. Chromatography in ethanol-water-ammonia showed two spots (R_f 0.35, 0.67), identical with those obtained on chromatography of the lactone. In this solvent system the lactone undergoes instantaneous hydrolysis. It thus seems likely that the culture component is γ -hydroxy- α -methylmuconic acid.

Accumulation of other intermediates. Very few cultures developed a significantly positive Folin reaction. The slight phenol accumulation that did occur disappeared soon after building up. At no time was a positive Evans reaction given by cultures. To detect the possible presence of small

amounts of other organic components, a series of cultures were extracted at various stages of growth, to give acid, neutral and continuous ether extracts. Components in these were then investigated by chromatography. The use of a variety of detection reagents and elution of spots into ethanol for u.v. examinations enabled partial characterization of the major constituents.

Examination of acid-ether extracts

Those cultures that still contained MCPA showed few other acidic components on chromatography, but extracts from cultures in which MCPA utilization was complete contained several acidic substances. The major components were separated by a countercurrent distribution between butanol and 0.1M- Na_2HPO_4 - KH_2PO_4 buffer, pH 7.0. The acidic extract from 100 litres of culture medium (harvested when MCPA disappearance was just complete) was evaporated to dryness and the residue dissolved in 10ml of buffer saturated with butanol. This was introduced into the first tube of a countercurrent apparatus and a 50-tube separation carried out. The distribution of extract components was determined by u.v. absorption and the results are shown in Table 1.

Isolation of γ -chloro- α -methylmuconic acid. The aqueous phases from the first three tubes of the countercurrent separation were combined, acidified and extracted with ether. After washing, the extract was dried over anhydrous sodium sulphate and evaporated to dryness. The brownish solid residue was recrystallized from ether-light petroleum (b.p. 40–60°C) to give light-brown crystals (2mg), m.p. 150–230°C, equiv.wt. 97. After titration, the material was recovered and recrystallized from water, with charcoal treatment, to give small white needles (1mg). Titration indicated a dicarboxylic acid, equiv.wt. 94.5; u.v. spectrum gave λ_{max} at 275nm (acid) (ϵ 22 000, based on mol.wt. 190) and λ_{max} at 271nm (alkaline). It did not react with 2,4-dinitro-

phenylhydrazine, it rapidly decolorized permanganate and was Beilstein positive. The isolate thus appears to be an unsaturated dibasic halogen-containing acid. The high extinction coefficient is of the order expected for a muconic acid and the slight acid-alkali shift in λ_{max} is comparable with that noted for other muconic acids [e.g. α -methylmuconic acid: λ_{max} at 272nm (acid), λ_{max} at 267nm (alkaline)]. The presence of chlorine and the equivalent weight are consistent with the view that this compound is α -methyl- γ -chloromuconic acid (equiv.wt. 95). A study of atomic models shows considerable steric interference in the *cis-cis* isomer of γ -chloro- α -methylmuconate, suggesting instability of this isomer. Isomerization to a *cis-trans* or *trans-trans* configuration seems likely and probably occurred in the culture medium before extraction (see Gaunt & Evans, 1971, for further discussion). MCPA-grown cells were not adapted to oxidize the isolated acid, further suggesting that it is not an intermediate in this form.

Isolation of 4-chloro-6-hydroxy-2-methylphenoxyacetic acid. Both phases of tubes 39–46 (inclusive) of the countercurrent separation contained the Folin-positive component of acid-ether extracts. They were combined and the buffer was acidified to below pH 3 and ether extracted. The ether-butanol extract was evaporated to dryness to give a brownish solid residue. This was dissolved in 1ml of hot water and treated with charcoal. After filtration and cooling, white needles were deposited (2mg), m.p. 98°C, undepressed on admixture with authentic 4-chloro-6-hydroxy-2-methylphenoxyacetic acid; equiv.wt. 218 (Calc. for $\text{C}_9\text{H}_9\text{ClO}_4$, 216.5); u.v. spectrum shows λ_{max} at 276 and 282nm (acid) and at 243 and 293nm (alkaline), identical with those of authentic material.

Isolation of 2-methylphenoxyacetic acid. The acid-ether extracts from two cultures showed a chromatographic spot with R_F 0.7 and λ_{max} at 272nm. These extracts were combined and treated with 0.5M-sodium hydroxide to hydrolyse any lactone present.

Table 1. Countercurrent separation of acid ether extracts of culture medium

The acid ether extract from 100 litres of culture medium was evaporated to dryness and dissolved in 10ml of 0.1M-phosphate buffer, pH 7.0. This was then subjected to a 50-tube countercurrent distribution between this buffer and butanol.

Tube nos.	λ_{max} (nm)	
	Aqueous phase	Butanol phase
1–3	272	No distinct spectrum
4 and 5	250 (no spectrum at acid pH)	No distinct spectrum
6–9	No distinct spectrum	No distinct spectrum
10–25	278 (250 at pH 13)	278 (250 at pH 13)
26–38	No distinct spectrum	No distinct spectrum
39–46	Slight peak at 289	280–285 (Folin-positive)
47–50	No spectrum	No spectrum

The solution was then acidified and extracted twice with ether, and the extract was washed with water to remove all γ -hydroxy- α -methylmuconic acid. After drying over anhydrous sodium sulphate the extract was evaporated to dryness. The solid residue was treated with hot water and charcoal; after filtration and cooling, white needles were deposited (3.5mg), m.p. 142–147°C, undepressed by admixture with authentic 2-methylphenoxyacetic acid (m.p. 149–153°C); equiv.wt. 170 (Calc. for $C_9H_{10}O_3$, 166); $\lambda_{\max}$. at 271 and 277nm, identical with those of 2-methylphenoxyacetic acid; chromotropic acid-positive and Beilstein-negative.

Isolation of oxalic acid. Evaporation of the solvent from the continuous ether extract of the 55-litre culture from which the lactone was first isolated yielded a yellow oil. On cooling to -20°C large colourless prismatic crystals were slowly deposited (400mg). These were washed with ether and purified by sublimation under high vacuum at 80–120°C to give a white powder, m.p. 101°C with loss of water and resolidification; at 150°C some sublimation occurred and the remaining solid finally melted at 184–186°C. The compound was very hygroscopic, strongly acidic, decolorized acid permanganate and had no u.v. absorption. Reactions and melting-point behaviour were identical with those of oxalic acid monohydrate. On admixture of the two materials no melting-point depression was observed. This was the only extract in which oxalic acid was found. MCPA-grown cells were unable to oxidize the compound.

Examination of neutral-ether extracts

In several extracts investigated only four neutral compounds were found. Three of these were pheno-

lic and readily separable by chromatography as their azo-dyes in butan-2-one-water. The properties of these compounds and of some authentic neutral substances related to MCPA are shown in Table 2. These results suggest that two of the components are 5-chloro-*o*-cresol and either *o*- or *m*-cresol. This was confirmed by reference to the u.v. spectra of certain extracts. The spectrum of one extract showing only one chromatographic spot, corresponding to 5-chloro-*o*-cresol, had $\lambda_{\max}$. at 280–285nm (acid) and $\lambda_{\max}$. at 243 and 297nm (alkaline). The spectrum of 5-chloro-*o*-cresol shows $\lambda_{\max}$. at 284nm (acid) and $\lambda_{\max}$. at 245 and 298nm (alkaline). A second extract, with the chromatographic spot corresponding to *o*- or *m*-cresol as the major component, had a spectrum with $\lambda_{\max}$. at 274nm (acid), identical with that of both *o*- and *m*-cresol. The third phenol present in neutral extracts is not 5-chloro-*m*-cresol, as was thought possible. It appears to be present in most cultures but remains unidentified. Similarly the non-phenolic aldehyde has also eluded identification.

DISCUSSION

Most of the MCPA culture components detected by chromatography have been identified, and these results can be summarized as follows: γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide, 4-chloro-6-hydroxy-2-methylphenoxyacetate, 2-methylphenoxyacetate and oxalate have been identified by crystalline isolation and comparison with synthetic samples. A γ -chloro- α -methylmuconate was tentatively identified on the basis of physical and chemical properties after isolation. 5-Chloro-*o*-cresol, either *o*- or *m*-cresol and γ -hydroxy- α -methylmuconic acid were tentatively identified on the basis of chromato-

Table 2. *Major components of neutral-ether extracts of culture medium*

The neutral-ether extracts from several cultures harvested at various stages of growth were evaporated to dryness and either chromatographed directly or after reaction with diazotized sulphanilic acid. The results below show a composite pattern of components observed. Data for some standard compounds are shown for comparison. The chromatographic solvent used for the compounds was ethanol-water-aq. ammonia (sp.gr. 0.88) (16:3:1, by vol.) and that for the azo-dyes was butan-2-one saturated with water.

Sample applied to chromatogram	R_F	Reactions			R_F of azo-dye	Colour of azo-dye	
		Folin	Diazotized <i>p</i> -nitroaniline	<i>o</i> -Dianisidine		Acid	Sodium salt
Neutral-ether extract	0.8	—	—	+	—	—	—
	0.85	+	Purple	—	0.1	Yellow	Red
	0.9	+	Purple	—	0.5	Orange	Orange
	0.95	+	Yellow spot and purple ring	—	0.9	Yellow	Red
<i>o</i> -Cresol	0.9	+	Purple	—	0.5	Orange	Orange
<i>m</i> -Cresol	0.9	+	Purple	—	0.5	Orange	Orange
5-Chloro- <i>o</i> -cresol	0.95	+	Yellow spot and purple ring	—	0.9	Yellow	Red
5-Chloro- <i>m</i> -cresol	0.9	+	Orange-brown	—	0.5		Orange

graphic and u.v. comparisons with authentic compounds.

The analysis of adaptation patterns clearly suggests that metabolism of MCPA involves 5-chloro-*o*-cresol and 5-chloro-3-methylcatechol as early intermediates. Cells were not induced to oxidize any other likely phenolic intermediates. It thus appears that ring fission mainly occurs with the 2-methyl and 4-chloro groups intact on the aromatic ring.

Identification of the lactone is very informative with regard to the metabolic pathway. This compound was detected in cultures at neutral pH by means of its characteristic u.v.-absorption properties, before extraction was attempted, and its identification as an intermediate cannot be doubted. It was rapidly metabolized in cultures, probably via γ -hydroxy- α -methylmuconate, and MCPA-grown cells were fully adapted to its oxidation. The metabolic precursor of the lactone is probably *cis-cis*- γ -chloro- α -methylmuconate, a theory that is supported by the tentative identification of an isomer of this compound in extracts. Lactonization by dehydrochlorination is thus pro-

posed as the mechanism for removal of chlorine from this molecule. This would explain the two double bonds of the lactone, an unusual feature in the bacterial metabolism of aromatic compounds. From a consideration of known methods of ring fission (Dagley, Evans & Ribbons, 1960) γ -chloro- α -methylmuconate could only arise by an *ortho*-cleavage of 5-chloro-3-methylcatechol. Although no catechol was ever detected in MCPA cultures, the implication of this compound as an intermediate is a logical deduction, corroborated by induction evidence. 5-Chloro-3-methylcatechol could arise from either 5-chloro-*o*-cresol or 4-chloro-6-hydroxy-4-methylphenoxyacetate. The detection of both compounds in cultures suggests that both routes from MCPA may operate. However, the non-adaptation of bacteria to 4-chloro-6-hydroxy-4-methylphenoxyacetate casts doubts on the role of this compound in MCPA metabolism. Its presence in cultures could be explained as a minor pathway of MCPA metabolism, possibly analogous to the hydroxylations observed by Faulkner & Woodcock (1961, 1964) for various chlorinated phenoxyacetates with *Aspergillus niger*. Alternatively it

Scheme 1. Proposed pathway for the metabolism of MCPA by *Pseudomonas* N.C.I.B. 9340.

could be an artifact arising in cultures from a true metabolite. If the mechanism for side-chain loss and hydroxylation involves diol formation (4-chloro-6-hydroxy-1, 6-dihydroxy-2-methylphenoxyacetate) non-enzymic loss of water could give 4-chloro-6-hydroxy-2-methylphenoxyacetate. A third possibility is that there are permeability barriers that prevent its entry into the bacterial cell, in which case the compound could still be an intermediate in MCPA metabolism.

From these arguments the metabolic pathway proposed for MCPA oxidation is that shown in Scheme 1.

The isolation of other compounds from cultures must now be considered. Oxalate could arise from oxidation of the ether-linked substituent or from further metabolism of γ -hydroxy- α -methylmuconic acid. However, the inability of cells to oxidize it, coupled with its detection in only one culture, suggest that it is either an artifact produced by the isolation procedure or a blocked metabolic product of the side chain. 2-Methylphenoxyacetate and the dehalogenated cresol could arise in two ways. (a) 2-Methylphenoxyacetic acid may be present in the MCPA used; commercial MCPA frequently contains this impurity, which is not completely removed by recrystallization. (b) Dechlorination of the aromatic ring may be a minor pathway of metabolism; in view of the aromatic dehalogenation implicated in 2,4-D metabolism (Fernley & Evans, 1959), this could be a plausible explanation.

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REFERENCES

- Audus, L. J. (1964). *The Physiology and Biochemistry of Herbicides*, p. 164. London and New York: Academic Press.
- Bell, G. R. (1960). *Can. J. Microbiol.* **6**, 325.
- Bollag, J. M., Helling, C. S. & Alexander, M. (1967). *Appl. Microbiol.* **15**, 1393.
- Bray, H. G., Thorpe, W. V. & White, K. (1950). *Biochem. J.* **46**, 271.
- Dagley, S., Evans, W. C. & Ribbons, D. W. (1960). *Nature, Lond.*, **188**, 560.
- Dickman, S. R. (1952). *Analyt. Chem.* **24**, 1064.
- Elliott, D. C. (1954). *J. exp. Bot.* **5**, 15.
- Evans, W. C. (1947). *Biochem. J.* **41**, 376.
- Evans, W. C., Gaunt, J. K. & Davies, J. I. (1961). *Proc. 5th int. Cong. Biochem., Moscow*, p. 306.
- Evans, W. C., Smith, B. S. W., Moss, P. & Fernley, H. N. (1971). *Biochem. J.* **122**, 509.
- Faulkner, J. K. & Woodcock, D. (1961). *J. chem. Soc.* p. 5397.
- Faulkner, J. K. & Woodcock, D. (1964). *Nature, Lond.*, **203**, 865.
- Feigl, F. (1954a) *Spot Tests: II Organic Applications*, p. 148. Amsterdam: Elsevier Publishing Co.
- Feigl, F. (1954b). *Spot Tests: II Organic Applications*, p. 171. Amsterdam: Elsevier Publishing Co.
- Fernley, H. N. (1959). Ph.D. Thesis; University of Wales, Bangor.
- Fernley, H. N. & Evans, W. C. (1959). *Biochem. J.* **73**, 22p.
- Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
- Gaunt, J. K. (1962). Ph.D. Thesis: University of Wales, Bangor.
- Gaunt, J. K. & Evans, W. C. (1961). *Biochem. J.* **79**, 25p.
- Gaunt, J. K. & Evans, W. C. (1971). *Biochem. J.* **122**, 533.
- Hossfield, R. L. (1951). *J. Am. chem. Soc.* **73**, 852.
- LeTourneau, D. & Krog, N. (1952). *Pl. Physiol., Lancaster*, **27**, 822.
- Lugg, J. W. H. & Overell, B. T. (1948). *Aust. J. sci. Res.* **1**, 98.
- MacDonald, D. L., Stanier, R. Y. & Ingraham, J. L. (1954). *J. biol. Chem.* **210**, 809.
- Milton, R. F. & Waters, W. A. (1955). *Methods of Quantitative Micro-analysis*, 2nd ed., p. 595. London: Edward Arnold (Publishers) Ltd.
- Mitchell, F. L. & Davies, R. E. (1954). *Biochem. J.* **56**, 690.
- Moss, P. (1956). M.Sc. Thesis: University of Wales, Bangor.
- Nordmann, J. & Nordmann, R. (1960). In *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 279. Ed. by Smith, I. London: W. Heinemann (Medical Publishers) Ltd.
- Rothera, A. C. H. (1908). *J. Physiol., Lond.*, **37**, 491.
- Shewan, J. M., Hobbs, G. & Hodgkiss, W. (1960). *J. appl. Bact.* **23**, 379.
- Shriner, R. L., Fuson, R. C. & Curtin, D. Y. (1956). *The Systematic Identification of Organic Compounds*, 4th ed. p. 64. New York: John Wiley and Sons Inc.
- Steenson, T. I. & Walker, M. (1957). *J. gen. Microbiol.* **16**, 146.
- Strouts, C. R. N., Gilfillan, J. H. & Wilson, H. N. (1955). *Analytical Chemistry* vol. 2, p. 522. Oxford: Clarendon Press.