Bacterial Metabolism of 2,4-Dichlorophenoxyacetate

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(Received 21 December 1970)

1. Two *Pseudomonas* strains isolated from soil metabolized 2,4-dichlorophenoxyacetate (2,4-D) as sole carbon source in mineral salts liquid medium. 2. 2,4-Dichlorophenoxyacetate cultures of *Pseudomonas* I (Smith, 1954) contained 2,4-dichlorophenol, 2-chlorophenol, 3,5-dichlorocatechol and α -chloromuconate, the last as a major metabolite. 3. Dechlorination at the 4(*p*)-position of the aromatic ring must therefore take place at some stages before ring fission. 4. *Pseudomonas* N.C.I.B. 9340 (Gaunt, 1962) cultures metabolizing 2,4-dichlorophenoxyacetate contained 2,4-dichloro-6-hydroxyphenoxyacetate, 2,4-dichlorophenol, 3,5-dichlorocatechol and an unstable compound, probably $\alpha\gamma$ -dichloromuconate. 5. Cell-free extracts of the latter organism grown in 2,4-dichlorophenoxyacetate cultures contained an oxygenase that converted 3,5-dichlorocatechol into $\alpha\gamma$ -dichloromuconate, a chlorolactonase that in the presence of Mn^{2+} ions converted the dichloromuconate into γ -carboxymethylene- α -chloro- $\Delta^{a\beta}$ -butenolide, and a delactonizing enzyme that gave α -chloromaleylacetate from this lactone. 6. Pathways of metabolism of 2,4-dichlorophenoxyacetate are discussed.

2,4-D,* possibly the most widely used of the hormone herbicides, is moderately persistent in soils; effective phytotoxic doses disappear within 3-4 weeks (Audus, 1964), though subsequent applications are degraded more quickly. The work on the bacterial metabolism of 2,4-D described here was carried out with two different organisms, obtained from enrichment cultures of various soils; one of these, *Pseudomonas* sp. N.C.I.B. 9340 (Gaunt, 1962), was isolated for its ability to metabolize MCPA, but was found to be equally effective in the utilization of 2,4-D.

MATERIALS AND METHODS

Micro-organisms. *Pseudomonas* sp. I (Smith, 1954) was isolated from enrichment cultures of a soil from a coniferous site and a concentration of 0.01% (w/v) 2,4-D in the standard basal mineral salts medium as perfusion fluid. It resembled *Pseudomonas subcreta* and *Pseudomonas pictorum* (Bergey, 1957) in cultural characteristics; an electron micrograph ($\times 28000$) showed polar flagella and a well-defined capsule. The isolation of *Pseudomonas* sp. N.C.I.B. 9340 is described by Gaunt & Evans (1971a).

Cultural conditions. Two inorganic salts media, differing particularly in their nitrogen source were used at various stages in this work (g/l): medium A: KNO_3 , 1; K_2HPO_4 ,

0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; medium B: $(NH_4)_2SO_4$, 1; K_2HPO_4 , 1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaSO_4$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.05. 2,4-D (0.05-0.1%) was added as the sodium salt and the pH adjusted to 7.4. Batch cultures (10 and 50 litres) were grown under forced aeration, at 25°C, with a starter inoculum (500 ml). For sequential induction experiments cells were also grown on glucose (0.1%) in the mineral salts medium B. Cells were harvested with a Sharples Superspeed (air-driven) centrifuge, washed once by suspension in 50 mM-sodium phosphate buffer, pH 7, centrifuged and stored frozen (-25°C) until required.

Manometric experiments, determinations, spectrometry, preparation of cell-free extracts, extraction of cultures etc. were performed as described by Gaunt & Evans (1971a,b) unless otherwise stated. Some of the i.r. spectra were obtained with a Perkin-Elmer model 137 Infracord with a pressed KCl disc medium. Acid production during enzyme reactions and equivalent weights of acids were determined with an automatic titrator (Radio-meter Ltd., Copenhagen, Denmark).

Materials. 2,4-D (m.p. 138-139°C) was obtained from Monsanto Chemicals Ltd., Ruabon, Denbighshire, U.K.; it was recrystallized with charcoal treatment from ethanol-water (1:1, v/v) until chromatographically pure and free from phenolic impurities.

2,4-Dichloro-6-hydroxyphenoxyacetic acid (m.p. 132°C) was obtained from two sources: one was an authentic sample supplied by Dr Cavill and Dr Ford, School of Applied Chemistry, New South Wales University of Technology, Sydney, N.S.W., Australia (Cavill & Ford, 1954); another sample was synthesized via an unambiguous route by Dr Brown and Dr McCall, Monsanto

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

Chemicals Ltd. (Brown & McCall, 1955), who also kindly supplied the following: 3,5-dichloro-2-hydroxyphenoxyacetic acid (m.p. 154°C), 2-chloro-4-hydroxyphenoxyacetic acid (m.p. 146°C), 3-chloroguaiacol (6-chloro-2-methoxyphenol, m.p. 54°C), 5-chloroguaiacol (4-chloro-2-methoxyphenol, m.p. 16–17°C), 3,5-dichloroguaiacol (4,6-dichloro-2-methoxyphenol, m.p. 64–65°C) and 4,6-dichloroguaiacol (3,5-dichloro-2-methoxyphenol, m.p. 63–64°C).

2,4-Dichloro-5-hydroxyphenoxyacetic acid (m.p. 169°C) was a gift from Dr D. Woodcock, Long Ashton Research Station, Bristol, U.K. 6-Chloroguaiacol (3-chloro-2-methoxyphenol, m.p. 32–33°C) was synthesized by the method of Jona (1912). 3-Chlorocatechol (m.p. 46°C) was prepared by the method of Willstätter & Pfannenstiel (1904) improved by Willstätter & Muller (1911). 3,5-Dichlorocatechol (m.p. 84°C) was synthesized by the method of Dakin (1909) from 3,5-dichloro-2-hydroxybenzaldehyde (Biltz & Stepf, 1904).

α -Chloromuconic acid (m.p. 160°C) was synthesized as follows. Acetic acid (54g), hydrogen peroxide (85%, 37g) and H_2SO_4 (0.87g) were mixed slowly at 0°C and allowed to stand overnight (Greenspan, 1947). *o*-Chlorophenol (14g) was added, in portions, to the peracetic acid reagent at 25°C; after several hours the mixture turned cherry red, and was allowed to stand at room temperature for 1 week. No solid precipitate had formed during this time. The solvents were slowly removed in a vacuum desiccator over conc. H_2SO_4 and KOH pellets, contained in separate dishes. The semi-crystalline syrup that remained was triturated with chloroform, and the insoluble residue taken up in ether, filtered and diluted with light petroleum (b.p. 40–60°C). A white crystalline solid separated with a sharp m.p. 160°C (decomp.). Microanalysis gave C, 40.7; H, 3.06; Cl, 20.3; $\text{C}_6\text{H}_5\text{O}_4\text{Cl}$ requires C, 40.7; H, 2.84; Cl, 20.1%. Boeseken & Metz (1935) describe an α -chloromuconic acid, prepared by using a much weaker solution of peracetic acid, with m.p. 190°C; this may be a typographical error, or they may have prepared another isomer of the acid. The acid that we obtained (m.p. 160°C) was metabolized by 2,4-D-grown cells, consonant with the view that it is the *cis-cis*-isomer. The syntheses of β -chloromuconate and γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide are described elsewhere (Evans, Smith, Moss & Fernley, 1971; Gaunt, 1962).

Chromatography. One-dimensional descending chromatograms were most frequently used to resolve mixtures extracted from culture, and to explore the nature of products formed in enzymic reactions. Whatman paper no. 1, no. 4 and no. 54 and monophasic solvents were preferred because of their rapidity. The solvent mixtures were: (a) ethanol-aq. ammonia (sp.gr. 0.88)-water (16:1:3, by vol.); (b) sodium formate (5%, w/v)-formic acid (200:1, v/v); (c) propan-2-ol-water-aq. ammonia (sp.gr. 0.88) (20:2:1, by vol.); (d) butan-1-ol-ethanol-aq. ammonia (0.5M) (7:1:2, by vol.); (e) pyridine-butan-1-ol-saturated NaCl-aq. ammonia (sp.gr. 0.88) (8:4:5:3, by vol.); (f) water-saturated butan-1-ol-aq. ammonia (sp.gr. 0.88) (16:1, v/v); (g) benzene-acetic acid-water (1:1:2, by vol.); (h) butan-1-ol-acetic acid-water (12:3:5, by vol.); (i) 80%-saturated $(\text{NH}_4)_2\text{SO}_4$ -propan-2-ol (49:1, v/v). Volatile phenols were run as the sodium salts of their azo-dyes, formed by coupling with diazotized sulphanilic acid (Hossfield, 1951), on paper previously

saturated with Na_2CO_3 (4%) in solvent (j), butan-2-one-water (5:1, v/v).

A Chromatolite u.v. lamp was used to locate substances that absorbed or fluoresced in u.v. light. Phenolic compounds were detected with the Folin-Ciocalteu reagent and diazotized *p*-nitroaniline or sulphanilic acid, acids by Bromocresol Green or the alinine-xylose reagent, and carbonyl compounds by 2,4-dinitrophenylhydrazine.

Countercurrent separation was performed in an apparatus consisting of 50 extraction tubes, each with a total volume of 20 ml. The stationary (0.1M-sodium phosphate buffer, pH 7) and mobile (butan-1-ol) phases were saturated with respect to each other by shaking and allowing them to stand for 24 h; the mixture to be fractionated was dissolved in 10 ml of buffer phase and placed in the first extraction tube.

Microanalysis was by Dr Weiler and Dr Strauss, Oxford, U.K.

RESULTS

Pseudomonas sp. I

The following results were obtained with *Pseudomonas* sp. I (Smith, 1954).

Isolation of intermediates transiently appearing in 2,4-D cultures. (a) 2,4-Dichlorophenol. Preliminary observations showed that, during growth on 2,4-D, release of Cl^- ion was accompanied by a pH change from an initial value of 7.4 to 5. A strong Folin reaction for phenols was given at this stage, which rapidly disappeared on readjustment of the culture back to neutrality. A 10-litre culture was grown in the normal way and, when the 2,4-D had practically gone, fresh substrate (0.05%, w/v) was added and the production of the Folin-positive substance followed quantitatively. The culture was harvested when the concentration of the intermediate was at a maximum (equivalent to about 0.1 mol of phenol/litre). The intermediate was steam-distilled from acidified culture fluid. The distillate (2 litres) was extracted with ether (3 $\times$ 200 ml), the combined ether extracts were dried over sodium sulphate and the solvent was carefully removed. A pungent-smelling oil remained, characteristic of 2,4-dichlorophenol. The azo-dye, formed on coupling with diazotized sulphanilic acid, co-chromatographed with an authentic sample. Condensation of the phenol with chloroacetic acid gave a derivative, m.p. 138°C, underpressed by authentic 2,4-D; the toluene-*p*-sulphonyl derivative was identical with authentic toluene-*p*-sulphonyl 2,4-dichlorophenate, m.p. 123–125°C.

(b) 2-Chlorophenol. A 10-litre culture (0.05% 2,4-D) was allowed to exhaust the substrate (this took about 14 days at room temperature of 15–18°C). 2,4-D (5g) was then added, and the pH readjusted to 8; 2 days later the pH had fallen to 6.5, but the Folin reaction increased. The culture fluid, after the cells had been harvested, was readjusted to pH 7 and concentrated in a

climbing-film evaporator; most of the Folin-positive products passed over into the distillate (2 litres), which was extracted with ether. On drying of the combined ether extracts with sodium sulphate and removal of the solvent an oil remained; tests for an *o*-dihydroxyphenol were negative. Chromatography showed that it was different from 2,4-dichlorophenol, 4,6-dichloroguaiacol, guaiacol and 3-, 4- or 6-chloroguaiacol. The sodium salt of the azo-dye formed by coupling with diazotized sulphanic acid, when chromatographed in parallel with those from 2-chlorophenol, 3-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol, ran in a manner identical with that of the azo-dye of 2-chlorophenol. The absorption spectrum of the azo-dye, as free acid and sodium salt, also corresponded to that obtained from authentic 2-chlorophenol.

(c) 3,5-Dichlorocatechol. The neutral ether extract from several 2,4-D cultures harvested shortly before the final disappearance of the substrate, gave a positive Evans test for *o*-dihydroxyphenols, and reduced ammoniacal silver nitrate (Tollens test). The substance responsible was chromatographically identical with 3,5-dichlorocatechol, and different from 3- and 4-chlorocatechol and catechol [solvents (e) and (i)].

(d) α -Chloromuconate. Eight 10-litre cultures (0.05% 2,4-D) were set up at room temperature, and when the u.v.-absorption maximum had shifted from 283 to 270 nm (after about 12 days) they were harvested and processed individually. The clear culture supernatant fluid (10 litres) was adjusted to pH 7 and concentrated to 1 litre in a climbing-film evaporator at 30°C. The neutral-ether-soluble material was first removed; the acid-ether fraction afforded a solid residue (50 mg). This was triturated with chloroform and then light petroleum, and finally recrystallized from 1,2-dichloroethane, giving a white micro-crystalline powder, m.p. 159°C, undepressed by admixture with a synthetic sample of α -chloromuconic acid (m.p. 160°C). The yield of pure isolate from a 10-litre culture was about 40 mg (Microanalysis gave C, 40.7; H, 3.1; Cl, 20.3%; equiv.wt. 88. Calc. for $C_6H_5ClO_4$: C, 40.8; H, 2.8; Cl, 20.1%; equiv.wt. 88.3). The titration curve showed that it was a dibasic acid. Unsaturation was indicated on catalytic hydrogenation (Milton & Waters, 1955), since 2 μ mol of H_2 was absorbed/ μ mol of substance (mol.wt. 176). The culture isolate gave a negative test for a lactone (Hestrin, 1949). The u.v. absorption maximum was 277 nm (ϵ 23000) in acid, shifting to 268 nm (ϵ 19000) in alkali. The culture isolate and synthetic acid co-chromatographed in a variety of solvent mixtures, and they had identical u.v. and i.r. spectra.

Sequential induction data. Cells harvested in the exponential phase of their growth on 2,4-D were capable of rapidly oxidizing 2-chlorophenoxy-

acetate, CPA, 4-chloro-2-hydroxyphenoxyacetate, 4-chlorophenol and 2,4-dichlorophenol without an intervening lag phase. The *o*-dihydroxyphenols, catechol, 3- and 4-chlorocatechol and 3,5-dichlorocatechol were oxidized initially at a rapid rate and then more slowly. Both synthetic and isolated samples of α -chloromuconate stimulated respiration rates but chlorosuccinate, chloromaleate and chlorofumarate had no significant effect. Similarly guaiacol and its 3- and 4-chloro-derivatives were slightly stimulatory but 2,4-dichloroanisole was not.

Pseudomonas N.C.I.B. 9340

At this point in the investigation *Pseudomonas* sp. I (Smith, 1954) was lost, but the work was continued with *Pseudomonas* N.C.I.B. 9340.

Investigations on 2,4-D cultures. 2,4-Dichlorophenol and 3,5-dichlorocatechol were identified in the neutral-ether fraction from 2,4-D cultures of this organism, by means of similar procedures to those described above. No 2-chlorophenol or α -chloromuconate was detected.

(a) Isolation of 2,4-dichloro-6-hydroxyphenoxyacetic acid. Acid-ether extracts of cultures harvested at the end of the exponential growth phase frequently gave a blue colour with the Folin-Ciocalteu reagent. Acid-ether extracts from five 10-litre cultures were evaporated to dryness, the residue was dissolved in the minimum volume of boiling water, treated with charcoal, filtered and allowed to crystallize in the cold-room. Three recrystallizations from water gave long colourless needles (5 mg), m.p. 130°C undepressed by admixture with authentic 2,4-dichloro-6-hydroxyphenoxyacetic acid. Micro-titration gave an equivalent weight of 240.2; $C_8H_6Cl_2O_4$ requires 237. The isolated compound gave an i.r. spectrum identical with that of 2,4-dichloro-6-hydroxyphenoxyacetic acid, but different from that of 2,4-dichloro-5-hydroxyphenoxyacetic acid. The melting point of the isolated compound was depressed on admixture with 2,4-dichloro-5-hydroxyphenoxyacetic acid (m.p. 169°C).

(b) Detection of ring-fission products. Acid-ether extracts of cultures contained small quantities of two presumed ring-fission intermediates that were chromatographically separable.

One had λ_{max} at 275 nm in buffer at pH 7 and R_F values of 0.63 in solvent (a) and 0.0 in solvent (f); the other had λ_{max} at 248 nm in aqueous solution at pH 7–13, which vanished on acidification. This latter compound had R_F values of 0.26 in solvent (a) and 0.27 in solvent (f) and gave a 2,4-dinitrophenylhydrazone on paper. Maintenance of the pH of the culture fluid at 8.0–8.5 appeared to favour the accumulation of these compounds although the

yields per 10-litre culture were never more than 0.5mg; often they were not detectable.

The residue from acid-ether extracts from which the solvent had been removed by evaporation was dissolved in 0.1M-phosphate buffer, pH7, and subjected to a 50-tube separation in a counter-current apparatus with the phosphate buffer and butan-1-ol phases. Spectrophotometric examination of the butanol and buffer phases showed that the '275' compound was localized in tubes 1-3 and the '248' compound in tubes 4-16, whereas 2,4-D and 2,4-dichloro-6-hydroxyphenoxyacetate had moved to tubes 25-50. A yellow pigment produced by this organism was distributed over almost the entire system.

(c) Isolation of the '275' compound. The aqueous phases of tubes 1-3 were pooled, acidified to Congo Red and extracted with ether (3×10ml). The ether extract was washed once with water (5ml) before being dried over anhydrous sodium sulphate. Finally the solvent was removed *in vacuo*. The residue was washed with cold chloroform, followed by light petroleum (b.p. 40-60°C); a yellowish-white solid remained (3mg), m.p. 142-145°C (decomp.). In phosphate buffer, pH7, $\lambda_{\max}$ was at 275nm (ϵ 17800); the compound showed a reversible shift in absorption maxima, at pH1 it was at 277nm and at pH13 at 274nm. The compound was Beilstein-positive. Its chromatographic and electrophoretic behaviour was compared with that of various muconic acids. In solvents (a) and (h) it had R_F values of 0.63 and 0.65, closely resembling α -chloromuconic acid (R_F values of 0.68 and 0.61 respectively). It was slightly less mobile in solvent (f) than α -chloromuconic acid, from which it was separated by descending chromatography. Attempted recrystallization from ethanol-benzene led to decomposition. Its absorption and chromatographic characteristics suggest that it may be an isomer of $\alpha\gamma$ -dichloromuconic acid, but unfortunately there was not enough material for a microanalysis.

Attempted isolation of the '248' compound from the relevant tubes of the countercurrent apparatus

by using a similar procedure to that employed for the '275' compound yielded an oil, the extinction of which rapidly diminished during manipulation, indicating extensive decomposition.

(d) Sequential induction data. Cells grown on 2,4-D and harvested during the exponential phase of growth oxidized the following substances without a lag: 2,4-D, MCPA, CPA, 4-chlorophenol, 2,4-dichlorophenol, catechol, 3-chlorocatechol, 4-chlorocatechol and 3,5-dichlorocatechol. Phenoxyacetate, 2-chlorophenoxyacetate and 2-chlorophenol were oxidized slowly by 2,4-D-grown cells, but oxygen consumption in the presence of 2,4-dichloro-6-hydroxyphenoxyacetate, phenol, chloroquinol, chlorofumarate, chloromaleate, chlorosuccinate, *cis-cis*-muconate, muconolactone and α -chloromuconate was not significantly higher than in controls. Glucose-grown cells were unable to oxidize any of these substrates.

With each of the catechols tested, the very rapid initial consumption of about 1 μ mol of oxygen per μ mol of substrate was followed by a phase of considerably slower respiration. When the very rapid oxidation had ceased, the cells were separated from the incubation media by centrifugation and spectrophotometric examination of the supernatants showed that substances had accumulated with strong u.v. absorptions, the details of which are shown in Table 1. It is clear that the products derived from 4-chlorocatechol and 3,5-dichlorocatechol were decomposed after acidification.

Rapid extraction, after careful acidification, of the compounds produced by microbial action, followed by chromatography in solvents (a), (f) and (h), showed the products derived from catechol, 3-chlorocatechol and 4-chlorocatechol to be muconate, α -chloromuconate and β -chloromuconate by comparison with authentic samples. The u.v. absorption of the substance formed in incubations with 3,5-dichlorocatechol is shown in Fig. 1 together with those of the products formed on successive additions of acid and alkali. Analogy with the substance produced in incubations with 4-chlorocatechol suggests that the product derived

Table 1. *Products accumulated by the action of 2,4-D-grown cells on various catechols*

Cells grown on 2,4-D were incubated with 1 μ mol of each catechol derivative until the rapid phase of oxygen consumption had ceased. The cells were then removed by centrifugation and the supernatant examined spectrophotometrically at the pH values indicated.

Substrate	$\lambda_{\max}$ of product (nm)			
	pH7	pH13	pH1	pH13 (after acidification to pH 1)
Catechol	257-258	257-258	257-258	256-258
3-Chlorocatechol	272-273	272-273	276-278	270-272
4-Chlorocatechol	264-266	264-266	276-278	242-244
3,5-Dichlorocatechol	266-268	264-266	280-282	249-251

Fig. 1. U.v. spectra of the ring-fission products of 3,5-dichlorocatechol. $\alpha\gamma$ -Dichloromuconate (---) was formed by the action of 2,4-D-grown cells on 3,5-dichlorocatechol as described in the text. This was converted into γ -carboxymethylene- α -chloro- $\Delta^{\alpha\beta}$ -butenolide (— · —) by acidification with 6M-HCl and subsequent neutralization to pH 7 with 2M-NaOH. The chlorolactone was converted into α -chloromaleylacetate (—) by brief boiling. The absorption of α -chloromaleylacetate disappeared on acidification to pH 1 with 6M-HCl (.....).

from 3,5-dichlorocatechol is $\alpha\gamma$ -dichloromuconate, though no authentic sample is available for comparison.

Action of cell-free extracts on 3,5-dichlorocatechol. Crude cell-free extracts were prepared by sonication of cells grown in 2,4-D-mineral salts medium B suspended in 4 volumes of 50mM-phosphate buffer, pH 7, in a double-walled vessel with circulating water at 0°C, followed by centrifugation (20000g at 0°C).

Incubation of such cell-free extracts with 3,5-dichlorocatechol led to the accumulation of a substance with the same u.v. absorption characteristics as that obtained with whole cells (Fig. 1) and presumed to be $\alpha\gamma$ -dichloromuconate.

(a) Isolation of the product derived by acidification of presumed $\alpha\gamma$ -dichloromuconate. 3,5-Dichlorocatechol (100mg) was incubated with cell-free extract (130ml; from 13g wet wt. of cells) in 50mM-phosphate buffer, pH 7 (500ml), at 30°C until all the substrate had disappeared (Evans reagent). The reaction product was extracted with ether (2 × 200ml) to remove neutral-ether-soluble components, acidified to Congo Red and re-extracted with ether (3 × 200ml). The combined acid-ether extract was washed with water (50ml), dried over anhydrous sodium sulphate and the solvent removed. During these stages there was an unavoidable progressive loss of the enzymically

derived material as shown by the migration of $\lambda_{\max}$ from 267nm towards 282nm. Therefore it was decided to continue with the isolation of the degradation product. The solid left after removal of solvent was dissolved in warm benzene, treated with charcoal and recrystallized from this solvent as white needles (11mg), m.p. 127–129°C (micro-analysis gave C, 41.76; H, 2.64; Cl, 19.5. $C_6H_3ClO_4$ requires C, 41.3; H, 1.7; Cl, 20.3%). Except for water, in which hydrolysis occurred on warming to a product with $\lambda_{\max}$ at 250nm, the product was too soluble in other solvents for recrystallization. The isolated product had the following properties: $\lambda_{\max}$ 282nm (aq.) ϵ 17100 (assuming the formula $C_6H_3ClO_4$); at pH 13 (0.1M-sodium hydroxide) the maximum shifted to 249–252nm. Re-acidification did not reverse this change, but caused a severe loss of absorption in this region. The i.r. spectrum is completely consistent with the structure γ -carboxymethylene- α -chloro- $\Delta^{\alpha\beta}$ -butenolide, showing only the expected minor differences from that of γ -carboxymethylene- α -methyl- $\Delta^{\alpha\beta}$ -butenolide, a metabolite of 5-chloro-3-methylcatechol. Thus, despite the lack of an authentic sample of the chlorolactonic acid, the above evidence and the nature of the products formed by its further enzymic dissimilation represent very strong evidence for the proposed structure.

(b) Enzymic hydrolysis of γ -carboxymethylene- α -chloro- $\Delta^{\alpha\beta}$ -butenolide. Incubation of γ -carboxymethylene- α -chloro- $\Delta^{\alpha\beta}$ -butenolide with a crude cell-free enzyme preparation resulted in a loss of absorption at 282nm and the formation of a product with $\lambda_{\max}$ at 249–251nm. The same change occurred instantaneously when the chlorolactonic acid was dissolved in 0.1M-sodium hydroxide or briefly boiled in buffer at pH 7. In all these hydrolytic reactions there was no detectable release of Cl^- ions. The reaction product gave an acidic 2,4-dinitrophenylhydrazone and a pH-dependent u.v. absorption similar to that encountered with compounds capable of undergoing keto-enol tautomerism [cf. maleylacetoacetate (Knox & Edwards, 1955), maleylpyruvate (Lack, 1959) and α -methylmaleylacetate (Gaunt & Evans, 1971b)]. It is therefore proposed that the hydrolytic product of the chlorolactonic acid is α -chloromaleylacetate; it is very water-soluble, difficult to extract into ether or ethyl acetate and behaves chromatographically in a manner identical with that of a compound with $\lambda_{\max}$ 248nm detected in 2,4-D culture fluid.

(c) Further evidence for the identity of the ring-fission product. The action of crude cell-free extracts upon 3,5-dichlorocatechol led to the accumulation of a substance with $\lambda_{\max}$ at 268nm (Fig. 1). The reaction involved consumption of 1 μ mol of O_2 and the formation of 2 μ equiv. (actual values

obtained were 1.75 and 1.9 μ equiv.) of acid per μ mol of 3,5-dichlorocatechol as shown by manometric and micro-titration techniques respectively. Determination of Cl^- ion (which involves acidification and therefore conversion of the ring-fission product into the chlorolactonic acid) showed that 1 μ equiv. of Cl^- was released/ μ mol of 3,5-dichlorocatechol metabolized. Thus the initial ring-fission product, having λ_{max} at 268nm, is either a dicarboxylic acid containing two chlorine atoms or a monocarboxylic acid from which one chlorine atom has been removed as Cl^- ion.

This evidence, together with the identification of its degradation product as γ -carboxymethylene- α -chloro- $\Delta^{a\beta}$ -butenolide, supports the provisional identification of the ring-fission product as $\alpha\gamma$ -dichloromuconate, presumably the *cis-cis*-isomer.

(d) Further dissimilation of $\alpha\gamma$ -dichloromuconate. Crude cell-free enzyme preparations were incapable of dissimilating $\alpha\gamma$ -dichloromuconate. However, addition of manganous sulphate (1mM) caused its spectrum to be replaced by that of α -chloromaleylacetate.

Further analysis of this reaction was achieved by applying to the crude cell-free extract the method developed for the separation of muconate-lactonizing and muconolactone-delactonizing enzymes by Sistrom & Stanier (1954) and used successfully in the work on MCPA (Gaunt & Evans, 1971b). The 0–37%-saturated-ammonium sulphate precipitate is referred to as the lactonizing preparation and the 50–75%-saturated precipitate as the delactonizing preparation.

To study these enzyme preparations, fresh solutions of the unstable $\alpha\gamma$ -dichloromuconate were prepared daily as follows. Washed 2,4-D-grown cells were incubated with the maximum tolerated concentration of 3,5-dichlorocatechol (this varied in the range 0.5–2 μ mol/ml and had to be determined for each batch of cells) in the buffer of choice, until the substrate disappeared, as shown with the Evans reagent. The cells were removed by centrifugation and the supernatant solution was extracted with ether to remove neutral compounds and coagulate the protein at the interphase. Any ether remaining in the aqueous phase was blown off with nitrogen gas; the solution had λ_{max} at 267–269nm, which persisted for several hours.

Lactonizing enzyme activity was detected spectrophotometrically as a migration of the λ_{max} from 268nm for $\alpha\gamma$ -dichloromuconate to 282nm for γ -carboxymethylene- α -chloro- $\Delta^{a\beta}$ -butenolide. Similarly, delactonizing enzyme activity was detected as a loss of absorption of the chlorolactonic acid (formed by brief acidification of the above preparation of $\alpha\gamma$ -dichloromuconate) at 282nm and its substitution with the λ_{max} of α -chloromaleylacetate at 250nm.

By using these techniques it was shown that crude cell-free extracts converted $\alpha\gamma$ -dichloromuconate into α -chloromaleylacetate on addition of manganous sulphate (1mM). When the crude cell-free preparation was replaced by the lactonizing enzyme fraction, again in the presence of manganous sulphate (1mM), the u.v. spectrum of $\alpha\gamma$ -dichloromuconate was replaced by that of γ -carboxymethylene- α -chloro- $\Delta^{a\beta}$ -butenolide, which was further converted into α -chloromaleylacetate by introducing the delactonizing-enzyme fraction. Thus it appears that the chlorolactonic acid and α -chloromaleylacetate, though initially identified as chemical degradation products of $\alpha\gamma$ -dichloromuconate, are in fact also intermediates in its enzyme-catalysed dissimilation.

The lactonizing enzyme obtained from 2,4-D-grown cells that converts $\alpha\gamma$ -dichloromuconate into the chlorolactonic acid was inactive towards *cis-cis*-muconate, α -chloromuconate and synthetic β -chloromuconate (which is probably the *cis-trans* isomer), but was active on the β -chloromuconate (presumably the *cis-cis* isomer) freshly prepared by the action of whole cells on 4-chlorocatechol as described above for 3,5-dichlorocatechol. A preparation containing muconate-lactonizing enzyme (obtained from bacterium N.C.I.B. 8250 after growth on benzoate), which in the presence of Mn^{2+} ion (1mM) converted muconate into β -oxoadipate, was without activity on any of the chloromuconates.

(e) Isolation of α -chloromuconate as the product of ring-fission of 3-chlorocatechol with a cell-free extract of *Pseudomonas* N.C.I.B. 9340 grown on 2,4-D. With the incubation mixture from which γ -carboxymethylene- α -chloro- $\Delta^{a\beta}$ -butenolide was isolated, but with 3-chlorocatechol replacing 3,5-dichlorocatechol, α -chloromuconate was isolated from the acid-ether extract. The product after recrystallization from 1,2-dichloroethane had m.p. 159°C, undepressed by admixture with authentic α -chloromuconic acid (cf. behaviour of *Pseudomonas* I; Smith, 1954). The chlorine substituent here acts as a marker, providing strong evidence for the position of ring fission in this micro-organism.

DISCUSSION

The sequential-induction technique alone was not found to be very reliable as a criterion for participation in 2,4-D metabolism among groups of related compounds. Thus 2,4-D-grown cells of both organisms were also immediately capable of metabolizing MCPA, 2-chlorophenoxyacetate and CPA; similarly they were able to metabolize all the *o*-dihydroxyphenols tested: catechol, 3-chlorocatechol, 4-chlorocatechol and 3,5-dichlorocatechol. Some of the guaiacols stimulated oxygen uptake

slightly, e.g. guaiacol, 3-chloroguaiacol and 4-chloroguaiacol, but 2,4-dichloroanisole had little effect. We interpret this phenomenon to be due to the relatively unspecific nature of the inducible enzymes that cause side-chain cleavage of the phenoxyacetates, aromatic hydroxylation and *ortho*-fission of the catechols.

Pseudomonas sp. I (Smith, 1954) gave rise to the following substances in culture: 2,4-dichlorophenol, 2-chlorophenol, 3,5-dichlorocatechol and α -chloromuconate, which was further metabolized with release of Cl^- ion.

The appearance in cultures of 2-chlorophenol suggests that non-oxidative elimination of chlorine occurred from 2,4-dichlorophenol or possibly 2,4-D itself. The accumulation of α -chloromuconate is probably a further manifestation of this phenomenon since it is probably formed by the action of the ring-cleavage enzyme on 3-chlorocatechol, the latter in turn being derived from either 2-chlorophenol or 3,5-dichlorocatechol.

The non-oxidative elimination of a substituent group on an aromatic ring is known to occur in microbial biochemistry; e.g. Perez-Silva, Rodrigues & Perez-Silva (1966) have reported briefly that they have isolated from rat faeces a strain of *Pseudomonas* sp. that converted caffeate (3,4-dihydroxycinnamate) into *m*-coumarate (3-hydroxycinnamate) and 3-hydroxyphenylpropionate. Moreover, Dacre, Scheline & Williams (1968) have shown that in the case of homoprotocatechuate (3,4-dihydroxyphenylacetate) the gut flora of rabbit and rat are capable of *meta*- and *para*-dehydroxylation. The exact mechanisms of these eliminations await elucidation. In the present example of aromatic dehalogenation it is impossible to tell precisely at which stages the eliminations take place. The yield of α -chloromuconate isolated from culture fluid together with the capacity of the organism for its dissimilation suggest that it forms part of a major pathway of 2,4-D oxidation in this organism.

Isolation of compounds from culture is also not incontrovertible proof that they are major metabolites; a knowledge of turnover rates would be helpful in assessing the extent of their participation in the metabolic pathway. This would be particularly helpful in view of the accumulation of 3,5-dichlorocatechol in cultures of *Pseudomonas* sp. I and the information now available concerning the dissimilation of 3,5-dichlorocatechol in *Pseudomonas* sp. N.C.I.B. 9340 which suggests that the cleavage of the aromatic ring may precede the elimination of Cl^- ion in this organism.

Pseudomonas sp. N.C.I.B. 9340 (Gaunt, 1962) growing on 2,4-D as substrate contained at some stage of growth of the culture: 2,4-dichloro-6-hydroxyphenoxyacetate, 2,4-dichlorophenol, 3,5-dichlorocatechol and two ring-fission products very

tentatively identified as $\alpha\gamma$ -dichloromuconate and α -chloromaleylacetate. The absence of α -chloromuconate in these culture media and the inability of the harvested cells to utilize it suggests that non-oxidative elimination of chlorine does not feature in the pathway of 2,4-D metabolism in this organism.

3,5-Dichlorocatechol is the only dihydroxyphenol that fulfils the criteria of sequential induction, presence in culture and enzymic transformation into ring-fission products apparently identical with those detected in culture. The ring-fission product is thought to be *cis-cis*- $\alpha\gamma$ -dichloromuconate, but in the absence of an authentic sample of this unstable acid the evidence is indirect, including analogy with results obtained in MCPA metabolism (Gaunt & Evans, 1971b).

3,5-Dichlorocatechol oxygenase extracted from 2,4-D-grown cells is unusual in its lack of specificity; this crude enzyme preparation was also capable of the *ortho*-cleavage of catechol, 3- and 4-chlorocatechol. A lactonizing enzyme with a requirement for Mn^{2+} ions converted $\alpha\gamma$ -dichloromuconate into γ -carboxymethylene- α -chloro- $\Delta^{\alpha\beta}$ -butenolide. A similar enzyme fraction, which converted the above acid and α -methyl- γ -chloromuconate into their respective lactones, was prepared from MCPA-grown cells. It is proposed that it be named γ -chloromuconate lactonase. Since muconate and α -chloromuconate were not attacked, the γ -chlorine may be an essential structural requirement for this enzyme.

2,4-Dichloro-6-hydroxyphenoxyacetate accumulated in cultures of *Pseudomonas* sp. N.C.I.B. 9340, but since it is not dissimilated by this organism its significance remains undecided.

Scheme 1 summarizes the evidence brought forward in this paper on the metabolic pathways of 2,4-D by these particular soil pseudomonads. It is noteworthy that the pathway in which there is no elimination of chlorine from the aromatic ring has also been proposed for an *Arthrobacter* sp. (Tiedje & Alexander, 1969; Tiedje, Duxbury, Alexander & Dawson, 1969).

Degradation of aromatic-ring-containing pesticides synthesized by man, but foreign to other living organisms in the biosphere, depends on the ability of certain microbes to synthesize enzymes that convert these strange compounds into simple aliphatic molecules, suitable for funnelling into the respiratory cycles of the cell. [Microbial halometabolites have been discussed by Petty (1961). Ando, Kato & Suzuki (1970) have claimed that a soil *Penicillium* sp. biosynthesizes 2,4-dichlorophenol and some unidentified derivatives.] Stage 1 requires their transformation into *o*- or *p*-dihydroxyphenols, the obligatory substrates for aerobic nuclear fission; stage 2, their *ortho* or *meta* cleavage, can be accomplished by a wide variety of micro-organisms

Scheme 1. Proposed pathways of 2,4-D degradation.

(Evans, 1969). Under natural conditions it is not necessary that the ability to perform stages 1 and 2 should reside together in a single microbial species, since we are dealing with mixed populations. Divergent pathways between pure culture isolates

of various bacteria, all capable of metabolizing the chlorophenoxyacetate herbicides, are also likely to be found, within the constraining conditions imposed by stages 1 and 2. The present work illustrates this, the variety being introduced at stage 1.

It is significant that Tiedje *et al.* (1969) and ourselves have encountered only the *ortho*-cleavage pathway among microbes performing stage 2, but Horvath (1970) has reported the co-metabolism of 4-chlorocatechol and 3,5-dichlorocatechol by a *meta*-cleaving oxygenase from an *Achromobacter* sp.

Although the phenomena described in this paper are dependent on aerobic conditions, afforded by free-draining soils, it is conceivable that some aromatic pesticides may be degraded in anaerobic environments, presumably by a reductive cleavage of the benzene ring (cf. Dutton & Evans, 1969).

Aspergillus niger hydroxylates 2,4-D in the 5-position (Faulkner & Woodcock, 1964); hydroxylation coupled with a chlorine shift to 2,5-dichloro-4-hydroxyphenoxyacetate also occurs (Faulkner & Woodcock, 1965). The fungus is incapable of metabolizing these products further. This mechanism of 2,4-D metabolism is also found in higher plants preliminary to detoxication by conjugation (Loos, 1969).

We thank Monsanto Chemicals Ltd., Ruabon, Denbighshire, U.K., for the gift of chemicals. B.S.W.S., H.N.F. and J.I.D. are indebted to the Agricultural Research Council for support.

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