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Bacterial Metabolism of 4-Chloro-2-methylphenoxyacetate

FORMATION OF GLYOXYLATE BY SIDE-CHAIN CLEAVAGE

BY Y. GAMAR* AND J. K. GAUNT
Department of Biochemistry and Soil Science, University College of North Wales, Bangor, Caerns., U.K.

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Crude extracts of *Pseudomonas* sp. grown on 4-chloro-2-methylphenoxyacetate as sole source of carbon were shown to oxidize 4-chloro-2-methylphenoxyacetate to 5-chloro-*o*-cresol and glyoxylate. A labelled 2,4-dinitrophenylhydrazone was isolated from an incubation mixture in which 4-chloro-2-methylphenoxy[*carboxy*-^{14}C]acetate was used. The hydrazone was shown to behave identically on thin-layer chromatograms with the authentic 2,4-dinitrophenylhydrazone of glyoxylate. Radioactivity assay showed that 82% of the side chain of 4-chloro-2-methylphenoxyacetate was recovered as glyoxylate.

Several investigations have been made of the microbial degradation of MCPA† Bollag, Helling & Alexander, 1967; Loos, Bollag & Alexander, 1967; Gaunt & Evans, 1971*a*,*b*) but little is yet known of the initial steps involved in this degradation. The mechanism concerned in the side-chain removal and the nature of the product derived from the side chain, for instance, have so far not been clarified. The object of the present report is to characterize the product of the metabolism of the side chain.

MATERIALS AND METHODS

MCPA was obtained from Marks and Co. Ltd. (Wyke Lane Works, Wyke, Bradford, U.K.) and crystallized from aq. ethanol (m.p. 120–121°C). [*carboxy*-^{14}C]MCPA (specific radioactivity 12.8 μCi/mg) and [1-^{14}C]glyoxylic acid, sodium salt (specific radioactivity 8.44 mCi/mmol), were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The latter was checked for radiochemical purity by paper chromatography of the free acid by using butan-1-ol–acetic acid–water (125:75:3, by vol.), and ether–acetic acid–water (13:3:1, by vol.), followed by radioautography. No impurities were detected on the radioautogram.

The 2,4-dinitrophenylhydrazone of glyoxylic acid was prepared by adding 2.5 ml of freshly prepared 2,4-dinitrophenylhydrazine solution (0.2% in 2 M-HCl) to 1 ml of an aqueous solution of glyoxylic acid (2 mg/ml) until a precipitate was just formed. After standing for approx. 3 h the mixture was extracted with ethyl acetate. The hydrazone was re-extracted into the aqueous phase with 20 ml of 10% (w/v) Na_2CO_3. The carbonate extract was acidified to pH 1.0 with 10 M-H_2SO_4 and the acidic solution was extracted twice with ethyl acetate. The combined ethyl acetate layers were washed twice with water, concentrated and kept for analysis.

Assay procedures. MCPA was measured by the chromotropic acid method of LeTourneau & Krog (1952) which was used by Gaunt (1962), and by spectrophotometric measurements. The u.v.-absorption spectrum of the solution was recorded and the E_{278} was measured ($E^{1\%}_{1cm}$ for MCPA 75.5 found by experiment). Phenolic substances were determined colorimetrically by the method of Folin & Ciocalteu (1927).

Glyoxylic acid was measured by a modification of the method of Paget & Berger (1938). In a test tube, 1 ml of test solution (containing not more than 0.5 μmol of glyoxylic acid), 0.1 ml of 1% (w/v) phenylhydrazine hydrochloride (freshly prepared) and 0.5 ml of 2 M-HCl were placed in a boiling-water bath for 1 min. The mixture was then cooled, 0.5 ml of conc. HCl and 1 ml of 1% (w/v) potassium ferricyanide were added and the volume was made up to 10 ml with water. The intensity of the red colour at 515 nm was determined against a reagent blank. This procedure gives a linear response from 0.02 to 0.5 μmol of glyoxylic acid and appears to be specific for glyoxylic acid. Glyceric acid, oxalic acid, citric acid, malonic acid, glycollic acid and MCPA itself produced no colour in this test.

Protein was determined by the method of Gornall, Bardawill & David (1949).

Chromatography. Both paper- and thin-layer-chromatography techniques were used. For the separation of C_2 acids on paper the solvents used were: (*a*) butan-1-ol–acetic acid–water (125:75:3, by vol.) (Nordmann & Nordmann, 1960) and (*b*) diethyl ether–acetic acid–water (13:3:1, by vol.) (Denison & Phares, 1952).

For t.l.c., plates were prepared from either silica gel G or silica gel H (nach Stahl; E. Merck A.-G., Darmstadt, Germany). Those made from silica gel G were allowed to dry in air for 15 min and were activated for 30 min at 110°C before use. Plates prepared from silica gel H were allowed to dry overnight in air before use.

* Present address: Faculty of Agriculture, University of Khartoum, Republic of the Sudan.

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

For the separation of 2,4-dinitrophenylhydrazones of keto acids the following solvents were used: (*c*) methanol–butan-1-ol–25% (v/v) NH_3 (25:10:2, by vol.) (Rasmussen, 1967); (*d*) 3-methylbutan-1-ol–0.5 M-NH_3 (2:1, v/v) (Dancis, Hutzler & Mortimer, 1963).

For the separation of neutral phenols, the following solvents were used: (*e*) benzene–methanol (19:1, v/v) (Randerath, 1964); (*f*) 96% (v/v) ethanol–25% (v/v) NH_3–water (25:4:3, by vol.) (Braun & Geenen, 1962).

Acidic components were detected with either 0.1% Bromocresol Green in 99.5% (v/v) ethanol (Lugg & Overell, 1948) or aniline–xylose reagent followed by heating (Nordmann & Nordmann, 1960).

Phenolic compounds were detected with diazotized *p*-nitroaniline or diazotized sulphanilic acid followed by 10% (w/v) $NaHCO_3$ (Bray, Thorpe & White, 1950).

Radioautography and radioassay. Thin-layer chromatograms containing labelled samples were radioautographed by being placed in contact with X-ray films (8in × 10in; Ilford Ltd., Ilford, Essex, U.K.) in lead-backed holders and stored in the dark for several days or weeks depending on the amount of radioactivity present. The X-ray films were then developed and the positions of the radioactive spots located on the chromatogram. Quantitative determinations of radioactivity were carried out with a NE8304 automatic liquid-scintillation spectrometer (Nuclear Enterprises Ltd., Edinburgh, U.K.). The scintillation fluids used were NE213 and NE220 (Nuclear Enterprises Ltd.). The maximum efficiency for counting of ^{14}C was approx. 90%, corrections for quenching being made when necessary.

Enzyme preparation. The bacterium used was *Pseudomonas* N.C.I.B. 9340 originally isolated from soils enriched with MCPA by Gaunt (1962). The organism was cultured on a basal medium consisting of 0.1% KNO_3, 0.05% K_2HPO_4, 0.035% $MgSO_4,7H_2O$, 0.1% trace elements solution (Barnett & Ingram, 1955) and either 0.05 or 0.1% MCPA sodium salt. The medium was made up with water and adjusted to pH 7.2–7.4 with NaOH. For solid media, 2.2% (w/v) of agar (Japanese) was added to the above medium. All media were autoclaved for 20min at 20lb/in^2. The organism was maintained by subculturing on MCPA–basal medium–agar slopes. For growth in liquid medium, baffled flasks (1 litre) containing 200ml of medium were inoculated from agar plates and after 3–4 days in a rotary shaker (Gallenkamp Orbital Shaker) (130 strokes/min) the contents were transferred to 5 or 10 litres of sterile medium in Pyrex bottles. After 3–4 days of forced aeration at 25°C, the cells were harvested in the Sharples Super centrifuge, taken up in 10 vol. of 0.05 M-phosphate buffer, pH 7.0, homogenized for 10s in a Silverson homogenizer and centrifuged at 15000*g* for 10min. The washed cells were either used immediately or stored at −15°C. The phosphate buffers used throughout were prepared from the appropriate mixtures of KH_2PO_4 and Na_2HPO_4.

Cell suspensions (5g wet wt. in 12ml of 0.05 M-phosphate buffer, pH 7.5) were maintained at 0–4°C and disrupted with an ultrasonic disintegrator (MSE–Mullard apparatus, 16–24kHz, 60W) for periods of 10–15min. The homogenate was centrifuged at 22000*g* for 20min at 0–5°C and the clear supernatant (crude cell-free extract) was either used as such or treated, at 2°C, with solid $(NH_4)_2SO_4$ to 30% saturation and centrifuged at 2000*g* for 30min. The supernatant was then dialysed at 2°C against 0.05 M-phosphate buffer, pH 7.5, for 3h. This preparation is referred to as the partially-purified enzyme preparation.

Incubation methods. Oxygen uptake was followed in the Warburg constant-volume apparatus at 30°C. The incubation mixture consisted of 0.2ml of MCPA solution (3–5μmol) in the side arm and 0.2ml of NADH solution (3–5μmol), 0.2ml of GSH or neutralized L-cysteine hydrochloride solution (3μmol), 1ml of cell-free enzyme preparation and 1.3ml of 0.05 M-phosphate buffer (pH adjusted to 7.5) in the main compartment. The centre-well contained a filter paper soaked in 0.1ml of 20% (w/v) KOH. The substrate was omitted from control flasks. For anaerobic incubations the flask contents and the manometers were thoroughly flushed with O_2-free N_2.

Ordinary incubations were set up in 25 or 50ml flasks. The incubation mixture, in a total volume of 5ml, consisted of 5μmol of MCPA, 12μmol of NADH, 5μmol of L-cysteine plus 3ml of crude cell-free extract and 1ml of 0.05 M-phosphate buffer (pH adjusted to 7.5). CMPA was omitted from control flasks. Incubations were performed in a metabolic shaker (Gallenkamp) at 30°C.

In both cases the reaction was stopped by acidifying to a pH below 3.0 with dil. H_2SO_4 and the precipitated proteins were removed by centrifugation. The supernatant was extracted twice with diethyl ether and the combined extracts were washed twice with water. Acidic components in the ether phase were extracted into 5ml of 10% (w/v) $NaHCO_3$ and this solution was analysed spectroscopically for residual MCPA. The ether was washed twice with water and the solvent was evaporated to dryness under vacuum at room temperature. The residue (referred to as the neutral-ether extract) was then dissolved in ethanol for spectrophotometric and chromatographic analysis. The aqueous residues left after ether extraction were kept for the analysis of water-soluble reaction products.

Enzyme assay. The side-chain cleavage activity of the system was assayed by the spectrophotometric determination of MCPA remaining after each incubation. The activity was expressed in terms of μmol of MCPA removed/min.

Incubations with oxalate. Incubations with oxalate were performed in 50ml flasks. The incubation mixture consisted of 3ml of crude cell-free extract (active towards MCPA), 3μmol of GSH, 5μmol of oxalate (disodium salt) and the final volume was made up to 5.0ml with 0.05 M-phosphate buffer, pH adjusted to 7.5. The contents were incubated at 30°C for 90min. Oxalate was omitted from control flasks.

The reaction was terminated by the addition of 5.0ml of hot ethanol. Samples were centrifuged and the supernatant was evaporated to dryness under reduced pressure. The residue was dissolved in 0.5ml of aq. 20% (v/v) ethanol and brought to 0.1 M with HCl. Samples (0.2ml) were analysed by paper chromatography in solvent (*b*). Glyoxylic acid, oxalic acid and glycollic acid were used as reference compounds.

RESULTS

Requirements of the system. The activity of the partially purified preparation towards MCPA seemed to be dependent on the presence of a reduced

Table 1. *Cofactors required for the side-chain-cleavage system*

Incubations were conducted in 25ml flasks, each containing, in a total volume of 4ml, 5μmol of MCPA, 1ml of partially purified enzyme preparation and 2ml of 0.05M-phosphate buffer, pH adjusted to 7.5. Cofactors were added as indicated below.

Cofactors added (μmol)	Activity (μmol of MCPA removed/min)
None	0.01
NADH (6)	0.10
NADPH (6)	0.10
NADH (6) + GSH (3)	0.11
NADH (6) + L-cysteine (3)	0.12

Table 2. *MCPA converted under aerobic and anaerobic conditions*

Incubations were performed in the Warburg apparatus as described in the Materials and Methods section. NADH (4μmol) and GSH (3μmol) were added as cofactors. 1ml of crude cell-free extract was used.

Condition	MCPA added (μmol)	Residual MCPA (μmol)	Oxygen uptake (μmol)	Phenol detected (μequiv.)
Aerobic	3.8	0.0	3.4	3.2
Anaerobic	3.8	4.0	0.0	0.0

nicotinamide nucleotide, since only a small amount of MCPA was removed in the absence of a reduced nicotinamide nucleotide. There was no difference between the effects of NADH and NADPH (Table 1). Activity was increased by the addition of GSH or L-cysteine, the latter being slightly more effective.

The breakdown of MCPA by crude cell-free preparations was found to require aerobic conditions. In the presence of NADH and GSH, but under anaerobic conditions, the activity of the preparation towards MCPA was negligible (Table 2). In the aerobic experiment shown, an equivalent amount of a neutral phenolic compound was found to accumulate. In this case approximately 1mol of oxygen was taken up/mol of MCPA removed.

Effect of pH. A set of enzyme assays was performed in a series of 0.05M-phosphate buffers, with pH values in the range 6.0–8.5. The pH of each incubation mixture was checked before the initiation of the reaction.

The system, in 25ml conical flasks, consisted of 5μmol of MCPA, 10μmol of NADH, 3μmol of L-cysteine and 1ml of crude cell-free extract (5mg of protein/ml). The final volume was made up to 5ml with 0.05M-phosphate buffer of the required pH. Incubation was at 30°C for 30min. The activity was assayed by measuring the removal of MCPA. The results showed an optimum pH between 7.5 and 7.8.

Table 3. *Effect of metal ions and inhibitors on the partially purified system*

Incubations were performed in Warburg flasks each containing MCPA (3μmol), NADH(5μmol), GSH(3μmol), partially purified enzyme preparation (1ml) plus ion or inhibitor as indicated below. Other conditions were as described in the Materials and Methods section.

Addition		Activity (μmol of MCPA removed/min)
None		0.12
Mg^{2+} (sulphate)	(1mM)	0.10
Mn^{2+} (sulphate)	(1mM)	0.13
MoO_4^{2+} (sodium salt)	(1mM)	0.10
Co^{2+} (chloride)	(1mM)	0.08
Fe^{2+} (sulphate)	(1mM)	0.07
Zn^{2+} (sulphate)	(1mM)	0.05
Cu^{2+} (sulphate)	(1mM)	0.00
EDTA	(1mM)	0.00
8-Hydroxyquinoline	(1mM)	0.00
p-Chloromercuribenzoate	(0.1mM)	0.00

Effect of centrifuging at 100000*g*. The crude cell-free extract was centrifuged at 100000*g* for 1h. The activities of the supernatant, the pellet and the original crude cell-free extract towards MCPA were assayed as before. Whereas the 100000*g* pellet showed no activity towards MCPA, 56% of the original activity was found in the supernatant.

Effect of inhibitors and cations. The system was inhibited by 0.1mM-*p*-chloromercuribenzoate, suggesting a requirement for free thiol groups. Other thiol group-complexing agents were not tried. The system was also inhibited by EDTA (1mM) and 8-hydroxyquinoline (1mM), suggesting a requirement for metal ions. However, the addition of several cations, including Mn^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+} (all at 1mM), did not reverse the inhibition and no significant increase in activity of the uninhibited enzyme was observed on the addition of the various metal ions (Table 3).

Identity of the reaction products. With crude cell-free extracts occasional accumulation of a phenol from MCPA was noted. This phenol, detected in neutral-ether extracts, was identified by t.l.c., by using silica gel G in solvents (*e*) and (*f*). This phenol was identical in its chromatographic behaviour [R_F 0.72 and 0.48 in solvents (*e*) and (*f*) respectively] with 5-chloro-*o*-cresol [R_F 0.73 and 0.5 in solvents (*e*) and (*f*) respectively]. The colour reactions with diazotized *p*-nitroaniline and diazotized sulphanilic acid were also identical. The absorption spectrum of the unknown phenol and that of authentic 5-chloro-*o*-cresol under neutral and acidic conditions were found to be identical and exhibited characteristic shifts under alkaline

conditions (absorption peaks at pH 7.0 or 3.0, 228 and 284nm; absorption peaks at pH 10.0, 248 and 302nm).

Examination of the aqueous residues. The aqueous residues from MCPA reaction mixtures were examined qualitatively to identify the products derived from the side chain. The residues were evaporated to dryness under vacuum at 60°C, the solid residues dissolved in aq. 95% (v/v) ethanol and any insoluble components were removed by filtration. The ethanolic solution was then subjected to chromatographic analysis. Acidic components were separated with solvent (*a*). Authentic glycollic acid, glyoxylic acid and oxalic acid (R_F 0.58, 0.66 and 0.72 respectively) were used as reference samples. Co-chromatography was not attempted. The unknown acidic component (R_F 0.7–0.74) gave a positive phenylhydrazine hydrochloride–ferricyanide reaction, specific for glyoxylic acid. For quantitative analysis an incubation mixture consisting of MCPA (10μmol), NADH (15μmol) and GSH (5μmol) was incubated for 1h with the crude cell-free extract (4ml) in 0.05M-phosphate buffer, pH 7.5. The reaction was stopped as described above and residual MCPA was determined. After concentration, the aqueous residue was examined for acidic components with solvent (*a*). The zone parallel to the glyoxylic acid standard was removed and eluted with 95% (v/v) ethanol. When treated with the phenylhydrazine hydrochloride–ferricyanide reagent (as described in the Materials and Methods section), this sample gave the red–violet colour characteristic of authentic glyoxylic acid. The amount of the glyoxylic acid present was estimated and was found to account for 72% of the amount calculated from the experimentally determined removal of MCPA, on the assumption of 1mol of glyoxylic acid formed/mol of MCPA removed.

A 2,4-dinitrophenylhydrazone was prepared from the MCPA reaction mixture. This was analysed by t.l.c. with solvents (*c*) and (*d*). The isolated hydrazone [R_F 0.68 in solvent (*c*) and 0.11 in solvent (*d*)] was found to give the same R_F values as the 2,4-dinitrophenylhydrazone of glyoxylic acid [R_F 0.67 in solvent (*c*) and 0.09 in solvent (*d*)].

Quantitative formation of glyoxylic acid from the side chain of MCPA was confirmed by the isolation of a labelled 2,4-dinitrophenylhydrazone after incubation of the enzyme preparation with [*carboxy*-^{14}C]MCPA. The experiment was performed in a 50ml flask containing 3.2μmol of MCPA, 1μmol of labelled MCPA (432600c.p.m.), 3μmol of neutralized L-cysteine hydrochloride and 6μmol of NADH in 3ml of 0.05M-phosphate buffer, pH 7.5. The contents were incubated with 2ml of partially purified enzyme preparation (6.2mg of protein/ml) at 30°C for 2h. The reaction was stopped and the reaction mixture was treated as described above. Of the added radioactivity 92% was recovered; 75% was found in the aqueous residue and 17% as residual MCPA. No radioactivity was detected in the neutral ether extract. The aqueous residue was treated with 2,4-dinitrophenylhydrazine and the hydrazone formed was extracted in the usual manner. This 2,4-dinitrophenylhydrazone fraction contained 82% of the radioactivity of the aqueous residue.

Further evidence for the identity of the side-chain product of MCPA was obtained by t.l.c. in solvent (*c*) followed by radioautography of the labelled hydrazone. The radioactivity appeared as one spot with an R_F (0.66) identical with that of authentic glyoxylic acid 2,4-dinitrophenylhydrazone.

Oxalate incubations. Analysis of the samples from the experiment in which oxalate was incubated with crude cell-free extract showed the presence of oxalate (R_F 0.9). Glyoxylic acid (R_F 0.29) and glycollic acid (R_F 0.63) were not detected. Quantitative determinations from chromatograms indicated the inactivity of the system towards oxalate.

DISCUSSION

Cleavage of the ether-linkage of the side chain of MCPA could take place by at least three mechanisms. These are: reductive cleavage giving acetate; hydrolytic cleavage giving glycollate; or oxidative cleavage giving glyoxylate. In all three cases the phenol would be produced.

From the present work it is probable that the initial step in the metabolism of MCPA by this organism is an oxidative cleavage of the ether-linked side chain giving glyoxylate and 5-chloro-*o*-cresol. The fact that the system is strictly aerobic makes it unlikely that either acetate or glycollate is the direct product of the side chain of MCPA. Further, glycollate and acetate have not been detected in MCPA incubation mixtures. The possibility that oxalate is involved in the metabolic pathway is eliminated by the fact that oxalate, under the conditions of the experiment, is not metabolized by the system. Thus the glyoxylate identified could not have been derived from oxalate.

With the MCPA-grown *Pseudomonas*, Gaunt (1962) reported the occasional accumulation of oxalate in MCPA cultures. Thus it is possible to assume that the oxalate accumulated in MCPA cultures is derived from the glyoxylate obtained from the side chain of MCPA. Such conversion of glyoxylate into oxalate is known to occur in micro-organisms (Quayle, Keech & Taylor, 1960, 1961) and is facilitated by a high pH (8.6) (Quayle & Taylor, 1961). Conditions of high pH are known to occur in cultures of the MCPA-grown organism,

where the pH rises markedly towards the end of MCPA metabolism (Gaunt, 1962).

A significant feature of the present work is the similarity of the system to the aromatic ether-cleavage system of mammalian liver (Axelrod, 1956) and to the *Pseudomonas fluorescens* vanillate demethylase (Cartwright & Smith, 1967). In all systems there is a requirement for oxygen, reduced nicotinamide nucleotides and free thiol groups.

Whereas the rabbit liver system described by Axelrod (1956) requires Mg^{2+} ions, the bacterial demethylase system described by Cartwright & Smith (1967) and the MCPA side-chain-cleavage system studied in the present work do not show clear-cut requirements for metal ions. Although the MCPA side-chain-cleavage system was completely inhibited by the addition of EDTA (1mM), suggesting a metal ion requirement, this inhibition was not reversed by the addition of various cations.

After centrifuging at 100000*g* for 1h, 56% of the original MCPA-cleavage activity of the crude cell-free preparation was found in the supernatant, whereas no activity was detected in the pellet. This reflects a further difference between the aromatic ether-cleavage enzymes in mammalian liver, which were shown to be localized in the microsomal fraction (Axelrod, 1956), and the bacterial MCPA side-chain-cleavage enzyme, which seems to be soluble.

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