1063. *Fungal Detoxication. Part V.* Metabolism of* o- *and* p-*Chlorophenoxyacetic Acids by* Aspergillus niger.

By J. K. FAULKNER and D. WOODCOCK.

In the metabolism of *p*-chlorophenoxyacetic acid by *Aspergillus niger* (replacement culture technique), 4-chloro-3-hydroxyphenoxyacetic acid, hitherto unknown, is formed in addition to the corresponding 2-hydroxy-acid. When *o*-chlorophenoxyacetic acid is the sole carbon source, hydroxylation takes place at all vacant ring positions, the main products being 2-chloro-4-hydroxyphenoxyacetic acid, together with 2-chloro-5-hydroxyphenoxyacetic acid. The corresponding 3- and 6-hydroxy-compounds are produced in much smaller amounts, and the additional formation of *o*-hydroxyphenoxyacetic acid appears to be the first example of fungal replacement of chlorine by hydroxyl.

EARLIER papers in this series [1-3] have shown important differences between the metabolism of aryloxyacetic acids by fungi on the one hand and by micro-organisms and in some cases plants, on the other. In the present paper, the absence of ring fission of *o*- and *p*-chlorophenoxyacetic acid is again a feature of fungal metabolism. This contrasts with the complete breakdown of *p*-chlorophenoxyacetic acid by way of 4-chloro-2-hydroxyphenoxyacetic acid, 4-chlorocatechol, and β-chloromuconic acid, which has been reported by Evans and his co-workers [4,5] using a gram-negative *Pseudomonas*-type soil organism.

Whilst the metabolism of phenoxyacetic acid by *Aspergillus niger* (replacement culture technique) was notable for nuclear hydroxylation without ring fission, substitution being exclusively in *ortho*- and *para*-positions,[2] it has now been shown that with both *p*- and *o*-chlorophenoxyacetic acid hydroxylation takes place at all available positions in the benzene nucleus. Thus the products from the *p*-chloro-acid were, in addition to the expected 2-hydroxy-compound, the hitherto unknown 4-chloro-3-hydroxyphenoxyacetic acid. The latter was identified by an unambiguous synthesis from 4-chloro-3-nitrophenol.

The major metabolic products from *o*-chlorophenoxyacetic acid were 2-chloro-4-hydroxyphenoxyacetic acid and the hitherto unknown 2-chloro-5-hydroxyphenoxyacetic acid, the latter being identical with the compound obtained from 2-chloro-5-ethoxyphenol by successive condensation with ethyl bromoacetate, hydrolysis, and de-ethylation with hydrobromic acid. Two minor metabolities were identified chromatographically as the hitherto unknown 2-chloro-3- and -6-hydroxyphenoxyacetic acid. The former was synthesised from 2-chloro-3-methoxyphenol by a route similar to that used for the 5-hydroxy-acid; 2-chloro-6-hydroxyphenoxyacetic acid was obtained by demethylation of its methyl ether. A fifth acidic compound, which gave a light brown-mauve spot on chromatograms after spraying with *p*-nitrobenzenediazonium fluoroborate, had R_F values indistinguishable from those of *o*-hydroxyphenoxyacetic acid in three solvent systems. Moreover, the infrared absorption spectra were very similar, the absence of the C–Cl peak at 685 cm.$^{-1}$ in the case of the fungal product being significant. This formation of *o*-hydroxy- from *o*-chloro-phenoxyacetic acid appears to be the first example of fungal replacement of chlorine by hydroxyl, though the replacement of carboxyl by hydroxyl is known to occur with *Mycobacterium smegmatis* [6] and with *Penicillium* sp.[7]

* Part IV, Byrde, Downing, and Woodcock, *Biochem. J.*, 1959, **72**, 344.

[1] Byrde, Harris, and Woodcock, *Biochem. J.*, 1956, **64**, 154.
[2] Byrde and Woodcock, *Biochem. J.*, 1957, **65**, 682.
[3] Byrde and Woodcock, *Biochem. J.*, 1958, **69**, 19.
[4] Evans and Smith, *Biochem. J.*, 1958, **57**, xxx.
[5] Evans and Moss, *Biochem. J.*, 1958, **65**, 8P.
[6] Sloane, Crane, and Mayer, *J. Biol. chem.*, 1951, **193**, 453.
[7] Henderson, *J. Gen. Microbiol.*, 1957, **16**, 686.

Experimental

Infrared absorption spectra were determined by using a Perkin-Elmer Infracord spectrophotometer.

Replacement Culture Technique and Detection of Metabolites.—Cultures of *Aspergillus niger* van Tiegh. (Mulder strain) were grown as previously described.[1] After the medium had been poured from the fungal mat, the under-surface of the latter was washed with water, and a 0·0001M-solution of the acid in 0·01M-disodium hydrogen phosphate introduced underneath it. After incubation for 3 days at 25°, the substrate (6 l.) was strained through muslin and evaporated to 400 ml. under reduced pressure at 40° in a rotary film evaporator. It was then acidified to pH 2 and continuously extracted with ether for 16 hr., the extract washed with water, and acidic material removed by sodium hydrogen carbonate solution. After acidification of the alkaline solution the products were extracted with ether, the extract dried (Na_2SO_4), and the solvent removed.

Chromatography was on Whatman no. 1 paper by downward solvent flow, with (A) butanol–ethanol–ammonia (4 : 1 : 5), (B) propanol–ammonia (7 : 3) or (C) benzene–acetic acid–water (3 : 1 : 1) as eluants, system (A) being used for routine examinations. After drying at laboratory temperature chromatograms were sprayed with *p*-nitrobenzenediazonium fluoroborate (1% solution in 20% w/v aqueous sodium acetate) for the detection of phenolic compounds.

Isolation of Metabolites.—Separation of small amounts of metabolites was possible by chromatography as a band on Whatman no. 3MM paper, a marker strip being cut and sprayed for location and the required band then extracted with ether saturated with 50% hydrochloric acid. For larger-scale separations partition chromatography with silica gel as supporting medium for the stationary aqueous phase was used. The mobile phase was saturated aqueous chloroform, and approximately 2 ml. fractions were collected automatically.

Metabolism of p-*Chlorophenoxyacetic Acid.*—Separation of the acidic material (0·51 g.) obtained from the fungal substrate (30 l.) on a silica column gave unchanged acid (0·39 g.; m. p. and mixed m. p. 156°) and two phenolic acids. The first (0·031 g.), m. p. 127—128°, had R_F 0·43 (solvent A) and was undepressed on admixture with authentic 4-chloro-2-hydroxyphenoxyacetic acid, m. p. 130—131°; the infrared absorption spectra (808, 1180, 1750, 1770 cm.$^{-1}$) were identical. The second acid (0·041 g.) (R_F 0·14) crystallised from aqueous methyl alcohol (10 : 1), m. p. 208° alone or mixed with 4-chloro-3-hydroxyphenoxyacetic acid (Found: C, 47·3; H, 3·7. Calc. for $C_8H_7ClO_4$: C, 47·4; H, 3·5%), $\nu_{max.}$ 685, 833, 1180, 1740, 1760, 3300 cm.$^{-1}$.

Metabolism of o-*Chlorophenoxyacetic Acid.*—The acidic material (0·46 g.) from 30 l. of fungal substrate was separated on a silica column and the compounds listed were eluted in the order shown in the Table. 2-Chloro-6-hydroxyphenoxyacetic acid which was obtained mixed with

Metabolism of *o*-chlorophenoxyacetic acid incubated with *A. niger*.

Subst. in phenoxyacetic acid	Wt. (g.)	R_F in solvent A	B	C	$\nu_{max.}$ (cm.$^{-1}$)	Colour with diazo-reagent	Identification
2-Chloro	0·25	—	—	—	687, 755, 1200, 1750, 1770	—	Mixed m. p.; I.R.
2-Chloro-6-hydroxy	Trace	0·66	0·75	—		Salmon-pink	R_F
2-Hydroxy	0·008	0·45	0·60	0·19	755, 1200, 1750, 1770	Mauve-brown	R_F, I.R.
2-Chloro-3-hydroxy	Trace	0·13	0·31	—		Deep pink	R_F
2-Chloro-5-hydroxy	0·046 *	0·35	0·48	—	835, 1185, 1740, 1760, 3300	Reddish-purple (yellow centre)	Mixed m. p.; I.R.
2-Chloro-4-hydroxy	0·078 *	0·35	0·50	—	805, 1200, 1750, 1770, 3300	Purple (yellow centre)	Mixed m. p.; I.R.

* An intermediate mixed fraction of these two acids amounted to 0·07 g.

o-chlorophenoxyacetic acid was separated by its greater solubility in cold water. 2-Chloro-5- and -4-hydroxyphenoxyacetic acid, which were inseparable chromatographically on paper in eight solvent systems, were sufficiently separated on the silica column to enable pure specimens to be isolated.

4-*Chloro*-2-*hydroxyphenoxyacetic Acid.*—Prepared as directed by Brown and McCall,[8] this

[8] Brown and McCall, *J.*, 1955, 3681.

compound crystallised from water in needles, m. p. 130—131° (Found: C, 47·4; H, 3·5; Cl, 17·5. Calc. for $C_8H_7ClO_4$: C, 47·5; H, 3·5; Cl, 17·5%) [lit., 124—130° (rapid heating)].

Ethyl 4-Chloro-3-nitrophenoxyacetate.—A solution of 4-chloro-3-nitrophenol [9] (4·3 g.) in ethyl alcohol (50 ml.) containing sodium (0·58 g.) was refluxed with ethyl bromoacetate (2·5 ml.) for 0·5 hr., then most of the alcohol was distilled off, the solution was cooled and diluted with water, and the *product* extracted with ether. It crystallised from light petroleum (b. p. 60—80°) in pale yellow rhombic plates (3·8 g.), m. p. 57—58° (Found: C, 46·4; H, 3·8; N, 5·5. $C_{10}H_{10}ClNO_5$ requires C, 46·2; H, 3·85; N, 5·4%). Alkaline hydrolysis gave *4-chloro-3-nitrophenoxyacetic acid*, yellow felted needles (from aqueous methyl alcohol), m. p. 137—137·5° (Found: C, 41·7; H, 2·4; N, 6·0. $C_8H_6ClNO_5$ requires C, 41·5; H, 2·6; N, 6·0%).

Ethyl 3-Amino-4-chlorophenoxyacetate.—A solution of the above nitro-ester (2 g.) in tetrahydrofuran (20 ml.) was shaken in hydrogen in the presence of Raney nickel until further uptake of gas ceased (671 ml.). After removal of catalyst and solvent the product was distilled *in vacuo*. The *ester* crystallised from benzene–light petroleum (b. p. 40—60°) in stout prisms, m. p. 62—63° (Found: C, 52·6; H, 5·4. $C_{10}H_{12}ClNO_3$ requires C, 52·3; H, 5·2%). Alkaline hydrolysis followed by benzoylation gave *3-benzamido-4-chlorophenoxyacetic acid*, monoclinic prisms (from aqueous methyl alcohol), m. p. 172—173° (Found: C, 59·0; H, 4·0. $C_{15}H_{12}ClNO_4$ requires C, 58·9; H, 3·9%).

4-Chloro-3-hydroxyphenoxyacetic Acid.—A suspension of the above amino-ester (0·46 g.) in 10% sulphuric acid (10 ml.) was stirred during dropwise addition of sodium nitrite (0·15 g.) in water (2 ml.). The filtered solution was decomposed by gradual addition to a boiling solution of copper sulphate pentahydrate (10 g.) in water (10 ml.), the solution being then cooled and extracted with ether. The acidic *product* was set free from 10% aqueous sodium hydroxide by acidification and extracted with ether. The ethereal solution was dried (Na_2SO_4), the solvent removed, and the residue crystallised from aqueous methyl alcohol (10 : 1) (charcoal). It had m. p. 207—208° (Found: C, 47·4; H, 3·6. $C_8H_7ClO_4$ requires C, 47·4; H, 3·5%).

2-Chloro-5-ethoxyphenol.—A solution of 2-chloro-5-ethoxyaniline [10] (5·8 g.) in water (27 ml.) and sulphuric acid (3 ml.) was stirred at 0° during dropwise addition of sodium nitrite (2·3 g.) in water (6 ml.). After being stirred for a further 0·25 hr. the solution was filtered and the filtrate gradually added to copper sulphate pentahydrate (50 g.) in boiling water (50 ml.), and the product was continuously steam-distilled off. Extraction of the distillate with ether gave 2-chloro-5-ethoxyphenol (2·2 g.), b. p. 230°/762 mm. It formed a *3,5-dinitrobenzoate*, m. p. 152—152·5° (from aqueous methyl alcohol) (Found: C, 49·4; H, 3·2; N, 7·5. $C_{15}H_{11}ClN_2O_7$ requires C, 49·1; H, 3·0; N, 7·6%), and a *phenylurethane*, m. p. 125—126° [from light petroleum (b. p. 100°)] (Found: C, 61·4; H, 4·6; N, 5·2; Cl, 11·7. $C_{15}H_{14}ClNO_3$ requires C, 61·8; H, 4·8; N, 4·8; Cl, 12·2%).

2-Chloro-5-ethoxyphenoxyacetic Acid.—This was prepared from 2-chloro-5-ethoxyphenol (0·9 g.) and ethyl bromoacetate. The *product* (1·1 g.) crystallised from aqueous methyl alcohol in rhombic prisms, m. p. 145—146° (Found: C, 52·3; H, 4·7; Cl, 15·4. $C_{10}H_{11}ClO_4$ requires C, 52·1; H, 4·8; Cl, 15·4%).

2-Chloro-5-hydroxyphenoxyacetic Acid.—The above 5-ethoxy-compound (0·85 g.) was refluxed with hydrobromic acid (*d* 1·48; 10 ml.) for 1 hr. and then the solution was cooled, diluted with water, and extracted with ether. The acidic *product* (0·73 g.) obtained by washing the extract with sodium hydrogen carbonate solution, acidification, and re-extraction, crystallised from water in needles, m. p. 135—136° (Found: C, 45·5, 45·8; H, 3·6, 3·7; Cl, 17·2, 17·3%; equiv., 212. $C_8H_7ClO_4,\frac{1}{2}H_2O$ requires C, 45·45; H, 3·8; Cl, 16·8%; equiv., 211·5). The ethyl ether, prepared by using diethyl sulphate, crystallised from aqueous methyl alcohol in rhombic prisms, m. p. 146—147°, undepressed on admixture with the ethoxy-acid prepared earlier.

2-Chloro-3-methoxyphenol.—This was prepared in 20% yield from 2-chloro-3-methoxyaniline [9] by the method used for 2-chloro-5-ethoxyphenol. It had b. p. 227—228°/762 mm., m. p. (prisms) 56—58° (Found: C, 53·1; H, 4·4. $C_7H_7ClO_2$ requires C, 53·0; H, 4·4%). The *3,5-dinitrobenzoate* crystallised from aqueous methyl alcohol in pale yellow prisms, m. p. 148° (Found: C, 47·5; H, 2·9; N, 8·1. $C_{14}H_9ClN_2O_7$ requires C, 47·7; H, 2·6; N, 7·95%).

2-Chloro-3-methoxyphenoxyacetic Acid.—This *acid* was prepared from the corresponding phenol by condensation with ethyl bromoacetate. It crystallised from aqueous methyl alcohol in plates, m. p. 188—189° (Found: C, 49·9; H, 4·1. $C_9H_9ClO_4$ requires C, 49·9; H, 4·2%).

[9] Clemo and Daglish, *J.*, 1950, 1481.
[10] Van Erp, *J. prakt. Chim.*, 1930, **127**, 31.

2-*Chloro*-3-*hydroxyphenoxyacetic Acid*.—2-Chloro-3-methoxyphenoxyacetic acid (0·4 g.) was demethylated by refluxing it with hydrobromic acid (*d* 1·48; 10 ml.) for 1 hr. The *product* (0·3 g.) crystallised from water in prisms, m. p. 176—177·5° (Found: C, 47·9; H, 3·6; Cl, 17·7. $C_8H_7ClO_4$ requires C, 47·7; H, 3·5; Cl, 17·5%).

Ethyl 2-*Chloro*-3-*nitrophenoxyacetate*.—Prepared from 2-chloro-3-nitrophenol [10] (1·75 g.) by condensation with ethyl bromoacetate, the *product* (1·9 g.) crystallised from aqueous methyl alcohol in monoclinic prisms, m. p. 82—83° (Found: C, 46·5; H, 4·0; N, 5·5. $C_{10}H_{10}ClNO_5$ requires C, 46·2; H, 3·85; N, 5·4%). Alkaline hydrolysis gave the corresponding *acid*, m. p. 177—178° (from aqueous ethyl alcohol) (Found: C, 41·7; H, 2·5; N, 6·0. $C_8H_6ClNO_5$ requires C, 41·5; H, 2·6; N, 6·0%).

2-*Chloro*-6-*methoxyphenoxyacetic Acid*.—A solution of 3-chloroguaiacol [8] (1·6 g.) in ethyl alcohol (20 ml.) containing sodium (0·23 g.) was refluxed for 4 hr. with ethyl bromoacetate (1·7 g.). After the addition of 10% sodium hydroxide (20 ml.) and refluxing for a further 0·5 hr. the solution was cooled, acidified, and extracted with ether. The required acid (1·6 g.) crystallised from benzene–light petroleum (b. p. 40—60°) in prisms, m. p. 120—121° (Found: C, 49·3; H, 3·9. Calc. for $C_9H_9ClO_4$: C, 49·9; H, 4·2%). Brown and McCall state in a personal communication that their m. p. 130° is an error.

2-*Chloro*-6-*hydroxyphenoxyacetic Acid*.—The above methoxy-acid (1·04 g.) was refluxed with hydrobromic acid (*d* 1·48; 10 ml.) for 1 hr. The solution was cooled, diluted with water, and extracted with ether. After being washed with water, the extract was dried (Na_2SO_4) and the solvent removed. The *product* crystallised from light petroleum (b. p. 40—60°) in prisms, m. p. 98—99° (Found: C, 47·3; H, 3·2; Cl, 17·4. $C_8H_7ClO_4$ requires C, 47·4; H, 3·5; Cl, 17·5%). Refluxing this with acetic anhydride for 1 hr. gave 5-*chloro*-2-*oxo*-1,4-*benzodioxan*, m. p. 46—48°, purified by sublimation *in vacuo* (Found: C, 51·7; H, 2·4. $C_8H_5ClO_3$ requires C, 52·0; H, 2·7%).

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Department of Agriculture and Horticulture, University of Bristol,
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