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Fungal Detoxication

2. THE METABOLISM OF SOME PHENOXY-*n*-ALKYLCARBOXYLIC ACIDS BY *ASPERGILLUS NIGER**

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The earlier paper in this series (Byrde, Harris & Woodcock, 1956) described the metabolism of ω -(2-naphthyl-oxo)-*n*-alkylcarboxylic acids by *Aspergillus niger* van Tiegh. By using a replacement culture technique it was found that 2-naphthyl-oxo-acetic and β -(2-naphthyl-oxo)propionic acids were hydroxylated in the 6-position; higher members of the homologous series underwent both nuclear hydroxylation and β -oxidation of the side chain.

Further evidence of β -oxidation by a micro-organism has been presented by Webley, Duff & Farmer (1955), who studied the metabolism of ω -phenyl-substituted fatty acids by *Nocardia opaca* Waksman & Henrici. Microbiological nuclear hydroxylation of these acids was also reported by these workers, and of 4-chlorophenoxyacetic acid by Evans & Smith (1954).

The importance of β -oxidation in the plant-growth-regulating activity of the phenoxyalkylcarboxylic acids has been clearly demonstrated by Fawcett, Ingram & Wain (1954) and has led to the development of highly selective weed-killers (Wain, 1954). Though the side chains of these acids are β -oxidized by the flax plant (Fawcett *et al.* 1954), Fawcett, Spencer & Wain (1955) found no indication from fungitoxicity tests that this degradation could be effected by the fungi *Botrytis fabae* Sardinia and *Pythium ultimum* Trow.

In addition to their use as plant-growth regulators phenoxyalkylcarboxylic acids have been studied as possible systemic fungicides (Fawcett *et al.* 1955). The present paper describes the metabolism of four members of this series by *A. niger*.

* Part 1: Byrde, Harris & Woodcock (1956).

EXPERIMENTAL

Materials and reference compounds

Phenoxyacetic acid. This was prepared by the condensation of sodium phenoxide and ethyl bromoacetate. It crystallized from benzene-light petroleum (b.p. 60–80°) in prisms, m.p. 97–98°. Fritzsche (1879) found m.p. 96°.

β -Phenoxypropionic acid. β -Propiolactone (3.6 g.) was added dropwise with stirring during 5 min. to a boiling solution of phenol (4.7 g.) and NaOH (2 g.) in water (30 ml.). The solution was boiled for a further 20 min., cooled, acidified and extracted with ether. The acid crystallized from aqueous methanol in prisms, m.p. 94–95°. (Found: C, 64.9; H, 6.0. Calc. for $C_9H_{10}O_3$: C, 65.0; H, 6.0%.) Bischoff (1900) found m.p. 97.5–98°.

*γ -Phenoxy-*n*-butyric acid*. Crude γ -phenoxybutyronitrile (11.2 g.), obtained by refluxing a solution of γ -phenoxypropyl chloride (11.4 g.) (see below) and sodium cyanide (4.4 g.) in ethanol (70 ml.) overnight, was hydrolysed by refluxing for 18 hr. with a solution of KOH (5.4 g.) in water (5 ml.) and ethanol (70 ml.). The acid, crystallized from light petroleum (b.p. 40°), had m.p. 64–65°. (Found: C, 66.6; H, 6.7. Calc. for $C_{10}H_{12}O_3$: C, 66.7; H, 6.7%.) Lohmann (1891) found m.p. 60°.

γ -Phenoxypropylmalonic acid. A solution of sodium phenoxide, prepared from phenol (18.8 g.) and sodium (4.6 g.) in ethanol (50 ml.), was added dropwise over a period of 1 hr. to a boiling solution of 1-bromo-3-chloropropane (40 ml.) in ethanol (40 ml.). After heating under reflux for a further 4 hr., the mixture was cooled, diluted with water and extracted with ether. After removal of the solvent, distillation of the residue gave γ -phenoxypropyl chloride, b.p. 90–100°/0.5 mm. (30 g.). This was refluxed for 6 hr. with a solution of ethyl sodiomalonate, prepared from diethyl malonate (33.6 g.) and sodium (4.8 g.) in ethanol (120 ml.). After the addition of 40% NaOH (40 ml.) the mixture was heated for a further 30 min., cooled, and acidified with conc.

HCl. The product was extracted with ether, and the extract washed with water and dried (Na_2SO_4). Removal of the solvent gave a product, m.p. 75–80°, which after drying at 100° crystallized from ether–light petroleum (b.p. 60–80°) in prisms (15 g.), m.p. 110–112°. (Found: C, 60.6; H, 5.7. Calc. for $\text{C}_{12}\text{H}_{14}\text{O}_5$: C, 60.5; H, 5.9%.) Gabriel (1892) found m.p. 75–80°.

δ-Phenoxyvaleric acid. γ -Phenoxypropylmalonic acid (15 g.) was heated at 140° for 1 hr. The product crystallized from light petroleum (b.p. 60–80°) in plates, m.p. 66°. (Found: C, 67.9; H, 7.1. Calc. for $\text{C}_{11}\text{H}_{14}\text{O}_3$: C, 68.0; H, 7.2%.) Gabriel (1892) found m.p. 66°.

o-Hydroxyphenoxyacetic acid. Catechol (11 g.) was added to a solution of sodium (2.3 g.) in ethanol (150 ml.) and refluxed with ethyl bromoacetate (11.2 ml.) for 18 hr. After hydrolysis of the ester by refluxing for a further 1 hr. with 40% NaOH solution (26 ml.) the required acid was isolated, with NaHCO_3 solution. Several crystallizations from acetone–benzene (with charcoal treatment) gave a product, m.p. 135–136°. (Found: C, 57.4; H, 4.9. Calc. for $\text{C}_8\text{H}_8\text{O}_4$: C, 57.1; H, 4.8%.) Carter & Lawrence (1900) found m.p. 152°.

p-Hydroxyphenoxyacetic acid. This was prepared from quinol, as described above for the corresponding *ortho* compound. It crystallized from acetone–benzene in prisms, m.p. 151–152°. (Found: C, 57.0; H, 4.7%.) Carter & Lawrence (1900) found m.p. 152°.

β-(p-Hydroxyphenoxy)propionic acid. This was prepared, with quinol and β -propiolactone, by a procedure similar to that described for β -phenoxypropionic acid. It crystallized from acetone–benzene (with charcoal) in minute prisms, m.p. 169.5–170.5. (Found: C, 59.1; H, 5.4. Calc. for $\text{C}_9\text{H}_{10}\text{O}_4$: C, 59.3; H, 5.5%.) Gottesman (1933) found m.p. 168–169°.

γ-(p-Hydroxyphenoxy)-n-butyric acid. This was prepared by hydrolysis of the corresponding nitrile. It crystallized from water (with charcoal) in aggregates of prisms, m.p. 121–122°. (Found: C, 60.8; H, 6.1. Calc. for $\text{C}_{10}\text{H}_{12}\text{O}_4$: C, 61.2; H, 6.1%.) Sobotka & Austin (1952) found m.p. 120°.

METHODS

Replacement culture technique and isolation of metabolites. The following minor modifications were made to the general methods previously described (Byrde *et al.* 1956):

Penicillin culture flasks were used throughout, with 500 ml. of substrate.

As a spray reagent for identifying acidic spots on paper chromatograms, B.D.H. Universal Indicator (Long, Quayle & Stedman, 1951) was used instead of bromocresol green. The former has the advantage that it can be used on papers run in an ammoniacal solvent.

Owing to the presence of more than one product from the metabolism by *A. niger* of the acids studied, separation was always effected by large-scale paper chromatography as described previously (Byrde *et al.* 1956).

Fungitoxicity in vitro. Fungistatic activity was assessed by rate of mycelial growth of *A. niger* on an agar medium in which the compounds under test were incorporated. The following medium was used: glucose, 50 g.; KNO_3 , 5 g.; KH_2PO_4 , 3.4 g. (equivalent to 0.025 M); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g.; Na_2SO_4 , 1 g.; agar, 20 g.; water to 1 l. It was found unnecessary to add trace metals for adequate growth.

The medium was dispensed in 50 ml. lots in 100 ml. flasks, autoclaved at 15 lb./in.² for 15 min., and held at 45–50° in a water bath. Phosphoric acid was then added to give a final phosphate concentration of 0.05 M. The pH of this medium was approximately 2.2, sufficiently low to ensure that the acids were predominantly in the undissociated form (pK_a of phenoxyacetic acid, 3.12). The acids were incorporated as ethanolic solutions of appropriate concentration by means of a sterile pipette; the final concentration of ethanol in the medium was adjusted to 1% (v/v) throughout.

Four replicate Petri dishes were poured from each of the flasks, which included a control. On cooling, the plates were inoculated at a central point with a 4.5 mm. disk cut from a day-old culture of *A. niger*, and incubated at 25°. Mycelial growth along two predetermined diameters at right angles was measured on the second and fourth days after inoculation. The mean growth rate (mm./day) over this arbitrary period was used to measure fungistatic activity, and was subjected to statistical analysis. Where acids were tested at a series of concentrations, toxicity data were computed by the probit method as adapted for quantitative responses (Finney, 1952, p. 183).

RESULTS

Identification of metabolites

The metabolism of ω -phenoxy-*n*-alkylcarboxylic acids by *A. niger* gave hydroxy acids detected on paper chromatograms with 1% (w/v) *p*-nitrobenzene diazonium fluoroborate solution in 20%

Table 1. *Metabolites of ω-phenoxy-n-alkylcarboxylic acids incubated with A. niger*

Conditions were as described in the text and by Byrde *et al.* (1956). β -(*o*-Hydroxyphenoxy)propionic acid was identified by its R_F value, the remainder by mixed m.p.'s.

Substrate	Principal metabolites		
	Identity	M.p.	R_F
Phenoxyacetic acid	<i>o</i> -Hydroxyphenoxyacetic acid	130–135°	0.45
	<i>p</i> -Hydroxyphenoxyacetic acid	140–150	0.23
β -Phenoxypropionic acid	β -(<i>o</i> -Hydroxyphenoxy)propionic acid	—	0.59
	β -(<i>p</i> -Hydroxyphenoxy)propionic acid	168–169	0.43
γ -Phenoxy- <i>n</i> -butyric acid	<i>o</i> -Hydroxyphenoxyacetic acid	125–130	0.42
	<i>p</i> -Hydroxyphenoxyacetic acid	145–150	0.24
δ -Phenoxy- <i>n</i> -valeric acid	β -(<i>o</i> -Hydroxyphenoxy)propionic acid	—	0.58
	β -(<i>p</i> -Hydroxyphenoxy)propionic acid	169–171	0.43

(w/v) aqueous sodium acetate. This reagent gave a dark-brown colour with *o*-hydroxyphenoxy-acetic and -propionic acids and a light reddish-brown colour with the corresponding *p*-hydroxy acids. Table 1 summarizes the identification of the principal metabolites. In a typical experiment in which 76 mg. of phenoxyacetic acid in 500 ml. of 0.01M- Na_2HPO_4 was incubated with *A. niger*, 31 mg. of *p*-hydroxyphenoxyacetic acid and 7 mg. of *o*-hydroxyphenoxyacetic acid were isolated.

Hydroxylation with a model system

The ascorbic acid system of Udenfriend, Clark, Axelrod & Brodie (1954) used previously (Byrde *et al.* 1956) was again employed to investigate the hydroxylation of phenoxyacetic acid. A solution of this acid (0.09 g.), ascorbic acid (0.25 g.), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.04 g.) and sodium ethylenediaminetetraacetate (0.23 g.) in 0.2M phosphate buffer, pH 6.8 (30 ml.), was aerated at 37° for 2 hr. The solution was then acidified with HCl, extracted with ether and acidic products were re-isolated by shaking with NaHCO_3 solution. Chromatographic examination of the acidified bicarbonate-soluble fraction gave spots identical with phenoxyacetic acid (R_f 0.51), *o*- and *p*-hydroxyphenoxyacetic acids (R_f values 0.46 and 0.28 respectively) and a further unidentified spot (R_f 0.65) which gave a dark-brown colour with the diazonium reagent. The *o*-

hydroxy compound, which had been present in the fungal extracts to only a small extent compared to the *para*-isomer, here appeared to be the major product.

Fungitoxicity in vitro

Table 2 shows the mean growth rate (mm./day) of cultures of *A. niger* in the presence of unsubstituted and hydroxylated phenoxyacetic and β -phenoxypropionic acids. The *p*-hydroxy derivative of each acid, even at the relatively high concentrations tested, produced no significant inhibition of fungal growth. Probit analysis of the data for the unsubstituted and *o*-hydroxy acids was carried out, and the results are summarized in Table 3. The ratio of the potency to *A. niger* of phenoxyacetic acid to that of its *o*-hydroxy derivative was 7.6:1; no overall figure for potency can be quoted for the corresponding β -propionic acids, owing to non-parallelism of dose-response lines, but the ED_{50} values were in the ratio of 17:1, the unsubstituted acid again being the more toxic.

Inspection of the data of Table 2 shows that, with the agar-plate method described above, it was possible to detect differences in growth which were of the order of 10% of that of the control plates to which ethanol only had been added. By contrast, in the studies on ω -(2-naphthyl)-*n*-alkylcarboxylic acids where mycelial growth in liquid medium was estimated gravimetrically, the differences between

Table 2. Mean growth rate of *A. niger* mycelium in the presence of phenoxyacetic and β -phenoxypropionic acids and their *o*- and *p*-hydroxy derivatives

Conditions were as described in the text.			
Test no.	Compound added	$10^4 \times \text{concn.}$ (M)	Mean growth rate (mm./day)
1	Phenoxyacetic acid	20.0	4.92
		10.0	8.31
		5.0	9.44
		2.5	9.94
	<i>o</i> -Hydroxyphenoxyacetic acid	100.0	7.00
		50.0	9.25
		25.0	9.56
	<i>p</i> -Hydroxyphenoxyacetic acid	100.0	10.88
		50.0	10.83
	None	—	10.38
	Least significant difference between means	($P=0.05$)	0.98
2	β -Phenoxypropionic acid	10.0	0.25
		5.0	2.38
		2.5	9.19
		1.25	11.06
	β -(<i>o</i> -Hydroxyphenoxy)propionic acid	50.0	6.50
		25.0	9.13
		12.5	10.44
	β -(<i>p</i> -Hydroxyphenoxy)propionic acid	50.0	9.50
		25.0	11.44
	None	—	10.69
	Least significant difference between means	($P=0.05$)	1.42

Table 3. Dose-response data derived from Table 2

Test no.	Compound	Equation of probit regression line	10 ⁴ ED ₅₀ * (molar)	Slope of probit regression line	Relative potency*
1	Phenoxyacetic acid	$Y = 8.48 - 2.65x$	20.6 (17.6-24.1)	-2.65 ± 0.39	100.0
	<i>o</i> -Hydroxyphenoxyacetic acid	$Y = 10.81 - 2.65x$	156.3 (127.6-191.5)	-2.65 ± 0.39	13.2 (10.8-16.0)
2	β -Phenoxypropionic acid	$Y = 8.39 - 5.91x$	3.8 (3.2-4.2)	-5.91 ± 0.91	†
	β -(<i>o</i> -Hydroxyphenoxy)propionic acid	$Y = 9.58 - 2.54x$	63.7 (49.8-148.5)	-2.54 ± 0.88	†

* Approximate 5% fiducial limits are quoted in parentheses.

† No estimate possible, owing to non-parallelism of lines. Ratio of ED₅₀ values, 17:1.

transformed treatment means required to be about 20% of the control in order to attain statistical significance (Byrde *et al.* 1956; Tables 2 and 3). The solid-medium technique was thus more sensitive, and also obviated the need for an angular transformation of the data.

DISCUSSION

The results obtained with the phenoxy-*n*-alkyl-carboxylic acids followed the general pattern of those in the 2-naphthyl-oxy series, both nuclear hydroxylation and β -oxidation of the side chain being demonstrated. Whereas hydroxylation by *A. niger* was limited to the 6-position in the naphthalene nucleus, it occurred in both the *para* and to a lesser extent in the *ortho* position in the benzene nucleus. Parke & Williams (1956), studying the detoxication of aniline by animals, also found that such preferential *parahydroxylation* occurred in the majority of species. Hydroxylation of phenoxyacetic acid with the Fe²⁺ ions-ascorbic acid system of Udenfriend *et al.* (1954), on the other hand, took place primarily in the *ortho* position, though *para*-substitution also occurred to some extent.

Whereas γ -phenoxy-*n*-butyric and δ -phenoxy-*n*-valeric acids were metabolized, forming mixtures of *o*- and *p*-hydroxyphenoxyacetic and *o*- and *p*-hydroxy- β -phenoxypropionic acids respectively, the side chain of the two latter acids remained intact. This resistance of the side chain to further oxidation has already been noted with the corresponding β -(6-hydroxy-2-naphthyl-oxy)propionic acid, but this is in sharp contrast with the results of Fawcett *et al.* (1954), who found that β -phenoxypropionic acid is converted into phenol in the flax plant.

The biological-assay data presented show that the hydroxylation of phenoxyacetic and β -phenoxypropionic acids is accompanied by a decrease in toxicity to the fungus. It was also found that the *o*-hydroxy acids were both more toxic than the corresponding *p*-hydroxy compounds, probably due to the more lipophilic nature of the former.

The significant difference between the slopes of the dose-response lines for β -phenoxypropionic and β -(*o*-hydroxyphenoxy)propionic acids suggests a dissimilar mode of action (Finney, 1952, p. 124). A difference in slope of dose-response lines after hydroxylation was also noted in the naphthyl-oxy acid series (Byrde *et al.* 1956). Recent findings, however, suggest that caution is needed in interpreting such differences in slope as necessarily implying dissimilarity in mode of action of fungicides (McCallan, unpublished work).

SUMMARY

1. By the use of a replacement culture technique it was demonstrated that phenoxyacetic and β -phenoxypropionic acids were hydroxylated in the *para* and to a lesser extent the *ortho* positions by *Aspergillus niger*, both products in each case being less toxic to the fungus than the original acid.

2. γ -Phenoxy-*n*-butyric acid likewise gave mainly *p*-hydroxyphenoxyacetic acid by hydroxylation and β -oxidation.

3. δ -Phenoxy-*n*-valeric acid was similarly metabolized to β -(*p*-hydroxyphenoxy)propionic acid (and probably also to the *o*-hydroxy acid).

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The Oxidative Pathway of Carbohydrate Metabolism in *Escherichia coli*

5. ISOLATION AND IDENTIFICATION OF RIBULOSE PHOSPHATE PRODUCED FROM 6-PHOSPHOGLUCONATE BY THE DEHYDROGENASE OF *E. COLI**

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In early enzymic studies on the oxidative decarboxylation of 6-phosphogluconate by a crude yeast system, a complex mixture of end products had been obtained (Warburg, Christian & Griese, 1935; Dickens, 1938). Work in this Laboratory demonstrated the presence of ribose phosphate and the presence of unknown Bial-reactive sugar phosphate among these products (Cohen & Scott, 1950; Scott & Cohen, 1951). The use of a considerably purified enzyme system from yeast permitted the further dissection of this reaction sequence by Horecker, Smyrniotis & Seegmiller (1951). These workers thus discovered the formation of ribulose 5-phosphate as a primary product of the oxidative decarboxylation of 6-phosphogluconate, and the subsequent isomerization of ribulose 5-phosphate to ribose 5-phosphate.

Subsequent work on this system in our Laboratory was concerned primarily with the role of this pathway in ribose formation in *Escherichia coli* under conditions of normal growth and virus infection. Nevertheless, it appeared desirable to demonstrate unequivocally that the oxidation of 6-phosphogluconate by bacterial enzymes was comparable

with that observed with yeast enzymes. Having purified the 6-phosphogluconate dehydrogenase from *Esch. coli* (Scott & Cohen, 1953), the nature of the primary reaction product using this bacterial enzyme was demonstrated to be ribulose phosphate as described below.

MATERIALS AND METHODS

The barium salt of 6-phosphogluconic acid was prepared from synthetic glucose 6-phosphate by the method of Seegmiller & Horecker (1951). Phosphogluconate (PG) was determined by the reduction of triphosphopyridine nucleotide (TPN) in the presence of phosphogluconic dehydrogenase. The barium salt of ribose 5-phosphate was prepared by the method of Groth, Mueller & LePage (1952) and always contained about 11% of ribulose 5-phosphate. Sodium pyruvate was prepared immediately before use by neutralization of freshly distilled pyruvic acid. Glycylglycine buffer and tris buffer (aminotrihydroxymethylmethane) were obtained from Sigma Chemical Co. TPN was prepared by the method of LePage & Mueller (1949) and was measured by reduction in the presence of PG and PG dehydrogenase. It was 74% pure. Pure ribulose was regenerated from the *o*-nitrophenylhydrazones of ribulose by treatment with freshly distilled benzaldehyde in the presence of benzoic acid. This derivative had been prepared from ribulose obtained

* Part 4: Scott (1956).