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FUNGAL METABOLISM—III.

THE HYDROXYLATION OF ANISOLE, PHENOXYACETIC ACID, PHENYLACETIC ACID AND BENZOIC ACID BY *ASPERGILLUS NIGER*

SHEILA M. BOCKS

Dyson Perrins Laboratory, Oxford

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Abstract—The hydroxylation of anisole and phenoxyacetic acid by several strains of *Aspergillus niger* has shown that the *ortho*-isomer was the main monohydroxylated product. Benzoic acid was found to be rapidly utilized by this organism and small amounts of protocatechuic acid and traces of *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid and catechol were the metabolites identified. Phenylacetic acid was found to be converted mainly to *o*-hydroxyphenylacetic acid and homogentisic acid.

INTRODUCTION

A PRELIMINARY investigation of the hydroxylation of anisole and phenoxyacetic acid by a wild strain of *Aspergillus niger*[1] has shown that the main monohydroxylated product was the *o*-isomer. This result was in contrast to a previous report that *p*-hydroxyphenoxyacetic acid was the main product obtained by the action of *A. niger* (Mulder) on phenoxyacetic acid.[2] It was concluded that the difference may be due to the difference in the strains used. In the studies reported here the hydroxylating activity of several strains of *A. niger* on anisole and phenoxyacetic acid have been compared.

Anisole and phenoxyacetic acid are both aromatic ethers and it was considered worthwhile to compare the action of *A. niger* under similar conditions on the two aromatic acids, benzoic and phenylacetic acid.

RESULTS AND DISCUSSION

All experiments were carried out using the replacement culture technique of Kluyer and van Zijp,[3] in which pregrown mycelial mats of *Aspergillus niger* were incubated with aqueous solutions of the substrate in stationary flasks. Samples were removed from the incubation mixtures at intervals and analysed by u.v. spectroscopy. At the end of the incubation period analyses of the concentrated ether extracts were carried out by thin-layer, paper and gas chromatography.

Anisole is both toxic and insoluble in aqueous media and it was necessary to modify the technique in this case; the substrate was dissolved in seven times its volume of carbon tetrachloride and this solution was placed at the bottom of each culture flask beneath the aqueous buffer solution and gave a two-phase incubation system. The cultures were incubated for

[1] S. M. BOCKS, J. R. LINDSAY SMITH and R. O. C. NORMAN, *Nature* **201**, 398 (1963).
[2] D. WOODCOCK, In *Phenolics in Plants in Health and Disease* (Edited by J. B. PRIDHAM). Pergamon Press, Oxford (1960).
[3] A. J. KLUYER and J. C. M. VAN ZIJP, *J. Microbiol. Serol.* **17**, 47 (1951).

7 days at 25° after which the ether extracts of the incubation mixtures were analysed for phenolic products.

Four strains of *A. niger* were used in this study and the results of experiments in which anisole was used as the substrate are shown in Table 1. It appears that with all four strains, *o*-hydroxyanisole was the main monohydroxylated product. Gas chromatography also revealed that phenol was produced in almost as large a quantity as *o*-hydroxyanisole (guaiacol). This is particularly interesting as *O*-dealkylation of anisole also occurs with the liver microsomal hydroxylating system.[4]

TABLE 1. HYDROXYLATED METABOLITES PRODUCED BY THE ACTION OF *A. niger* STRAINS ON ANISOLE

Substrate	Metabolite identified (%)			
Anisole	Phenol	Guaiacol	*m*-Hydroxy-anisole	*p*-Hydroxy-anisole
Strain				
A. niger (wild)	+	over 95	—	—
A. niger (11394)	+	81·8	trace	18·2
A. niger (Mulder)	+	69·1	13·3	17·6
A. niger (A_2)	+	65·7	15·4	18·9

Ratios of the monohydroxylated products of anisole were obtained by GLC analyses of ether extracts of incubation mixtures containing anisole.

A study of the hydroxylation of phenoxyacetic acid using three strains of *A. niger* confirmed the previous observation[1] that *o*-hydroxyphenoxyacetic acid was obtained as the main product (approx. values obtained were 72–84%). In addition, the presence of *p*-hydroxyphenoxyacetic acid and *m*-hydroxyphenoxyacetic acid were identified as minor products. It was frequently noted that although *p*-hydroxyphenoxyacetic was identified when the pH of the substrate solution was adjusted to pH 7, or buffered at this pH value before incubation with the organism, it was absent, or present in very low concentrations, when unbuffered solutions of phenoxyacetic acid (0·01 M) were incubated with the organism. The formation of *o*-hydroxyphenoxyacetic acid as the main product by *A. niger* (Mulder) was also described by Clifford and Woodcock.[5] Experiments carried out at varying pH values, and substrate concentrations and in the presence of (1) ascorbic acid, (2) ethylenediaminetetraacetic acid, (3) an active preparation of catalase, and (4) metal ions—Cu^{++}, Fe^{++}, Mn^{++} and Zn^{++}, showed that under all conditions the *o*-isomer was the main monohydroxylated product. Hydroxylation was found to be considerably inhibited in incubation mixtures containing zinc sulphate and manganese sulphate at a concentration of 10^{-3} M. Incubation mixtures to which an active solution of catalase was added daily for 5 days, showed no difference in the rate of hydroxylation as indicated by u.v. measurements and it was concluded that the formation of free hydrogen peroxide may not be involved in the process of hydroxylation.

The purification procedure used in the preparation of the authentic *o*-hydroxyphenoxyacetic acid involved steam-distillation of the corresponding lactone[6] which readily re-

[4] J. AXELROD, *J. Pharmacol. Exp. Therap.* **115**, 259 (1955).
[5] D. R. CLIFFORD and D. WOODCOCK, *Nature* **203**, 763 (1964).
[6] *Beilstein's Handbuch der Organischen Chemie* **6**, 777 (1923).

hydrolyses in water to give the acid; and this suggested that the predominance of the *p*-isomer noted in the earlier studies of the previous workers[2] may have been due to the loss of the *o*-isomer as the lactone. In the studies reported here, such a loss was avoided by freeze-drying the incubation mixture and redissolving it in a known volume of solvent for chromatography. Phenoxyacetic acid did not appear to be metabolized further than the monohydroxylated stage to any appreciable extent and the concentrations of hydroxylated products in the incubation mixtures remained constant for periods of 3–5 weeks. However, an unidentified spot was always present in most chromatograms: this may be a dihydroxylated product but owing to difficulties in the preparation of the authentic dihydroxylated products this has not yet been verified. It has always been noted that in the presence of phenoxyacetic acid the mycelial mats of *A. niger* did not undergo the rapid autolysis which occurred in the control cultures.

When *A. niger* was incubated with benzoic acid a different picture was obtained. Unlike phenoxyacetic acid and anisole, benzoic acid was found to be readily metabolized and, in cultures incubated for 7 days with 0·001 M benzoic acid, no hydroxylated products could be identified, but after 72 hr × 2 incubation small amounts of protocatechuic acid, *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid and a trace of catechol were obtained. The metabolism of benzoic acid by *A. niger* resembles that of cinnamic acid[7] in being rapidly utilized. Traces of *p*-hydroxybenzoic acid and catechol were also identified as products in the degradation of cinnamic acid and were considered to be β-oxidation products of the corresponding hydroxy-*trans*-cinnamic acids.

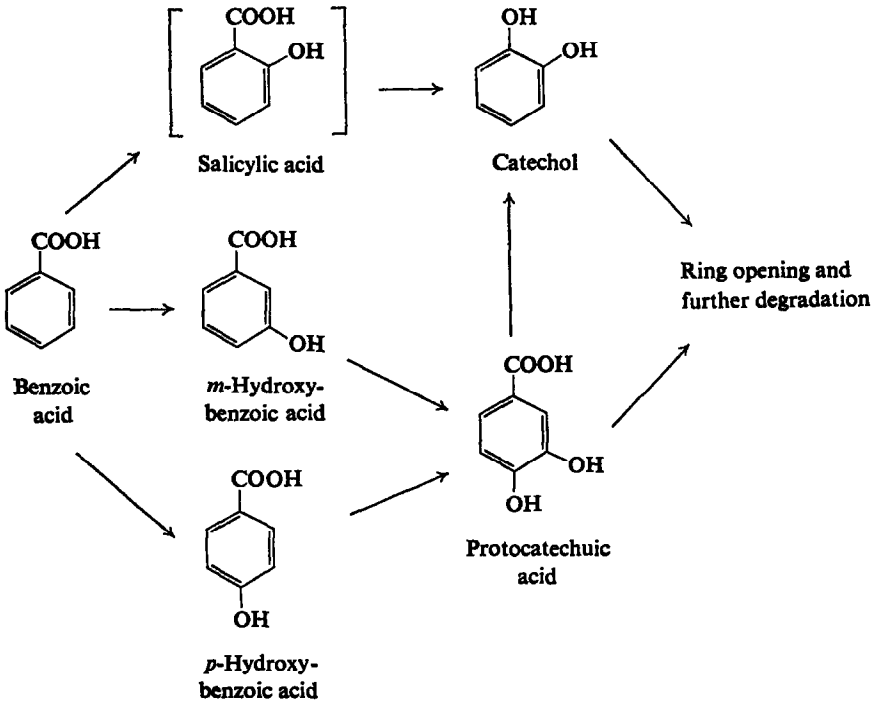

FIG. 1. REACTION SEQUENCES IN THE METABOLISM OF BENZOIC ACID BY *A. niger*.

[7] S. M. BOCKS, *Phytochem.* 6, 127 (1967).

The hydroxylation of phenylacetic acid by *A. niger* (Mulder) was carried out under conditions similar to those used for phenoxyacetic acid and benzoic acid. *o*-Hydroxyphenylacetic acid was identified as the main mono-hydroxylated product (approx. 76%). *m*-Hydroxyphenylacetic acid and *p*-hydroxyphenylacetic acid were identified as minor products. In this case, a dihydroxylated product—homogentisic acid (2,5-dihydroxyphenylacetic acid)—was also identified. The formation of homogentisic acid by *A. niger* was noted by Kluyer and van Zijp,[3] but they have not recorded the presence of the monohydroxylated products. Homogentisic acid was found to accumulate in substantial amounts in the incubation mixtures and it is yet uncertain whether the metabolism of phenylacetic acid proceeds beyond this stage. In 1950, Utkin[8] also reported the formation of homogentisic acid when tyrosine was incubated with *A. niger*.

It appears from these studies that the presence of the substituents in anisole, phenoxyacetic acid and phenylacetic acid inhibit the further metabolism of the hydroxylated products to a considerable extent. In contrast, the hydroxylated products of benzoic acid were rapidly degraded further. Hence, the enzyme system responsible for the hydroxylation of aromatic compounds in these organisms appears to be less specific compared with enzymes catalysing ring-opening and further degradation. This condition could be of value in the development of microbiological methods for the large-scale preparation of the *o*-hydroxylated derivatives, in particular, of these and other suitably hindered aromatic substrates.

EXPERIMENTAL

The four strains of *Aspergillus niger* used in this study were obtained from the Centraalbureau voor schimmel cultures, Baarn-Netherlands. For experimental purposes, stationary liquid cultures of *A. niger* were grown in 250-ml conical flasks on the medium used by Byrde *et al.*,[9] for 3 days at 25°. The mycelial mats were then washed and reincubated at 25° with an aqueous solution of the substrate (0·001 M, pH 7) dissolved in 0·001 M solution of potassium dihydrogen phosphate (50 ml per flask). In a typical experiment 1 l. of the substrate solution was used. Routine determinations of u.v. spectra were carried out on aliquots removed at intervals, using an Unicam S.P. 800 recording spectrophotometer. At the end of the incubation period (3, 5, or 7 days), the substrate solution was filtered through muslin. In the case of anisole, the substrate solutions were acidified and extracted with ether. The concentrated ether extracts were analysed for phenolic products by gas chromatography and paper chromatography as previously described.[1] When phenoxyacetic acid, benzoic acid and phenylacetic acid were used as substrates, the incubation mixtures were filtered and freeze-dried. The freeze-dried products were dissolved in 10 ml ether for analysis by gas chromatography, paper chromatography and thin-layer chromatography. Preparative paper chromatography was used in the isolation of products on sheets of Whatman No. 3 MM paper. The approximate concentrations of the products were estimated spectroscopically by comparison of the products eluted from the chromatograms with known concentrations of the authentic compounds using an S.P. 500 spectrophotometer.

Paper chromatograms were developed by downward migration of the solvent and Whatman No. 1 and 4 paper were used for most purposes. The solvent systems used were (1) *n*-butanol–ethanol–3 N ammonia (4:1:5), (2) 2% acetic acid, (3) ethanol–ammonia–water (20:1:4), and (4) benzene–acetic acid–water (125:72:3). Thin-layer chromatography

[8] L. M. UTKIN, *Biokhimiya* **15**, 330 (1950).
[9] R. J. W. BYRDE, J. F. HARRIS and D. WOODCOCK, *Biochem. J.* **64**, 154 (1956).

was carried out using solvents (3) and (4) with cellulose powder plates. Compounds were detected by examination of chromatograms under u.v. light. The position of phenolic spots were also detected with diazotized *p*-nitroaniline spray and ferric chloride-ferricyanide spray.[10] Acids were detected by spraying chromatograms with a 95% ethanolic solution of bromocresol green.[11]

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[10] G. M. BARTON, R. S. EVANS and J. A. F. GARDNER, *Nature* **170**, 239 (1952).
[11] In *Data for Biochemical Research* (Edited by R. M. C. DAWSON, D. C. ELLIOT, W. H. ELLIOT and K. M. JONES), p. 210. Clarendon Press, Oxford (1959).