Phytochemistry, 1969, Vol. 8, pp. 549 to 552. Pergamon Press. Printed in England.

METABOLISM OF CINNAMIC ACID BY *ASPERGILLUS NIGER**

D. R. CLIFFORD, J. K. FAULKNER,† J. R. L. WALKER‡ and D. WOODCOCK

Long Ashton Research Station, University of Bristol

(*Received* 18 *October* 1968)

Abstract—In a study of the metabolism of cinnamic acid by *Aspergillus niger* using a replacement culture technique, the facile formation of styrene has been confirmed, and phenylethylene glycol has been shown to be a metabolite for the first time in any system.

INTRODUCTION

THE METABOLISM of cinnamic and hydroxycinnamic acids has been comprehensively reviewed by Pridham.[1] In higher plants cinnamic acid is an intermediate in the biosynthesis of hydroxycinnamic acid derivatives (e.g. chlorogenic acid),[2–4] whilst soil *Pseudomonas* spp. under aerobic conditions convert it to 2,3-dihydroxyphenylpropionic acid prior to ring fission and further degradation,[5,6] the metabolic steps being thought to involve phenyl, *m*-hydroxyphenyl- and 2,3-dihydroxyphenylpropionic acids.[6] By contrast, *Lactobacillus pastorianus* var. *quinicus* is able to reduce the side-chain of cinnamic, 4-hydroxycinnamic and 3,4-dihydroxycinnamic acids and decarboxylate the products to varying extents.[7]

The present work was carried out in order to compare the metabolism of cinnamic acid—a potent inhibitor of spore germination[8]—by *Aspergillus niger* van Tiegh, with that observed in higher plants, yeasts and bacteria. Whilst it was in progress, Bocks[9] reported on the metabolism of cinnamic acid by the same fungus.

RESULTS AND DISCUSSION

The formation of styrene after as little as 30 min confirms earlier reports[10,11] and it was further shown, using a u.v. spectrophotometric method, that at least 5 per cent of the substrate underwent this conversion, apart from the formation of polymeric material. Of the non-acidic metabolites formed, the most interesting is undoubtedly phenylethylene glycol which

* Part X in the series "Fungal Detoxication".

† Present address: Research Dept., Pfizer Ltd., Sandwich, Kent.

‡ A.R.C. Underwood Research Fellow: on leave from the Cawthron Institute, Nelson, New Zealand.

[1] J. B. PRIDHAM, *Ann. Rev. Plant Physiol.* **16**, 13 (1965).
[2] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 537 (1959).
[3] C. C. LEVY and M. ZUCKER, *J. Biol. Chem.* **235**, 2418 (1960).
[4] P. N. AVADHANI and G. H. N. TOWERS, *Can. J. Biochem. Physiol.* **39**, 1605 (1961).
[5] C. B. COULSON and W. C. EVANS, *Chem. Ind.* **543** (1959).
[6] E. R. BLAKLEY and F. J. SIMPSON, *Can. J. Microbiol.* **10**, 175 (1964).
[7] G. C. WHITING and J. G. CARR, *Nature* **184**, 1427 (1959).
[8] R. J. W. BYRDE, A. H. FIELDING and A. H. WILLIAMS, In *Phenolics in Plants in Health and Disease* (edited by J. B. PRIDHAM), pp. 95–99, Pergamon Press, Oxford (1960).
[9] S. M. BOCKS, *Phytochem.* **6**, 127 (1967).
[10] R. O. HERZOG and O. RIPKE, *Z. Physiol. Chem.* **57**, 43 (1908).
[11] S. L. CHEN and H. J. PEPPLER, *J. Biol. Chem.* **221**, 101 (1956).

is reported as a cinnamic acid metabolite for the first time in any system as far as we know. This compound, which may be derived from cinnamic acid either by decarboxylation and subsequent hydroxylation or vice versa, was separated with difficulty from a very small amount of a carbonyl-containing impurity, which it was not possible to isolate. Bocks[9] makes no mention of either styrene or styrene glycol.

The acidic metabolite mixture, which contained a very large proportion of unchanged cinnamic acid, also showed traces of at least five "diazo-positive" compounds, of which only two were not present in the corresponding extract from *Aspergillus niger* mycelium which had been incubated with buffer solution alone. A TLC evaluation using authentic specimens failed to identify the two latter compounds, though it appears unlikely that one is *m*- or *p*-hydroxycinnamic acid, *p*-hydroxyphenylpropionic, *p*-hydroxybenzoic or salicylic acids; the second may be *p*-hydroxybenzoic acid. Bocks[9] has reported the presence of only traces of *o*- and *p*-coumaric, *o*-hydroxyphenylpropionic (melilotic) and *p*-hydroxybenzoic acids. This limited formation of mono-hydroxy acids contrasts with the extensive hydroxylation observed in previous work with *A. niger* and phenoxy-acetic acids,[12–14] with the production of *o*-hydroxyphenylpropionic acid from cinnamic acid by a strain of *Pseudomonas* isolated from soil,[6] and with the formation of *p*-coumaric acid by *Polystictus versicolor*.[15]

Of the other "diazo-positive" compounds, one which showed a pale blue fluorescence in u.v. light was identified as 2,3-dihydroxybenzoic acid (DHB), and this was subsequently shown to be metabolized at the same rate as the original substrate. Its formation can be explained by nuclear hydroxylation followed by β-oxidation, an order of precedence which has been observed earlier in the metabolism of ω-(2-naphthyloxy)-*n*-alkyl carboxylic acids by *A. niger*.[16] Although dihydroxylation has not been previously observed with *A. niger* (except in the case of salicylic acid),[17] the present findings are in agreement with those of Coulson and Evans[5] and Blakley and Simpson,[6] both of whom showed that soil *Pseudomonas* spp. were able to convert cinnamic acid to DHB. However, the position is complicated by the fact that DHB has also been detected when only buffer solution is placed under the mycelial mats in the replacement culture technique, though not in the glucose mineral salts medium after the initial growth of the mycelium. This observation together with the known production of DHB in the "low-iron fermentation" of *Streptomyces griseus*[18] and in the submerged culture of *Claviceps paspali*[19,20] suggested that DHB might be formed by an abnormal pathway caused by lack or deficiency of some essential element. The addition of Fe–EDTA (10^{-3} M) to the buffer solution, however, failed to alter the metabolic pattern.

EXPERIMENTAL

I.r. spectra were determined as films (unless otherwise stated) using a Perkin-Elmer Infracord Spectrophotometer Model 237. Proton magnetic resonance spectra were determined at 100 Mc/s with a Varian Model HA-100. Mass spectra were measured on an LKB Model 9000.

[12] J. K. FAULKNER and D. WOODCOCK, *J. Chem. Soc.* 5397 (1961).
[13] D. R. CLIFFORD and D. WOODCOCK, *Nature* **203**, 763 (1964).
[14] J. K. FAULKNER and D. WOODCOCK, *J. Chem. Soc.* 1187 (1965).
[15] V. C. FARMER, M. E. K. HENDERSON and J. D. RUSSELL, *Biochem. Biophys. Acta* **35**, 202 (1959).
[16] R. J. W. BYRDE, J. F. HARRIS and D. WOODCOCK, *Biochem. J.* **64**, 154 (1956).
[17] G. TERUI, T. ENATSU and S. TABOTA, *J. Ferment. Technol.* **39**, 224 (1961).
[18] J. R. DYER, H. HEDING and C. P. SCHAFFNER, *J. Org. Chem.* **29**, 2802 (1964).
[19] F. ARCAMONE, E. B. CHAIN, A. FERRETTI and P. PENNELLA, *Nature* **192**, 552 (1961).
[20] D. GROEGER, D. ERGE and H. G. FLOSS, *Z. Naturforsch* **20b**, 856 (1965).

Isolation and Identification of Metabolites

The replacement culture technique previously described[16] was used, cinnamic acid (2×10^{-3} M) in Na_2HPO_4 buffer (10^{-2} M) being incubated at 26° for varying times with mycelial mats of *A. niger* which had been grown in penicillin flasks on a glucose-mineral salts medium. The formation of styrene was established by aspiration of the air in the flasks after the appropriate incubation period, through a solution of Br_2 in CCl_4. Concentration of the solution yielded a product, m.p. 71–72°, identical (mixed m.p. and i.r. spectrum) with an authentic specimen of styrene dibromide.

After concentration *in vacuo* in a cyclone evaporator, the fungal liquor was acidified (pH 1–2), extracted continuously with ether for 16 hr and the ether-soluble material separated into acidic and non-acidic components using aqueous $NaHCO_3$. Examination of the latter fraction was carried out by TLC using silica gel G (thickness 0·25 cm) and developing for 10 cm with 2% formic acid in ether, spots being located with chromic acid. This showed the presence of a major metabolite (A) of medium R_f, together with another compound (B) of higher R_f which was present in very small amount. Separation on a florisil column by eluting with ether and collecting 20 ml fractions, gave (B) in the first fraction (not further examined) followed by (A) in fractions 3–7. The latter still showed a carbonyl peak at 1710 cm^{-1} in the i.r. spectrum and was further purified by preparative TLC using Kieselgel HF_{254} (Merck) (thickness 0·60 cm) on 20×20 cm plates and developing for 15 cm in 2% formic acid in ether. Examination of the plate in u.v. light showed the metabolite and impurities as dark blue bands on a white fluorescent background. The appropriate band was scraped off the plate, placed in a soxhlet thimble and the compound isolated by continuous extraction with ether. Repetition of this procedure twice finally gave the chromatographically pure metabolite, m.p. 53°, ν_{max} (cm^{-1}) 3250 (OH str), 2925, 2860 (CH_2 str.), 1595, 1495 (conjugated C=C str.), 1340, 1050 (primary OH), 1340, 1100 (secondary OH), 760, 700 (aromatic mono-substitution). The NMR spectrum (in $CDCl_3$) showed singlets (τ 2·72, 6·9) and multiplets (τ 5·25, 6·3) assigned to aromatic, OH, CH and CH_2 protons respectively, and the major peaks in the mass spectrum were at 138, 107, 120, 108. The identity of the metabolite was established by comparison of the i.r., NMR and mass spectra with those of an authentic specimen of phenylethylene glycol prepared as described below. It is likely that the carbonyl peak in the i.r. spectrum is due to loss of water from phenylethylene glycol under the influence of acidic reagents, followed by rearrangement of the ω-hydroxy styrene so formed to give phenylacetaldehyde, which has been noted as a rearrangement product of styrene glycol by Zincke.[21] Whilst it could be a metabolic intermediate in the formation of the glycol, it seems more likely to be an artefact since it also appears to be associated with the synthetic styrene glycol.

A TLC examination of the acidic fraction of the fungal extract using silica gel G plates (0·25 cm thickness) developed to 10 cm with light petroleum (b.p. 40–60°)/ether/formic acid (60/40/2), showed a metabolite with a pale blue fluorescence in u.v. light when exposed to ammonia vapour. This compound was isolated by striping on thick layer plates and developing in the same solvent. The appropriate band was scraped off the plate and continuously extracted with ether in a soxhlet. Evaporation of the solvent left a crystalline solid which had the same R_f as an authentic specimen of 2,3-dihydroxybenzoic acid when examined by paper chromatography (see Table). The i.r. spectra, [ν_{max} (cm^{-1}) 3400–2500 (OH Str. ex.-COOH), 1650 (C=O), 1600, 1500 (conjugated C=C str.), 1250, 1230 (phenolic OH)] determined as KCl discs were also identical.

IDENTIFICATION OF 2,3-DIHYDROXYBENZOIC ACID (DHB)

Compound	R_f (×100) in			Fluorescence	Spray reagent colour	
	Solvent A	Solvent B	Solvent C		Diazo	$FeCl_3$
Unknown	61	31	14	Light blue	Brick red	Blue
DHB	61	31	16	Light blue	Brick red	Blue

Solvent A, Benzene/acetic acid/water (125:72:3); Solvent B, Methyl ethyl ketone/*t*-butanol/3 N Ammonia (70:20:10); Solvent C, Isopropanol/·880 Ammonia/water (20:1:2). Diazo Spray: Diazotized *p*-nitraniline fluoroborate.

[21] T. ZINCKE, *Annalen* **216**, 293 (1883).

Phenylethylene Glycol

This was prepared by the method of Nystrom and Brown.[22] A solution of mandelic acid (1·52 g) in dry ether (15 ml) was added dropwise over 15 min to a suspension of $LiAlH_4$ (0·47 g) in dry ether (18 ml) cooled to 0°. After refluxing for 30 min the mixture was cooled in ice, water added dropwise, followed by dil. H_2SO_4, and extracted with ether. After washing with dil. H_2SO_4 and water the extract was dried and the solvent removed. The i.r. spectrum of the crude product showed a similar carbonyl peak at 1710 cm^{-1} to the crude metabolite, probably due to phenylacetaldehyde formed by rearrangement of the glycol. Purified by crystallization from light petroleum (b.p. 60–80°), it had m.p. 54° and further purification by TLC failed to raise the m.p. Zincke[21] gives m.p. 67–68°; neither Nystrom and Brown,[22] nor Evans and Morgan[23] who prepared this compound from styrene dibromide via the diacetate, record a m.p. It is significant that a spot of R_f corresponding to the carbonyl-containing impurity reappeared on chromatographing the pure glycol in 2% formic acid in ether.

Acknowledgements—We thank Drs. R. J. Goodfellow and R. L. S. Patterson for the NMR and mass spectra respectively, and Mr. A. Deacon for valuable technical assistance. One of us (J. R. L. W.) is indebted to the Agricultural Research Council for financial assistance.

[22] R. F. NYSTROM and W. G. BROWN, *J. Am. Chem. Soc.* **69**, 2548 (1947).
[23] W. L. EVANS and L. H. MORGAN, *J. Am. Chem. Soc.* **35**, 54 (1913).