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SHORT COMMUNICATION

FUNGAL METABOLISM—I.

THE TRANSFORMATIONS OF COUMARIN, *O*-COUMARIC ACID AND *TRANS*-CINNAMIC ACID BY *ASPERGILLUS NIGER*

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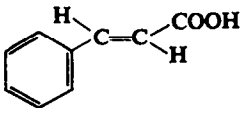
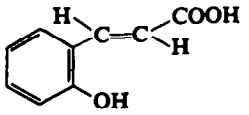
Abstract—The metabolism of coumarin, *o*-coumaric acid and *trans*-cinnamic acid by *A. niger* (mulder strain) has been investigated. Melilotic acid was obtained as the main product of the degradation of coumarin, while *o*-coumaric acid was converted to 4-hydroxycoumarin. *trans*-Cinnamic acid was found to be metabolized rapidly under the conditions used, and only traces of *p*-coumaric acid, melilotic acid, *o*-coumaric acid and *p*-hydroxybenzoic acid were identified.

INTRODUCTION

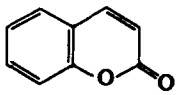
trans-Cinnamic acid (I), *o*-coumaric acid (II) and coumarin (III) and their derivatives are known to occur in free and bound form in many plants. Recent work on the biosynthesis of coumarin and umbelliferone indicates that *ortho*-hydroxylation of a cinnamic acid occurs at some stage in the formation of these compounds.[1] The studies reported here were carried out as a result of the finding that different strains of *A. niger*, when incubated with anisole and phenoxyacetic acid produced the *o*-isomer as the main monohydroxylated product,[2–4] some also producing small amounts of *p*- and *m*-isomers.



(I) *trans*-Cinnamic acid



(II) *o*-Coumaric acid



(III) Coumarin

RESULTS

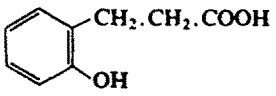
Experiments in which neutral aqueous solutions of *trans*-cinnamic acid (I) were incubated with pregrown mycelial mats of *A. niger* (mulder strain) showed that this compound was being rapidly removed from the medium as judged by the decrease in absorptivity at 273 mμ. After

[1] D. J. AUSTIN and M. B. MEYERS, *Phytochem.* **4**, 245 (1965).
[2] S. M. BOCKS, J. R. LINDSEY-SMITH and R. O. C. NORMAN, *Nature* **201**, 398 (1963).
[3] S. M. BOCKS, unpublished results.
[4] D. WOODCOCK and D. R. CLIFFORD, *Nature* **203**, 763 (1964).

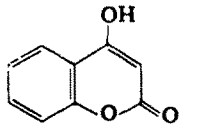
5 days' incubation at 25°, analysis of the concentrated chloroform extracts by thin-layer and paper chromatography showed the presence of very small amounts of melilotic acid (IV), *p*-coumaric acid and a trace of *o*-coumaric acid (II) and *p*-hydroxybenzoic acid.

Similar experiments in which *o*-coumaric acid (II) was incubated with *A. niger* showed that approximately 72 per cent of the substrate was converted to 4-hydroxycoumarin (V). 4-Hydroxycoumarin itself was not found to be further metabolized by this organism during 5 days' incubation.

When coumarin (III) was used as substrate, large amounts of melilotic acid were isolated; in addition, the presence of small amounts of *o*-coumaric acid and traces of 4-hydroxycoumarin and catechol were also detected. Traces of two other products were also noted on paper chromatograms, but have not been identified.



(IV) Melilotic acid



(V) 4-Hydroxycoumarin

DISCUSSION

Hydroxylation of the aromatic nucleus is now generally recognized as being one of the initial steps in the degradation of aromatic compounds by bacteria and this appears to be the case with the fungus *A. niger*. Studies of the metabolism of anisole and phenoxyacetic acid by this organism have indicated that hydroxylation occurred predominantly in the *o*-position.[2] In this respect the results are similar to those obtained when these compounds are hydroxylated by the ascorbic acid–ferrous ion system of Udenfriend[5] and Fenton's reagent.[6, 7] Such comparisons were valid in the case of anisole and phenoxyacetic acid, as the metabolism of these compounds by *A. niger* does not appear to be continued beyond the monohydroxylated stage. This has not been found to be the case with *trans*-cinnamic acid, where a large portion of the substrate was found to be degraded beyond the phenolic acid stage and only small amounts of melilotic acid, *p*-coumaric acid, *o*-coumaric acid and *p*-hydroxybenzoic acid were detected. But it is still possible that the main product is the *o*-isomer which rapidly undergoes β-oxidation to salicylic acid.

Salicylic acid itself has been found to be readily utilized by *A. niger*.[3] The *p*-hydroxybenzoic acid detected probably arises by β-oxidation of *p*-coumaric acid. *p*-Hydroxybenzoic acid was converted to protocatechuic acid by *A. niger*.[3]

The metabolism of *o*-coumaric acid by *A. niger* resembles that of the rabbit liver system described by Mead *et al.*[8] in the formation of 4-hydroxycoumarin, but 7-hydroxycoumarin was not obtained under the conditions used. However, the formation of some 7-hydroxycoumarin cannot be ruled out as this compound was found to be further degraded by *A. niger* and is not an end product. Mead *et al.*[8] suggested that *o*-coumaric acid undergoes the normal process of β-oxidation but is not finally oxidized to salicylic acid, instead, part of the end form of *o*-hydroxyphenyl-β-oxopropionic acid, which is one of the intermediates, cyclizes to give 4-hydroxycoumarin. The only evidence against this is that intermediates in the oxidation

[5] R. J. W. BYRDE and D. WOODCOCK, *Biochem. J.* **65**, 682 (1957).
[6] G. A. HAMILTON and J. P. FRIEDMAN, *J. Am. Chem. Soc.* **85**, 1008 (1963).
[7] R. O. C. NORMAN and G. K. RADDA, *Proc. Chem. Soc.* 138 (1962).
[8] J. A. R. MEAD, J. N. SMITH and R. T. WILLIAMS, *Biochem. J.* **68**, 67 (1958).

of fatty acids are not usually known to accumulate and substrates do not appear to leave the fatty acid spiral until β-oxidation is complete. However, the cyclization may occur while the compound is still attached to the enzyme and the 4-hydroxycoumarin so formed is released as it no longer forms a substrate for the next "enzymic receiver".

The conversion of *o*-coumaric acid to 4-hydroxycoumarin by *A. niger* is also interesting for other reasons. *A. niger* strains are almost ubiquitous in distribution, and probably contribute to the formation of 4-hydroxycoumarin and thence dicoumarol[9] the active principle[10] in "spoiled" sweet clover (*Melilotus alba*) hay. Derivatives of 4-hydroxycoumarin and dicoumarol are used clinically as anticoagulants and the ready conversion of *o*-coumaric acid to 4-hydroxycoumarin by *A. niger* may provide an easy microbiological method for their preparation.

The initial steps in the metabolism of coumarin by *A. niger* differs from results obtained with the model systems and animal systems which give 3-, 5-, 6-, 7- and 8-hydroxycoumarins.[8] In none of these cases was hydroxylation at the 4-position found to occur even in trace amounts, and β-oxidation products and melilotic acid were also not found. The traces of 4-hydroxycoumarin probably arises as a metabolite of *o*-coumaric acid which was also identified in trace amounts. It is unlikely that direct hydroxylation would occur at the 4-position, a position of low-charge density in the coumarin molecule,[8] since the evidence obtained by the action of *A. niger* on anisole and phenoxyacetic acid resembles the action of an electrophilic hydroxylating species.

The traces of catechol detected in the metabolism of coumarin could arise from the oxidative decarboxylation of salicylic acid. Salicylic acid itself was not detected but would arise from the β-oxidation of melilotic acid, *o*-coumaric acid or from *cis*-*o*-hydroxycinnamic acid formed by the opening of the lactone ring of coumarin. The presence of a trace of *o*-coumaric acid indicates that some isomerization of *cis*-isomer has occurred. None of these transformations were detected in the control experiments carried out in the absence of the fungus.

EXPERIMENTAL

Stationary cultures of *A. niger* van Tiegh (mulder strain) were grown in 250-ml conical flasks on the medium used by Byrde *et al.*,[11] for 3 days at 25°. The fungal mats were then washed and reincubated with an aqueous (0·001 M, pH 7) solution of the substrate (50 ml per flask). Routine determinations of the u.v. spectra of the incubation medium were carried out on aliquots removed at intervals, using an Unicam S.P. 800 automatic recording spectrophotometer. After 5 days' incubation at 25°, the substrate solution was filtered through muslin, acidified and extracted with chloroform. For isolation of substrate and products, the medium was acidified to pH 2 and extracted with 3 × 200 ml chloroform. The chloroform was removed on a rotary evaporator, and the residue in 2 ml ethanol for chromatography.

Paper chromatograms were developed by downward migration of the solvent. Sheets of Whatman No. 3 MM paper were used for preparative work in the isolation of melilotic acid and 4-hydroxycoumarin. For other purposes, Whatman No. 1 and No. 4 were used. The solvent systems used were: (1) 2% acetic acid, (2) *n*-butanol–ethanol–3 N ammonia (4:1:5), (3) ethanol–ammonia–water (20:1:4) and (4) benzene–acetic acid–water (125:72:3).

[9] R. Anschutz, *Ber. Deut. Chem. Ges.* **36**, 463 (1903).
[10] K. P. Link, *Harvey Lectures* **39**, 162 (1944).
[11] R. J. W. Byrde, J. F. Harris and D. Woodcock, *Biochem. J.* **64**, 154 (1956).

Thin-layer chromatography was carried out using two solvent systems: (1) chloroform–acetic acid–water (4:1:1) with silica gel plates and (2) *n*-butanol–ethanol–3 N ammonia (4:1:5) with cellulose powder plates. Compounds were detected by examination of the plates or papers under u.v. light and fluorescent spots marked before and after spraying with 2 N sodium hydroxide. The position of phenols and coumarins were also detected with diazotized *p*-nitroaniline[12] and ferric chloride–ferricyanide spray[13] and acids with bromocresol green.[12]

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