Mechanism of Aromatic Hydroxylation in Fungi. Evidence for the Formation of Arene Oxides

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Aromatic and aliphatic hydroxylations as well as *O*-demethylations occurred during metabolism of aromatic substrates by a range of fungi. *para*-Hydroxylation of monosubstituted aryl rings by fungi proceeded with migration and retention of deuterium label (NIH shift) and appeared to involve only arene 3,4-oxide intermediates, in common with plant and animal metabolism. The possibility of contributions from arene 1,2- or 2,3-oxide intermediates during *ortho*-hydroxylation is examined in the light of the results of oxygen-18 incorporation, the lack of deuterium–hydrogen primary kinetic isotope effects, and the wide range of deuterium migration and retention values. From these last experimental data no evidence for an arene oxide isomerase enzyme was found.

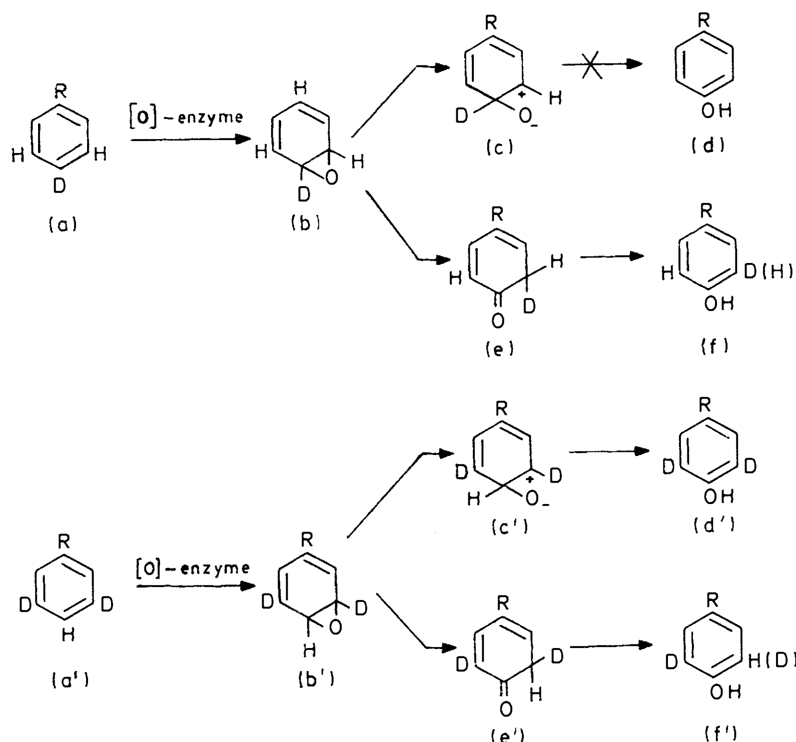
THE intermediacy of arene oxides in mono-oxygenase-catalysed hydroxylation of natural aromatic substrates has previously been suggested for animals,[1] plants,[2] and the micro-organisms *Pencillium cyclopium* [3] and a *Pseudomonas* species.[4] However, despite many examples of aromatic hydroxylation by fungi,[5] prior to the preliminary publication of the present work [6] investigations on the mechanism of aromatic hydroxylation during metabolism of xenobiotic substances by fungi had not been reported.

An initial objective of this study was the identification of suitable aromatic substrates with low toxicity towards fungi. Such substrates could then be used to screen a range of fungi for the potential to catalyse aromatic hydroxylation without problems of toxicity. From studies of the dose-dependence of the effects of several potential substrates on the mycelium of the fungi, anisole was found to be suitable. A series of comparative experiments established that a shake culture method, involving a 2—3 day culture and then a transformation period of 2 days, was most convenient for aromatic hydroxylation. The dependence of total yields and relative ratios of phenol, *ortho*-, *meta*-, and *para*-hydroxylation products upon pH,[7] fungal strain,[8] and incubation time [7] had been established previously. The relative yields of *o*-, *m*-, and *p*-hydroxylated anisoles with ten fungi are shown in Table 1. *ortho*-Hydroxylation was found to be generally preferred to *para*-hydroxylation. In addition, the present experiments showed that *O*-demethylation occurred in preference to aromatic hydroxylation during the early stages (spores and germlings) of fungal growth. All the fungi selected for the present studies had previously been shown to effect several oxygen atom transfer reactions in addition to the aromatic hydroxylation of anisole shown in Table 1.

The initial enzymic formation of arene oxides as intermediates in the biosynthesis of phenolic products has been inferred from the intramolecular migration of deuterium from the position at which hydroxylation occurs to an adjacent carbon atom (NIH shift;[9] Scheme 1). This pathway normally involves rearrangement of the arene oxide [(b) or (b′)] to the keto tautomer [(e) or (e′)] *via* a 1,2-shift of the substituent, which the enolizes to the phenol [(f) or (f′)]. The initial extent of migration

[1] G. Guroff, J. W. Daly, D. M. Jerina, J. Renson, S. Udenfriend, and B. Witkop, *Science*, 1967, **157**, 1524.
[2] M. H. Zenk, *Z. Pflanzenphysiol.*, 1967, **57**, 477.
[3] L. Nover and M. Luckner, *F.E.B.S. Letters*, 1969, **3**, 292.
[4] G. Guroff and J. Daly, *Arch. Biochem. Biophys.*, 1969, **122**, 212.
[5] G. S. Fonken and R. A. Johnson in 'Chemical Oxidation with Microorganisms,' Dekker, New York, 1972, p. 127.
[6] B. J. Auret, D. R. Boyd, P. M. Robinson, C. G. Watson, J. W. Daly, and D. M. Jerina, *Chem. Comm.*, 1971, 1585.
[7] S. M. Bocks, J. R. Lindsay-Smith, and R. O. C. Norman, *Nature*, 1964, **201**, 398.
[8] S. M. Bocks, *Phytochemistry*, 1967, **6**, 785.
[9] J. W. Daly, D. M. Jerina, and B. Witkop, *Experientia*, 1972, 1129.

[(b) ⟶ (e) or (b′) ⟶ (e′)] relative to the proportion of direct loss of a proton or a deuteron [(b) ⟶ (c) ⟶ (d) or (b′) ⟶ (c′) ⟶ (d′)], as well as the magnitude of the kinetic isotope effect during enolization of (e) or (e′), controls the degree to which the migrant atom is retained in the phenolic product.



SCHEME 1 *para*-Hydroxylation

para-Hydroxylation of [4-^{2}H]anisole (a) was found to occur in the presence of fungi 1—5 (Table 1) and g.l.c.–mass spectrometric analysis of the products revealed that the deuterium atom had undergone the NIH shift to the extent of *ca.* 70%. This value is close to that obtained after *para*-hydroxylation of anisole by liver microsomes from mammals.[9] The similarity in magnitude of the NIH shift for *para*-hydroxylation of fungi 1—5 (69—72%), taken in conjunction with a similar result recently obtained for the fungus *Cunninghamella bainieri* (73%),[10] supports the case for the formation of an arene 3,4-oxide intermediate during *para*-hydroxylation of anisole by fungi [(a) ⟶ (b) ⟶ (e) ⟶ (f)].

TABLE 1

Migration and retention of deuterium in phenolic metabolites of selectively labelled anisole with various fungi [a]

Fungus	Relative proportions of o-OH anisole	Relative proportions of p-OH anisole	% ^{2}H retained in *o*-OH product from [2-^{2}H]-anisole	% ^{2}H retained in *p*-OH product from [4-^{2}H]-anisole
1, *Aspergillus niger*	+	+++	65	71
2, *Aspergillus foetidus* [b]		+	*c*	69
3, *Rhizopus arrhizus*	+++	+	53	72
4, *Cunninghamella elegans*	++	+	57	71
5, *Mucor parasiticus*	+	+	35	72
6, *Helicostylum piriforme*	+		40	*c*
7, *Rhizopus stolonifer*	++	+	39	*c*
8, *Sporotrichum sulphurescens*	+		52	*c*
9, *Curvularia falcata*	+		40	*c*
10, *Trichothecium roseum*	+		67	*c*

[a] The deuterium retention results are corrected to account for the original deuterium incorporation being <100%. [b] Originally supplied from the culture collection as *Aspergillus niger* NRRL 337. [c] The quantity of hydroxylated product isolated was insufficient to provide a meaningful ^{2}H analysis. Values are generally accurate to within ±5%.

When *para*-hydroxylation of [3,5-$^2H_2$]anisole (a′) was effected by *Aspergillus niger*, one deuterium atom was entirely retained in the product, while 36% of the other deuterium atom was lost; *i.e.* 64—72% deuterium retention occurred on *para*-hydroxylation irrespective of whether the deuterium atom was initially at position 3 or 4. These results suggest that hydrogen or deuterium migrate equally well to form (e) or (e′) from the arene 3,4-oxide which is common to both substrates. Loss of hydrogen or deuterium from the site of hydroxylation to form a phenol directly from the carbocation ion [(c) ⟶ (d) or (c′) ⟶ (d′)] therefore must be minimal in these fungi.

The stereospecificity of isomerization of the intermediate arene oxide to *p*-hydroxylated product is attributed to the electronic influence of the methoxy-group.

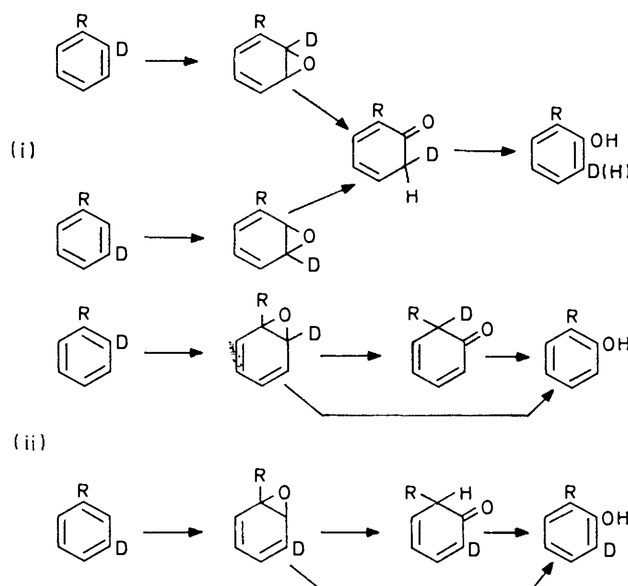
[10] J. P. Ferris, M. J. Fasco, F. L. Stylianopoulou, D. M. Jerina, J. W. Daly, and A. M. Jeffery, *Arch. Biochem. Biophys.*, 1973, **156**, 97.

It has been proposed that stereospecific isomerization of an arene oxide intermediate with specific removal of the hydrogen atom attached to the carbon atom adjacent to the position of hydroxylation occurs in aromatic hydroxylation during the biosynthesis of the alkaloids capsaicin and norpluviine.[11] It was further suggested [11] that an arene oxide isomerase enzyme might render these isomerizations stereospecific. The intact metabolizing system in the fungus *A.niger* appeared well suited for an examination of this interesting possibility in micro-organisms.

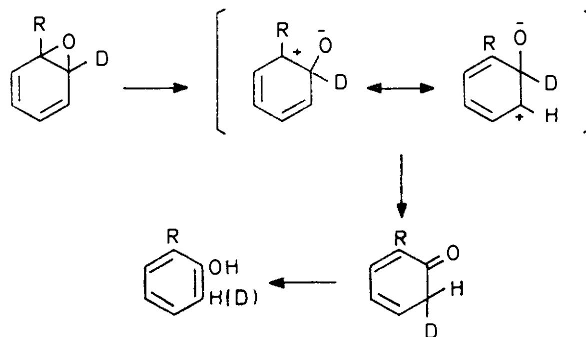
In the presence of an isomerase enzyme, total removal of a labelled hydrogen atom from the carbon atom adjacent to the position of hydroxylation during metabolism of [3,5-$^2H_2$]anisole by *A. niger* would have been anticipated. In practice only 36 and 15% of one of the adjacent deuterium atoms was lost during *para*- and *ortho*-hydroxylation, respectively. A similar experiment with *R. arrhizus* unfortunately gave insufficient 4-hydroxyanisole, and only 18% loss of deuterium was observed in the isolated 2-hydroxyanisole. Thus, it may be concluded that the migration and retention of label during hydroxylation of anisole by *A. niger* and *R. arrhizus* is not controlled by an arene oxide isomerase capable of stereospecific abstraction of a hydrogen atom adjacent to the position of hydroxylation. This result, allied to analogous observations with *C. bainieri* [10] and a *Pseudomonas* culture,[12] suggests that micro-organisms do not possess arene oxide isomerase enzymes. Studies with plant and animal systems have also failed to produce evidence for an arene oxide isomerase enzyme.[13]

The literature on the mechanism of enzymic aromatic hydroxylation deals primarily with *para*-hydroxylation,[9,14] owing to a preference for *para*- rather than *ortho*-hydroxylation during metabolism of aromatic rings by animals. For *para*-hydroxylation a single arene oxide intermediate is possible. The results in Table 1 show that *ortho*-hydroxylation is more often preferred in fungi. For *ortho*-hydroxylation either of two different arene oxides may be involved: a 2,3- or a 1,2-oxide. Isomerization studies of 1-methyl- and 3-methyl-benzene oxide have shown that *ortho*-cresol is obtained exclusively from both arene oxides.[15] From a comparison of the liver microsomal metabolites of alkyl-substituted benzenes with the rearrangement products of the possible arene oxides, it was concluded that *ortho*-hydroxylation of methyl-substituted benzene rings (*e.g.* toluene) by mammals would occur predominantly *via* arene 2,3-oxides.[15] This proposal, if applicable both to fungi and to other substituted benzenes, would lead to a significant retention of deuterium label originally at either the 2- or the 3-position during *ortho*-hydroxylation [Scheme 2 (i)].

The proportion of deuterium retained during *ortho*-hydroxylation of [2-^{2}H]anisole by fungi 1—10, although variable (35—67%), is consistent with a pathway involving a 2,3-oxide intermediate. A 60% retention was obtained with liver microsomes.[9] The lower values



SCHEME 2 NIH shift during *ortho*-hydroxylation



SCHEME 3 Possible mechanism for NIH shift during isomerization of 1,2-oxide

with fungi may be due to an additional contribution from a pathway involving a 1,2-oxide [Scheme 2 (ii)] or to a small amount of direct hydroxylation.[16,17] Isomerization of a 1,2-oxide would probably lead to complete loss of deuterium. Although a migration to the 3-position is theoretically possible (Scheme 3), such a migration has yet to be demonstrated. This point will be further discussed during consideration of the results of fungal metabolism of aromatic carboxylic acids.

Since the NIH shift is a feature of both *ortho*- and *para*-hydroxylation of anisole with fungi, a more detailed examination of one micro-organism, *Aspergillus niger*, was carried out with a range of substrates. *A. niger* was

[11] W. R. Bowman, I. T. Bruce, and G. W. Kirby, *Chem. Comm.*, 1969, 1075.
[12] W. R. Bowman, W. R. Gretton, and G. W. Kirby, *J.C.S. Perkin I*, 1973, 218.
[13] D. J. Reed, J. Vimmerstedt, D. M. Jerina, and J. W. Daly, *Arch. Biochem. Biophys.*, 1973, **154**, 642.
[14] D. M. Jerina and J. W. Daly, *Science*, 1974. **185**, 573.
[15] N. Kaubisch, J. W. Daly, and D. M. Jerina, *Proc. Nat. Acad. Sci. U.S.A.*, 1971, **68**, 2545.
[16] J. E. Tomazewski, D. M. Jerina, and J. W. Daly, *Biochemistry*, 1975, **14**, 2024.
[17] H. G. Selander, D. M. Jerina, and J. W. Daly, *Arch. Biochem. Biophys.*, 1975, **168**, 309.

selected since it is a common fungus and capable of a wide range of metabolic transformations. Thus oxygen atom transfer enzymes in *A. niger* catalyse the conversions of methyl [18] and methylene [19] groups into alcohol functions, of olefins into diols,[20] of sulphides into sulphoxides,[21] of sulphoxides into sulphones,[22] of aldehydes into carboxylic acids,[23] and of alcohols into carboxylic acids,[23] as well as *O*-demethylation of ethers [7] and *S*-demethylation of thioethers.[24] Since *A. niger* appears capable of effecting many mono-oxygenase-catalysed transformations with a wide range of substrates and since, in contrast to most of the other fungi studied (Table 1), *A. niger* has a preference for *para*-hydroxylation of anisole, it shows a close similarity to the mono-oxygenases present in mammalian liver.

Metabolism studies with *A. niger* and a number of specifically deuteriated aromatic compounds (most of which were previously reported to be acceptable substrates [7,8]) were carried out. The results (Table 2) indicate that *O*-demethylation occurred during metabolism of anisole by *A. niger* and toluene was metabolized in part to benzyl alcohol, *i.e.* aliphatic hydroxylation occurred. All the substrates in Table 2 were hydroxylated on the aromatic ring. The low deuterium retention values obtained after *ortho*-hydroxylation of [2-^{2}H]-toluene (11%), [2,4-$^2H_2$]- and [2,6-$^2H_2$]-phenoxyacetic acid (5 and 14%, respectively), [6-^{2}H]-2-naphthyloxyacetic acid (8%), and [2-^{2}H]phenylacetic acid (7%) deserve further comment. The intermediacy of an arene 1,2-oxide would, as shown in Scheme 2 (ii), provide one explanation for the low deuterium retention which occurs during *ortho*-hydroxylation of toluene, phenoxyacetic acid, and phenylacetic acid. A similar explanation could not however be used for the low deuterium retention observed on 6-hydroxylation of [6-^{2}H]-2-naphthyloxyacetic acid by *A. niger* (Table 2).

TABLE 2

Migration and retention of deuterium in phenolic metabolites of selectively deuteriated substrates with *Aspergillus niger* *

	^{2}H retained on *o*-hydroxylation	^{2}H retained on *p*-hydroxylation	Additional metabolites
[4-^{2}H]Anisole	65	71	PhOH
[2-^{2}H]Anisole			PhOH
[4-^{2}H]Toluene		49	$PhCH_2OH$
[2-^{2}H]Toluene	11		$PhCH_2OH$
[2,4-$^2H_2$]Phenoxy-acetic acid	4,† 5	64	
[3,5-$^2H_2$]Phenoxy-acetic acid	97	69	
[2,6-$^2H_2$]Phenoxy-acetic acid	14	94	
[2-^{2}H]Phenylacetic acid	7 †	‡	
[4-^{2}H]Phenylacetic acid	89	‡	
[3-^{2}H]Phenylacetic acid	93	‡	
[6-^{2}H]-2-Naphthyl-oxyacetic acid	13 †		

* Deuterium analysis was carried out by g.l.c.–mass spectrometry of the phenolic components; phenolic acids were prepurified by paper chromatography and analysed as trimethylsilyl derivatives. † Deuterium analysis was carried out by direct mass spectrometry of product isolated by paper chromatography. ‡ A large impurity peak was consistently found to coincide with that of the phenolic product, and prevented a meaningful ^{2}H analysis.

Phenoxyacetic acid and phenylacetic acid were not substrates for aromatic hydroxylation by liver microsomes and thus it was not possible to compare the deuterium retention values for *ortho*-hydroxylation with results obtained with *A. niger*. Aromatic hydroxylation of [2,4-$^2H_2$]phenoxyacetamide and [6-^{2}H]-2-naphthyloxyacetamide could, however, be effected by liver microsomes; retentions of 77% (*para*-hydroxylation) and 45% (6-hydroxylation), respectively, were observed. Hydroxylation at the 6-position of the naphthalene ring proceeded with a considerably higher migration and retention of deuterium in the hepatic system as compared with to the very low retention obtained on 6-hydroxylation of [6-^{2}H]-2-naphthyloxyacetic acid by *A. niger* (13%). A possible rationalization for the abnormally low retention values associated with the fungus is that a different or additional enzymic mechanism is operating both during *ortho*-hydroxylation of aryl acids and during 6-hydroxylation of 2-naphthyloxyacetic acid.

The *ortho*-hydroxylation of phenoxyacetic acid by *A. niger* was established as an oxygenase reaction by the quantitative incorporation of molecular oxygen (18% $^{18}O_2$ enriched) into the product phenol (17% $^{18}O_2$ enriched). Furthermore, a metabolic pathway involving enzymic or nonenzymic hydration of an arene oxide followed by dehydration to a phenol [25] with incorporation of solvent water and loss of label from the site of hydroxylation can now be excluded.

In addition to the arene oxide intermediate route to phenols, a direct hydroxylation pathway involving insertion of an oxygen atom into the carbon–hydrogen bond has previously [16,17] been considered as a possible mechanism for enzymic aromatic hydroxylation. Direct hydroxylation necessarily occurs without migration and retention of deuterium, and would account partially for the unusually low retention values during hydroxylation of the aromatic acids.

In order to test whether the incorporation of one atom of molecular oxygen on *ortho*-hydroxylation of aryl-substituted carboxylic acids by *A. niger* was associated with a primary isotope effect, mixtures (1.0 : 1.0) of [$^2H_7$]phenylacetic acid ($C_6D_5 \cdot CD_2 \cdot CO_2H$) with unlabelled

[18] H. L. Holland and B. J. Auret, *Canad. J. Chem.*, 1975, **53**, 845.

[19] J. Fried, R. W. Thoma, J. R. Gerke, J. E. Hertz, M. N. Donin, and D. J. Perlman, *J. Amer. Chem. Soc.*, 1952, **74**, 3962.

[20] D. R. Clifford, J. K. Faulkner, J. R. L. Walker, and D. Woodcock, *Phytochemistry*, 1969, **8**, 549.

[21] B. J. Auret, D. R. Boyd, H. B. Henbest, and S. Ross, *J. Chem. Soc.* (*C*), 1968, 2371.

[22] B. J. Auret, D. R. Boyd, and H. B. Henbest, *J. Chem. Soc.* (*C*), 1968, 2374.

[23] F. Challenger, V. Subramaniam, and T. K. Walker, *Nature*, 1927, **119**, 674.

[24] H. Kexel and H. L. Schmidt, *Biochem. Pharmacol.*, 1972, **21**, 1009.

[25] G. J. Kasperek, T. C. Bruice, H. Yagi, N. Kaubisch, and D. M. Jerina, *J. Amer. Chem. Soc.*, 1972, **94**, 7876.

phenylacetic acid and of [2,6-$^2H_2$]phenoxyacetic acid with normal phenoxyacetic acid were examined as substrates. The *ortho*-hydroxylated product mixture showed no significant isotope effect in either case (1.0 : 1.0 and 1.0 : 1.1, respectively). Although a positive isotope effect can be taken as evidence for an insertion pathway, the lack of an isotope effect in the present context, while compatible with the formation of an arene oxide intermediate, does not exclude a direct insertion mechanism.[16]

As with the results of metabolism of anisole with *A. niger*, on *para*-hydroxylation of [2,4-$^2H_2$]phenoxyacetic acid and [3,5-$^2H_2$]phenoxyacetic acid, the magnitude of deuterium retention was almost the same (64 and 69%, respectively) regardless of whether the deuterium was initially at the 3- or the 4-position. This result is only consistent with an arene oxide pathway. The presence of a carboxy-substituent on the aromatic substrate does not therefore necessarily eliminate an arene oxide intermediate mechanism for aryl hydroxylation. A similar conclusion may also be drawn from the report[26] of *ortho*-hydroxylation of [2-^{3}H]cinnamic acid in higher plants, where a high proportion of migration and retention of tritium at the *ortho*-position led to *o*-coumaric acid with >90% tritium retained. It is of interest however that salicylic acid, again derived from *ortho*-hydroxylation of [2-^{3}H]-cinnamic acid in higher plants, showed a large (65—74%) loss of tritium label.[26]

In summary, pathways involving arene 1,2-oxide intermediates or direct insertion, while unusual in mammalian metabolism, seem to provide the most attractive explanations for the low retentions on *ortho*-hydroxylation of the phenyl- and phenoxy-acetic acids by *A. niger*. Similarly, an insertion pathway appears to be a major route for 6-hydroxylation of 2-naphthyloxyacetic acid.

TABLE 3

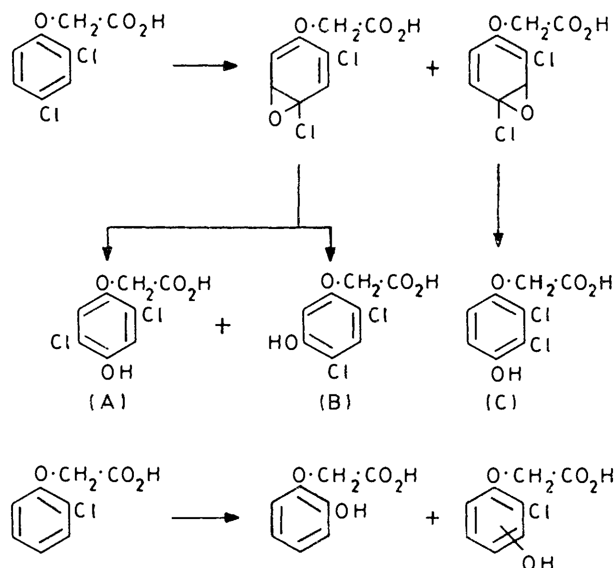
Metabolites from 2,4-dichlorophenoxyacetic acid †

	Metabolites	Ref.
1, *Aspergillus niger* (fungus)	(A) + (B)	*a*
2, *Phaseolus vulgaris* (bean)	(A) + (B)	*b*
	(A) + (C)	*c*
	(A) + (C)	*d*
3, *Zea mays* (corn)	(A) + (C)	*d*
4, *Poa praten* (blue grass)	(A) + (C)	*d*
5, *Glycine max* (soybean)	(A) + (C)	*e*

† See Scheme 4 for structures (A)—(C).

[a] J. F. Faulkner and D. Woodcock, *J. Chem. Soc.*, 1965, 1187. [b] E. W. Thomas, B. C. Loughman, and R. G. Powell, *Nature*, 1964, 884. [c] R. H. Hamilton, J. Hunter, J. K. Hall, and C. D. Ercegovich, *J. Agric. Food Chem.*, 1971, **19**, 480. [d] M. L. Montgomery, Y. L. Chang, and V. H. Freed, *J. Agric. Food Chem.*, 1971, **19**, 1219. [e] C. S. Feung, R. H. Hamilton, F. H. Whitham, and R. O. Mumma, *Plant Physiol.*, 1972, **50**, 80.

This study of the metabolism of phenoxyacetic acid by *Aspergillus niger* provides an opportunity for a brief summary and, based upon the present conclusions, an interpretation of the literature reports on the related and widely used herbicide 2,4-dichlorophenoxyacetic acid and other chlorinated phenoxyacetic acids (Table 3). In plants, 2,4-dichlorophenoxyacetic acid is metabolized almost exclusively by 4-hydroxylation and concomitant chlorine migration to the 3- or 5-position. In the fungus *A. niger*, 5-hydroxylation without chlorine migration is preferred, although 4-hydroxylation and chlorine migration do occur. The metabolic products (A)—(C) could all be derived from the two arene oxides shown in Scheme 4.



SCHEME 4 Products of aromatic hydroxylation of chlorinated phenoxyacetic acids

The loss of a halogen atom during hydroxylation does not appear to be a major pathway, but has been reported for other aromatic hydroxylations, *e.g.* formation of tyrosine as a minor product from *p*-chlorophenylalanine with phenylalanine hydroxylase from a *Pseudomonas* species.[27] The metabolism of 2-chlorophenoxyacetic acid by *A. niger* parallels that of 2,4-dichlorophenoxyacetic acid in the preferential hydroxylation without chlorine migration to yield 3-, 4-, 5-, and 6-monohydroxylated 2-chlorophenoxyacetic acids.[28] The isolation of the additional product 2-chlorophenoxyacetic acid, being an example of *ortho*-hydroxylation with total loss of *ortho*-substituent, is noteworthy in view of the loss of deuterium atom from [2-^{2}H]phenoxyacetic acid which is observed in the present work. *ortho*-Hydroxylation with loss of chlorine atom was confirmed by g.l.c.–mass spectrometric analysis when 2-chlorophenoxyacetic acid was metabolized by *A. niger* 382 under our standard experimental conditions.

Although, by analogy with our studies on phenoxyacetic acid metabolism by *A. niger*, an arene 1,2-oxide at first seems to be a reasonable intermediate, Cl^+ must act as a leaving group to achieve the loss of chlorine, and this seems rather improbable. The mechanisms for hydroxylation and concomitant loss of halogen from

[26] B. E. Ellis and N. Amrhein, *Phytochemistry*, 1971, **10**, 3069.
[27] G. Guroff, K. Kondo, and J. Daly, *Biochem. Biophys. Res. Comm.*, 1966, **25**, 622.
[28] J. K. Faulkner and D. Woodcock, *J. Chem. Soc.*, 1961, 5397.

aromatic rings in this and other biological systems will thus require further investigation.

EXPERIMENTAL

All substrates and products described in this paper had previously been synthesized in either deuterium-labelled or non-labelled form. All mass spectrometric analyses were carried out on A.E.I. MS902, A.E.I. MS30 (g.l.c.–mass spectrometer), or LKB 9000 (g.l.c.–mass spectrometry) spectrometers operated at 70 eV unless otherwise stated. In paper chromatographic separations the descending technique, and Whatman no. 1 (analytical) or Whatman no. 3 (preparative) paper were used. T.l.c. analysis was carried out on silica gel (Merck $_{254-366}$ fluorescent 5 × 20 cm) plate.

Microbiological Techniques.—The strain of fungi used in the present work were: 1, *Aspergillus foetidus* NRRL 337 (originally purchased as *A. niger*, NRRL 337); 2, *Aspergillus niger* 382 (previously [6] incorrectly described as NRRL 382); 3, *Rhizopus arrhizus* ATCC 11145; 4, *Cunninghamella elegans* ATCC 9245; 5, *Mucor parasticus* ATTCC 647b; 6, *Helicostylum piriforme* ATCC 8992; 7, *Rhizopus stolonifer* ATCC 6277-b; 8, *Sporotrichum sulphurescens* ATCC 7159; 9, *Curvularia falcata* CBS; 10, *Trichothecium roseum* ATCC 8685. Fungi 3, 4, 5, 6, 7, and 8 were obtained from the American Type Culture Collection, Washington, D.C. Fungus 1 was obtained from the Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois. Fungus 2 was obtained from the Laboratory of Industrial Microbiology, Faculty of Technology, University of Zagreb 4100, Yugoslavia. Fungus 9 was obtained from the Central-bureau voor Shimmelcultures, Baarn, Holland. The growth, substrate addition, biotransformation, and dichloromethane extraction were typically carried out in the manner previously described.[22]

Synthesis of Deuterium-labelled Molecules.—This was carried out by two standard methods: (I) reductive deuteriolysis of the corresponding aryl halide with deuterium gas (99.8% $^2H_2$; Isomet) and Pd–C catalyst as described previously;[10,29,30] (II) decomposition of the Grignard reagent formed from aryl bromides by D_2O (99.8%; Norsk Hydro). The percentage deuterium incorporations achieved by method (I) were: [2-^{2}H] anisole, >97%; [3,5-$^2H_2$]-anisole, >97%; [4-^{2}H]anisole, >97%; [2,4-$^2H_2$]phenoxyacetic acid, 97% $^2H_2$; [3,5-$^2H_2$]phenoxyacetic acid, 88% $^2H_2$; [2,4-$^2H_2$]phenoxyacetamide, 97% $^2H_2$; [6-^{2}H]-2-naphthyloxyacetic acid, >97%; [6-^{2}H]-2-naphthyloxyacetamide, >97%. The deuterium incorporations obtained by method (II) were generally lower: [2-^{2}H]toluene, 81%; [3-^{2}H]toluene, 77%; [4-^{2}H]toluene, 90%. [2-^{2}H]-, [3-^{2}H]-, [4-^{2}H]-, and [$^2H_8$]-phenylacetic acids were formed from the corresponding labelled toluenes by the literature route,[31] and contained the same percentages of deuterium atoms, *i.e.* 81, 77, 90, and 97%, respectively. Samples of the pure phenolic products were obtained commercially or by synthesis according to literature methods.[32,33]

Separation and Identification of Hydroxylated Products.—Hydroxylated anisoles and cresols were isolated from the dichloromethane extracts of the acidified mycelium and culture medium by basic extraction (2N-NaOH). Acidification, extraction, and concentration of the basic extracts yielded a crude mixture of phenols. T.l.c. analysis of hydroxyanisoles and cresols was carried out on silica gel plates with benzene–chloroform–ethyl acetate (1 : 5 : 1) and benzene–ethyl acetate (19 : 1) as eluants, respectively. An analysis of the hydroxylated phenoxy-, 2-naphthyloxy-, and phenyl-acetic acids was obtained by paper chromatography with the solvent systems reported.[8]

The phenolic products were detected by u.v. light (Hanovia Chromatolite), and spraying with *N*,2,6-trichlorobenzoquinone imine (1% in ethanol) followed by exposure to ammonia vapour, or *p*-nitrobenzenediazonium tetrafluoroborate (1% in acetone) followed by exposure to KOH (10% in MeOH).

Hydroxyanisoles and cresols were further separated by g.l.c. on a 3% ECNSS-M column at 160 °C or a 3% XE-60 column at 160 °C (hydroxyanisoles), or a 3% tricresyl phosphate column at 135 °C (cresols).

Hydroxylated products from metabolism of phenoxy-, 2-naphthyloxy-, and phenyl-acetic acids were purified initially by preparative paper chromatography and then analysed directly by mass spectrometry. The *ortho*-phenols from phenoxy- and phenyl-acetic acids were predominant and clearly separated from the *meta*- and *para*-isomers. 6-Hydroxy-2-naphthyloxyacetic acid was the sole phenolic product from 2-naphthyloxyacetic acid.

The phenolic products from the carboxylic acid substrates were also analysed by g.l.c.–mass spectrometry, after treatment with bis(trimethylsilyl)acetamide in pyridine to produce the volatile trimethylsilyl ether and trimethylsilyl ester derivatives. The silylated derivatives of the hydroxylated phenoxy-, naphthyloxy-, and hydroxyphenylacetic acids were separated on a 3% OV-1 column at 170, 230, and 180 °C, respectively.

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