Side Chain Hydroxylation of Aromatic Hydrocarbons by Fungi. Part 2.[1] Isotope Effects and Mechanism

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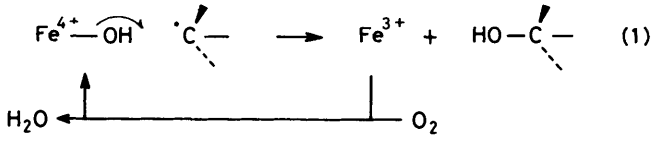
The benzylic hydroxylation of ethylbenzene, *p*-diethylbenzene, tetralin, indane, and toluene by the fungi *Mortierella isabellina*, *Cunninghamella echinulata*, and *Helminthosporium* species has been investigated by the use of deuterium-labelled substrates. An intermolecular primary isotope effect k_H/k_D 1.04 ± 0.05 was observed for conversion of ethylbenzene into 1-phenylethanol. The secondary deuterium isotope effect for this conversion was large and positive (k_H/k_D averaging 1.27 ± 0.05). *p*-Diethylbenzene, tetralin, and indane were hydroxylated at the benzylic position with an intramolecular primary deuterium isotope effect averaging 2.6 ± 0.2. The use of *R*-(−)- and *S*-(+)-1-deuterioethylbenzenes as substrates with the fungus *M. isabellina* show that hydrogen removal is specific for the *pro-R* position of substrate. Toluene is converted into benzyl alcohol by the fungi *Mortierella isabellina* and *Helminthosporium* species; in the latter case, the product is further metabolized. [α-^{2}H]-, [αα-$^2H_2$]-, and [ααα-$^2H_3$]-toluene have been used with *M. isabellina* in a series of experiments to determine both primary and secondary deuterium kinetic isotope effects for the enzymic benzylic hydroxylation reaction. The values obtained, intermolecular primary k_H/k_D = intramolecular primary k_H/k_D = 1.02 ± 0.05, and secondary k_H/k_D = 1.37 ± 0.05, lead to the suggestion of a mechanism for benzylic hydroxylation involving benzylic proton removal from a radical-cation intermediate in a non-symmetrical transition state.

In Part 1[1] we reported the benzylic hydroxylation of ethylbenzene and some of its *para*-substituted derivatives, and that of tetralin and indane, by several common fungi. These processes serve both as models for mammalian metabolism,[2] and as indicators for the possible use of these fungi in synthetic or degradative applications with aromatic organic compounds.[3] We have now extended these studies to include toluene, and report herein the results of our experiments with isotopically labelled substrates. The fungal side-chain hydroxylation of toluene has not hitherto been reported, although fungi are capable of oxidizing the methyl group of methyl-substituted naphthalenes,[4] benz[*a*]anthracenes,[5] and thioxanthones,[6] and the fungal hydroxylation of an *N*-acyl-*p*-aminotoluene derivative at the methyl group has recently been reported.[7] The mammalian metabolism of toluene, for which the fungal hydroxylation serves as a model, produces benzoic acid as the ultimate major metabolite,[8] but proceeds by way of cytochrome P-450-dependent hydroxylation of toluene to give benzyl alcohol as the initial metabolite.[9]

Our initial experiments[1] suggested that all these benzylic hydroxylations are performed by cytochrome P-450-dependent hydroxylase enzymes whose normal mode of action is thought to involve a radical reaction between substrate and an activated ferryl ion of the enzyme's active site [equation (1)].[10]

The use of substrate deuterium isotope effects has been pivotal in investigating the mechanism of action of these enzymes.[11,12] In general, primary intermolecular effects are



(1)

small (k_H/k_D < 3), whereas primary intramolecular effects are large (k_H/k_D > 3).[13] Secondary isotope effects have received much less attention, but in a related example (benzylic hydroxylation by the copper-containing enzyme dopamine β-mono-oxygenase), are positive (k_H/k_D 1.19[14]).

The difference in magnitude between isotope effects measured for inter- *versus* intra-molecular competitions in the P-450-dependent hydroxylation reaction has been attributed to the possibility that hydrogen removal is only partly rate-limiting in the catalytic cycle of the enzyme,[15,16] and to the existence of isotope fractionation in substrate binding and other non-rate-limiting steps.[17] The intramolecular effects, which can arise from competition within a single catalytic cycle, are generally regarded as a better estimate of the intrinsic isotope effect for product formation than are the intermolecular effects, which involve competition between successive cycles of the enzyme.[18]

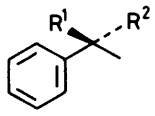
In view of the usefulness of isotopic probes in the elucidation of the mode of action of the hydroxylase enzymes, both from the kinetic and stereochemical viewpoints, we have undertaken a study of the hydroxylation of ethylbenzene, *p*-diethylbenzene, tetralin, indane, and toluene using deuterium-labelled analogues. In view of the severe problems associated with the isolation of mono-oxgenases of fungal origin, we have employed the standard techniques of fungal biotransformation in this study, namely the use of whole-cell methodology. Our earlier data[1] suggest that a single enzyme of *M. isabellina*, readily accessible to the aqueous growth medium, is responsible for the benzylic hydroxylation of a range of alkyl-substituted aromatic compounds. If this is true, then comparison of data obtained for different substrates (*e.g.* ethyl benzene and toluene) is valid. If, however, different enzymes are responsible for the hydroxylation of these substrates, then such comparison is of lesser value. In the absence of purified enzyme preparations, and consequently genetic information, the cross-induction experiments described below were performed in an attempt to address this problem. In any event, in the case of competition experiments between molecules or sites of molecules which differ only in isotopic substitution, the question of transport differences or metabolism by different enzymes does not arise unless isotopically sensitive metabolic branching, giving rise to different product distributions from labelled *versus* non-labelled sub-

strates, is observed.[18] No evidence for this phenomenon was detected during the present study.

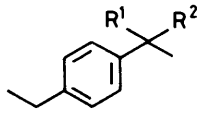
Results and Discussion

Preparation of Substrates.—The syntheses of [1,1-$^2H_2$]ethylbenzene (**12**), [1,1-$^2H_2$]indane (**19**), and [1,1-$^2H_2$]tetralin (**24**) were carried out by parallel routes involving lithium aluminium deuteride reduction of the corresponding ketone, giving the $^2H_1$ alcohols [(**8**)—(**9**), (**15**), (**20**), and (**25**), respectively]. These were then converted into chlorides, and the latter treated with lithium triethylborodeuteride (Super Deuteride) to give the corresponding labelled hydrocarbons in high yield. Racemic [1-^{2}H]ethylbenzene was similarly prepared from 1-phenylethyl chloride.

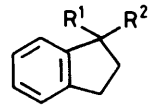
(*S*)-(+)-1-Deuterioethylbenzene (**4**) was synthesized by an analogous route starting from (*S*)-(−)-1-phenylethanol (**7**), and (*R*)-(−)-1-deuterioethylbenzene (**3**) from the corresponding labelled alcohol (**8**) by a route involving reduction of the intermediate chloride (**11**) with Super Hydride. These transformations have been described by others,[19–21] but the reagents and/or conditions used resulted in considerable loss of optical purity during the reaction sequence. The syntheses of (**4**) and its *R*-(−) enantiomer (**3**) described herein, in optical purities of 66 and 60%, respectively, represent the most efficient route yet reported for their direct preparation. The key features are rapid, low-temperature conversion of (**7**) or (**8**) into the corresponding chloride by $POCl_3$, and their stereospecific reaction with Super Deuteride or Super Hydride.[21] In our hands, the intermediate chlorides (**10**) and (**11**) were not optically stable at room temperature for prolonged periods. Their generation at lower temperature and immediate use is therefore indicated in the preparation of labelled ethylbenzenes of high optical purity. The labelled toluenes (**31**) and (**32**) were similarly prepared from benzyl chloride and benzaldehyde, respectively.



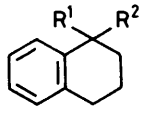
(**1**) $R^1 = R^2 = H$
(**2**) $R^1 = R^2 = D$
(**3**) $R^1 = H, R^2 = D$
(**4**) $R^1 = D, R^2 = H$
(**5**) $R^1R^2 = H,D$
(**6**) $R^1 = H, R^2 = OH$
(**7**) $R^1 = OH, R^2 = H$
(**8**) $R^1 = OH, R^2 = D$
(**9**) $R^1 = D, R^2 = OH$
(**10**) $R^1 = H, R^2 = Cl$
(**11**) $R^1 = D, R^2 = Cl$
(**12**) $R^1R^2 = O$



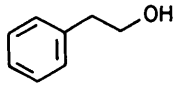
(**13**) $R^1 = R^2 = H$
(**14**) $R^1 = R^2 = D$
(**15**) $R^1 = OH, R^2 = D$
(**16**) $R^1 = OH, R^2 = H$
(**17**) $R^1R^2 = O$



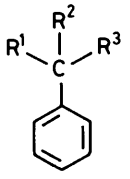
(**18**) $R^1 = R^2 = H$
(**19**) $R^1 = R^2 = D$
(**20**) $R^1 = OH, R^2 = D$
(**21**) $R^1 = OH, R^2 = H$
(**22**) $R^1R^2 = O$



(**23**) $R^1 = R^2 = H$
(**24**) $R^1 = R^2 = D$
(**25**) $R^1 = OH, R^2 = D$
(**26**) $R^1 = OH, R^2 = H$
(**27**) $R^1R^2 = O$



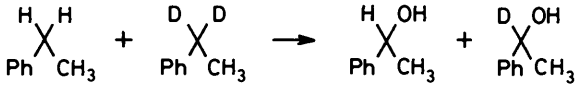
(**28**)



(**29**) $R^1 = R^2 = R^3 = H$
(**30**) $R^1 = R^2 = H, R^3 = OH$
(**31**) $R^1 = R^2 = H, R^3 = D$
(**32**) $R^1 = H, R^2 = R^3 = D$
(**33**) $R^1 = R^2 = R^3 = D$
(**34**) $R^1 = H, R^2 = D, R^3 = OH$
(**35**) $R^1 = R^2 = D, R^3 = OH$

Isotope Effect Studies.—The incubations performed are summarized in Table 1. The transformations of [$^2H_2$]ethylbenzene (**2**) to [^{2}H]-1-phenylethanol, and of [$^2H_3$]toluene (**33**) to [$^2H_2$]-benzyl alcohol, with the loss of only a single deuterium atom, establish that there is no significant further metabolism of product with consequent exchange of label, although some oxidation (presumably irreversible) does occur with *C. echinulata* and *H.* species.[1] Since at least one other P-450-catalysed reaction occurs with loss of deuterium from a non-labile centre[22] the establishment of the integrity of residual label is essential in the interpretation of the isotope effect data discussed below.

The results of the simple intermolecular competitive incubations of ethylbenzenes (**1**) + (**2**) [equation (2)] are summarized in Table 1. The measured isotopic ratio of products, corrected for the isotopic distribution of starting materials, are listed in Table 2, and are the result of a combination of primary (p) and



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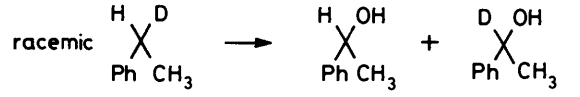


(3)

secondary (s) isotope effects. Also listed in Tables 1 and 2 are the results of incubation of racemic [^{2}H]ethylbenzene (**5**) [equation (3)]. In this latter case, the isotope ratio of products reflects p divided by s, as the isotope removed during reaction now differs from that which remains. These two sets of data are compatible in that the transformation of (**5**) may be considered an inter- rather than an intra-molecular process if hydrogen removal is stereospecific. This has been demonstrated, as discussed below. In this event, if the assumption is made that the primary and secondary isotope effects are expressed independently of each other, then each can be calculated separately: the results are listed in Table 2. The calculation of intermolecular isotope effects directly from product ratios is valid at low degrees of conversion,[23] the situation which pertains here (<10%). A more detailed treatment, using a kinetic analysis which assumes pseudo-first-order behaviour,[23] gives a quantitatively identical result.

The values obtained for p and s from transformations of ethylbenzene by *M. isabellina* and *C. echinulata* are in good agreement. Those from incubation with *H.* species are distorted by the further oxidation of product to acetophenone [to the

Table 1. Summary of incubations

Substrate	% ^2H	Fungus	Products (%)	% ^2H
(1)		M.i.	(6) + (7) (10)	
(1)		C.e.	(6) + (7) (10)	
			(12) (1)	
			(28) (1)	
(1)		H.s.	(6) + (7) (9)	
			(12) (1)	
(2)	100,$^2H_2$	M.i.	(8) + (9) (8)	100,^2H
(2)	100,$^2H_2$	C.e.	(8) + (9) (5)	100,^2H
			(12) (1)	
			(28) (1)	
(2)	100,$^2H_2$	H.s.	(8) + (9) (8)	100,^2H
			(12) (1)	
(3)	100,^2H	M.i.	(6)—(9) (10)	17,^2H
(4)	100,^2H	M.i.	(6)—(9) (12)	80,^2H
(5)	50,^2H	M.i.	(6)—(9) (10)	44,^2H
	50,^2H	C.e.	(6)—(9) (16)	46,^2H
			(12) (1)	
			(28) (1)	
	50,^2H	H.s.	(6)—(9) (8)	45,^2H
			(12) (1)	
(1) + (2)	36,^2H	M.i.	(6)—(9) (10)	29,^2H
	47,^2H	C.e.	(6)—(9) (11)	40,^2H
			(12) (0.5)	
			(28) (2)	
	47,^2H	H.s.	(6)—(9) (10)	55,^2H
			(12) (3)	
(13)		M.i.	(16) (3)	
		C.e.	(16) (12)	
			Diols (25)	
		H.s.	(16) (10)	
			(17) (15)	
(14)	100,$^2H_2$	M.i.	(15) + (16) (10)	21,^2H
	100,$^2H_2$	C.e.	(15) + (16) (2)	32,^2H
			Diols (22)	
(18)		M.i.	(21) (12)	
		C.e.	(21) (3)	
(19)	100,$^2H_2$	M.i.	(20) + (21) (15)	23,^2H
	100,$^2H_2$	C.e.	(20) + (21) (2)	25,^2H
(23)		M.i.	(26) (17)	
		C.e.	(26) (23)	
			Diol (1.5)	
(24)	100,$^2H_2$	M.i.	(26) (4)	23,^2H
	100,$^2H_2$	C.e.	(26) (15)	23,^2H
(29)		M.i.	(30) (10)	
		H.s.	(30) (3)	
(30)		M.i.	(30) (76)	
		H.s.	(30) (7)	
(31)	100,^2H	M.i.	(30) + (34) (4)	60,^2H
(32)	100,$^2H_2$	M.i.	(34) + (35) (5)	63,(^2H + $^2H_2$)
(33)	100,$^2H_3$	M.i.	(35) (10)	100,$^2H_2$
(29) + (33)	44,$^2H_3$	M.i.	(30) + (35) (5)	29,$^2H_2$
(29) + cycloheximide		M.i.		
(29) + cycloheximide†		M.i.	(30) (3)	

† Enzymes induced by ethylbenzene.

extent of *ca.* 30% in the case of (**5**)], a process which would be subject to a primary isotope effect and thus result in isotope enrichment in the remaining alcohol.

Since *M. isabellina* is the only fungus studied which transforms ethylbenzene to 1-phenylethanol without the formation of side products or further oxidation,[1] this micro-organism was selected for use in the transformation of (*R*)-(−)- and (*S*)-(+)-1-deuterioethylbenzenes. The *R*-(−)-enantiomer (**3**) (e.e. 60%, *R*:*S* 80:20) gave product containing 17% deuterium, whereas the *S*-(+)-enantiomer (**4**) (e.e. 66%, *R*:*S* 17:83) gave product which retained 80% of the original label. These results clearly

Table 2. Kinetic isotope effects for benzylic hydroxylation

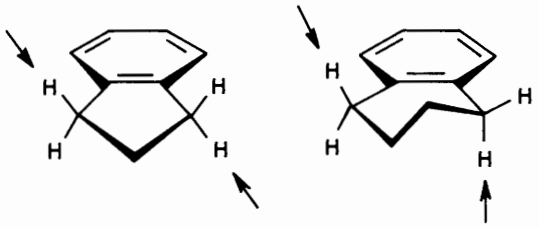
Substrate	Fungus	Product ratio, loss of H:loss of D	Composed of	p^a	s^a
(5)	M.i.	0.786	*p*/*s*	1.04 ± 0.05	1.32 ± 0.05
	C.e.	0.851	*p*/*s*	1.04 ± 0.05	1.22 ± 0.05
	H.s.[b]	0.818	*p*/*s*	0.92 ± 0.05	1.12 ± 0.05
(1) + (2)	M.i.	1.37	*p*.*s*		
	C.e.	1.27	*p*.*s*		
	H.s.[b]	1.03	*p*.*s*		
(14)	M.i.	3.76	*p*.*s*	3.0 ± 0.2[c]	
	C.e.[b]	2.12			
(19)	M.i.	3.35	*p*.*s*	2.6 ± 0.2[c]	
	C.e.	3.0	*p*.*s*	2.4 ± 0.2[c]	
(24)	M.i.	3.35	*p*.*s*	2.6 ± 0.2[c]	
	C.e.	3.35	*p*.*s*	2.6 ± 0.2[c]	

[a] p = primary k_H/k_D, s = secondary k_H/k_D. [b] Values may be distorted by further metabolism of the alcohols; see text. [c] Calculated assuming that s = 1.27.

indicate that hydrogen removal is stereospecific for the *pro-R* position (asterisked in Scheme 2), and suggest that hydrogen removal preceeds and is stereochemically distinct from the step which controls product formation; *M. isabellina* gives a 2:1 ratio of (*R*)- to (*S*)-1-phenylethanol from ethylbenzene.[1] A similar phenomenon (following preferential removal of the *pro-S* hydrogen) is observed in the benzylic hydroxylation of ethylbenzene using enzymes of mammalian origin.[24] In the latter case, the optical purity of product varies with isotopic substitution, a phenomenon which we have also observed with *M. isabellina*[25] and which will be discussed in detail elsewhere. Of the other labelled substrates used in this study, (**14**), (**19**), and (**24**) gave products whose enantiomeric purities were identical, within experimental error, to those previously reported by us for the products from unlabelled material.[1]

Also listed in Table 2 are the product ratios obtained from intramolecular experiments using [1,1-$^2H_2$]-*p*-diethylbenzene (**14**), [1,1-$^2H_2$]indane (**19**), and [1,1-$^2H_2$]tetralin (**24**). In view of the propensity of *H.* species to oxidize product alcohols to ketones,[1] and the complications arising therefrom, this organism was not used with the above substrates. The metabolism of diethylbenzene by *C. echinulata* is complex, giving a series of oxidative products including ketones;[1] data obtained from this reaction differ quantiatively from those of the other intramolecular competition experiments listed in Table 2, and was not analysed further. The product ratios listed in Table 2 follow directly from the analyses of deuterium contents presented in Table 1. These ratios are the product of primary and secondary isotope effects. The primary deuterium isotope effects listed in Table 2 were calculated assuming an average secondary effect of 1.27, obtained from data on ethylbenzene with the omission of the value for *H.* species for the reasons outlined above.

The values for the intermolecular primary deuterium isotope effect for ethylbenzene hydroxylation listed in Table 2 are consistent with those for other intermolecular effects for P-450-catalysed hydroxylations, where effects between 1 and 1.5 are commonly reported.[13,26] The low magnitude of these effects is attributable to the factors discussed in the introduction. The existence of a large, positive secondary isotope effect is suggestive of the existence of considerable bond rehybridization at the benzylic carbon in the transition state, and essentially rules out the once classical mechanism for enzymic hydroxylation involving direct insertion of oxygen into a C–H bond.[27] This effect could arise from either cation or radical formation at the benzylic carbon, values of 1.10—1.25[28] and 1.13—1.19[14,29–32] per deuterium having been reported for these



(18) (23)

Figure. Stereochemical preference for hydroxylation

processes, respectively. The intramolecular isotope effects for the hydroxylation of (**14**), (**19**), and (**24**) differ substantially from those reported for other substrates, such as the benzylic hydroxylation of 1,1-dideuterio-1,3-diphenylpropane (k_H/k_D 11[16]), *N*-demethylation (*via* hydroxylation) of *N*-methyl-*N*-trideuteriomethylaniline (k_H/k_D 8.6—10.1[33]), and *O*-demethylation (*via* hydroxylation) of [$^2H_3$]-*p*-methoxyanisole (k_H/k_D 10[34]). This difference may be accounted for in two ways: firstly, true intramolecular effects are normally only fully expressed when the active site of the enzyme can select for a single site of reaction on the substrate (H or D) following irreversible or slow binding of the latter. If substrate binding is reversible, and more rapid than subsequent reaction, then the situation can resemble the intermolecular case in which *either* H *or* D can be presented to the enzyme's active site, as determined by the mode of binding.[35]

This analysis requires that a single catalytic site of the enzyme be capable of reacting at *either* labelled *or* unlabelled position of the symmetrically labelled substrate. This can be easily envisaged for $PhN(CH_3)CD_3$ or $PhCH_2CH_2CD_2Ph$. It is less easy to see in the case of *p*-$CH_3OC_6H_4OCD_3$, but not impossible, as a single reactive centre sitting above the aromatic ring can come within 1.6 Å of either H or D (based on Drieding model estimation). Using this argument, [1,1-$^2H_2$]-*p*-diethylbenzene could behave as an *inter*molecular substrate if (a) the enzyme's active site cannot simultaneously approach both *para*-benzylic positions closely enough to select for either, and (b) its binding is reversible. In this situation, the primary isotope effect would reflect an inter- rather than an intra-molecular value. A similar argument applies both tetralin and indane, where the absolute stereochemical preference for hydroxylation (giving predominantly the *R* absolute configuration[1]), would require attack from one of the two directions shown in the Figure, evidently stereochemically difficult for a single active-site reagent.

A second, completely separate explanation for the low values of the intramolecular isotope effects observed for (**14**), (**19**), and (**24**) is based on the mechanism of the reaction. The high values of these effects reported in the literature and discussed above have all been associated with reactions involving hydrogen-atom abstraction.[16,33,34,36–38] The deprotonation of cation radicals involves much lower intrinsic isotope effects (k_H/k_D < 3.6),[29,38–42] opening the possibility that the effects listed in Table 2 are indeed normal (intrinsic) intramolecular effects, but of low magnitude since they result from deprotonation of a cation radical rather than hydrogen-atom abstraction. It was in order to explore this latter possibility further that the examination of labelled toluene substrates was performed. The labelled toluenes (**31**) and (**32**) clearly fulfil the requirements outlined above for the normal expression of intramolecular primary isotope effects, namely that the enzyme's active site be able to access H or D without substrate reorientation.

The fungi *M. isabellina* and *H.* species can transform toluene to benzyl alcohol, and we present below the deuterium kinetic isotope effects obtained from the use of [α-^{2}H]-, [αα-$^2H_2$]-, and [ααα-$^2H_3$]-toluene as substrates with *M. isabellina*. We have restricted the isotope effect studies to this fungus, as *H. species*, a fungus known to oxidize alcohols to carbonyl,[1] gave benzyl alcohol in only low and variable yield from toluene. This latter micro-organism appears to be capable of further biotransformation of the initially formed alcohol, as evidenced by the low recoveries obtained in control experiments using (**30**) as substrate for *H. species* biotransformation (Table 1). The incubations performed are summarized in Table 1.

The first step in the mammalian metabolism of toluene has been investigated in detail by Hanzlik and his co-workers, using hydroxylase enzymes of rat liver microsomes.[9] They studied the hydroxylation of labelled substrates (**31**)—(**33**), but were unable to obtain definitive values for the primary and secondary isotope effects involved, the ^2H and $^2H_2$ substrates (**31**) and (**32**), respectively, being hydroxylated with *different* isotope effect ratios. Their data analysis, outlined in Scheme 1, requires the value of r_1/r_2 to be 4; their experimentally determined value of 2.13 reflects the variable p/s ratios of 3.01 and 5.64 obtained for hydroxylations of (**31**) and (**32**) respectively.[9] Our data, presented in the Tables and Scheme 1, are internally self-consistent. Hydroxylations of both (**31**) and (**32**) proceed with a deuterium isotope effect ratio (p/s) of 0.75, and the ratio r_1/r_2 is 4, as required by the method of analysis.

(**31**) $PhCH_2D \longrightarrow$ $PhCH_2OH$ + $PhCHDOH$

product composition = x% + $(100 - x)$%

product benzylic ^1H (from n.m.r.) content = 1.40 hydrogens

$= 2x + (100 - x)$

$= 100 + x$

$x = 40$%

product ratio (loss of H:loss of D) $= \frac{100 - x}{x} = \frac{2p}{s} = 1.50$ (Table 2) $= r_1$

(**32**) $PhCD_2H \longrightarrow$ $PhCHDOH$ + $PhCD_2OH$

product composition = y% + $(100 - y)$%

product benzylic ^1H content from ^1H n.m.r. = y = 0.73 hydrogens

product ratio (loss of H:loss of D) $= \frac{100 - y}{y} = \frac{p}{2s} = 0.37$ (Table 2) $= r_2$

(**33**) $PhCD_3 + PhCH_3 \longrightarrow$ $PhCD_2OH$ + $PhCH_2OH$

product composition from ^1H n.m.r., benzylic H content = z% + $(100 - z)$%

= 1.42 hydrogens

$100 - z = 71$%

$z = 29$%

product ratio (loss of H:loss of D) $= \frac{100 - z}{z} = 2.45$

corrected product ratio† $= \frac{2.45 \times 44}{56} = 1.92 = p^*s^2$ (Table 2)

Scheme 1. Isotope effect ratios for hydroxylation of (**31**)—(**33**).† See footnote in Table 3.

Our data do not permit unequivocal deconvolution of p, p^*, and s (Table 3) without the assumption of a limiting value for at least one of these terms, since inter- and intra-molecular primary kinetic isotope effects may not be identical. However, the assumption of a *minimum* value of 1.0 for the intramolecular primary k_H/k_D gives a value of 1.33 ± 0.06 for the secondary k_H/k_D. If the assumption is made that p and p^* are similar, as the data suggest, then the values of $p = p^* = 1.02 \pm 0.05$ and $s = 1.37 \pm 0.06$ are consistent with all the ratios of Scheme 1. Although inter- and intra-molecular primary kinetic isotope effects for cytochrome P_{450}-catalysed hydroxylations are usually different,[16,34] precedent exists for intrinsic (intra-

Table 3. Kinetic isotope effects for toluene hydroxylation by *M. isabellina*

Substrate	Product (%)	Ratio H loss : D loss	Composed of [b]
(**31**)	(**30**) (40) + (**34**) (60)	1.50 ± 0.05	$2p/s$
(**32**)	(**34**) (73) + (**35**) (27)	0.37 ± 0.015	$p/2s$
(**29**) + (**33**) (56:44)	(**30**) (71) + (**35**) (29)		
	(**30**) (66.5) + (**35**) (34.5)[a]	1.92 ± 0.06	p^*s^2

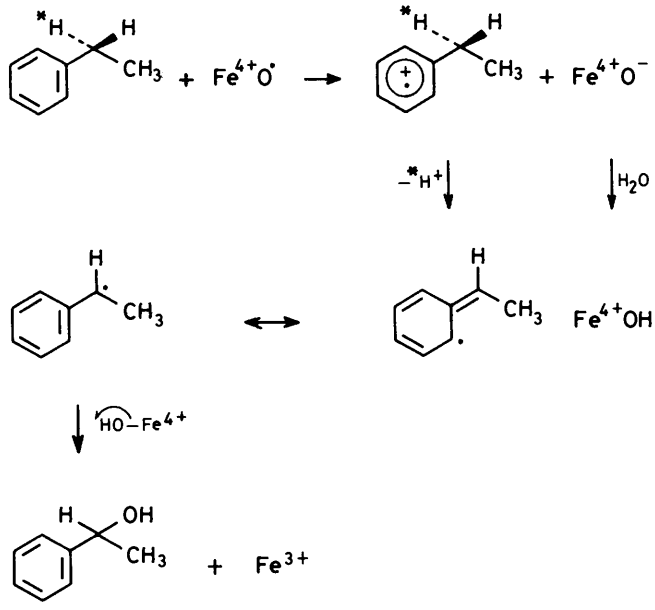
[a] Corrected for 1:1 substrate ratio. [b] p = intramolecular primary k_H/k_D, s = secondary k_H/k_D, p^* = intermolecular primary k_H/k_D.

molecular) primary isotope effects which are very low[43] and identical to the intermolecular effect.[44] In any event, it is clear that the secondary effect s for toluene hydroxylation is larger than p and probably larger than p^*.

Although in the absence of a purified enzyme preparation we cannot state definitively that the same enzyme metabolizes both toluene and ethyl benzene, we feel that this is likely in view of the similarity in substrate structure, the observation that both substrates are transformed with similar rates,[45] and the results of the induction experiments presented in Table 1. The addition of the protein synthesis inhibitor cycloheximide to the incubation medium at the end of the growth period, but prior to the addition of toluene, produces conditions under which no hydroxylation occurs. The toluene-hydroxylating enzyme is thus substrate inducible, as is the ethylbenzene hydroxylase in the same fungus.[1] Furthermore, the use of ethylbenzene as an enzyme inducer during the growth period followed by incubation with toluene in the presence of cycloheximide leads to normal product formation, suggesting that the ethylbenzene hydroxylase is also capable of accepting toluene as substrate.

Our data may thus be summarized as follows. Benzylic hydroxylation involves a small primary intramolecular deuterium isotope effect, together with a relatively large secondary deuterium isotope effect. These data collectively suggest a product-like transition state for the reaction in which C–H or C–D bond breaking is nearing completion, resulting in a low primary effect, with concomitant substantial rehybridization of carbon from sp^3 to sp^2 being responsible for the expression of a positive secondary effect. The differences in the magnitude of the effects observed for toluene and the other substrates may thus reflect a small difference in the degree of C–H or C–D bond breaking in the transition state, the process being more complete in the case of the former substrate.

The nature of the intermediates in benzylic hydroxylation, alluded to above, must now be considered in detail. The body of data on the enzymic hydroxylation reaction favour a radical intermediate (see Introduction). This may be formed by direct hydrogen-atom abstraction, or alternatively by electron abstraction followed by proton loss, as in Scheme 2. We favour the latter route for the following reasons. First, it is consistent with the magnitude of the intramolecular isotope effects discussed above, whereas hydrogen-atom removal is not; although intrinsic primary deuterium isotope effects for enzymic hydroxylation *via* hydrogen-radical abstraction are normally high,[46] effects of low magnitude (k_H/k_D 1—3) have been reported for *N*-demethylation *via* hydroxylation involving proton removal from a radical cation.[33,44] Secondly, our proposal provides an explanation for the observation that initial hydrogen loss can be stereospecific (by loss of the *pro-R* benzylic proton to a basic group of the enzyme following substrate binding) when subsequent reaction with oxygen is not. Furthermore, it parallels the known mechanism of oxidation of thioanisole by the same enzymes of *M. isabellina* which involves a one-electron oxidation at sulphur as the rate-determining



Scheme 2.

step;[1,47,48] and it is entirely consistent with some recent data on cytochrome P-450-dependent reactions, such as the hydroxylation of the hydrocarbon quadricyclane,[49] epoxidation by hydroxylase enzymes,[50] and the oxidation of heteroatom-substituted cyclopropanes,[51] all of which processes are thought to involve one-electron abstraction from the substrate, proceeding *via* a radical-cationic intermediate. Our proposal has chemical precedent;[52] and it has biochemical precedent in the mechanism of oxidation of aromatic compounds by the enzyme ligninase.[53] Our data therefore suggest a new possibility for the mode of action of benzylic hydroxylating enzymes, involving a one-electron abstraction from the aromatic ring followed by proton loss, giving a benzylic radical, the stereochemistry of whose subsequent oxidation may not be related to the stereochemistry of hydrogen loss.

Experimental

Materials and Methods.—The techniques used were those previously described.[1] The deuterium contents listed in Table 1 were determined by mass spectral analysis of molecular ion clusters (for substrates) and ^{1}H n.m.r. integration of the signal due to benzylic hydrogen, using the aromatic hydrogen signal as internal standard (for products). Values are accurate to ±3%. [ααα-$^2H_3$]Toluene was a commercial sample (Merck, Sharpe and Dohme), and contained >99% $^2H_3$ species.

Maintenance of Micro-organisms.—*Mortierella isabellina* NRRL 1757 (M.i.), *Cunninghamella echinulata* var. *elegans*, ATCC 26269 (C.e.), and *Helminthosporium* species NRRL 4671 (H.s.) were maintained on agar slopes as previously described.[1]

Synthesis of Substrates.—(S)-(+)-1-*Deuterioethylbenzene* (**4**). A stirred solution of (*S*)-(−)-1-phenylethanol (6 g obtained from the racemate *via* brucine resolution of the phthalate half ester,[54] e.e. >98%) in dry pyridine (12 ml) and pentane (32 ml) was cooled to −20 °C, and then $POCl_3$ (4.5 ml) added dropwise at such a rate that the temperature of the mixture did not exceed −10 °C (during *ca.* 1 h). The mixture was then treated with ice and extracted with ether (3 × 50 ml). The ether was washed, dried, and evaporated. The residue was dissolved in pentane (200 ml), washed sequentially with 85% H_2SO_4 and water,

dried, and evaporated to give (*R*)-(+)-1-phenylethyl chloride (3.75 g), $[\alpha]^{21}$ +82.4° (EtOH) (e.e. 66% based on $[\alpha]_D$ +125°[55]). The ^1H n.m.r. spectrum was identical with that of racemic material.

The above chloride was placed in a flame-dried flask fitted with a P_2O_5 drying tube, a magnetic stirring bar, and a septum inlet. The flask was cooled in ice and a solution of lithium triethylborodeuteride (Super Deuteride) (50 ml; 1M in THF) added *via* a syringe. The mixture was stirred at room temperature overnight, and then placed in an ice-bath. Following the addition of water to decompose excess of reagent, aqueous sodium hydroxide (30 ml, 3M) was added all at once to the rapidly stirred reaction mixture, and then hydrogen peroxide solution (30 ml; 30%) added dropwise (care!). The resulting mixture was then extracted with light petroleum (4 × 50 ml, redistilled; b.p. 30—45 °C), washed with 50% H_2SO_4 (4 × 50 ml), followed by water (2 × 50 ml), dried (Na_2SO_4), filtered through silica gel (1 in) in a Buchner funnel, and then evaporated at room temperature to give (**4**) (1.5 g), $[\alpha]^{20}$ +0.80° (neat) (e.e. 68% based on $[\alpha]^{21}$ +1.17°[21]). The ^1H n.m.r. spectrum was identical with that of racemic material (**5**).

(R)-(−)-1-*Deuterioethylbenzene* (**3**). This was prepared from 1-deuterio-(*S*)-(−)-1-phenylethanol (**8**) (obtained by resolution of the product of the lithium aluminium deuteride reduction of acetophenone, as described above) by the procedure outlined above. The intermediate chloride showed $[\alpha]^{20}$ +73.5° (e.e. 60%[55]). This was treated with Super Hydride to give product (**3**) $[\alpha]^{20}$ −0.71° (neat) (e.e. 61%[21]).

(±)-1-*Deuterioethylbenzene* (**5**). This was prepared from (±)-1-phenylethyl chloride by reaction with Super Deuteride as described above.

1,1-*Dideuterioethylbenzene* (**2**). This was prepared from (±)-1-deuterio-1-phenylethanol (obtained by $LiAlD_4$ reduction of acetophenone), *via* conversion into the chloride using $SOCl_2$ in the normal way, and subsequent reaction with Super Deuteride as described above.

1,1-*Dideuterio*-1-(4-*ethylphenyl*)*ethane* (**14**), 1,1-*dideuterioindane* (**19**), *and* 1,1-*dideuteriotetralin* (**24**). These were prepared from the corresponding ketones (**17**), (**22**), or (**27**), respectively, by the route outlined above for the preparation of (**2**). In all cases, the intermediates and products exhibited spectral data consistent with their structures.

[α-^{2}H]*Toluene* (**31**). This was prepared from benzyl chloride and Super Deuteride as described above. The yield was quantitative and the product, which contained >99% ^2H species, showed the anticipated spectral and physical properties.

[αα-$^2H_2$]*Toluene* (**32**). This was prepared from [α-^{2}H]benzyl alcohol (**34**) (obtained from $LiAlD_4$ reduction of benzaldehyde), *via* conversion into [α-^{2}H]benzyl chloride and subsequent reaction with Super Deuteride in an overall yield of 80%. The product contained >99% $^2H_2$ species and was analytically pure (^1H, ^2H n.m.r.; mass spectrum).

Incubations with Fungi.—All incubations were performed as previously described for unlabelled material[1] using 72 h old cultures, for a further period of 72 h, at substrate concentrations of 0.75—1.0 mg ml^{-1} of medium. Products were isolated by continuous extraction (CH_2Cl_2) and purified by flash column chromatography (silica gel, benzene–ether 10% stepwise gradient). Isolated yields and deuterium content of products are listed in Table 1. In all cases, the identify of products was confirmed by spectral analysis. Enantiomeric composition of products were determined as previously described.[1] Deuterium contents and enantiomeric compositions were determined following pooling of homogeneous chromatographic fractions. No evidence for isotope discrimination during chromatography was observed.

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