Mechanism of hydroxylation of biphenyl by *Cunninghamella echinulata*

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The hydroxylation of [U-²H]biphenyl and [2,2',3,3',5,5',6,6'-²H]biphenyl by *Cunninghamella echinulata* A.T.C.C. 9244 has been studied. G.l.c.–mass-spectrometry analyses indicate the lack of an isotope effect during the hydroxylation of the perdeuterated substrate. Both g.l.c.–mass spectrometry and ¹H n.m.r. were used to definitively demonstrate the presence of a 1,2-hydride-shift during the microbiological hydroxylation of [2,2',3,3',5,5',6,6'-²H]biphenyl.

The metabolism of foreign organic compounds by numerous micro-organisms (principally fungi) parallels that observed in higher organisms. These correlations serve as a basis for the study of 'microbial models of mammalian metabolism' (Smith & Rosazza, 1974a; Rosazza & Smith, 1979). Biphenyl is actively hydroxylated in mammalian systems and this compound has been used as a model substrate in microbiological studies.

The microbiological hydroxylation of biphenyl has been studied by several workers (Smith & Rosazza, 1974b; Dodge *et al.*, 1979; Smith *et al.*, 1980). A number of fungi have been found that preferentially hydroxylate the substrate in either the 2- or 4-positions and this matches results obtained with certain mammalian systems (Smith & Rosazza, 1981). One organism previously studied in our laboratories (Smith & Rosazza, 1974a; Smith *et al.*, 1980), *Cunninghamella echinulata* A.T.C.C. 9244, converts biphenyl primarily into 4-hydroxybiphenyl, though 4,4'-dihydroxybiphenyl is also formed to a small extent. Conjugation (*e.g.* O-glucuronidation) of these products was not studied. Dodge *et al.* (1979), however, have observed conjugation of phenolic biphenyl analogues by a strain of *Cunninghamella elegans*. By analogy with mammalian mono-oxygenases (Billings & McMahon, 1978), the mechanism of 4-hydroxylation by *C. echinulata* could involve a direct oxygen insertion reaction or the intermediacy of an arene oxide. These mechanisms can be differentiated by study of a deuterium isotope effect and the occurrence of a 1,2-hydride shift during hydroxylation respectively. An investigation employing [U-²H]biphenyl and [2,2',3,3',5,5',6,6'-²H]biphenyl has been undertaken to elucidate the mechanism of

hydroxylation of biphenyl by *C. echinulata*, and is described in the present report.

Materials and methods

[U-²H]Biphenyl was purchased from ICN, Irvine, CA, U.S.A. After recrystallization from n-hexane, it was found to contain 83.9 atom-% excess deuterium. [2,2',3,3',5,5',6,6'-²H]Biphenyl was prepared by the procedure of Clark *et al.* (1980) and contained 96.7 atom-% excess deuterium. Other chemicals used were the purest available from Aldrich Chemicals, Milwaukee, WI, U.S.A., Fisher Chemicals, Houston, TX, U.S.A., and Pierce Chemical, Rockford, IL, U.S.A.

Deuterium isotope effect

Stage I cultures of *C. echinulata* A.T.C.C. 9244 were prepared in a soya-bean meal/glucose medium adjusted to pH 5.5, as described previously (Smith *et al.*, 1980) and were incubated on a rotary shaker (250 rev./min; 2.54 cm/stroke). After growth for 72 h, 2 ml portions were used to inoculate 250 ml Erlenmeyer (Stage II) flasks containing 50 ml of identical soya-bean meal/glucose medium (Smith *et al.*, 1980). After addition of 12.5 mg of biphenyl/[U-²H]biphenyl (1:1) in 50 μ l of dimethylformamide to each flask, the resulting cultures were allowed to continue growing. At 2 and 4 h, individual cultures were homogenized and 2 ml portions of the homogenates were diluted with 2 ml of 0.1 M-phosphate buffer (pH 6.5) and extracted with 2 ml of ethyl acetate. After centrifugation at approx. 2000 g at 22°C for 15 min, 1 ml portions of the ethyl acetate layers were evaporated to dryness with N₂. The residues were redissolved in 100 μ l of benzene/acetonitrile (1:1) and derivatives were formed with

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25 μ l of heptafluorobutyric anhydride for 15 min at 65°C (Davis *et al.*, 1978). Residual biphenyl, [U-²H]biphenyl and the 4-hydroxy-metabolites (as their corresponding heptafluorobutyrate) were analysed by g.l.c.-mass spectrometry using a Finnigan 4023 system complete with model 9610 gas chromatograph, model 4000 quadrupole mass spectrometer and model 2300 INCOS data system. Residual biphenyl and [U-²H]biphenyl were determined through peak-area measurements of ions at m/e 154 and 164; peak areas of the ions 366 and 375 were used in the analysis of the unlabelled and labelled metabolite, 4-hydroxybiphenyl. An ionization voltage of 70 eV was used throughout. All compounds were chromatographed on a glass column (2 m $\times$ 2 mm int. diam.) packed with 3% OV-17 on Chromosorb W-HP (100–120 mesh) eluted with helium (20 ml/min) at 130°C.

1,2-Hydride-shift experiments

Stage II cultures of *C. echinulata* were prepared, 12.5 mg of [2,2',3,3',5,5',6,6'-²H]biphenyl in 50 μ l of dimethylformamide was added per flask, incubated for 72 h, and analysed by g.l.c.-mass spectrometry for phenolic metabolites as indicated above except that the ionization voltage was maintained at 20 eV. Peak-area measurements at m/e 374 and 373 ($M - 1$) were used in calculating the percentage H retention of the 4-hydroxy-metabolites; similar measurements at m/e 586, 585 ($M - 1$) and 584 ($M - 2$) were used to determine H retention in the 4,4'-dihydroxy-metabolites.

Isolation and identification of the 4-hydroxybiphenyl produced from [2,2',3,3',5,5',6,6'-²H]biphenyl

Ten Stage II cultures were incubated with [2,2',3,3',5,5',6,6'-²H]biphenyl for 72 h as indicated above. The cultures were homogenized, combined and extracted with three separate 200 ml portions of ethyl acetate. The combined extracts were dried over anhydrous Na₂SO₄ and evaporated *in vacuo* leaving a dark yellow oil, which was streaked on to 1 mm-thick silica gel PF₂₅₄ (Brinkmann Instruments, Westbury, NY, U.S.A.) t.l.c. plates and eluted with chloroform/acetone (4:1, v/v). A band corresponding to 4-hydroxybiphenyl was made visible under 254 nm radiation and scraped off; the scrapings were extracted with three 12 ml portions of acetone containing a few drops of ethanol and centrifuged at 1000 g for 4 min at 22°C after each elution. The combined acetone extracts were reduced to dryness *in vacuo* and the residue crystallized from n-hexane/diethyl ether to give 1.8 mg of white needles having an m.p. of 160°C (authentic 4-hydroxybiphenyl melts at 163°C); an additional crop of 6.0 mg of yellow needles melting at 153°C was obtained from the mother liquor. The isolated

product displayed identical t.l.c. and g.l.c. behaviour (Davis *et al.*, 1978) to authentic 4-hydroxybiphenyl. The white needles were dissolved in deuterated methanol and analysed by ¹H n.m.r. using a 200 MHz Nicolet FT-n.m.r. spectrometer. The percentage H retention was also determined after mass-spectral analysis with a Dupont model 491 mass spectrometer with solid probe inlet and operated at 12 eV.

Results and discussion

The mechanism of hydroxylation of biphenyl by *C. echinulata* was studied through use of two specifically deuterated forms of the substrate. After incubation with a 1:1 mixture of biphenyl and [U-²H]biphenyl, substrate and product analyses indicate K_H/K_D ratios (mass spectral peak ratio of 4-hydroxybiphenyl/[U-²H]4-hydroxybiphenyl) of 1.07 ± 0.02 ($n = 3$) and 0.92 ± 0.00 ($n = 3$) (means $\pm$ S.D.) respectively after 4 h incubations. Essentially identical ratios were obtained from analyses performed after 2 h incubations. These results suggest the lack of a direct insertion mechanism, which shows a K_H/K_D of 1.27–1.45

Table 1. 1,2-Hydride-shift studies with [2,2',3,3',5,5',6,6'-²H]biphenyl

After incubation of [2,2',3,3',5,5',6,6'-²H]biphenyl with *C. echinulata* for 72 h, the 4-hydroxy- and 4,4'-dihydroxy-metabolites were analysed by g.l.c.-mass spectrometry as their heptafluorobutyrate derivatives. Results are means $\pm$ S.D. for the numbers of observations in parentheses.

Product	H retention (%)
4-Hydroxy[2,2',3,3',5,5',6,6'- ² H]biphenyl	20.3 ± 6.3 (14)
4,4'-Dihydroxy-[2,2',3,3',5,5',6,6'- ² H]biphenyl	37.6 ± 4.5 (8)
	(calc. = 32%*)
4,4'-Dihydroxy-[2,2',5,5',6,6'- ² H]biphenyl	2.8 ± 0.8 (6)
	(calc. = 4%*)

* Calculated, based on sequential hydroxylation and H retention of approx. 20% during the initial hydroxylation reaction. That is, the 4-hydroxy-product consists of 20% 4-hydroxy[2,2',3,3',5,5',6,6'-²H]biphenyl (¹H₂-4-HBP) and 80% 4-hydroxy[2,2',3,3',5,5',6,6'-¹H]biphenyl (¹H₁-4-HBP). During the formation of the 4,4'-dihydroxy metabolite, 80% of ¹H₂-4-HBP substrate is converted into 4,4'-dihydroxy[2,2',3,3',5,5',6,6'-²H]biphenyl (¹H₁-4,4'-DiHBP) ($80\% \times 20\% = 16\%$ ¹H₁-4,4'-DiHBP); 20% of ¹H₁-HBP is converted into ¹H₁-4,4'-DiHBP ($20\% \times 80\% = 16\%$). The total theoretical amount of ¹H₁-4,4'-DiHBP formed = $16\% + 16\% = 32\%$. Similarly, 20% of ¹H₂-4-HBP should be converted into 4,4'-dihydroxy[2,2',5,5',6,6'-²H]biphenyl (¹H₂-4,4'-DiHBP); thus, $20\% \times 20\% = 4\%$ ¹H₂-4,4'-DiHBP. The convincing nature of these calculations is best understood by drawing the structures of all products formed.

Fig. 1. Partial 200 MHz ^1H n.m.r. spectrum (deutero-methanol) of 4-hydroxy[2,2',3,3',5,5',6,6'- ^2H]biphenyl and 4-hydroxy[2,2',3',5,5',6,6'- ^2H]biphenyl produced by *C. echinulata* from [2,2',3,3',5,5',6,6'- ^2H]biphenyl. Percentage H retention was determined by the ratio of integrations of the peaks at δ 6.84 (H ortho to the phenolic hydroxy group) and 7.23 p.p.m. (H in 4'-position).

during the 3-hydroxylation of biphenyl by certain mammalian preparations (Billings & McMahon, 1978) and is greater than 1.2 for a number of other substrates (Trager, 1980).

The absence of an isotope effect during the hydroxylation of biphenyl prompted a study of a possible 1,2-hydride shift during 4-hydroxylation, which would implicate the intermediacy of an arene oxide. Therefore, the hydroxylation of [2,2',3,3',5,5',6,6'- ^2H]biphenyl was studied by g.l.c.-mass spectrometry and ^1H n.m.r. In one set of experiments, the ring-deuterated 4-hydroxy- and 4,4'-dihydroxy-metabolites were analysed by g.l.c.-mass spectrometry as their heptafluorobutyrate derivatives after incubation of the specifically octadeuterated biphenyl with *C. echinulata*. As indicated in Table 1, 20.3% retention of ^1H was measured for the 4-hydroxy-product, whereas the 4,4'-dihydroxy-metabolite showed retentions of 37.6% (heptadeutero-product) and 2.8% (hexadeutero-product). It is noteworthy that the latter values are close to calculated ^1H retentions based on sequential hydroxylation of the substrate. Overall

these data suggest that the [2,2',3,3',5,5',6,6'- ^2H]biphenyl undergoes a 1,2-hydride shift during hydroxylation, thus implicating an intermediary arene oxide and mono-oxygenase activity in *C. echinulata*. Similar hydrogen (deuterium) retentions have been observed for a variety of substrates in mammalian and microbial systems containing mono-oxygenase activities (Daly *et al.*, 1972).

After a preparative-scale incubation of [2,2',3,3',5,5',6,6'- ^2H]biphenyl with *C. echinulata*, a crystalline product was isolated that displayed m.p., t.l.c. and g.l.c. behaviour indicative of 4-hydroxybiphenyl (Davis *et al.*, 1978). Mass-spectral analysis (solid probe) revealed a 23.3% retention of hydrogen. ^1H n.m.r. analysis with a Fourier-transform 200 MHz instrument showed a retention of hydrogen of 30.1% (see Fig. 1). These data confirm an NIH-shift in the hydroxylation of the octadeutero-biphenyl by *C. echinulata*. To our knowledge, this work represents the first isolation of a 1,2-hydride-shift product where isotope retention was determined by n.m.r. spectroscopy. Similar studies are required for organisms that regioselectively hydroxylate biphenyl in different positions (Smith *et al.*, 1980).

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