Fungal Metabolism of Biphenyl

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Cunninghamella elegans grown on Sabouraud dextrose broth transformed biphenyl to produce 2-, 3- and 4-hydroxybiphenyl, as well as 4,4'-dihydroxybiphenyl as free phenols. A compound tentatively identified as 2,4'-dihydroxybiphenyl was also produced. When 4-hydroxybiphenyl or 2-hydroxybiphenyl replaced biphenyl as the substrate, *C. elegans* produced 4,4'-dihydroxybiphenyl and 2,5-dihydroxybiphenyl respectively. The compound identified as 2,4'-dihydroxybiphenyl was produced from both substrates. A survey of 11 species of fungi known to degrade hydrocarbons revealed two species that were comparable to *C. elegans* in their ability to convert biphenyl into free phenols. In addition to free phenolic metabolites, deconjugation experiments indicated that 44% of the known metabolites present in the culture filtrate were present in the form of conjugates. These results suggest that the transformation of biphenyl by *C. elegans* is similar to that found in mammalian systems.

The metabolic fate of biphenyl in biological systems has been widely investigated since Klingenberg (1891) first reported the conversion of biphenyl into 4-hydroxybiphenyl by the dog [for a review, see Sundstrom *et al.* (1976)]. Recent interest in the metabolism of biphenyl stems in part from its extensive commercial use as a fungistat in the shipping of citrus fruits (Hakkinen *et al.*, 1973), from its relationship to the problem of polychlorinated biphenyl degradation in the environment (Gibson *et al.*, 1973), from its use as a model compound for the study of biotransformation of aromatic compounds in mammals (Smith & Rosazza, 1974) and from its application in an indicator system for detecting the metabolic activation of carcinogenic compounds (McPherson *et al.*, 1974).

Biphenyl is initially oxidized by mammals to produce phenols (Raig & Ammon, 1970; Meyer & Scheline, 1976; Wiebkin *et al.*, 1976; Meyer *et al.*, 1976). The major site of hydroxylation is at the 4-position, with significantly less hydroxylation occurring at the 2- and 3-positions. Additional oxidation of these monohydroxylated products can lead to the formation of 4,4'-dihydroxybiphenyl, 3,4'-dihydroxybiphenyl, 3,4-dihydroxybiphenyl, 2,5-dihydroxybiphenyl and 3,4,4'-trihydroxybiphenyl (Meyer & Scheline, 1976). In addition to the free phenols a high percentage of the metabolites may be present in the form of conjugates (Meyer *et al.*, 1976; Wiebkin *et al.*, 1976).

In contrast with mammals, bacteria metabolize aromatic hydrocarbons by incorporating two atoms of oxygen into the aromatic substrate to form *cis*-dihydrodiols. The latter compounds can be further oxidized to catechols (Gibson, 1977). These reactions

have been demonstrated in biphenyl degradation (Lunt & Evans, 1970; Catelani *et al.*, 1971; Gibson *et al.*, 1973).

There is some evidence that fungi metabolize biphenyl to metabolites similar to those formed by mammalian systems. Smith & Rosazza (1974) showed that species of several genera of fungi grown in the presence of biphenyl formed 4-hydroxy-, 2-hydroxy- and 4,4'-dihydroxy-biphenyl, although no single species produced all three compounds. Wiseman *et al.* (1975) presented evidence that microsomal preparations from *Candida tropicalis* oxidized biphenyl to 4-hydroxybiphenyl.

The present paper describes the isolation and identification of oxidation products formed from biphenyl by *Cunninghamella elegans*.

Materials and Methods

Organisms

The isolation and characterization of *C. elegans* has been reported previously (Cerniglia & Perry, 1973). *Absidia* sp., *Aspergillus niger*, *C. echinulata*, *C. japonica*, *Gilbertella* sp., *Penicillium zonatum*, *Saccharomyces cerevisiae* and *Syncephalastrum racemosum* were obtained as described previously (Cerniglia *et al.*, 1978).

Growth conditions

The inoculum was prepared by aseptically homogenizing a 4-day-old Sabouraud dextrose/agar-plate culture in 30 ml of sterile Sabouraud dextrose broth. In large-scale experiments, 10 ml samples of inoculum were added to 500 ml of Sabouraud dextrose

broth in each of eight 2800ml Fernbach flasks. For small-scale experiments, 1ml samples were added to 30ml of Sabouraud dextrose broth in 125ml Erlenmeyer flasks. In both cases the flasks were incubated at 27°C for 48h on a rotary shaker operating at 250 rev./min.

After 48h the spent medium was removed from the flask by filtration through sterile cheesecloth, and the mycelial pellets were resuspended in the original amount of fresh sterile Sabouraud dextrose broth. Biphenyl (2mg/ml) was added to the flasks in powdered crystalline form. These flasks were incubated as described above for 48 or 120h. Two control flasks were also incubated; one contained sterile Sabouraud dextrose broth, and the other contained sterile Sabouraud dextrose broth and biphenyl (2mg/ml).

Extraction of transformation products

After the second incubation period, the flask cultures were examined by phase-contrast microscopy for bacterial contamination. The mycelial pellets were removed by cheesecloth filtration and the filtrate was extracted with three equal volumes of ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and the ethyl acetate was removed under reduced pressure at 30°C. Analysis of the residue was performed by t.l.c., silica-gel column chromatography, and high-pressure liquid chromatography (h.p.l.c.).

Deconjugation experiments

Conjugated metabolites (glucuronides and/or sulphates) were deconjugated before organic extraction and quantification by using a procedure modified from Wiebkin *et al.* (1976). A small-scale transformation experiment was carried out in which the addition of biphenyl (2mg/ml) was accompanied by the addition of 27 nmol of [^{14}C]biphenyl dissolved in 100 μl of dimethylformamide. After 48h incubation the mycelial pellets were removed and the filtrate was extracted with three equal volumes of ethyl acetate. The filtrate was divided into two 10ml portions. The first portion received 5ml of 0.2M-acetate buffer (Gomori, 1955) and 10mg of β -glucuronidase (type H-1; Sigma Chemical Co., St. Louis, MO, U.S.A.), equivalent to 3600 Fishman units. The second portion received 5ml of acetate buffer and served as a control. Both portions were incubated for 8h at 37°C on a rotary shaker operating at 150 cycles/min. After incubation, each portion was extracted with three equal volumes of ethyl acetate, which were pooled and examined by h.p.l.c. for the presence of free phenolic metabolites.

Experiments with [^{14}C]biphenyl were performed by collecting 0.75ml fractions and adding 5ml of scintillation fluid (1 litre of 1,4-dioxan, 100g of naphthalene, 6g of 2,5-diphenyloxazole, 0.3g of 1,4-bis-(5-phenyloxazol-2-yl)benzene to each fraction before liquid-scintillation counting. In some experiments, Aquasol-2 (New England Nuclear Corp.,

Boston, MA, U.S.A.) served as the scintillation fluid. Appropriate corrections were made for machine efficiency and quenching.

Trimethylsilyl ether derivatives of authentic biphenyl phenols and metabolites were prepared by dissolving 1mg of each compound in 1ml of benzene and adding 5 drops of *NO*-bis(trimethylsilyl)acetamide.

Analytical methods

U.v. spectra were determined on a Beckman model 25 recording spectrophotometer. Melting points were determined by using a Fisher-Johns melting-point apparatus and were uncorrected. Low-resolution mass spectra were determined on a DuPont model 21-491 mass spectrometer, and high-resolution mass spectra were determined by using a DuPont model 21-110C instrument. P.m.r. spectra were recorded on a Varian A-60, HA-100, or a Perkin-Elmer R-12 spectrometer.

Analytical t.l.c. was performed on Polygram Sil G/UV₂₅₄ sheets (Macherey-Nagel & Co., Düren, Germany). Preparative t.l.c. was performed by using glass plates coated with silica 60 F-254 (E. Merck, Darmstadt, Germany). The t.l.c. solvent pair was chloroform/acetone (4:1, v/v). Metabolites were identified by their absorbance of u.v. radiation (254nm) and by their characteristic colour reaction with Gibbs reagent [2% (w/v) 2,6-dichloroquinone-4-chloroimide in methanol].

Analytical separation of metabolites was also performed by using a Waters model 440 high-pressure liquid chromatograph equipped with a 254nm and a 281nm photometer and a solvent-gradient programmer. To resolve the more polar metabolites, a C_{18} μ Bondapak column (3.9mm $\times$ 30cm) was used in conjunction with a solvent gradient (curve 8) beginning with acetonitrile/water (3:7, v/v) and terminating after 30min with acetonitrile/water (7:3, v/v). The solvent flow rate was 1.5ml/min. To resolve the less-polar metabolites, a μ Porasil column (3.9mm $\times$ 30cm) was used with dichloromethane/chloroform (19:1, v/v) and a flow rate of 2.0ml/min. This system was run isocratically for 15min.

^{14}C radioactivity determinations were carried out by using either a Beckman model LS-250 or LS-1000 liquid-scintillation counter.

Gas-chromatographic analysis of metabolites was carried out on a Perkin-Elmer model 270B gas chromatograph equipped with a 5% SE-30 column (0.31cm $\times$ 210cm). The oven and injector temperatures were 200 and 220°C respectively. The carrier gas was helium, with a flow rate of 25ml/min.

Chemicals

Biphenyl was from Mallinckrodt Chemical Works, St. Louis, MO, U.S.A. 2-Hydroxy-, 4-hydroxy- and 4,4'-dihydroxy-biphenyl were from Aldrich Chemical

Company, Milwaukee, WI, U.S.A. 3-Hydroxy- and 2,5-dihydroxy-biphenyl were obtained from RFR Corp., Hope, RI, U.S.A. All chemical standards were used without further purification. *NO*-Bis(trimethylsilyl)acetamide was from Pierce Chemical Company, Rockford, IL, U.S.A. [^{14}C]Biphenyl with a sp. radioactivity of 37 mCi/mmol was from Amersham/Searle, Arlington Heights, IL, U.S.A.

Results

Preliminary results

Small-scale (30 ml) biotransformation experiments were carried out in which cells of *C. elegans* were incubated with biphenyl. After 48 h, the flask contents were extracted to recover transformation products. T.l.c. analysis of the concentrated extract revealed the presence of two compounds with R_F values of 0.35 and 0.58, which gave purple and orange spots respectively when sprayed with Gibbs reagent. When this experiment was repeated with [^{14}C]biphenyl (27 nmol) and unlabelled biphenyl (2 mg/ml), h.p.l.c. analysis of the culture filtrate showed the presence of four transformation products and an unresolved group of more polar compounds (Fig. 1).

Two major products, compounds (I) and (III), were observed, having retention times of 4 and 18 min respectively. Two minor products, compounds (II) and (IV), had retention times of 10 and 19 min respectively. A control flask containing [^{14}C]biphenyl and sterile Sabouraud dextrose broth showed no autooxidation of the substrate in 48 h.

Isolation of metabolites

A large-scale transformation experiment with 5 litre of Sabouraud dextrose broth resulted in 3.2 g of extracted material, which was applied to the top of a silica-gel column (2.3 cm $\times$ 35 cm). A total of 170 fractions of 4 ml each were collected; fractions 1–105 were eluted with chloroform/methanol (19:1, v/v). Examination of the fractions by t.l.c. showed compound (I) present in fractions 142–166 (68 mg) and compound (III) in fractions 32–90 (455 mg). Both compounds required further purification before characterization.

Identification of compound (I)

The residue obtained from fractions 142–166 was subjected to preparative t.l.c. and 17 mg of compound (I) was obtained in purified form. Compound (I) was identified as 4,4'-dihydroxybiphenyl, having u.v., p.m.r. and mass spectra identical with that of the authentic compound. A mass determination gave a calculated mass for $^{12}\text{C}_{12}^{1}\text{H}_{10}^{17}\text{O}_2$ of 186.0681; the observed mass was 186.0674. The m.p. of 278–279°C for compound (I) was identical with that of 4,4'-dihydroxybiphenyl, and a mixed m.p. was un-depressed.

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Fig. 1. Elution profile of metabolites formed from biphenyl by *C. elegans*

Separation was achieved with a μ Bondapak column as described in the Materials and Methods section. (a) Elution profile of authentic hydroxybiphenyls; (b) elution profile of biphenyl metabolites showing compounds (I)–(IV). —, A_{254} ; ----, radioactivity (d.p.m.).

Identification of compound (III)

The residue from fractions 32–90 was washed with hexane (20 ml) to remove biphenyl, followed by preparative t.l.c. Compound (III) (5 mg) was isolated and identified as 4-hydroxybiphenyl on the basis of u.v., p.m.r. and mass spectra. The calculated mass for $^{12}\text{C}_{12}^{1}\text{H}_{10}^{17}\text{O}$ was 170.0732; the observed mass was 170.0736. The melting point of compound (III) (167–168°C) was identical with that of 4-hydroxybiphenyl, as was a mixed m.p.

Identification of compound (IV)

Although compound (IV) was not detectable in fractions from the silica-gel column, a small amount of this compound was isolated by using the h.p.l.c.

separation procedure shown in Fig. 1. A sample of the initial extraction residue was dissolved in acetonitrile and resolved by using h.p.l.c. After several repetitions of this procedure, the fractions containing compound (IV) were pooled and re-extracted with ethyl acetate, which was then removed *in vacuo*. Compound (IV) was identified as 2-hydroxybiphenyl, having a u.v. spectrum, R_F value on t.l.c., h.p.l.c. retention time, and colour reaction with Gibbs reagent identical with that of authentic 2-hydroxybiphenyl. When the initial extraction residue was treated with *NO*-bis(trimethylsilyl)acetamide to produce trimethylsilyl ether derivatives, examination by using a combination gas chromatograph-mass spectrometer indicated that a peak was present that was identical with that of the authentic 2-hydroxybiphenyl trimethylsilyl ether derivative with respect to g.l.c. retention time and mass spectrum.

Identification of compound (II)

Compound (II) was isolated by trapping fractions from the initial extract residue by using h.p.l.c., but an insufficient quantity was obtained for complete chemical characterization. Compound (II) was found to have a u.v. $\lambda_{\max.} = 255$ nm. The h.p.l.c. retention time of this compound is similar to that of 2,2'-dihydroxybiphenyl, but the u.v. spectra are dissimilar. In experiments described elsewhere (see under 'Replacement studies') in which 2- and 4-hydroxybiphenyl replaced biphenyl as the substrate, both compounds were transformed to produce a trace amount of a compound with an h.p.l.c. retention time and u.v. spectrum similar to that of compound (II). This suggests that compound (II) may be 2,4'-dihydroxybiphenyl.

Separation of monohydroxylated biphenyl isomers

As described in the Materials and Methods section, 3-hydroxybiphenyl was accumulated by repeated separations of the 4-hydroxybiphenyl fraction from silica-gel columns by using the μ Porasil system (Fig. 2) and identified from its u.v. spectrum, R_F value on t.l.c., and its characteristic colour reaction with Gibbs reagent. Identification was confirmed by g.l.c. separation of the trimethylsilyl ether derivative and comparison of retention time and mass spectrum with authentic material.

Time course of biphenyl transformation

The accumulation of the identified biphenyl metabolites over a period of time was evaluated by using the large-scale procedure. At intervals over a 5-day period, 5 ml samples were aseptically taken from a 500 ml shake culture of *C. elegans*. These samples were extracted three times with equal volumes of ethyl acetate, and the solvent removed *in vacuo*. The residue was redissolved in acetonitrile and examined

Fig. 2. Elution profile of monohydroxylated biphenyl isomers. Separation was achieved with a μ Porasil column as described in the Materials and Methods section. (a) Biphenyl and synthetic hydroxybiphenyls. (b) 3- and 4-hydroxybiphenyl produced by *C. elegans*.

Fig. 3. Kinetics of biphenyl transformation by *C. elegans*. Growth and assay conditions are described in the text. ●, 4,4'-Dihydroxybiphenyl; ○, 4-hydroxybiphenyl; △, 2-hydroxybiphenyl.

by h.p.l.c. for the amount of each metabolite present. The results are shown in Fig. 3. The amount of each metabolite was determined by the ratio of its peak

height (A_{254}) to that of a known amount of the authentic compound. The results indicate that under the defined growth conditions, maximal accumulation of these metabolites occurs at 48–60 h after addition of the substrate.

Replacement studies

To evaluate the ability of *C. elegans* to transform further the major monohydroxylated metabolites, small-scale experiments were carried out in which 2- and 4-hydroxybiphenyl (0.2 mg/ml) each replaced biphenyl as the substrate. As expected, metabolism of 4-hydroxybiphenyl gave rise to 4,4'-dihydroxybiphenyl. Transformation of 2-hydroxybiphenyl gave rise to a compound having a t.l.c. R_F value of 0.41 and a green colour reaction with Gibbs reagent followed by aq. ammonia treatment. Isolation by preparative t.l.c. yielded a compound identified as 2,5-dihydroxybiphenyl. The compound was identical with authentic 2,5-dihydroxybiphenyl with respect to u.v. and mass spectra, as well as h.p.l.c. retention time. The m.p. (100°C) was not depressed after admixture with the authentic compound.

As stated above, both 2- and 4-hydroxybiphenyl

were also transformed into a compound having physical properties similar to those of compound (II).

Fungal survey

Twelve species of fungi known to utilize hydrocarbons (Cerniglia & Perry, 1973) were tested for their ability to transform biphenyl. The transformation experiments were performed by using the small-scale procedure described for *C. elegans*, except that original inocula were 1 ml slant washes from test tube cultures of each organism. To allow for differences in growth rates, each species was grown to approximately the same density as a 2-day-old *C. elegans* culture before the introduction of biphenyl. The results are shown in Table 1. The amount of each metabolite produced was determined by the ratio of its peak height on h.p.l.c. relative to that of a known amount of the authentic compound. It may be seen that only four of the species tested showed measurable transformation of biphenyl, and only two showed transformation comparable with that of *C. elegans*.

Conjugated metabolites

When [^{14}C]biphenyl was incubated with *C. elegans*

Table 1. *Fungal survey of biphenyl transformation*

Growth and assay conditions are as described in the text. Results are expressed as percentages of *C. elegans* production at 48 h. For a small-scale (30 ml) biotransformation experiment, these values are: 4,4'-dihydroxybiphenyl, 5 nmol; 4-hydroxybiphenyl, 3 nmol; 2-hydroxybiphenyl, 0.5 nmol. Trace production (tr) of phenols is 2% or less of *C. elegans* production.

	Metabolite production (% of production in <i>C. elegans</i>)			
	4,4'-Dihydroxybiphenyl	Compound (II)	4-Hydroxybiphenyl	2-Hydroxybiphenyl
<i>Absidia</i> sp.	12	17	103	225
<i>C. echinulata</i>	13	17	13	106
<i>C. japonica</i>	tr	7	9	13
<i>Gilbertella</i> sp.	tr	—	3	13
<i>Syncephalastrum racemosum</i>	tr	10	tr	26
<i>Gliocladium</i> sp.	tr	10	tr	13
<i>Saccharomyces cerevisiae</i>	—	—	tr	tr
<i>Graphium ulmi</i>	—	—	—	—
<i>Penicillium zonatum</i>	—	—	tr	tr
<i>Penicillium ochro-chloron</i>	—	—	tr	—
<i>Aspergillus niger</i>	—	—	—	—

Table 2. *Metabolites present as conjugates*

Deconjugation and assay procedures are as described in the text. Resolution of 2- and 4-hydroxybiphenyl is insufficient for separate quantification, and the results are therefore pooled.

	Radioactivity (d.p.m./10 ml culture filtrate)			
	4,4'-Dihydroxybiphenyl	Compound (II)	4- and 2-Hydroxybiphenyl	Total
Free phenols	4400	136	7920	12456
Conjugated phenols	3400	1940	4400	9740
Conjugated phenols				
Total phenols (free and conjugated)	0.44	0.93	0.36	0.44

for 48 h a significant percentage of the radioactivity could not be extracted with ethyl acetate; a result that indicated the possible presence of conjugated metabolites. The culture filtrate was extracted with ethyl acetate before and after treatment with a commercial preparation of β -glucuronidase. This enzyme preparation also contained sulphatase activity. The phenols in the ethyl acetate extracts were separated by h.p.l.c. and quantified by measuring the radioactivity in each fraction (see the Materials and Methods section). The results are shown in Table 2. The free phenols are those that were present in the initial ethyl acetate extraction of the culture filtrate. The conjugated phenols represent those phenols that were extracted only after incubation of the culture filtrate with β -glucuronidase. The results indicate

that 44% of the known metabolites present in the culture filtrate were glucuronide or sulphate conjugates. One metabolite, compound (II), was present almost entirely in the conjugated state. Further characterization of the conjugates was not attempted.

Discussion

C. elegans and other fungal species preferentially hydroxylate biphenyl at the *para* positions, giving rise to 4-hydroxybiphenyl and 4,4'-dihydroxybiphenyl as the major metabolites. Substantially less hydroxylation occurs at the *ortho* position, giving rise to 2-hydroxybiphenyl in lesser amounts. The formation of 3-hydroxybiphenyl and 2,5'-dihydroxybiphenyl, previously unreported in fungi, is known

Scheme 1. Proposed pathway for the initial transformation of biphenyl by *C. elegans*

to occur in mammals (Meyer *et al.*, 1976; Meyer & Scheline, 1976). Also, the formation of 2,5-dihydroxybiphenyl from 2-hydroxybiphenyl by *C. elegans* parallels the result found in experiments with the rat (Ernst, 1965). Our results are therefore in accord with the observation of Smith & Rosazza (1974) that fungal transformation of biphenyl into phenols parallels that of mammals. The proposed pathway for the initial transformation of biphenyl by *C. elegans* is shown in Scheme 1.

The formation of water-soluble biphenyl metabolites suggested that conjugates, possibly glucuronides, were produced by *C. elegans*. Glucuronide conjugates account for 30–50% of the biphenyl metabolites produced by mammals (West *et al.*, 1956; Block & Cornish, 1959) and their formation is generally considered to be a mechanism for detoxification and rapid excretion of foreign compounds (Smith & Williams, 1966). Thus it is noteworthy that *C. elegans* forms a similar amount of conjugates. These cannot be identified definitively as glucuronides since the β -glucuronidase preparation used also contained sulphatase activity. Although no other attempt was made to identify the types of conjugates produced by *C. elegans* this observation warrants further investigation.

We have been unable to demonstrate the oxidation of biphenyl by microsomal fractions prepared from mycelium grown in the presence of biphenyl. However, the same microsomal fraction will oxidize naphthalene and benzo[a]pyrene (C. E. Cerniglia, R. H. Dodge & D. T. Gibson, unpublished work). In the light of the recent observation that biphenyl 2,3-oxide rapidly isomerizes to 2- and 3-hydroxybiphenyl in a ratio of 49:1 (Servé & Jerina, 1978) it seems possible that biphenyl 2,3-oxide could be the precursor of the 2- and 3-hydroxybiphenyl produced by *C. elegans*. By analogy 4-hydroxybiphenyl and possibly a trace amount of 3-hydroxybiphenyl could be formed by the isomerization of biphenyl 3,4-oxide. However, it should be emphasized that non-arene oxide pathways have been suggested to account for the formation of phenols in mammalian systems (Boyd *et al.*, 1972; Tomaszewski *et al.*, 1975; Billings & McMahon, 1978).

Although a large number of fungal species are capable of oxidizing naphthalene (Cerniglia *et al.*, 1978) most of the species in the present study failed to transform biphenyl. This may be due to the fungistatic properties of the biphenyl molecule. The mechanism of toxicity of biphenyl is still a matter for conjecture, although several hypotheses have been advanced (Georgopoulos & Vomvovanni, 1965). Whether activity is due to the parent molecule (Ramsey *et al.*, 1944) or its metabolically activated products remains to be determined.

In summary, fungi appear to metabolize biphenyl to a number of phenolic compounds. The fungi may

also convert free phenols into conjugates, possibly glucuronides. Further investigation into the fungistatic action of biphenyl is needed to clarify the mechanisms and consequences of fungal transformation of this hydrocarbon.

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