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Bacterial and Fungal Oxidation of Dibenzofuran

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Cunninghamella elegans and a mutant strain (B8/36) of *Beijerinckia* both oxidized dibenzofuran to 2,3-dihydroxy-2,3-dihydrodibenzofuran. The bacterial metabolite was extremely unstable and, in the presence of acid, was rapidly converted into a mixture of 2- and 3-hydroxydibenzofuran. In contrast, the 2,3-dihydroxy-2,3-dihydrodibenzofuran formed by *C. elegans* was stable and only yielded 2- and 3-hydroxydibenzofuran when heated under acidic conditions. The results suggest that *Beijerinckia* B8/36 and *C. elegans* form the respective *cis*- and *trans*-isomers of 2,3-dihydroxy-2,3-dihydrodibenzofuran. *C. elegans* also oxidized dibenzofuran to 2- and 3-hydroxydibenzofuran under conditions that would not lead to the dehydration of the *trans*-dihydrodiol. These observations implicate the initial formation of dibenzofuran- 2,3-epoxide in the fungal oxidation of dibenzofuran. *Beijerinckia* B8/36 also produced a second unstable dihydrodiol that was tentatively identified as *cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran. This compound gave 2-hydroxydibenzofuran as the major dehydration product and the *cis* relative stereochemistry was suggested by the isolation and characterization of an isopropylidine derivative. A preparation of *cis*-naphthalene dihydrodiol dehydrogenase and cell extracts of the parent strain of *Beijerinckia* oxidized both bacterial dihydrodiols to catechols. Cell extracts prepared from *C. elegans* catalysed an analogous oxidation of *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran to 2,3-dihydroxydibenzofuran. The latter product was also isolated and identified from culture filtrates. The results suggest that bacteria and fungi utilize different mechanisms to initiate the oxidation of dibenzofuran.

Dibenzofuran is a constituent of coal tar and its derivatives are found in many natural products such as lichen acids (Wachmeister, 1956), fungal pigments (Read & Vining, 1959; Gripenberg, 1960), and fruit (Anderson *et al.*, 1969). The ability of certain of its derivatives to lower lipid concentrations in animals is of pharmacological interest (Bondesson *et al.*, 1974). In addition, certain toxic chlorinated derivatives of dibenzofuran have been identified as trace contaminants in commercial preparations of polychlorinated biphenyls, pentachlorophenol and hexachlorobenzene (Fishbein, 1976). At this time there is little information available relating to the metabolism of dibenzofuran and its derivatives by micro-organisms. The present paper describes the isolation and identification of the oxidation products formed from dibenzofuran by a *Beijerinckia* species and *Cunninghamella elegans*.

Materials and Methods

Micro-organisms, maintenance and growth conditions

A *Beijerinckia* species originally isolated by its ability to grow with biphenyl as sole source of carbon and a mutant strain, *Beijerinckia* B8/36, that oxidizes several different aromatic hydrocarbons to form *cis*-dihydrodiols (Gibson *et al.*, 1973, 1975; Jerina *et al.*, 1976; Laborde & Gibson, 1977) were maintained on agar slants of mineral salts medium (Stanier *et al.*, 1966) containing 0.2% glucose. Before use as inocula, the bacteria were transferred from a slant to 100ml of glucose/mineral salts medium in a 500ml Erlenmeyer flask. Cultures were incubated at 25°C on a rotary shaker operating at 250rev./min. After 24h, 10ml quantities of the culture were used to inoculate six 2 litre Erlenmeyer flasks, each of which contained 400ml of glucose/mineral salts medium and solid dibenzofuran (0.4g). The flasks were incubated, as described above, for 17h and then extracted to recover the transformation products.

Stock cultures of *C. elegans* were maintained on Difco Sabouraud dextrose/agar medium. The isolation and characterization of *C. elegans* is described elsewhere (Cerniglia & Perry, 1973). The procedure for the fungal transformation of dibenzofuran was essentially similar to that described for naphthalene metabolism (Cerniglia & Gibson, 1977). The inoculum was prepared by aseptically homogenizing a 4-day-old Sabouraud dextrose/agar plate culture in 50ml of sterile 0.9% NaCl; 5ml quantities were dispensed in 2 litre Erlenmeyer flasks. Six flasks were used, each of which contained 400ml of Sabouraud dextrose broth. The flasks were incubated at 30°C

for 72h on a rotary shaker operating at 250rev./min. After 72h the medium was decanted, and the mycelial pellets were washed 3 times with sterile water. The washed pellets were resuspended in 400ml of Sabouraud dextrose broth that contained 0.4g of dibenzofuran. Six flasks were incubated under the same conditions as described above for 96h and then extracted to recover transformation products. For the production and isolation of transformation intermediates, two sets of controls were incubated. A substrate control was prepared by incubating dibenzofuran in Sabouraud dextrose broth without the micro-organism. A culture control was prepared by incubating the micro-organism in the medium in the absence of substrate. Samples of each culture were examined by phase-contrast microscopy before extraction and recovery of transformation products. No contamination was observed.

Extraction and detection of transformation products

After 17h incubation, the *Beijerinckia* B8/36 culture was filtered through glass-wool to remove the remaining dibenzofuran and the medium was extracted with 6 litres of ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4 and concentrated *in vacuo* to yield 1.30g of a brown residue.

After 96h, the mycelium from *C. elegans* was filtered, and the culture filtrate was extracted with ethyl acetate. Removal of the solvent gave 815mg of a brown oily residue. Each of the residues from the bacterial and fungal transformation of dibenzofuran were examined for metabolic products by thin-layer, high-pressure liquid, and silica-gel column chromatography.

Analytical methods

U.v.-and visible-absorption spectra were determined on a Cary model 14 recording spectrophotometer. All melting points were determined by use of a Fisher–Johns melting-point apparatus and are uncorrected. I.r. spectra were recorded on a Perkin–Elmer model 137 spectrophotometer. Crystalline samples were mulled in Nujol and placed between NaCl plates. All absorptions were referenced to the absorptions of polystyrene. Low-resolution mass spectra were determined on a Bell–Howell model 21-491 mass spectrometer. High-resolution spectra were determined on a Dupont–Consolidated Electrodynamics Corp. model 21-110 high-resolution mass spectrometer. Parent-ion molecular weights were determined by peak matching with assigned perfluoroalkane peak fragments. ^1H n.m.r. spectra were recorded on a Varian HA-100 spectrometer. Absorptions were assigned values at the midpoint of half-height and are referenced to tetramethylsilane. T.l.c. was carried out with Eastman chromatogram sheets (type K130R; silica gel with fluorescent indicator). The solvent used for chromatography was chloroform/acetone (4:1, v/v). Compounds were located on chromatograms by spraying with a 2% (w/v) methanolic solution of 2,6-dichloroquinone-4-chloroimide (Gibb's reagent) and also by use of u.v. light. Catechols were also detected colorimetrically with 4-aminoantipyrine (La Rue & Blakley, 1964). High-pressure liquid chromatography (h.p.l.c.) was used to separate dibenzofuran metabolites. H.p.l.c. was performed on a component system consisting of a Waters model 6000 A solvent-delivery system, model U-6K septumless injector and model 440 absorbance detector operated at 254nm. Separation was achieved with a μBondapak C_{18} column (3.9mm × 30cm). The metabolites were separated by gradient elution. The initial solvent was acetonitrile/water (3:7, v/v). The final solvent was acetonitrile/water (7:3, v/v). A gradient at curvature setting eight was used at a flow rate of 1.0ml/min. As metabolites eluted from the column samples were collected and immediately analysed by u.v. spectrophotometry and mass spectrometry. Absorption coefficients at 254nm were used to determine the relative amount of each metabolite produced by the different micro-organisms. Preparative separation of a 2- and 3-hydroxydibenzofuran was achieved on a Waters analytical μPorasil column (3.9mm × 30cm) with hexane/ethyl acetate (17:3, v/v) as the mobile phase. The flow rate was 1.0ml/min and a 280nm photometer was used to monitor the effluent.

Acid-catalysed dehydration of dihydrodiols

The dihydrodiols formed from the bacterial and fungal oxidation of dibenzofuran were dissolved in 3ml of methanol and the dehydration reaction initiated by the addition of 200μl of 3M-HCl. Formation of phenols from *cis*- and *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran were determined by measuring the increase in A_{251}. The phenols formed were extracted with ethyl acetate, dried over Na_2SO_4, concentrated *in vacuo* and separated by h.p.l.c.

Preparation of isopropylidine derivative

Crude dihydrodiol (200mg) residue from *Beijerinckia* B8/36 was dissolved in 5.0ml of 2,2-dimethoxypropane. The suspension was cooled in an ice bath before the addition of 1.0mg of toluene-*p*-sulphonic acid. After 180min the 2,2-dimethoxypropane was removed *in vacuo* to leave a solid residue that was dissolved in a small volume of chloroform and applied to the top of a column (1.5cm × 15cm) of basic alumina. Elution with chloroform gave 55mg of a yellow oil. T.l.c. in chloroform/acetone (4:1, v/v) gave a single spot (R_F 0.60).

Preparation of cell extracts

Cells of *Pseudomonas* sp. N.C.I.B. 9816 were grown with naphthalene as the sole source of carbon and energy. Washed cells (6.0g wet wt.) were suspended in 12.0ml of 0.05M-KH_2PO_4 buffer, pH7.2, and disrupted with the aid of a Biosonik II ultrasonic disintegrator. Deoxyribonuclease (6mg) was added to the suspension and the cell debris was removed by centrifugation at 30000*g* for 90min. The clear supernatant liquid was taken as a source of cell extract.

Cell extracts of the parent strain of *Beijerinckia* and *C. elegans* were prepared as described previously (Laborde & Gibson, 1977; Cerniglia & Gibson, 1978). In the case of *Beijerinckia* dibenzofuran replaced dibenzothiophen as the inducing substrate. Protein in cell extracts was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Chemicals

Dibenzofuran was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and was recrystallized from hexane before use. 2-Hydroxydibenzofuran, 3-hydroxydibenzofuran and 4-hydroxydibenzofuran were generously given by N. E. Stjernström, Astra Pharmaceuticals AB, Södertälje, Sweden. Ox heart lactate dehydrogenase (L-lactate–NAD^+ oxidoreductase, EC 1.1.1.27), sodium pyruvate, NAD^+ and $NADP^+$ were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Solvents used for h.p.l.c. analysis were purchased from Burdick and Jackson Laboratories Inc., Muskegon, MI, U.S.A. All other chemicals were as previously described (Laborde & Gibson, 1977).

Results

Bacterial oxidation of dibenzofuran

When *Beijerinckia* B8/36 was grown on glucose/mineral salts medium in the presence of dibenzofuran a yellow colour appeared in the culture medium. After 17h, cultures were filtered through glass-wool and centrifuged to remove the cells. The absorption spectrum of the clear supernatant solution before and after extraction with ethyl acetate is shown in Fig. 1. The yellow colour was due to a non-extractable product that gave an absorption maximum at 462nm. Ethyl acetate extraction of 2.4 litres of culture medium followed by removal of the solvent gave 1.33g of a solid residue. T.l.c. of a sample of the residue revealed the presence of two products [compounds (I) and (II)]. When the residue was acidified before chromatographic analysis compound (I) disappeared and only compound (II) was detected (Fig. 1, inset).

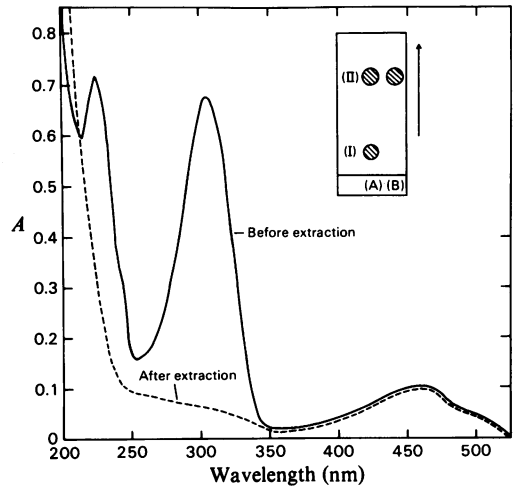


Fig. 1. *Absorption spectra of the oxidation products formed from dibenzofuran by Beijerinckia B8/36*

Results were obtained by diluting 0.1ml of clear culture supernatant fluid to 3.0ml with water. The spectra were recorded on a Cary model 14 recording spectrophotometer before (——) and after (----) extraction of the supernatant liquid with ethyl acetate. The inset shows the results of t.l.c. analysis of the residue obtained by extraction with ethyl acetate. (A) Before acidification; (B) after acidification with 2.5M-H_2SO_4.

Attempts to crystallize compound (I) from a variety of different solvents were unsuccessful. In every instance substantial decomposition to compound (II) was observed. Compound (I) (1.3g) was dissolved in a small amount of chloroform and applied to a column (34cm × 2.0cm) of 30% deactivated silica gel. The column was eluted with chloroform and 20ml fractions were collected. The contents of fractions 12–18 were pooled and the solvent removed to give an oily residue that had the chromatographic properties of compound (II). After 54 fractions had been collected the eluting solvent was changed to chloroform/methanol (49:1, v/v). The contents of tubes 40–82 were pooled and after removal of the solvent 1.0g of a yellow residue was obtained. All attempts to crystallize this material led to decomposition with the formation of compound (II).

In a separate experiment 635mg of compound (II) was applied to a column (25cm × 1.5cm) of activated silica gel. Chloroform was used as the eluting solvent and 10ml fractions were collected. The contents of each fraction were analysed by t.l.c. in chloroform/acetone (4:1, v/v). Fractions 5–22 each contained compounds with almost identical R_F values. However, differential colour reactions were observed when the chromatograms were sprayed with Gibb's

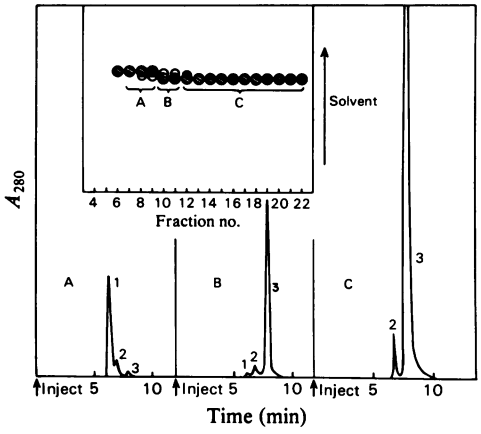


Fig. 2. *Resolution of compound* (II) *into three components by column chromatography on activated silica gel*
Each fraction eluted from the column was analysed by t.l.c. The solvent was chloroform/acetone (4:1, v/v). Compounds were located on the chromatogram by colour reactions with Gibb's reagent. Fractions 7–9 (A; blue), 10–11 (B; red-brown) and 12–22 (C; red-brown) were analysed by h.p.l.c. on a μPorasil column as described in the Materials and Methods section.

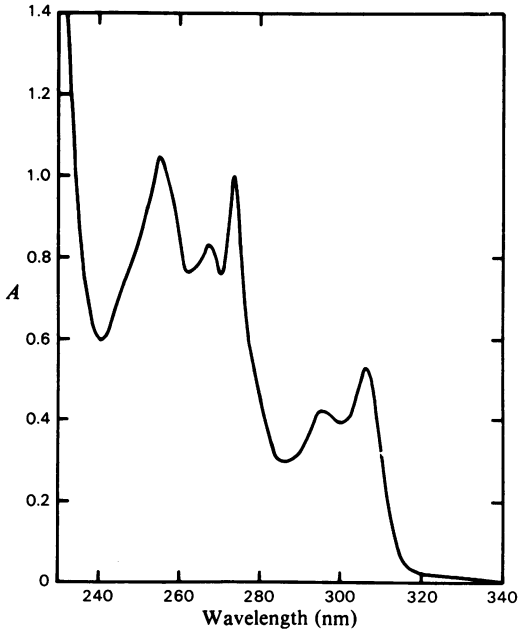


Fig. 3. *Absorption spectrum of the major component* (1-*hydroxydibenzofuran*) *in fractions* 7–9 (*Fig.* 2*A, peak* 1)
The compound was collected as it eluted from the high-pressure liquid chromatograph and its absorption spectrum was recorded on a Cary 14 recording spectrophotometer.

reagent. These results are represented schematically in the inset to Fig. 2. The contents of tubes 7–9, 10–11 and 12–22 were pooled and evaporated to dryness. The weights of each pooled fraction were 1.2, 49 and 279 mg respectively. Analysis of each fraction by h.p.l.c. gave the results shown in Fig. 2. The major component in fractions 7–9 gave the absorption spectrum shown in Fig. 3. Insufficient material was available for further characterization. It was tentatively identified as 1-hydroxydibenzofuran. Peaks 2 and 3 gave absorption spectra identical with those given by authentic samples of 2- and 3-hydroxydibenzofuran respectively. The residue obtained from the contents of tubes 12–22 was recrystallized from a mixture of diethyl ether/hexane. The crystals melted at 137–138°C and gave identical mass, i.r., u.v. and ^{1}H n.m.r. spectra to those given by synthetic 2-hydroxydibenzofuran.

Compound (I), isolated as described above, was analysed by h.p.l.c. The results obtained (Fig. 4*a*) indicated the presence of two components, which were designated (IA) and (IB). The absorption spectrum of component (IA) and its acid-catalysed degradation product is given in Fig. 5(*a*). The spectrum of the product formed by acid treatment is identical with that given by synthetic 2-hydroxydibenzofuran. Further confirmation was provided by h.p.l.c. (Fig. 4*b*), where 2-hydroxydibenzofuran was

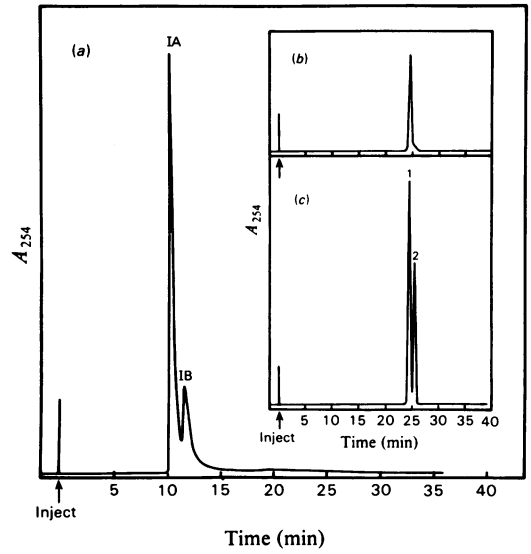


Fig. 4. (*a*) *Resolution of compound* (*I*) *into two components* (*IA and IB*) *by h.p.l.c.; (b) product formed after acidification of compound* (*IA*); (*c*) *products formed after acidification of compound* (*IB*)
Separations were achieved with a μBondapak column as described in the Materials and Methods section.

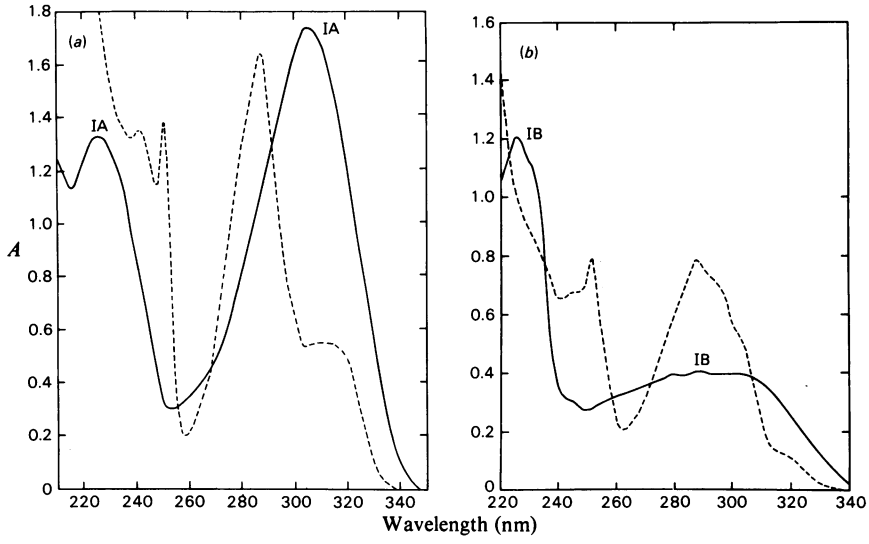


Fig. 5. *Absorption spectra of compounds (IA) (cis-1,2-dihydroxy-1,2-dihydrodibenzofuran) and (IB) (cis-2,3-dihydroxy-2,3-dihydrodibenzofuran) before (——) and after (----) acidification with H_2SO_4*

Each compound was isolated by h.p.l.c. as described in Fig. 4. The absorption spectra were recorded on a Cary model 14 recording spectrophotometer before acidification of the contents of each cuvette with 0.1 ml of 2.5 M-H_2SO_4.

found to be the only major component. The slight distortion of the peak at a retention time of 25 min did indicate the presence of trace amounts of a second phenol. In a separate experiment this component was found to have an identical absorption spectrum to that reported for 1-hydroxydibenzofuran (DeJongh & Van Fossen, 1972). Analogous experiments with compound (IB) gave the results shown in Fig. 5(*b*). The absorption spectrum obtained after acid treatment did not resemble that of any known dibenzofuranols. This anomaly was resolved by h.p.l.c. (Fig. 4*c*), where material from peaks 1 and 2 gave identical absorption spectra with those given by 2- and 3-hydroxydibenzofuran respectively. The i.r., u.v., mass and ^1H n.m.r. spectra of the phenols formed from compound (IB) were identical with those given by synthetic 2- and 3-hydroxydibenzofuran. Comparison of the relative peak area of each phenol (Fig. 4*c*) with the corresponding molar absorption coefficients showed that the ratio of 2- to 3-hydroxydibenzofuran was 61:39.

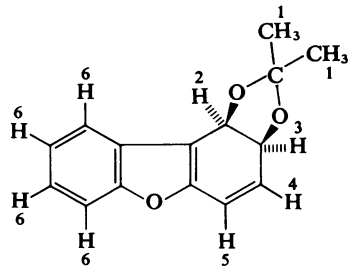
The results suggest that *Beijerinckia* B8/36 oxidizes dibenzofuran to a mixture of 1,2-dihydroxy-1,2-dihydrodibenzofuran and 2,3-dihydroxy-2,3-dihydrodibenzofuran. All attempts to crystallize the separated dihydrodiols led to decomposition to the corresponding dibenzofuranols. Thus it was not possible to assign the relative stereochemistry of the hydroxy groups in the two products. In an attempt to resolve this aspect of the problem an attempt was made to form the isopropylidine derivatives of compounds (IA) and (IB). The formation of such compounds from dihydrodiols is indicative of a *cis* relative stereochemistry (Brown & MacBride, 1964). A crude mixture of both dihydrodiols was treated with 2,2-dimethoxypropane under acidic conditions (see the Materials and Methods section). Although considerable dehydration occurred an isopropylidine derivative was obtained. The product was a colourless oil that had the following properties: mass spectrum, calculated for $^{12}C_{15}{}^1H_{14}{}^{16}O_3$, 242.0943, found mass 242.0944; $\lambda_{max.}^{oil}$ 3.35, 3.42, 6.15, 6.30 and 13.35 μm; $\lambda_{max.}^{methanol}$ 387 ($\varepsilon = 4600\ M^{-1}\cdot cm^{-1}$) 280 ($\varepsilon = 5150\ M^{-1}\cdot cm^{-1}$) 248 nm ($\varepsilon = 4800\ M^{-1}\cdot cm^{-1}$). The ^1H n.m.r. spectrum (Table 1) did not indicate the presence of two isopropylidine derivatives and in conjunction with the above evidence establishes the structure of the isolated product as 2,2-dimethyl-1,3-methylenedioxy-1,2-dihydrodibenzofuran. The results indicate that compound (IA) formed from dibenzofuran by *Beijerinckia* B8/36 is *cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran. Although the acidic conditions used to synthesize the isopropylidine derivative apparently caused complete dehydration of compound (IB) it appears probable that the structure of this compound is *cis*-2,3-dihydroxy-2,3-dihydrodibenzofuran.

Oxidation of bacterial dihydrodiols by cell extracts

cis-Naphthalene dihydrodiol dehydrogenase has been reported to oxidize a variety of *cis*-dihydrodiols (Patel & Gibson, 1974). An attempt was made to see

Table 1. *Analysis of the ^1H n.m.r. spectrum of the isopropylidine derivative formed from 1,2-dihydroxy-1,2-dihydrodibenzofuran*

The isolation procedure is described in the Materials and Methods section. The sample was dissolved in [^3H]chloroform and the spectrum recorded at 100MHz. Tetramethylsilane was used as the internal standard.



Proton	δ (p.p.m.)	Description
1	1.42	3H (s, methyl)
1	1.50	3H (s, methyl)
2	5.35	1H (d, alkoxymethine, $J_{2,3}$ 8.0Hz)
3	5.10	1H (double d, alkoxymethine, $J_{3,2}$ 8.0Hz, $J_{3,4}$ 3.5Hz, $J_{3,5}$ 1.0Hz)
4	5.96	1H (double d, olefinic, $J_{4,5}$ 10Hz, $J_{4,3}$ 3.5Hz)
5	6.66	1H (d, olefinic, $J_{5,4}$ 10Hz, $J_{5,3}$ 1.0Hz)
6	7.3–7.6	4H (m, aromatic)

if this enzyme would resolve the 1,2- and 2,3-dihydrodiols formed from dibenzofuran. A crude preparation of a deoxyribonuclease-treated extract of N.C.I.B. 9816 (100mg of protein) in 25ml of 0.5M-KH_2PO_4 buffer, pH7.2, was added to a serum bottle (70ml capacity). The continuous generation of NAD^+ in the reaction mixture was provided by the addition of 10μmol of NAD^+, 176μmol of sodium pyruvate and 560 units of lactate dehydrogenase (1.0mg of protein). The final reaction volume was 52.0ml. Before the addition of a crude mixture of both dibenzofuran dihydrodiols (approx. 100μmol in 0.2ml of dimethylformamide) the stoppered serum bottle was flushed with N_2 for 1h. Samples (0.3ml) were withdrawn at various time intervals with a 1.0ml hypodermic syringe and analysed by t.l.c. in chloroform/acetone (4:1, v/v). After 2h no dihydrodiol could be detected and the reaction mixture contained compound (I) (mixture of dibenzofuranols) and two components (IIIA) and (IIIB). These products were detected by t.l.c. in benzene/methanol (91:9, v/v). The contents of the reaction vessel were acidified with 5.0ml of 5M-H_2SO_4 and the precipitated protein was removed by centrifugation. The clear supernatant solution was extracted with ethyl acetate and the organic layer was dried over anhydrous Na_2SO_4. The residue remaining after removal of the solvent was dissolved in a small volume of acetone and purified by preparative t.l.c. in benzene/methanol (91:9). Compounds (IIIA) (R_F 0.53) and (IIIB) (R_F 0.43) were located with u.v. light and the areas of silica gel containing each compound were removed from the plate and extracted with methanol. The absorption spectrum of compound (IIIA) showed absorption maxima at 246, 258, 278 (shoulder), 282, and 320nm. Compound (IIIB) was formed in very small amounts and a definitive absorption spectrum was not obtained. Crude cell extracts of the parent strain *Beijerinckia* also catalysed the NAD^+-dependent oxidation of the dibenzofuran dihydrodiol mixture to compounds (IIIA) and (IIIB). Attempts to purify these products further were unsuccessful. However, from the known properties of *cis*-naphthalene dihydrodiol dehydrogenase (Patel & Gibson, 1974) they were assumed to be catechol derivatives. Compound (IIIA) was tentatively identified as 1,2-dihydroxydibenzofuran.

Crude cell extracts of *Beijerinckia* and *Pseudomonas* N.C.I.B. 9816 both oxidized 1,2-dihydroxydibenzofuran to a yellow ring-fission product whose absorption maximum was 462nm. Owing to the small amounts of catechol available no attempt was made to characterize the ring-fission product.

Fungal oxidation of dibenzofuran

C. elegans was grown with Sabouraud dextrose broth in the presence of dibenzofuran. After 96h the culture filtrate (2.4 litres) was extracted with ethyl acetate. Removal of the solvent gave 0.81g of a solid residue. T.l.c. of the residue in chloroform/acetone (4:1, v/v) revealed the presence of dibenzofuran (R_F 0.53) and three oxidation products (R_F 0.17, 0.27 and 0.46). Each of the dibenzofuran metabolites absorbed u.v. light and reacted with Gibb's reagent.

The solid residue was dissolved in a small volume of chloroform and applied to the top of a column (2.0cm × 40cm) of deactivated silica gel (20% water). The solvent used for elution was chloroform/acetone (4:1, v/v) and 15ml fractions were collected. Samples of each fraction were analysed for the presence of dibenzofuran metabolites by t.l.c. in chloroform/acetone (4:1, v/v). Fractions 5–9 contained a product with an R_F of 0.46 that gave a blue colour with Gibb's reagent. The contents of tubes 5–9 were pooled and the solvent removed leaving 19.5mg of a brown residue. This material, when analysed by h.p.l.c., was shown to contain two components [(I) and (II)] with retention times identical with those given by synthetic 2- and 3-hydroxydibenzofuran. Repeated injections of a portion of thin-layer residue into the high-pressure liquid chromatograph gave sufficient material for the chemical characterization of each component. Compound (I) melted at 134–135°C and the melting point was not depressed on admixture with a pure sample of synthetic 2-hydroxydibenzofuran. Its

absorption spectrum in methanol showed absorption maxima at 236, 242, 252, 284 (shoulder), 289 and 318nm and was superimposable on the spectrum given by synthetic 2-hydroxydibenzofuran. Additional evidence was provided by mass-spectral analysis (calculated mass for $^{12}C_{12}{}^{1}H_{8}{}^{16}O_{2}$, 184.0524; found mass 184.0528). The above data identify compound (I) as 2-hydroxydibenzofuran. Compound (II) melted at 138–141°C and a mixed melting point with synthetic 3-hydroxydibenzofuran showed no depression. Its absorption spectrum ($\lambda_{max.}$ 305, 298, 254 and 231nm) was identical with that given by authentic 3-hydroxydibenzofuran. The molecular weight of compound (II) was 184.0530, which is in good agreement with the calculated mass for a monohydroxylated dibenzofuran. These observations establish the structure of compound (II) as 3-hydroxydibenzofuran.

The contents of tubes 10–15 were pooled and the solvent removed *in vacuo*. The resulting brown oil (27mg) contained a major product [compound (III)] that was detected by t.l.c. in chloroform/acetone (4:1, v/v). The chromatographic properties (R_F 0.27), the brown colour formed with Gibb's reagent and the red colour formed with 4-aminoantipyrine suggested that compound (III) was a catechol. The brown oil was dissolved in acetonitrile and further purified by h.p.l.c. Multiple injections of the sample led to the isolation of a crystalline product, m.p. 166–170°C, that gave an absorption maxima at 308nm (ε 5559$M^{-1}\cdot cm^{-1}$), and 256nm (ε 5992$M^{-1}\cdot cm^{-1}$). Further evidence was provided by mass-spectral analysis where the determined mass (200.0478) was in close agreement with the calculated mass of 200.0472 for $^{12}C_{12}{}^{1}H_{8}{}^{16}O_{3}$. These properties are similar to those reported for 2,3-dihydroxydibenzofuran (Pettersson & Stjernström, 1963).

Fractions 13–20 were pooled and the solvent removed to leave 64mg of a white solid [compound (IV)]. This material, m.p. 128–133°C, was free from detectable impurities when analysed by t.l.c. (R_F 0.17; chloroform/acetone, 4:1) and h.p.l.c. Mass-spectral data (calculated for $^{12}C_{12}{}^{1}H_{10}{}^{16}O_{3}$, 202.0630; found mass, 202.0622) suggested that compound (IV) was a dihydrodihydroxy derivative of dibenzofuran. This was confirmed by acid-catalysed dehydration of compound (IV) to yield a mixture of 2- and 3-hydroxydibenzofuran (Fig. 6). The ratio of 2-/3-hydroxydibenzofuran was 57:43. The absorption spectra of compound (IV) before and after acidification are identical with the spectra given by the bacterial metabolite (Fig. 5*b*), which was identified as *cis*-2,3-dihydroxy-2,3-dihydrodibenzofuran. However, compound (IV) was relatively stable in the presence of acid, a property that suggested its structure to be *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran. Rates of dehydration have been shown to be reasonably reliable for the assignment of relative stereochemistry since

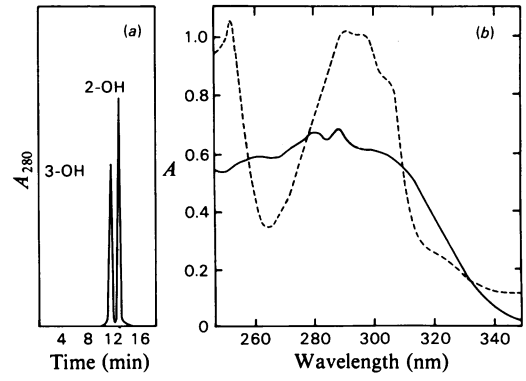


Fig. 6. *Absorption spectrum of compound* (IV) *produced from dibenzofuran by C. elegans*

(*b*) The sample was dissolved in methanol and the spectrum recorded on a Cary 14 recording spectrophotometer (——). The mixture was acidified with 0.1ml of 2.5M-H_2SO_4 and heated at 80°C for 15min. The absorption spectrum of the acidified reaction mixture was also recorded (----). (*a*) The resolution by h.p.l.c. of the products formed from compound (IV) by acid treatment. Separation was achieved with a μPorasil column as described in the Materials and Methods section except that the solvent composition was hexane/ethyl acetate (4:1, v/v). Calculated molar absorption coefficients for 2-hydroxydibenzofuran (2-OH) and 3-hydroxydibenzofuran (3-OH) at 280nm were 13600 and 12400$M^{-1}\cdot cm^{-1}$ respectively.

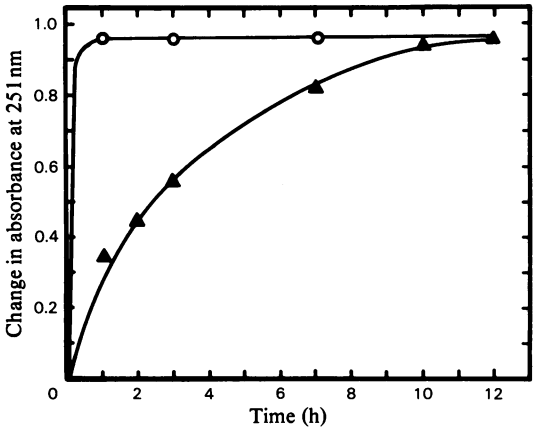


Fig. 7. *Relative rates of dehydration of the* 2,3-*dihydroxy*-2,3-*dihydrodibenzofuran produced by Beijerinckia B*8/36 (○) *and C. elegans* (▲)

Samples were dissolved in methanol and the reactions initiated by the addition of 0.2ml of 3M-HCl. The reaction was followed at 251nm where both 2- and 3-hydroxydibenzofuran show significant absorption.

cis-arene dihydrodiols dehydrate much faster than the corresponding *trans*-isomers (Jerina *et al.*, 1976). The rates of dehydration of the 2,3-dihydroxy-2,3-dihydrodibenzofuran produced by *Beijerinckia* B8/36 and *C. elegans* are shown in Fig. 7. The results suggest that compound (IV) is the *trans*-isomer.

Oxidation of trans-2,3-dihydroxy-2,3-dihydrodibenzofuran by cell extract

Owing to the low enzymic activity of cell extracts it was not possible to detect the oxidation of *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran by measuring the reduction of $NADP^+$ at 340nm. However in a long-term experiment the formation of 2,3-dihydroxydibenzofuran from the *trans*-isomer was clearly demonstrated (Fig. 8).

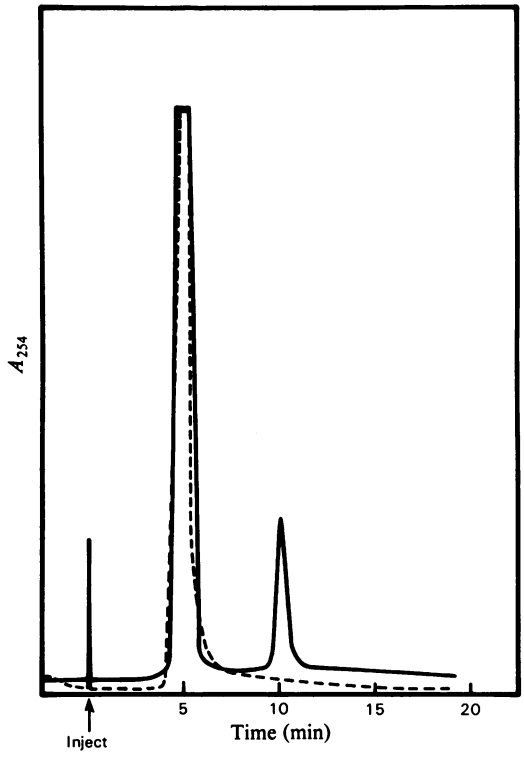


Fig. 8. *Oxidation of trans-2,3-dihydroxy-2,3-dihydrodibenzofuran by cell extracts of C. elegans*

The reaction mixture contained in a final volume of 11.0ml: Tris buffer, pH8.0, 500μmol; $NADP^+$, 6μmol; cell extract, 10mg of protein. The reaction was initiated by the addition of 4.95μmol of *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran in 200μl of dimethylformamide. After 1h the reaction was terminated by the addition of 1.0ml of 2.5M-H_2SO_4 and the reaction mixture was extracted with ethyl acetate. Reaction products were analysed by h.p.l.c. as described in the Materials and Methods section (——). A control experiment with boiled cell extract showed no oxidation of the substrate (– – – –). Retention times for *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran and its oxidation product were 5 and 10min respectively.

Quantification of dibenzofuran oxidation products

H.p.l.c. was used to separate and quantify each metabolite produced from dibenzofuran by *C. elegans*. The elution profile of a crude ethyl acetate extract obtained from *C. elegans* after growth in the presence of dibenzofuran is shown in Fig. 9. Absorption coefficients at 254nm were calculated for pure samples of each metabolite and the values were used to determine the relative amounts produced by *C.* elegans. The major metabolites were *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran (58%) and 2,3-dihydroxydibenzofuran (28%). Minor products isolated were 2-hydroxydibenzofuran (8%) and 3-hydroxydibenzofuran (6%).

Discussion

The reaction sequences shown in Scheme 1 are proposed for the metabolism of dibenzofuran by

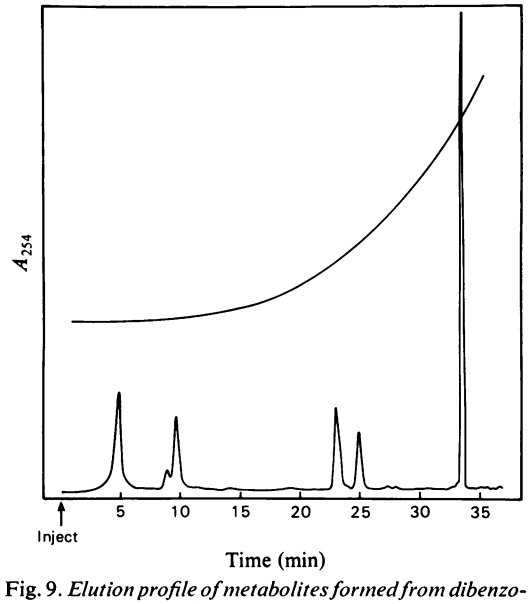
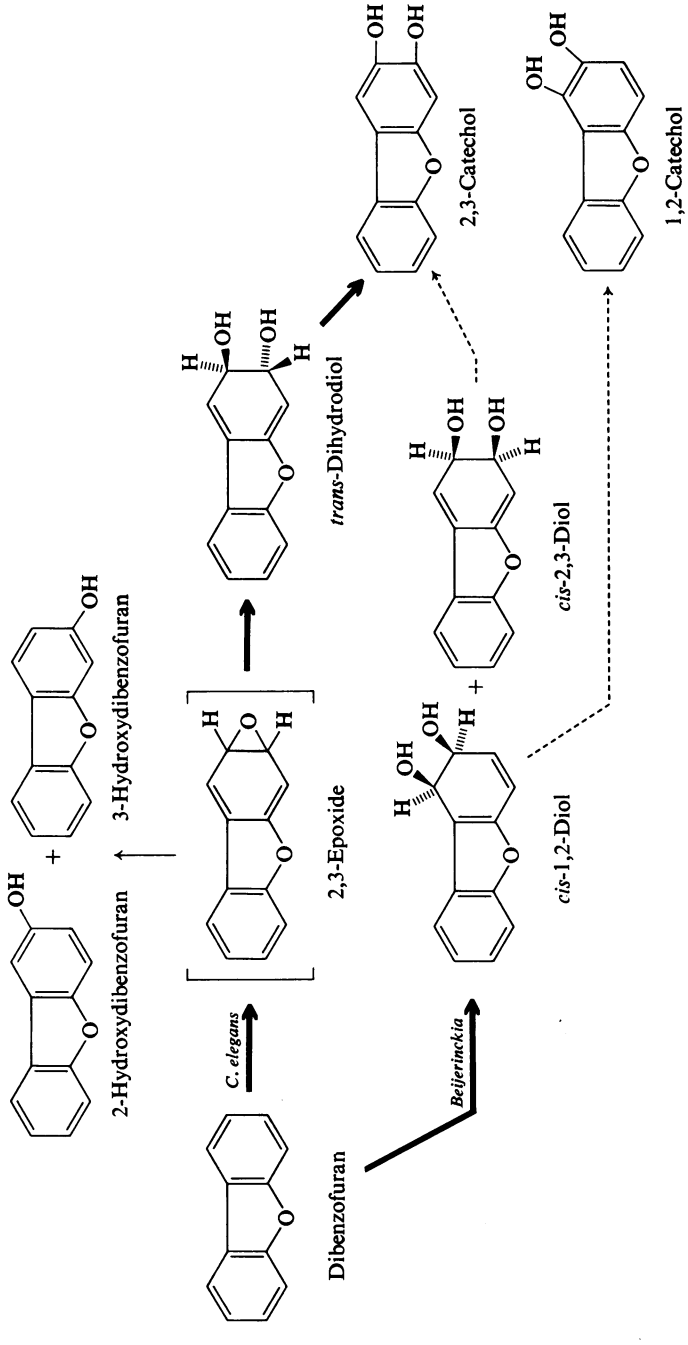


Fig. 9. *Elution profile of metabolites formed from dibenzofuran by C. elegans*

Procedures used for the separation and identification of metabolites are as described in the text. Retention times and absorption coefficients at 254nm are for *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran 5min and 5421 $M^{-1} \cdot cm^{-1}$, for 2,3-dihydroxydibenzofuran 10min and 5992 $M^{-1} \cdot cm^{-1}$, for 2-hydroxydibenzofuran 23min and 25214 $M^{-1} \cdot cm^{-1}$, and for 3-hydroxydibenzofuran 25min and 22403 $M^{-1} \cdot cm^{-1}$.



Scheme 1. *Proposed reaction sequences for the initial reactions in the metabolism of dibenzofuran by Beijerinckia and C. elegans*
The compound in brackets (dibenzofuran 2,3-epoxide) was not identified and its inclusion in the reaction sequence is based on the indirect evidence cited in the text. Postulated reactions are indicated by the dotted arrows.

Beijerinckia sp. and *C. elegans*. *Beijerinckia* B8/36 oxidized dibenzofuran to a mixture of *cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran and *cis*-2,3-dihydroxy-2,3-dihydrodibenzofuran. Both products were unstable and their identification was based on the isolation and identification of the dibenzofuranols formed by dehydration of the parent molecules. The *cis* relative stereochemistry was indicated by the reaction of 1,2-dihydroxy-1,2-dihydrodibenzofuran with 2,2-dimethoxypropane to form an isopropylidine derivative (Brown & MacBride, 1964). It was not possible to isolate an analogous derivative from 2,3-dihydroxy-2,3-dihydrodibenzofuran. However, the rapid rate of dehydration of this compound compared with the rate observed with the *trans*-isomer formed by *C. elegans* (Fig. 7) supports the assignment of a *cis* stereochemistry to the bacterial metabolite (Jerina *et al.*, 1976). Further indirect evidence for the formation of *cis*-isomers is provided by previous observations that *Beijerinckia* B8/36 forms *cis*-dihydrodiols from other aromatic substrates (Gibson *et al.*, 1973, 1975; Jerina *et al.*, 1976; Laborde & Gibson, 1977).

C. elegans also oxidized dibenzofuran to 2,3-dihydroxy-2,3-dihydrodibenzofuran. The fungal metabolite had identical spectral and chromatographic properties to those given by the compound formed from dibenzofuran by *Beijerinckia* B8/36. Also, the 2,3-dihydrodiols produced by each organism were subject to acid-catalysed dehydration and in each instance the ratios of the dehydration products (2-hydroxydibenzofuran/3-hydroxydibenzofuran) were similar. However, as mentioned previously, the compound formed by *C. elegans* was extremely stable and its rate of dehydration was much slower than that of the compound formed by *Beijerinckia* B8/36. These observations provide good evidence that *C. elegans* oxidizes dibenzofuran to *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran. In a previous report we have shown that *C. elegans* oxidizes naphthalene to *trans*-1,2-dihydroxy-1,2-dihydronaphthalene (Cerniglia & Gibson, 1977). No evidence was obtained for oxidative attack at the 2,3-position of naphthalene.

The formation of *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran suggests that the initial oxidation product in dibenzofuran metabolism is dibenzofuran 2,3-epoxide (Jerina & Daly, 1974). Arene oxides are formed from a variety of aromatic substrates by mammalian microsomal fraction and are known to be converted into *trans*-dihydrodiols by the enzyme epoxide hydratase (Oesch, 1973). The isolation of 2- and 3-hydroxydibenzofuran from culture filtrates of *C. elegans* also implicates the prior formation of dibenzofuran 2,3-epoxide since most arene oxides undergo spontaneous isomerization to form phenols. It could be argued that 2- and 3-hydroxydibenzofuran are formed by dehydration of *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran. However, control experiments showed phenol formation under conditions that did not lead to dehydration of the *trans*-dihydrodiol.

The incubation of *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran with cell extracts of *C. elegans* and $NADP^+$ led to the formation of a small amount of 2,3-dihydroxydibenzofuran. The latter compound was also isolated from culture filtrates. Although enzymic activity was extremely low the dehydrogenation reaction is analogous to the conversions of *trans*-dihydrodiols into catechols that are catalysed by the soluble dihydrodiol dehydrogenases of mammalian liver (Jerina *et al.*, 1970). In contrast, bacterial arene dihydrodiol dehydrogenases are specific for *cis*-isomers (Patel & Gibson, 1974; Axcell & Geary, 1975; Rogers & Gibson, 1977). When a mixture of 1,2-dihydroxy-1,2-dihydrodibenzofuran and 2,3-dihydroxy-2,3-dihydrodibenzofuran was incubated with NAD^+ and cell extracts from the parent strain of *Beijerinckia* or a crude preparation of *cis*-naphthalene dihydrodiol dehydrogenase two catechol derivatives were formed. One of these products had similar chromatographic properties to the 2,3-dihydroxydibenzofuran formed by *C. elegans*. By analogy the other product was assumed to be 1,2-dihydroxydibenzofuran.

The results of the present study illustrate the different mechanisms used by bacteria and fungi to initiate the metabolism of aromatic substrates. Bacteria utilize an enzyme system that incorporates both atoms of molecular oxygen into the aromatic substrate to form *cis*-dihydrodiols as the first detectable products (Gibson, 1977). *Beijerinckia* is an unusual organism in this respect since it can form positional isomers with different aromatic substrates (Gibson *et al.*, 1975; Jerina *et al.*, 1976). In contrast, fungi appear to incorporate one atom of molecular oxygen into the aromatic substrate to form arene oxides (Auret *et al.*, 1971; Daly *et al.*, 1972; Ferris *et al.*, 1973, 1976; Cerniglia & Gibson, 1977). The latter can undergo isomerization to yield phenols or the enzymic addition of water to form *trans*-dihydrodiols. In this respect the metabolism of aromatic compounds by *C. elegans* (Cerniglia & Gibson, 1977, 1978; Cerniglia *et al.*, 1978, and the present paper) is very similar to the mechanisms used by mammals for the detoxification and excretion of these substrates.

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3-hydroxydibenzofuran and 4-hydroxydibenzofuran. We thank Judith M. Davis for technical assistance and Roberta DeAngelis for assistance in preparing the manuscript.

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