Metabolism of Dibenzo[1,4]dioxan by a *Pseudomonas* Species

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Pseudomonas sp. N.C.I.B. 9816 strain 11, when grown on salicylate in the presence of dibenzo[1,4]dioxan, accumulated *cis*-1,2-dihydroxy-1,2-dihydrodibenzo[1,4]dioxan and 2-hydroxydibenzo[1,4]dioxan in the culture medium. Each metabolite was isolated in crystalline form and identified by a variety of conventional chemical techniques. Crude cell extracts prepared from the parental strain grown with naphthalene oxidized *cis*-1,2-dihydroxy-1,2-dihydrodibenzo[1,4]dioxan under both aerobic and anaerobic conditions to 1,2-dihydroxydibenzo[1,4]dioxan. Further degradation of this metabolite was not detected.

Heterocyclic aromatic compounds containing two atoms of oxygen, such as dibenzo[1,4]dioxan, have been identified in the neutral fractions of bituminous coal (Hayatsu *et al.*, 1975). Interest in chlorinated dibenzo[1,4]dioxans arose with the identification by X-ray diffraction of 1,2,3,7,8,9-hexachlorodibenzo[1,4]dioxan as the 'chick oedema factor' that caused the deaths of millions of broiler chickens in 1957 (Cantrell *et al.*, 1969). It is now known that conditions utilized in the synthesis of certain chlorinated fungicides and herbicides, such as pentachlorophenol, 2,4,5-trichlorophenol and 2,4,5-trichlorophenoxyacetic acid can result in the formation of chlorinated dibenzo[1,4]dioxans. The most potent isomer, 2,3,7,8-tetrachlorodibenzo[1,4]dioxan, has been termed the most potent low-molecular-weight toxin known to man, with a mean lethal dose in guinea pigs of 1.0 µg/kg body wt. (Schwetz *et al.*, 1973).

Little is known about the metabolism of these compounds by micro-organisms. Kearney *et al.* (1972) studied the metabolism of ¹⁴C-labelled 2,7-dichlorodibenzo[1,4]dioxan and 2,3,7,8-tetrachlorodibenzo[1,4]dioxan in soil samples. They reported that measurable ¹⁴CO₂ was detected from soils treated with 2,3,7,8-tetrachlorodibenzo[1,4]dioxan. Ethanol extracts of soil samples treated with 2,7-dichlorodibenzo[1,4]dioxan were found to contain one or more polar metabolites. However, attempts to identify the compounds were unsuccessful. No metabolites were detected in soils treated with 2,3,7,8-tetrachlorodibenzo[1,4]dioxan. Matsumura & Benezet (1973) screened over 100 microbial strains previously shown to be capable of degrading persistent pesticides for the ability to degrade ¹⁴C-labelled 2,3,7,8-tetrachlorodibenzo[1,4]dioxan. Only five strains were found to be capable of degrading this compound to unidentified polar metabolites.

The present paper describes the co-oxidation of dibenzo[1,4]dioxan by a *Pseudomonas* sp. Evidence is presented for the identification of metabolites before enzymic fission of the aromatic nucleus.

Materials and Methods

Organisms

Pseudomonas sp. N.C.I.B. 9816 was kindly provided by Professor W. C. Evans, University of North Wales, Bangor, Wales, U.K. This organism was isolated from soil by elective culture, and has the ability to grow on naphthalene as a sole source of carbon (Davies & Evans, 1964). A spontaneous mutant, *Pseudomonas* sp. N.C.I.B. 9816, strain 11, was isolated from the parent organism, which oxidized naphthalene to *cis*-1,2-dihydroxy-1,2-dihydronaphthalene when grown on succinate or salicylate as the carbon source (D. T. Gibson, unpublished work).

Growth conditions

Organisms were cultured in a liquid mineral-salts media as described by Stanier *et al.* (1966) with the exception that the phosphate buffering capacity was double strength (pH 7.25). Large-scale biotransformation cultures were conducted in a 50 litre carboy containing 10 litre of media with sodium salicylate (1.0 g/litre) and dibenzo[1,4]dioxan (0.5 g/litre). Cultures were incubated on a reciprocating shaker at 27°C.

Preparation of cell extracts

Pseudomonas sp. N.C.I.B. 9816 was grown with naphthalene (1.0 g/litre) as the carbon source in large-scale cultures (10 litre) under forced aeration in a New Brunswick model M14 Microferm fermentor at 30°C for 12 h. Air was supplied at a rate of 10 litre/min and the culture was stirred at 500 rev./min. The cultures were initially filtered to remove solid materials, and the cells were harvested with an air-driven Sharples continuous-flow centrifuge. The cells were washed twice with 0.05 M-KH₂PO₄ buffer, pH 7.2, and stored at -15°C until required. Frozen

cells were thawed and suspended in 0.05 M-KH₂PO₄ buffer, pH 7.2, containing 10% (v/v) acetone (3 g of cells/10 ml of buffer). The suspension was subjected to sonic oscillation at 40 kHz with a Bronwill Biosonik III ultrasonic disintegrator for 1.5 min (three 30 s exposures). Cell debris was removed after centrifugation at 30000g for 1 h at 5°C. The clear supernatant solution was used as a source of crude cell extract. Protein concentration in the extracts was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as the standard.

Enzyme assays

1,2-Dihydroxynaphthalene oxygenase was assayed by measuring the rate of O₂ uptake at 30°C in a Clark-type oxygen electrode, with 0.05 M-KH₂PO₄ buffer, pH 6.0, which was saturated with air before use. The reaction mixture, in a total volume of 1.4 ml, contained buffer and an amount of cell extract found to give a linear rate of O₂ consumption with respect to protein concentration. The reaction was initiated with the addition of 100 nmol of 1,2-dihydroxynaphthalene in 10 µl of anhydrous tetrahydrofuran. All rates were corrected for endogenous respiration and for the rate of the non-enzymic oxidation of 1,2-dihydroxynaphthalene.

Analytical methods

U.v. and visible spectra were determined on a Beckman model 25 recording spectrophotometer. I.r. spectra were recorded on a Perkin-Elmer model 137 spectrophotometer with polystyrene as the reference absorption. Crystalline samples were milled in Nujol and placed between NaCl discs. Mass spectra were determined on a Dupont-Consolidated Electrodynamics Corp. model 21-1000C 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 δ values at the midpoint of half-height with tetramethylsilane as reference compound. Optical activity was determined with a Perkin-Elmer model 141 polarimeter. Melting points were obtained with a Büchi melting-point apparatus and were uncorrected.

T.l.c. was performed by using type SIL G/UV Brinkman chromatogram sheets (Brinkman Instruments Inc., Westbury, NY, U.S.A.). Preparative t.l.c. was performed by using glass plates coated with silica gel 60 F-254 (E. Merck, Darmstadt, Germany). A chloroform/acetone (4:1, v/v) system was used as solvent for chromatography. Compounds were located by the use of u.v. light and also by spraying with a 2% (w/v) solution of 2,6-dichloroquinone-4-chloroimide in methanol (Gibb's reagent).

Chemicals

Dibenzo[1,4]dioxan was synthesized by the procedure described by Gilman & Dietrich (1957). 2-Hydroxydibenzo[1,4]dioxan was synthesized by a method analogous to that described by Hartough & Meisel (1954) for the synthesis of 2-hydroxydibenzothiophen. 1,2-Dihydroxynaphthalene was synthesized by the method of Corner & Young (1954). NAD⁺ was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Silica gel 60 F-254 from Brinkman Instruments and basic alumina, Brockman Activity I, from Fisher Scientific Co., Pittsburg, PA, U.S.A., were used in column chromatography. Organic solvents were purified by vacuum distillation. All other materials were of the highest purity commercially available and were used without further purification.

Results

Oxidation of dibenzo[1,4]dioxan by Pseudomonas sp. N.C.I.B. 9816 strain 11

Initial studies revealed that optimum co-oxidation was observed when *Pseudomonas* sp. N.C.I.B. 9816 strain 11 was grown in a mineral-salts medium containing salicylate (1.0 g/litre) and dibenzo[1,4]dioxan (0.5 g/litre). Under these conditions, two neutral products [compounds (I) and (II)] were excreted into the culture medium. Compound (I) (R_F 0.22) absorbed u.v. light and gave a brown colour with Gibb's reagent. Compound (II) (R_F 0.5) also absorbed u.v. light and gave a purple colour with Gibb's reagent. The accumulation of compound (I) in the culture medium with time was monitored spectrophotometrically (Fig. 1). After 36 h, the culture was filtered to remove unused dibenzo[1,4]dioxan and extracted with an equal volume of ethyl acetate. The organic layer was dried over anhydrous Na₂SO₄ and the solvent removed *in vacuo* at 35°C to yield 637 mg of a solid residue.

Isolation of compounds (I) and (II)

The residue was dissolved in a small volume of chloroform and applied to the top of a silica-gel column [1.9 cm × 22.0 cm; 25% (w/v) water]. The column was eluted with 700 ml of chloroform and then 700 ml of chloroform/methanol (49:1, v/v). The presence of compounds (I) and (II) in the column fractions (15 ml) was detected by t.l.c. Fractions 9–15 were found to contain compound (II). Compound (I) was eluted in fractions 63–75. Fractions containing each metabolite were pooled and the solvent removed *in vacuo* at 30°C. Compound (I) (242 mg) and compound (II) (276 mg) were further purified by two recrystallizations from hexane/acetone.

Fig. 1. Formation of compound (I) by *Pseudomonas* sp. N.C.I.B. 9816 strain 11

A 10 litre culture was grown as described in the text. At the times indicated (h) on the graph, 5.0ml samples were extracted with ethyl acetate (15.0ml). The solvent was removed at 30°C under a stream of air, and the residue dissolved in 1.0ml of acetone before chromatography on precoated glass t.l.c. plates (see the Materials and Methods section). Compound (I) (R_F 0.22) was detected by u.v. light. The region of the plate containing compound (I) was removed, and the silica gel was extracted with 5.0ml of methanol. Samples (0.1 ml) of the methanol extract were diluted to 1.0ml with the same solvent, and the u.v. spectrum recorded on a Beckman model 25 recording spectrophotometer. The reference cuvette contained methanol.

in methanol), light-absorption maxima in methanol at 227 and 298 nm ($\epsilon = 12600$ and $2800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ respectively) and i.r.-absorption maxima in Nujol at 3.05, 5.92, 6.3, 6.9, 7.95, 10.82, 12.0, 12.4 and 14.18 μm . A mass-spectral determination gave a calculated mass for $^{12}\text{C}_{12}^{1}\text{H}_{10}^{16}\text{O}_4$ of 218.0579 and a found mass of 218.0587. An analysis of the ^1H n.m.r. spectrum is given in Fig. 3.

The physical data suggest that compound (I) is 1,2-dihydroxy-1,2-dihydrodibenzo[1,4]dioxan. Ad-

Proton	δ (p.p.m.)	Description
1	6.77	1H (m, aromatic)
2	6.46	2H (m, aromatic)
3	6.82–7.02	4H (m, aromatic)
4	8.20–8.44	1H (broad, hydroxy)

Fig. 2. Analysis of the ^1H n.m.r. spectrum of compound (II) (2-hydroxydibenzo[1,4]dioxan)

The sample was dissolved in $[\text{D}_6]\text{acetone}$ and the spectrum was recorded at 100MHz. Tetramethylsilane was used as an internal standard.

Identification of compound (II)

The white crystals obtained after recrystallization had m.p. 144–145°C, light-absorption maxima in methanol at 221 and 292 nm ($\epsilon = 32000$ and $4300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ respectively) and i.r.-absorption maxima in Nujol at 2.82, 2.92, 6.15, 6.32, 7.53, 10.83, 12.5 and 13.4 μm . A mass-spectral determination gave a calculated mass for $^{12}\text{C}_{12}^{1}\text{H}_8^{16}\text{O}_3$ of 200.0473 and a found mass of 200.0468. An analysis of the ^1H n.m.r. spectrum of compound (II) is given in Fig. 2.

The results of the physical measurements suggest that compound (II) is 2-hydroxydibenzo[1,4]dioxan. Verification of this structure was obtained with the chemical synthesis of 2-hydroxydibenzo[1,4]dioxan from 2-bromodibenzo[1,4]dioxan by an alkali fusion reaction. Compound (II) exhibited i.r., u.v. and mass spectra identical with those of the synthetic compound. No decrease in melting point was observed when compound (II) was mixed with the authentic sample of 2-hydroxydibenzo[1,4]dioxan.

Identification of compound (I)

The white crystals obtained after recrystallization had m.p. 139–140°C, $[\alpha]_D^{25} + 110 \pm 1$ ($n = 10$) (c 0.405

Proton	δ (p.p.m.)	Description
1	4.55	1H (d, hydroxymethine, $J_{1,2}$ 6.5 Hz)
2	4.92	1H (m, hydroxymethine, $J_{2,1}$ 6.5 Hz)
3	5.80	1H (double d, olefinic, $J_{3,4}$ 10.0 Hz, $J_{3,2}$ 2.0 Hz)
4	6.06	1H (m, olefinic, $J_{4,3}$ 10.0 Hz)
5	6.70–7.00	4H (m, aromatic)

Fig. 3. Analysis of the ^1H n.m.r. spectrum of compound (I) (1,2-dihydroxy-1,2-dihydrodibenzo[1,4]dioxan)

The sample was dissolved in $[\text{D}_6]\text{pyridine}$ and the spectrum was recorded at 100MHz. Tetramethylsilane was used as an internal standard. The hydroxy protons were exchanged with $^2\text{H}_2\text{O}$ before recording the spectrum as they interfered with the signal given by H-5.

ditional support for the assigned structure was provided by the acid-catalysed dehydration of compound (I). Compound (I) (53 mg) was dissolved in 20 ml of diethyl ether. Conc. HCl (5.0 μ l) was added to the solution. After 1 h of incubation at 27°C the solvent was removed, leaving 46 mg of a solid residue. The physical properties of this compound were found to be identical with compound (II), previously identified as 2-hydroxydibenzo[1,4]dioxan. A minor product was also observed from the acid treatment of compound (I), although sufficient quantities were not obtained for characterization. This minor product was tentatively identified as 1-hydroxydibenzo[1,4]dioxan.

Although the results obtained establish the structure of compound (I) as 1,2-dihydroxy-1,2-dihydrodibenzo[1,4]dioxan, they do not distinguish between the possible *cis* and *trans* stereoisomers. Evidence for a *cis* configuration was obtained by synthesis of an isopropylidene derivative. Compound (I) (290 mg) was suspended in 20 ml of 2,2-dimethoxypropane. The suspension was chilled in an ice bath before the addition of 1.0 mg of *p*-toluenesulphonic acid. The reaction was monitored by t.l.c. After 4 h the presence of compound (I) in the reaction mixture could no longer be detected. However, a non-polar compound [compound (IA); R_f 0.64] was observed that absorbed u.v. light and gave a yellow-brown colour with Gibb's reagent. At this point, triethylamine (0.2 ml) was added to neutralize the acid and the solvent was removed *in vacuo* at 35°C. The oily residue (335 mg) was dissolved in a small volume of hexane and applied to the top of a column (1.0 cm $\times$ 16.0 cm) of basic alumina. Elution with benzene/hexane (1:1, v/v) yielded 90 mg of a white solid. Compound (IA) had m.p. 97–99°C, $[\alpha]_D^{25} + 232 \pm 1$ ($n = 10$) (c 0.41 in methanol), light-absorption maxima in methanol at 228 and 297 nm ($\epsilon = 13900$ and $2300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ respectively) and i.r.-absorption maxima in Nujol at 5.85, 6.27, 7.93, 6.9, 7.95, 8.67, 8.9, 10.8, 12.7 and $13.4 \mu\text{m}$. A mass-spectral determination gave a calculated mass for $^{12}\text{C}_{15}^{14}\text{H}_{14}^{16}\text{O}_4$ of 258.0892 and a found mass of 258.0886. An analysis of the ^1H n.m.r. spectrum of compound (IA) is given in Fig. 4. These observations establish the structure of compound (IA) as 2,2-dimethyl-1,3-methylenedioxy-1,2-dihydrodibenzo[1,4]dioxan and support the assignment of a *cis* stereochemistry to the hydroxy groups in 1,2-dihydroxy-1,2-dihydrodibenzo[1,4]dioxan (dibenzo[1,4]dioxan dihydrodiol).

Oxidation of dibenzo[1,4]dioxan dihydrodiol by cell extracts

When cell extracts prepared from the parent organism were incubated under anaerobic conditions with the dibenzo[1,4]dioxan dihydrodiol and NAD^+ , a new product [compound (III)] was detected by

Proton	δ (p.p.m.)	Description
1	1.44	3H (s, methyl)
1	1.49	3H (s, methyl)
2	4.71	1H (d, alkoxyethylene, $J_{2,3}$ 8.0 Hz)
3	4.90	1H (double d, alkoxyethylene, $J_{3,2}$ 8.0 Hz, $J_{3,4}$ 3.0 Hz)
4	5.68	1H (double d, olefinic, $J_{4,5}$ 10.0 Hz, $J_{4,3}$ 3.0 Hz)
5	5.83	1H (d, olefinic, $J_{5,4}$ 10.0 Hz)
6	6.62–6.90	4H (m, aromatic)

Fig. 4. Analysis of the ^1H n.m.r. spectrum of compound (IA) (2,2-dimethyl-1,3-methylenedioxy-1,2-dihydrodibenzo[1,4]dioxan)

The sample was dissolved in $[\text{D}_2]\text{chloroform}$ and the spectrum was recorded at 100 MHz. Tetramethylsilane was used as an internal standard.

t.l.c. (R_f 0.34) that absorbed u.v. light and gave an intense brown colour with Gibb's reagent. In a large-scale reaction, crude cell extract (80 mg of protein) was mixed with dibenzo[1,4]dioxan dihydrodiol (0.7 mmol) and NAD^+ (1.0 mmol) in 50 ml of 0.05 M- KH_2PO_4 buffer, pH 7.2, and incubated under anaerobic conditions in a sealed serum bottle. After 3 h the reaction mixture was acidified with 0.5 ml of 5 M-HCl and extracted with three equal volumes of ethyl acetate. The organic extracts were pooled, dried over anhydrous Na_2SO_4 , and the solvent removed *in vacuo* at 35°C to yield 185 mg of a solid residue. The residue was suspended in a small volume of chloroform and loaded on the top of a silica-gel column [1.0 cm $\times$ 20.0 cm; 25% (w/v) water]. The column was eluted with 500 ml of chloroform and 10.0 ml fractions were collected. The presence of compound (III) in fractions 16–28 was detected by t.l.c. These fractions were pooled and the solvent removed to yield 90 mg of compound (III), which was further purified by two recrystallizations from hexane/acetone. The resulting white crystals had m.p. 172–173°C, light-absorption maxima in methanol at 229 and 289 nm ($\epsilon = 30800$ and $3300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ respectively) and i.r.-absorption maxima in Nujol at 2.85, 3.06, 6.15, 6.3, 7.73, 10.8, 12.72 and $13.4 \mu\text{m}$. A mass-spectral determination gave a calculated mass for $^{12}\text{C}_{12}^{14}\text{H}_8^{16}\text{O}_4$ of 216.0422

and a found mass of 216.0422. An analysis of the ^1H n.m.r. spectrum of compound (III) is given in Fig. 5. Due to a rapid exchange rate the hydroxy protons were not observed at ambient temperature. However, they were observed at δ (p.p.m.) 8.65 when the spectrum was recorded at -15°C . The results of the physical measurements suggest that compound (III) is 1,2-dihydroxydibenzo[1,4]dioxan.

When cell extracts were incubated under aerobic conditions with the dibenzo[1,4]dioxan dihydrodiol and NAD^+ as described above, analysis of extracts of the reaction mixture by t.l.c. again revealed the accumulation of compound (III). Analysis of the visible spectrum of the reaction mixture at various time intervals did not reveal the formation of products with absorption in this region.

Polarographic experiments were performed to test the ability of 1,2-dihydroxydibenzo[1,4]dioxan to serve as a substrate for 1,2-dihydroxynaphthalene oxygenase (Fig. 6). An initial investigation revealed that cell extracts were extremely active on the physiological substrate 1,2-dihydroxynaphthalene (4724 nmol of O_2 consumed/min per mg of protein). However, only low activity was observed with 1,2-dihydroxydibenzo[1,4]dioxan (54 nmol of O_2 consumed/min per mg of protein). It was also noteworthy that incubation of cell extracts (0.15 mg of protein) with as low as 10 nmol of the dibenzo[1,4]dioxan catechol led to complete inhibition or inactivation of the 1,2-dihydroxynaphthalene oxygenase, as additions of 1.0 μmol of 1,2-dihydroxynaphthalene did not result in further enzymic activity.

Discussion

Initial attempts by enrichment culture failed to yield micro-organisms capable of utilizing dibenzo[1,4]dioxan as a sole source of carbon and energy. A preliminary screening of over 20 organisms previously shown to degrade aromatic hydrocarbons revealed that none of the organisms could utilize dibenzo[1,4]dioxan as a carbon source. However, co-oxidation was observed under certain conditions. *Pseudomonas* sp. N.C.I.B. 9816 strain 11 failed to co-oxidize dibenzo[1,4]dioxan when grown on sodium succinate (2.0 g/litre). However, the compound was readily oxidized when the organism was grown on sodium salicylate (1.0 g/litre). The latter observation is consistent with the report by Shamsuzzaman & Barnsley (1974) suggesting that in this organism salicylate is the co-ordinate inducer of a block of enzymes catalysing reactions in the conversion of naphthalene into salicylate. The results also indicate that dibenzo[1,4]dioxan fails to induce the enzymes responsible for the oxidation of the aromatic nucleus.

The present study has demonstrated that *cis*-1,2-dihydroxy-1,2-dihydrodibenzo[1,4]dioxan is the first detectable metabolite in the oxidation of dibenzo-

Proton	δ (p.p.m.)	Description
1	6.45	1H (d, aromatic, $J_{1,2}$ 8.0 Hz)
2	6.52	1H (d, aromatic, $J_{2,1}$ 8.0 Hz)
3	6.88–7.08	4H (m, aromatic)
4	8.25–8.96	2H (broad, hydroxy)

Fig. 5. Analysis of the ^1H n.m.r. spectrum of compound (III) (1,2-dihydroxydibenzo[1,4]dioxan)

The sample was dissolved in $[\text{D}_6]\text{acetone}$ and the spectrum was recorded at 100 MHz. Tetramethylsilane was used as an internal standard. The hydroxy protons were observed when the spectrum was recorded at -15°C (see the text).

Fig. 6. Polarographic assay of 1,2-dihydroxynaphthalene oxygenase activity in cell-free extracts of *Pseudomonas* sp. N.C.I.B. 9816

The reaction vessel contained, in a final volume of 1.4 ml, 70 μmol of phosphate buffer, pH 6.0, and cell extract (0.15 mg of protein). The reaction was initiated with the addition of 100 nmol of 1,2-dihydroxynaphthalene in 10 μl of anhydrous tetrahydrofuran (—), 10 nmol of 1,2-dihydroxydibenzo[1,4]dioxan (----), or 100 nmol of 1,2-dihydroxydibenzo[1,4]dioxan (— · —), all in 10 μl of anhydrous tetrahydrofuran. At the time indicated by the arrow 1.0 μmol of 1,2-dihydroxynaphthalene (in 10 μl of anhydrous tetrahydrofuran) was added. All rates are corrected for endogenous respiration and for the rate of autooxidation of 1,2-dihydroxynaphthalene. No autooxidation of 1,2-dihydroxydibenzo[1,4]dioxan was observed under the conditions described.

Scheme 1. Pathway proposed for the oxidation of dibenzo[1,4]dioxan by *Pseudomonas* sp. N.C.I.B. 9816. Only preliminary evidence was obtained for the compound shown in brackets.

[1,4]dioxan by a *Pseudomonas* sp. It is noteworthy that the *cis* stereochemistry of the hydroxy groups was inferred by the formation of an isopropylidene derivative with 2,2-dimethoxypropane (Brown & MacBride, 1964) and also from the fact that *cis*-dihydrodiols have been implicated as intermediates in the bacterial metabolism of a variety of aromatic hydrocarbons (Gibson, 1977). The *cis*-dibenzo[1,4]-dioxan dihydrodiol can subsequently undergo acid-catalysed dehydration to yield 2-hydroxydibenzo[1,4]dioxan as the major product, and a minor amount of a phenolic compound tentatively identified as 1-hydroxydibenzo[1,4]dioxan. The formation of two products on acid-catalysed dehydration provides further support for the structural assignment of the *cis*-dibenzo[1,4]dioxan dihydrodiol.

A pure sample of *cis*-1,2-dihydroxy-1,2-dihydrodibenzo[1,4]dioxan was oxidized in the presence of NAD^+ under both anaerobic and aerobic conditions to yield 1,2-dihydroxydibenzo[1,4]dioxan. This observation is consistent with the fact that several *cis*-dihydrodiols have been shown to be oxidized to catechols by nicotinamide nucleotide-dependent dehydrogenases (Reiner, 1972; Axcell & Geary, 1973; Patel & Gibson, 1974; Rogers & Gibson, 1977).

Negligible oxidation of 1,2-dihydroxydibenzo[1,4]dioxan was demonstrated with cell extracts of the parent strain. In addition, incubation of cell extracts with 1,2-dihydroxydibenzo[1,4]dioxan resulted in the inhibition or inactivation of the 1,2-dihydroxynaphthalene oxygenase. It is noteworthy that this

enzyme was recently purified from this organism (Patel & Barnsley, 1978). The enzyme was found to be sensitive to aerated buffers and iron-chelating reagents.

The results obtained support the metabolic pathway proposed in Scheme 1 for the oxidation of dibenzo[1,4]dioxan by *Pseudomonas* sp. N.C.I.B. 9816. Research is now in progress to determine the exact nature of the inhibitory effects exhibited by 1,2-dihydroxydibenzo[1,4]dioxan and to determine if these effects are observed in other organisms that possess the ability to degrade aromatic hydrocarbons.

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