

Metabolism of Biphenyl

2-HYDROXY-6-OXO-6-PHENYLHEXA-2,4-DIENOATE: THE *META*-CLEAVAGE PRODUCT FROM 2,3-DIHYDROXYBIPHENYL BY *PSEUDOMONAS PUTIDA*

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1. 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid was isolated and identified from washed suspensions of *Pseudomonas putida* incubated in the presence of 2,3-dihydroxybiphenyl. 2. Benzoic acid was isolated from reaction mixtures of crude cell-free extracts incubated with 2,3-dihydroxybiphenyl. 3. The presence in the same reaction mixtures of either 4-hydroxy-2-oxovalerate or 2-hydroxypenta-2,4-dienoate was suggested by mass spectrometry. 4. The degradative pathway of biphenyl is discussed.

During a study on the microbial decontamination of waste waters containing aromatic hydrocarbons used as nuclear reactor coolants, Catelani *et al.* (1970) isolated by enrichment culture a strain of *Pseudomonas putida* able to grow on biphenyl supplied as the sole carbon and energy source. Lunt & Evans (1970) isolated a Gram-negative bacterium able to metabolize biphenyl with the formation of 2,3-dihydroxybiphenyl, α -hydroxy- β -phenylmuconic semialdehyde and phenylpyruvic acid. Catelani *et al.* (1971) showed that cultures of *Ps. putida* grown on biphenyl yielded both 2,3-dihydro-2,3-dihydroxybiphenyl and benzoic acid; moreover the same cells grown on biphenyl were simultaneously induced to oxidize biphenyl and benzoic acid but not 3,4-dihydroxybiphenyl and phenylpyruvic acid; only the cells grown on phenylpyruvic acid were simultaneously induced to oxidize this compound.

In the present paper the metabolic pathway of 2,3-dihydroxybiphenyl in *Ps. putida* leading to the formation of benzoic acid is described.

Materials and Methods

Organism and growth conditions

A strain of *Ps. putida*, isolated by Catelani *et al.* (1970), was used. For maintenance, manometric experiments and preparations of cell-free extracts, the organism was cultured on Tausson (1927) mineral salts medium, solidified with 1.8% (w/v) Difco agar (Difco Laboratories, Detroit, Mich., U.S.A.) and supplemented with crystals of biphenyl (Merck A.-G., Darmstadt, Germany) placed in the lid of inverted plates. Approx. 10g (wet wt.) of cells were harvested from 50 plates of 15cm diam. after 24h incubation, and washed twice by centrifugation in 0.02M-potassium phosphate buffer, pH7. For manometric experiments the cells were resuspended in the same potas-

sium phosphate buffer to give a total N content of 0.3mg/ml. The measurement of O₂ uptake was performed in a Warburg respirometer at 30°C.

Cell-free extracts

Freshly harvested cells (10g wet wt.), suspended in 10ml of 0.02M-potassium phosphate buffer, pH7, were crushed at 4–5°C in an Aminco French Pressure Cell (American Instrument Co. Inc., Washington, D.C., U.S.A.) at 1.42×10^5 kPa. The crude extract was incubated with 10 μ g of deoxyribonuclease (Mann Research Laboratories, New York, N.Y., U.S.A.) for 15 min at 0°C and centrifuged. The supernatant solution, which usually contained about 40mg of protein/ml, was diluted with phosphate buffer to about 20mg of protein/ml.

Spectral analyses

U.v. spectra were recorded in a Zeiss PMQII spectrophotometer; an Infracord model 137 (Perkin-Elmer Ltd., Beaconsfield, Bucks., U.K.) was used to obtain i.r. spectra (in Nujol). Mass spectra were recorded on an LKB model 9000 spectrometer.

Chemical determinations

The presence of a free aldehyde group was assayed by the Tollens test (Vogel, 1970). Protein was determined by the method of Warburg & Christian (1941). Methyl esters were prepared by one of the following procedures: methanol-BF₃, methanol-toluene-*p*-sulphonic acid or diazomethane in diethyl ether. Hydrogenation was performed in the standard apparatus as described by Canonica *et al.* (1966). The catalyst, 10% (w/w) Pd on charcoal or 5% (w/w) Pd on CaCO₃, was obtained from Engelhard Minerals and Chemical Co., Newark, N.J., U.S.A.

Chromatography

T.l.c. was done on silica-gel plates (Baker-flex IB-F no. 5002; J. T. Baker Chemical Co., Deventer, Holland), activated by heating at 110°C for 30 min. The developing solvent system was acetic acid-hexane-chloroform (1:8:2, by vol.); spots were revealed by a u.v. lamp (at 254 nm).

G.l.c. of phenols and phenyl esters was performed by using a C. Erba model G.T. 200 gas chromatograph, with a flame-ionization detector. A stainless-steel column (2 m × 2 mm internal diam.) packed with 10% SE30 on Chromosorb W (60–80 mesh) was used at the following temperatures: column 190°C, injector 230°C, detector 220°C. The carrier gas was N₂ (30 ml/min). The flame was fed with H₂ at 70.7 kPa and air at 120 kPa. For g.l.c. of methyl esters, a stainless-steel column of the same dimensions was used, packed with 5% ethylene glycol succinate (C. Erba LAC886) on Chromosorb W (60–80 mesh) at the following temperatures: column 150°C, injector 210°C, detector 200°C. The N₂, H₂ and air were supplied as described above. He was used as carrier gas (30 ml/min) when the gas chromatograph was combined with the LKB model 9000 mass spectrometer; columns and temperatures were the same as reported above.

Chemical synthesis

2,3-Dihydroxybiphenyl. *o*-Hydroxybiphenyl (17 g) (Eastman-Kodak Co., Rochester, N.Y., U.S.A.) and 17.8 g of octanoyl chloride (Fluka AG, Buchs, Switzerland) were heated on a boiling-water bath for 6 h. The reaction mixture was cooled and poured into 200 ml of water and the resulting solution was boiled for 10 min to decompose the excess of acyl chloride. Three extractions (200 ml each) were performed with light petroleum (b.p. 40–60°C). The extract was washed with 5% (w/v) NaHCO₃, dried over anhydrous MgSO₄ and evaporated to dryness. 2-Hydroxybiphenyl octanoate (28 g) was obtained [b.p. 132°C at 6.6 Pa (0.05 mmHg)]. A mixture of this compound (24 g) and finely powdered AlCl₃ (20 g) was heated on an oil bath at 160–180°C for 3 h. The glassy reaction product was cooled, powdered and decomposed by gradual addition to 250 ml of well-stirred 5% (w/v) HCl. The decomposition was completed by warming on a steam bath for a short time. The solution was cooled and three extractions were performed with 200 ml each of diethyl ether. The dried extract was fractionated under reduced pressure. The fraction boiling at 160–170°C at 6.6 Pa (0.05 mmHg) was collected and redistilled. 2-Hydroxy-3-phenyl-octanoylphenone was collected as a viscous liquid [b.p. 167–168°C at 6.6 Pa (0.05 mmHg)]. 2,3-Dihydroxybiphenyl was obtained from 2-hydroxy-3-phenyl-octanoylphenone by the Baeyer-Villiger oxidation method as modified by Dakin (1909) by using H₂O₂,

KOH and pyridine. The reaction mixture was extracted with diethyl ether and decolorized with charcoal. Removal of the solvent left a grey solid which was sublimed at 110°C at 6.6 Pa (0.05 mmHg) to give a colourless microcrystalline powder which, after crystallization from light petroleum (b.p. 80–100°C), gave 2,3-dihydroxybiphenyl as colourless needles. This compound was identical (m.p. and mixed m.p. 114°C) with that obtained in very low yield by the time-consuming method of Bruce & Sutcliffe (1955).

Results

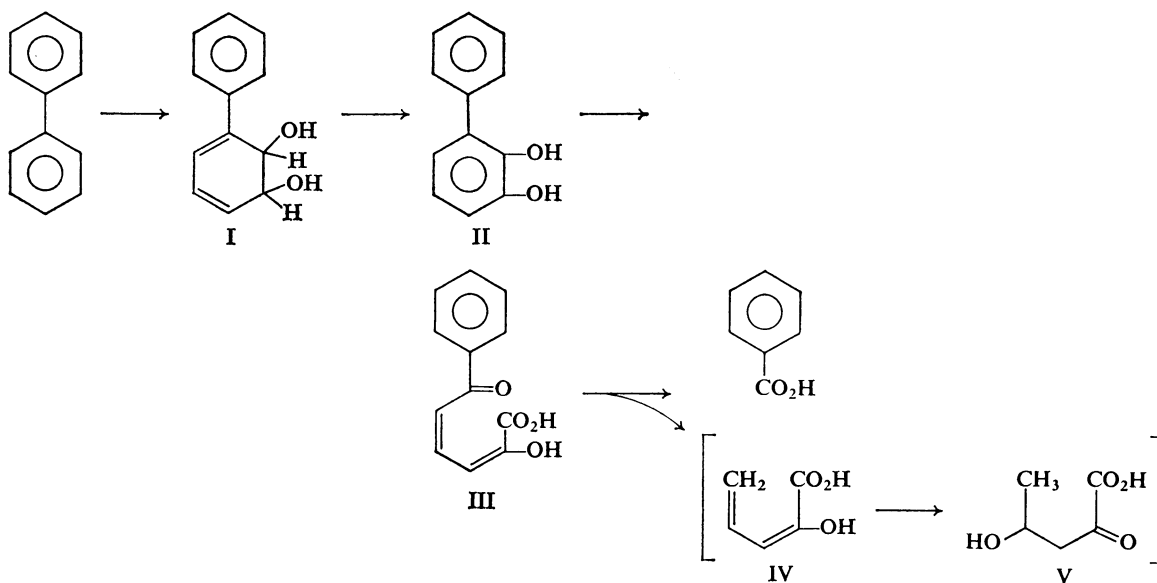
2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, the ring-fission product of 2,3-dihydroxybiphenyl

Washed suspensions of *Ps. putida* grown on biphenyl rapidly oxidized 2,3-dihydroxybiphenyl with the formation of a yellow compound, showing the spectrophotometric features of α -hydroxymuconic semialdehydes (λ_{\max} . 390–430 nm at pH 12, shifting to 310–330 nm at pH 3), which slowly disappeared in the reaction mixture. In order to isolate a sufficient amount of the yellow compound for identification 750 ml Erlenmeyer flasks containing 30 ml of a washed suspension of *Ps. putida* grown on biphenyl (equivalent to a total N content of 0.30 mg/ml), 60 ml of 0.02 M-potassium phosphate buffer, pH 7, and 10 mg of 2,3-dihydroxybiphenyl were incubated at 27°C by shaking. The yellow colour developed immediately. To check the cultures for accumulation of the yellow intermediate, 2 ml samples were taken at intervals, made alkaline (pH 12) with 1 M-NaOH and filtered. When the absorbance at λ_{\max} . 435 nm reached its maximum value, corresponding approximately to 0.45 mg of intermediate compound/mg of 2,3-dihydroxybiphenyl, the incubation mixture from several Erlenmeyer flasks was acidified (pH 3) with conc. HCl and cells were removed by centrifugation. The supernatant was saturated with NaCl and extracted five times with diethyl ether (200 ml). The ethereal solution was cooled to –20°C, the ice that formed was removed and the solvent was then evaporated in the cold under reduced pressure. T.l.c. of the crude residue revealed two spots, the first one with R_F 0.85, identical with that of benzoic acid, and the second with R_F 0.30. The crude residue was dissolved in diethyl ether, and light petroleum (b.p. 40–60°C) was slowly added with efficient stirring: the compound with R_F 0.30 was precipitated, whereas benzoic acid was almost completely soluble. The brown precipitate was recrystallized from diethyl ether-light petroleum (b.p. 40–60°C). The light-brown microcrystalline powder obtained was repeatedly washed with boiling light petroleum (b.p. 20–40°C) and stored at –20°C under N₂. Care was taken against contamination with moisture. This

substance (Found: C, 65.8; H, 4.5; $C_{12}H_{10}O_4$ requires C, 66.0; H, 4.6%; uncorr. m.p. 112°C, decomp.) was stable under these conditions and also in diluted ethereal or alkaline aqueous solutions in the cold (up to 5°C). In the dry state at room temperature it was rapidly decomposed, probably by a polymerization process. The u.v. spectrum in alkaline conditions (pH 12) showed λ_{max} . 435 nm ($\log \epsilon$ 4.5) and in acidic conditions (pH 2.5) λ_{max} . 336 nm ($\log \epsilon$ 4.3), on account of a keto-enol tautomerism. The mass spectrum showed a molecular-ion peak at m/e 218 and other peaks at m/e 173 ($M-CO_2H$), 105 ($C_6H_5CO^+$) and 77 ($C_6H_5^+$). Peaks also appeared at m/e 202 and 157: in repeated recordings, these peaks showed intensities in widely varying ratios with the other reported above. The compound gave a negative Tollens test, whereas the test for enols with $FeCl_3$ was positive. These properties are consistent with the structure of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (compound III in Scheme 1). Methylation of this compound afforded no definite product: in fact g.l.c.-mass spectrometry gave no evidence of the presence of any methyl ester. Attempted acetylation before and/or after methylation was unsuccessful. Probably polymerization occurred in the reaction mixture producing compounds with high molecular weights and consequently low volatility.

Action of crude cell-free extracts on 2,3-dihydroxybiphenyl and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate

Crude cell-free extracts of *Ps. putida* grown on biphenyl rapidly oxidized 2,3-dihydroxybiphenyl with an O_2 uptake of 1 mol/mol of substrate, with the formation of a yellow compound that rapidly disappeared. The same cell-free extracts incubated with pure 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, isolated as previously described, showed no O_2 uptake, but the yellow colour of the reaction mixture rapidly disappeared. To isolate and identify the further disimilation products of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, 30 ml of cell-free extract and 70 ml of 0.02 M-potassium phosphate buffer, pH 7, were incubated in the presence of 20 mg of 2,3-dihydroxybiphenyl; when the yellow colour had completely disappeared the incubation mixture was acidified to pH 3 and centrifuged. The supernatant was repeatedly extracted with diethyl ether and the crude oily residue from the evaporated ethereal fraction was heated with a few ml of light petroleum (b.p. 30–50°C), the liquid phase decanted and the solvent evaporated. White crystals were obtained after repeated crystallization from *n*-pentane (Found: C, 69.1; H, 5.0. Calc. for benzoic acid $C_7H_6O_2$: C, 68.8; H, 4.9%). This material was identical (m.p. and mixed m.p. 120°C)



Scheme 1. Proposed pathway of biphenyl degradation by *Ps. putida*

I, 2,3-Dihydro-2,3-dihydroxybiphenyl; II, 2,3-dihydroxybiphenyl; III, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid; IV, 2-hydroxypenta-2,4-dienoic acid; V, 4-hydroxy-2-oxovaleric acid. The square brackets denote that these compounds have not been isolated and identified, but that their presence has been suggested by mass spectrometry.

with an authentic sample of benzoic acid. The i.r., u.v. and mass spectra were wholly superimposable on those of benzoic acid. Mother liquors from the crystallization of benzoic acid were evaporated until dry. Mass spectrometry of the resulting solid residue showed that it was mainly benzoic acid; evidence was also obtained for the presence in traces of another compound responsible for peaks at m/e 114 ($C_5H_6O_3^+$), 97 ($C_5H_5O_2^+$) and 69 ($C_4H_5O^+$). These peaks are consistent with the structure of 4-hydroxy-2-oxovaleric acid [$114 = (M - H_2O)$] or of its probable precursor 2-hydroxypenta-2,4-dienoic acid ($M = 114$) (compounds IV and V respectively in Scheme 1).

Discussion

From washed suspensions of *Ps. putida* incubated with 2,3-dihydroxybiphenyl a crystalline compound was isolated whose u.v. features were similar to those of muconic semialdehydes. In addition the presence in the mass spectrum of a peak at m/e 105 indicated a partial structure C_6H_5CO . These properties are consistent with the structure of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid for the ring-fission product of 2,3-dihydroxybiphenyl. It thus appears that the ring fission of the first benzene ring of biphenyl occurs between the C-1 and C-2 positions (extra-diol vicinal cleavage, in the nomenclature reported by Nozaki *et al.*, 1970). A similar cleavage was also demonstrated for phenylpropionic acid by Dagley *et al.* (1965), for 3-methylcatechol by Catelani *et al.* (1968), for 4-methylcatechol by Canonica *et al.* (1966) and for 3-phenylpentane by Baggi *et al.* (1972).

The isolation and identification of benzoic acid and the evidence for the formation of either 4-hydroxy-2-oxovaleric acid or 2-hydroxypenta-2,4-dienoic acid, together with the results obtained from manometric experiments with cell-free extracts, allow us to correlate the degradative pathway of 2-hydroxy-6-oxo-6-

phenylhexa-2,4-dienoate to that of muconic semialdehydes (Dagley *et al.*, 1964).

From these results and those previously obtained (Catelani *et al.*, 1971) a pathway for biphenyl metabolism by *Ps. putida* may be proposed (Scheme 1). This pathway seems to be different from that reported by Lunt & Evans (1970); in this case the formation of phenylpyruvic acid accounts for a *meta*-cleavage of the first aromatic ring of biphenyl between the C-3 and C-4 positions (extra-diol distal cleavage).

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