Initial Reactions in the Oxidation of Anthracene by *Cunninghamella elegans*

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Cunninghamella elegans oxidized anthracene predominantly to *trans*-1,2-dihydroxy-1,2-dihydroanthracene and 1-anthryl sulphate. In addition, several unidentified metabolites were produced. The major compounds formed were isolated by TLC and HPLC, and identified by their UV-visible absorption and mass spectra. Experiments with [9-¹⁴C]anthracene indicated that the total amount of anthracene metabolized increased from 2.0% at 2 h to 26.9% at 48 h. The ratio of organic-soluble to water-soluble metabolites at 2 h was 85:15 while at 48 h it was 43:57. The results demonstrate that *C. elegans* has the ability to oxidize anthracene in a manner similar to that observed in mammals.

INTRODUCTION

There has been recent interest in the fate of anthracene and its derivatives in the environment since trace amounts are emitted into the environment during coal gasification and liquefaction processes (Blumer, 1976). Although anthracene and its metabolites are not acutely toxic, carcinogenic and/or mutagenic there is a considerable amount of information on the metabolism of these compounds since the anthracene structure is found in carcinogenic polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene, benz[*a*]anthracene and 3-methylcholanthrene.

Micro-organisms with the ability to metabolize anthracene as the sole source of carbon and energy are readily isolated from soil (Gray & Thornton, 1928; Rogoff & Wender, 1957; Colla *et al.*, 1958; Evans *et al.*, 1965; Akhtar *et al.*, 1975; Jerina *et al.*, 1976). Bacteria initially oxidize anthracene in the 1,2 position, to form 1,2-dihydro-1,2-dihydroxyanthracene. Jerina *et al.* (1976) showed that *Beijerinckia* B-836 and *Pseudomonas putida* accumulated (+)-*cis*-1,2-dihydroxy-1,2-dihydroanthracene (*cis*-anthracene dihydrodiol). Further studies have shown that three *Pseudomonas* strains and a *Nocardia* strain oxidize *cis*-anthracene dihydrodiol to 1,2-dihydroxyanthracene. This reaction is catalysed by (+)-*cis*-dihydrodiol dehydrogenase which requires NAD⁺ as an electron acceptor (Patel & Gibson, 1974). The 1,2-dihydroxyanthracene is further oxidized to ring cleavage products (Evans *et al.*, 1965).

In contrast, mammals metabolize anthracene predominantly in the 1,2 position to form a dihydrodiol with a *trans*-relative stereochemistry, (Akhtar *et al.*, 1975; Jerina *et al.*, 1976). Evidence has also been presented for an attack at the 9,10-position of anthracene to form 9,10-dihydroxy-9,10-dihydroanthracene, 9,10-dihydroxyanthracene, 9,10-anthraquinone, 2,9,10-trihydroxyanthracene and 2-hydroxy-9,10-anthraquinone (Boyland & Levi, 1935; Boyland & Shoppee, 1947; Sims, 1964).

Although there have been numerous studies on the bacterial and mammalian oxidation of anthracene, little is known about the metabolism of this compound by fungi. In this report, studies on the fungal metabolism of anthracene are described.

METHODS

Organism. *Cunninghamella elegans* ATCC 36112 was maintained on Sabouraud dextrose agar slants and stored at 4 °C.

Growth conditions and metabolic experiments. *Cunninghamella elegans* was grown in 125 ml Erlenmeyer flasks which contained 30 ml Sabouraud dextrose broth (Difco). Incubation was carried out at 25 °C for 48 h on a rotary shaker operating at 200 rev. min⁻¹. After 48 h the culture broth was filtered and cells of *C. elegans* (5.9 g dry wt) were resuspended in 30 ml Sabouraud dextrose broth that contained 2.0 mg anthracene dissolved in 0.3 ml dimethylformamide. Six flasks were incubated as described above for 24 h. Control experiments in which anthracene was incubated with Sabouraud dextrose broth in the absence of the organism showed no degradation of the substrate. All flasks were incubated in the dark at 25 °C for 24 h as described above. At the end of the experiment, the cultures were examined microscopically for bacterial contamination. No contamination was observed.

After 24 h the filtrates of each culture broth were pooled and extracted with three equal volumes of ethyl acetate. The combined organic extracts were dried over anhydrous Na₂SO₄ and the solvent was removed under vacuum. The residue was examined for metabolic products by TLC and HPLC, and compared to the residue obtained from control experiments.

Experiments with [9-¹⁴C]anthracene were conducted as described above with the exception that [9-¹⁴C]anthracene [82.5 nmol, 1.25 µCi (46.25 kBq)] in 25 µl dimethylformamide was added to each flask. After 0, 2, 4, 8, 12, 24 and 48 h incubation in the presence of [9-¹⁴C]anthracene, the contents of each flask were filtered and the filtrate was extracted with 6 vol. of ethyl acetate. The aqueous layer was extracted until no ethyl acetate soluble radioactivity was detected. The ethyl acetate soluble extracts were combined and the solvent was removed as described above, the residue being analysed by HPLC. Samples (0.1 ml) of the aqueous phase (water-soluble metabolites) were added to 5.0 ml Aquasol-2 and the amount of radioactivity was measured in a liquid scintillation counter.

Analytical methods. Absorption spectra were determined on a Beckman model 25 recording spectrophotometer. TLC was performed with Polygram Sil G/UV254 plastic sheets (Machery-Nagel & Co., Duren, Germany). Plates were developed in benzene/ethanol (9:1, v/v). Compounds were visualized by spraying with Gibbs reagent [2,6-dichloroquinone-4-chlorimide in methanol (2%, w/v)] or by viewing under UV light (254 nm).

A Beckman model 332 high-pressure liquid chromatograph with a model 155-10 absorbance detector operated at 254 nm was used to separate the anthracene metabolites. A C₁₈ Ultrasphere column (25 cm × 4.6 mm i.d.) was used for the separation of anthracene metabolites, which was achieved with a programmed 60 min linear gradient of methanol/water (1:1 to 19:1, v/v). The flow rate was 1 ml min⁻¹. In experiments with [9-¹⁴C]anthracene, 0.5 ml fractions were collected every 0.5 min and added to scintillation vials containing 5.0 ml Aquasol-2. The radioactivity present in each fraction was determined in a Beckman LS-250 liquid scintillation counter.

Mass spectra were obtained with a Finnigan model 4023 mass spectrometer at 70 eV ionizing voltage with a solid probe. Direct probe mass spectrometry analyses were performed on samples dissolved in 5 µl methanol and dried in glass sample cups. Spectra were recorded as the probe temperature was increased ballistically from 30 to 300 °C, monitoring the ion source temperature at 270 °C. Exact mass measurements were made with a Kratos MS-50 mass spectrometer operating at a nominal resolution of 10000.

Tests for the relative stereochemistry of the dihydrodiol (*cis/trans* isomer) were done by dissolving each compound in anhydrous acetone in the presence of anhydrous cupric sulphate; the reaction product was analysed by mass spectrometry (Yang *et al.*, 1977). A positive control of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene was used.

Deconjugation experiments. Compound II (Fig. 1) was added in equal amounts to three test tubes each containing 5 ml 0.2 M-sodium acetate buffer (pH 4.5). To one sample 3.0 × 10³ Fishman units of β-glucuronidase (type H-1, Sigma) were added. The second sample was incubated with 15 units of arylsulphatase (Type V, Sigma) and 12.5 µmol D-saccharic acid 1,4-lactone (to inhibit β-glucuronidase activity). The third sample served as a control and was incubated for 24 h at 37 °C on a rotary shaker operating at 150 rev. min⁻¹. Samples were then extracted with three equal volumes of ethyl acetate, dried under vacuum at 30 °C and analysed by HPLC as described above for the separation of anthracene metabolites.

¹⁸O₂ incorporation experiment. Cells of *C. elegans* (30 ml, 60 mg dry wt ml⁻¹) were incubated with anthracene in a 250 ml Erlenmeyer flask modified with a vacuum outlet at its top and with an ¹⁸O₂ bulb (99 atom %) fused to the side of the flask. The contents of the flask were degassed with a vacuum pump and by flushing with N₂, after which 100 ml ¹⁸O₂ was introduced. The isotopic composition of the oxygen portion of the atmosphere was monitored during the experiment and was 100% ¹⁸O¹⁸O as measured by mass spectrometry. The *trans*-1,2-dihydroxy-1,2-dihydroanthracene was isolated by HPLC and its isotopic abundance was calculated from the relative intensities of the molecular ions of the ¹⁶O and ¹⁸O species at *m/e* 212, 214 and 216, respectively, on a Finnigan model 4023 mass spectrometer.

Chemicals. Anthracene was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. [9-¹⁴C]Anthracene

[15.1 mCi mmol⁻¹ (559 MBq mmol⁻¹)] Amersham/Searle, Arlington Heights, Ill., U.S.A. was a generous gift from Dr D. T. Gibson, University of Texas at Austin, Tex., U.S.A. Solvents for HPLC were purchased from Burdick and Jackson Laboratories, Muskegon, Mich., U.S.A. All other chemicals were of reagent grade or the highest available purity.

RESULTS

Mycelial pellets of *C. elegans* were incubated with anthracene in Sabouraud dextrose broth for 24 h. TLC analysis of the organic solvent-soluble metabolites revealed the presence of anthracene (R_F 0.61) and two oxidation products (R_F 0.08 and 0.12). HPLC analysis of the metabolites formed during anthracene oxidation is shown in Fig. 1. The two major compounds were almost certainly anthracene metabolites since they did not appear in control samples. The absorption spectrum of compound I and its acid catalysed dehydration product is shown in Fig. 2. The UV spectral properties of compound I are identical to those reported for *trans*-1,2-dihydro-1,2-dihydroxyanthracene (Jerina *et al.*, 1976). Furthermore, the acid catalysed dehydration of compound I (Fig. 2) produced a phenol whose spectral and chromatographic properties are similar to 1-anthrol (Sims, 1964). HPLC analysis of the dehydration products indicated that compound I was converted to a mixture of 1-anthrol and 2-anthrol. A similar experiment with 9-¹⁴C-labelled compound I indicated that the ratio of 1-anthrol to 2-anthrol was 86:14. Mass spectral analysis of compound I (Fig. 3) showed a molecular ion at m/e 212, and the loss of H₂O ($M^+ - 18$) from the molecular ion gave an ion at m/e 194, which is indicative of a dihydrodiol. This molecular composition was confirmed by high-resolution mass measurement (calculated for C₁₄H₁₂O₂, 212.0837; observed, 212.0839). Although the absorbance and mass spectral data indicated that *C. elegans* formed 1,2-dihydro-1,2-dihydroxyanthracene, the data do not provide evidence for the relative stereochemistry of the hydroxyl groups. The *trans*-stereochemistry of compound I was assigned on the basis of its relative stability in acid compared to that reported for the *cis*-isomer (Jerina *et al.*, 1976). In addition, compound I gave a negative response when tested with potassium triacetylosmate (Criegee *et al.*, 1942) and did not form an acetonide (Yang *et al.*, 1977). Moreover, all dihydrodiols produced by *C. elegans* have been shown to have a *trans*-relative stereochemistry (Cerniglia, 1981). Further evidence was provided by an ¹⁸O₂ experiment in which *C. elegans* was grown in the presence of anthracene under ¹⁸O₂ for 24 h and the anthracene metabolites isolated by HPLC. The *trans*-1,2-dihydroxy-1,2-dihydroanthracene formed in the presence of ¹⁸O₂ was isolated; mass spectral analysis showed a molecular ion at m/e 214, indicating that one of the oxygen atoms of the dihydrodiol is derived from molecular oxygen.

The UV absorption spectrum (λ_{max} 252 nm) of the second metabolite, compound II, is shown in Fig. 4. TLC analysis of compound II in benzene/ethanol (9:1) showed the presence of a single spot with an R_F value of 0.08. These data indicate that compound II is a very polar metabolite. Since previous studies have shown that *C. elegans* has the ability to form glucuronide and sulphate conjugates of aromatic hydrocarbons (Cerniglia & Gibson, 1979; Dodge *et al.*, 1979) an experiment was done in which compound II was incubated with arylsulphatase and/or β -glucuronidase. Separation of the metabolites released after enzyme treatment by HPLC indicated that enzymic hydrolysis of compound II with arylsulphatase, but not with β -glucuronidase, released a compound which eluted at the same HPLC retention time (32.5 min) as 1-anthrol. Mass spectral analysis of the hydrolysis product gave a molecular ion at m/e 194, which indicated that compound II was a sulphate ester of 1-anthrol. The non-hydrolysed control sample showed no release of ethyl acetate-soluble products.

The metabolism of [9-¹⁴C]anthracene in cells of *C. elegans* was determined by measuring the amount of radioactivity in the ethyl acetate and water phases of the culture filtrate after 2, 4, 8, 12, 24 and 48 h incubation. The ethyl acetate soluble metabolites were separated and quantified by HPLC. Approximately 84% of the added [9-¹⁴C]anthracene was recovered from each incubation flask and 21% of the total radioactivity added adhered to the mycelia and was found to be mainly non-utilized anthracene. The results in Table 1 show that the total amount of [9-¹⁴C]anthracene metabolized increased from 2.0% at 2 h to 26.9% at 48 h. The compounds *trans*-

Fig. 1. HPLC elution profile of ethyl acetate-soluble metabolites formed from anthracene by *C. elegans*. HPLC conditions were as described in Methods. I represents compound I, and II, compound II.

Fig. 2

Fig. 3

Fig. 2. Absorption spectra of Compound I. Spectra were recorded on a Beckman model 25 recording spectrophotometer before acid and heat treatment (—), and after acid treatment with 6 M-HCl and boiling in water for 15 min (----).

Fig. 3. Mass spectrum of compound I.

1,2-dihydroxy-1,2-dihydroanthracene and 1-anthrlyl sulphate accounted for 42% and 27%, respectively, of total metabolites after 4 h incubation of [9- 14 C]anthracene with cells of *C. elegans*. After longer incubation (48 h), the ratio of ethyl acetate-soluble to water-soluble metabolites decreased: at 2 h it was 85:15 while at 48 h it fell to 42:7:57:3 (Table 1). The large amount of [9- 14 C]anthracene water-soluble metabolites is in accord with previous studies on the fungal metabolism of benzo[*a*]pyrene (Cerniglia & Gibson, 1979). These metabolites may be polyhydroxylated anthracene metabolites and/or their sulphate and glucuronide derivatives.

Fig. 4. Absorption spectrum of compound II.

Table 1. Distribution of radioactivity of metabolites formed from [9-¹⁴C]anthracene by *C. elegans*

Time (h)	Total metabolism (%)	Percentage distribution of metabolites			
		Water soluble*	Ethyl acetate soluble†		Total
			<i>trans</i> -1,2-Dihydroxy- 1,2-dihydroanthracene	1-Anthryl sulphate	
2	2.0	0.3	0.8	0.3	1.7
4	8.8	1.9	3.7	2.4	6.9
8	11.4	4.0	3.7	2.0	7.4
12	15.7	6.2	3.3	1.8	9.5
24	21.3	9.8	2.5	1.8	11.5
48	26.9	15.4	2.2	1.8	11.5

* Radioactivity recovered from the aqueous phase of the media after ethyl acetate extraction, expressed as percentage of added radioactivity.

† Radioactive ethyl acetate-soluble metabolites were determined by HPLC as described in Methods.

DISCUSSION

The results presented here show that *C. elegans* can oxidize anthracene. The metabolic scheme for the oxidation of anthracene by *C. elegans* is given in Fig. 5. The predominant metabolite formed was *trans*-1,2-dihydroxy-1,2-dihydroanthracene. The formation of a dihydrodiol with a *trans*-relative stereochemistry, and the ¹⁸O₂ experiment which indicated that one atom of oxygen was incorporated into the *trans*-1,2-dihydroxy-1,2-dihydroanthracene, suggest a mono-oxygenase catalysed reaction and the prior formation of anthracene-1,2-oxide (Daly *et al.*, 1972; Fu *et al.*, 1978). The metabolite *trans*-1,2-dihydroxy-1,2-dihydroanthracene could be formed from initial epoxidation of the 1,2 double bond of anthracene, followed by an epoxide hydrolase catalysed hydration to form the dihydrodiol. Recently it has been shown that cell extracts prepared from *C. elegans* have epoxide hydrolase activity (Wackett & Gibson, 1981). 1-Anthrol may arise by spontaneous isomerization of anthracene 1,2-oxide or by a direct insertion mechanism. The enzymic mechanism for the oxidation of anthracene is similar to that described for the fungal metabolism of naphthalene (Cerniglia & Gibson, 1977, 1978; Cerniglia *et al.*, 1978).

Similar results have been reported in mammals where the mono-oxygenase-epoxide hydrolase enzyme systems oxidize anthracene almost entirely to *trans*-1,2-dihydroxy-1,2-dihydroanthracene. The compounds 1- and 2-anthrol are minor oxidation products since anthracene 1,2-oxide has been shown to be relatively resistant towards isomerization to phenols, and to be a good substrate for epoxide hydrolase (Akhtar *et al.*, 1979). It will be interesting to see if the purified fungal epoxide hydrolase also has high specificity with respect to anthracene 1,2-oxide.

Fig. 5. Proposed pathway for the metabolism of anthracene by *C. elegans*. The compound in brackets was not isolated. Absolute stereochemistry is not implied.

1-Anthryl sulphate was also formed from anthracene by *C. elegans*. Sulphate conjugation reactions in mammals are thought to be important as detoxification mechanisms, but the role and enzymic basis of this reaction in fungi has yet to be determined.

It is clear from these results that *C. elegans* has the capacity to oxidize anthracene. Further investigation of the mechanism of anthracene hydroxylation and the role of enzymes involved in the metabolism of anthracene is evidently warranted.

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