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Biotransformation of Fluorene by the Fungus *Cunninghamella elegans*

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The metabolism of fluorene, a tricyclic aromatic hydrocarbon, by *Cunninghamella elegans* ATCC 36112 was investigated. Approximately 69% of the [9-^{14}C]fluorene added to cultures was metabolized within 120 h. The major ethyl acetate-soluble metabolites were 9-fluorenone (62%), 9-fluorenol, and 2-hydroxy-9-fluorenone (together, 7.0%). Similarly to bacteria, *C. elegans* oxidized fluorene at the C-9 position of the five-member ring to form an alcohol and the corresponding ketone. In addition, *C. elegans* produced the novel metabolite 2-hydroxy-9-fluorenone.

Fluorene, a tricyclic aromatic hydrocarbon which contains a five-member ring, is formed during the combustion of fossil fuels and has been detected in gasoline and diesel engine exhaust, cigarette smoke, urban air particulates, and a variety of coal-derived liquids and tars (11–13, 16). Although not carcinogenic, fluorene is highly toxic to fish (24) and aquatic algae (15) and is an important hydrocarbon pollutant of aquatic ecosystems (1, 23, 25).

Limited studies on the microbial degradation of fluorene have been reported. A pure culture of *Pseudomonas vesicularis* degraded fluorene (26); however, no metabolic pathways were described. Recently, Grifoll et al. (9) isolated, from oil refinery sludge, a fluorene-degrading *Arthrobacter* sp. which is able to grow on the compound as the sole source of carbon and energy. The bacterial degradation of fluorene proceeds via one of two enzymatic pathways, either with an attack on the aliphatic ring to form 9-fluorenol and 9-fluorenone or by ring oxidation via *meta* cleavage to form 3,4-dihydrocoumarin (9). Monna et al. (18) also demonstrated formation of 9-fluorenol, 9-fluorenone, 4-hydroxy-9-fluorenone, and 1-hydroxy-9-fluorenone and accumulation of 1,1a-dihydroxy-1-hydro-9-fluorenone when *Staphylococcus auriculans* was grown on fluorene as the sole source of carbon and energy. Less is known about the fungal metabolism of fluorene (14). A lignin-degrading fungus, *Phanerochaete chrysosporium*, degraded both fluorene and its by-product 9-fluorenone, which had been added to soil (8). Bumpus (2) found only 2% of added fluorene remaining in cultures of *P. chrysosporium* after 27 days of incubation. Our investigation describes the metabolism of fluorene by the fungus *Cunninghamella elegans* ATCC 36112 and the identification of the major metabolites.

Culture conditions and methods for physical and chemical analyses of metabolites have been reported previously (19). In this study the following modifications were made. After growth of *C. elegans* cultures in 30 ml of Sabouraud dextrose broth for 48 h, 10 mg of fluorene (Aldrich Chemical Co., Milwaukee, Wis.) was dissolved in 0.5 ml of dimethyl sulfoxide, and the solution was added to each culture. In additional experiments, 10 mg of 9-fluorenol and 9-fluorenone dissolved in 0.5 ml of dimethyl sulfoxide was added to each culture. Sterile control flasks (19) incubated with fluorene, 9-fluorenol, and 9-fluorenone showed no metabolism. Kinetic experiments were conducted with 0.81 μCi of [9-^{14}C]fluorene (specific activity, 14.2 mCi/mmol; radiochemical purity, >98%; Sigma Chemical Co., St. Louis, Mo.) and 10 mg of unlabeled fluorene added to culture flasks. The percent metabolism to various products was quantified as described previously (19).

Metabolites of fluorene were separated with a Shimadzu high-performance liquid chromatography (HPLC) system, consisting of a model SCL6B system controller, two model LC-600 pumps, and a model SPD-M6A diode array detector (Shimadzu Corp., Kyoto, Japan). The column was a Altex Ultrasphere C_{18} column (25 cm by 4.6 mm; Altex Scientific, Berkeley, Calif.), and the mobile phase was a 40-min linear gradient of methanol-water (from 30:70 to 95:5 [vol/vol]) at a flow rate of 1.0 ml/min.

UV–visible-light absorption spectra of the metabolites were determined in methanol with a Shimadzu model 2101 PC spectrophotometer system. A Finnigan gas chromatograph-mass spectrometer, upgraded with a model 4500 source and analyzer with a Finnigan Direct Exposure Probe (DEP) system, was used for mass spectral analyses (17). ^{1}H nuclear magnetic resonance (NMR) measurements and data acquisition were as described previously (19), and for an authentic sample of 9-fluorenone, ^{13}C NMR measurements were recorded. A long-range heteronuclear correlation experiment was also conducted (5).

Figure 1 shows the reversed-phase HPLC elution profile of the ethyl acetate-extractable metabolites formed by incubation of [9-^{14}C]fluorene with *C. elegans*. Fluorene was metabolized to three metabolites, referred to below as I, II, and III, which eluted at 26.1, 26.4, and 30.6 min, respectively. Fluorene eluted at 39.0 min.

Figure 2 illustrates the disappearance of fluorene and the production of ethyl acetate-extractable metabolites by *C. elegans* at 24-h intervals during 144 h of incubation. There was no apparent sequential pattern of metabolite production (Fig. 2). About 40% of the total radioactivity added to the culture was recovered in the organic phase, and the remainder was bound to the mycelia. At time zero, 95% of the recovered radioactivity was from unmetabolized fluorene. Thereafter, the percentage of fluorene decreased rapidly to about 10.5% of the total recovered within 120 h, when 69% of the metabolite formation had occurred. Metabolites I and II together accumulated to maximum levels at 72 h, and metabolite III reached the maximum level at 120 h (Fig. 2).

The UV–visible-light absorption and gas chromatograph-

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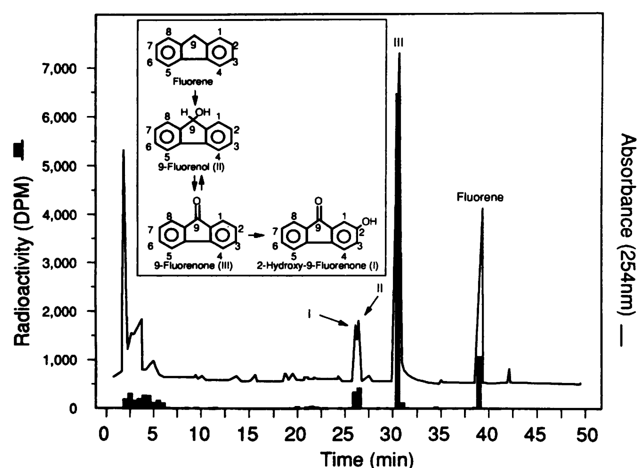

FIG. 1. HPLC elution profile and radioactivity of the ethyl acetate-soluble metabolites formed from [9-^{14}C]fluorene by *C. elegans*. (Inset) Proposed pathway for the metabolism of fluorene by *C. elegans*.

ic-mass spectral data for fluorene and the three metabolites isolated are given in Table 1. The UV–visible-light absorption and mass spectra of metabolites II and III were identical to those of authentic standards and to published spectra (9) of 9-fluorenol (for metabolite II) and 9-fluorenone (for metabolite III) (Table 1). Similarly, the UV–visible-light absorption and mass spectra of metabolite I were identical to those of authentic 2-hydroxy-9-fluorenone. The electron impact mass spectrum indicated two oxygens, with a molecular ion at m/z 196 [M^+] and characteristic fragment ions at m/z 168, 139, 98, 84, and 70 (Table 1).

The structural identification of metabolites I, II, and III was confirmed by NMR analyses. In spite of extensive HPLC and thin-layer chromatographic purification steps, the sample that contained metabolite I was a mixture of two components. On the basis mainly of the coupling constant pattern, including the presence or absence of long-range benzylic coupling (7), it was determined that the sample was a mixture of 2-hydroxy-9-fluorenone and 9-fluorenol, while metabolite II was 9-fluorenol. Metabolite III was identified as 9-fluorenone by ^{1}H NMR analysis, in part from the presence of only four resonances and the absence of benzylic couplings. The NMR spectra of authentic standards for 2-hydroxy-9-fluorenone and 9-fluorenol matched those of metabolites I and II (Table 1), respectively, which confirmed the proposed structures.

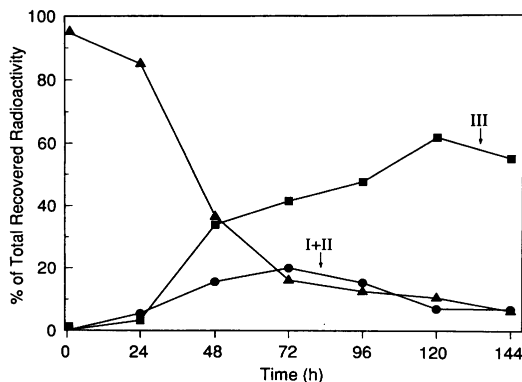

FIG. 2. Metabolism of [9-^{14}C]fluorene (▲) and formation over time by *C. elegans* of fluorene metabolites I and II (summed) (●) and III (■).

The ^{1}H NMR assignments for 9-fluorenone are based on long-range heteronuclear correlation experiments, which enabled unambiguous assignment of the H_1 resonance from the correlation with the carbonyl carbon resonance. The remaining ^{1}H assignments readily followed from homonuclear decoupling experiments. An apparent reversal in ^{1}H resonance assignments for the H_1 and H_4 resonances from those previously reported (6) was actually a solvent effect. The ^{1}H resonance assignments for 2-hydroxy-9-fluorenone were determined by comparison with the assignments of 9-fluorenone and by homonuclear decoupling experiments. The ^{1}H NMR spectral parameters for 2-hydroxy-9-fluorenone and 9-fluorenone in acetone-d_6 are presented in Table 1. We have previously characterized the ^{1}H NMR spectrum of 9-fluorenol (17).

The metabolism of fluorene appears to have occurred via hydroxylation of the aliphatic ring to produce hydroxylated and keto derivatives of fluorene (Fig. 1, inset). We propose that the initial oxidative attack on fluorene by *C. elegans* was on the C-9 position, resulting in the formation of the secondary alcohol 9-fluorenol (Fig. 1, inset). *C. elegans* also formed 9-fluorenone (Fig. 1, inset); however, the additional formation of a novel metabolite, 2-hydroxy-9-fluorenone, suggests that subsequent hydroxylation occurred at the C-2 position of the aromatic ring. The formation of 9-fluorenone and 2-hydroxy-9-fluorenone as the end product was further confirmed by replacement culture experiments with unlabeled 9-fluorenol and 9-fluorenone, which were metabolized to form 9-fluorenone and 2-hydroxy-9-fluorenone, respectively (data not shown). The identification of these two metabolites was based on HPLC retention times and UV–visible-light spectral data identical to those reported in Table 1 for compounds III (9-fluorenone) and I (2-hydroxy-9-fluorenone).

In fluorene metabolism by an *Arthrobacter* sp., formation of 9-fluorenol by monooxygenation is the initial oxidative step (9) and subsequent dehydrogenation forms 9-fluorenone as the end product (9). *Staphylococcus auriculans* also oxygenated C-9 of fluorene by monooxygenase(s) to form 9-fluorenol and 9-fluorenone (18) and formed 4-hydroxy-9-fluorenone as a result of dioxygenation (18). Even though Holland et al. (14) have reported the formation of 9-fluorenol from fluorenone by *C. elegans* ATCC 26269, they did not observe the formation of 2-hydroxy-9-fluorenone or the metabolism of fluorene, as found in our study.

In our previous study with acenaphthene (20), we reported that the initial oxidation by *C. elegans* occurred at the C-1 and C-2 bridge, which resulted in the formation of the secondary alcohol 1-acenaphthenol. Interestingly, the formation of 2-hydroxy-9-fluorenone in the present study appears to be analogous to the formation of 1-acenaphthenol from acenaphthene, with subsequent hydroxylation of the aromatic ring of acenaphthene to form 6-hydroxyacenaphthenone (20). The subsequent hydroxylation of 9-fluorenone to form 2-hydroxy-9-fluorenone (Fig. 1, inset) is likely, since previous studies of naphthalene (3) and acenaphthene (20) metabolism by *C. elegans* have indicated similar oxidation via cytochrome P-450 monooxygenase.

The persistence of fluorene and metabolites in soil and aquatic ecosystems poses a concern for human health, since the U.S. Environmental Protection Agency has included it on the list of priority pollutants (4). Microbial degradation

TABLE 1. HPLC retention times and spectrophotometric, mass spectral, and ^{1}H NMR data of metabolites formed from fluorene by *C. elegans*

Compound	Assignment	HPLC retention time (min)	Mass spectral properties {m/z(% relative intensity)[molecular ion]}	UV–visible-light absorption maxima (nm)	Proton NMR assignments, chemical shifts (δ), and coupling constants (J)[a]
I	2-Hydroxy-9-fluorenone	26.13	197(12), 196(100)[M$^+$], 195(1), 168(7), 140(10), 139(33), 113(4), 98(5), 84(10), 70(14), 63(6)	227, 234, 266, 296	δH_1, 7.01; δH_3, 7.07; δH_4, 7.5; δH_5, 7.59; δH_6, 7.51; δH_7, 7.2; δH_8, 7.54 ppm; J_{1-3}, 2.4; J_{1-4}, 0.4; J_{3-4}, 8.0; J_{5-6}, 7.4; J_{5-7}, 1.1; J_{6-7}, 7.4; J_{6-8}, 1.1; J_{7-8}, 7.4 Hz
II	9-Fluorenol	26.36	183(4), 182(37)[M$^+$], 181(54), 180(100), 165(7), 153(14), 152(54), 151(25), 150(12), 126(8), 91(11), 77(10), 76(56), 75(17), 63(23), 51(10)	227, 234, 271, 295	ND
III	9-Fluorenone	30.56	181(11), 180(100)[M$^+$], 152(29), 151(14), 150(5), 126(4), 76(20), 75(8), 74(4), 63(11)	248, 256, 293, 361	δH_1, 7.62; δH_2, 7.38; δH_3, 7.5; δH_4, 7.75; δH_5, 7.75; δH_6, 7.5; δH_7, 7.38; δH_8, 7.62 ppm; J_{1-3}, 1.1; J_{3-4}, 7.5; J_{5-6}, 7.5; J_{5-7}, 1.1; J_{6-7}, 7.5; J_{6-8}, 1.1; J_{7-8}, 7.5 Hz
Parent	Fluorene	39.00	167(13), 166(100)[M$^+$], 165(90), 164(11), 163(14), 139(7), 115(4), 83(22), 82(33), 74(2), 70(4), 69(6), 63(5)	261, 289, 300, 320	ND

[a] ND, not determined.

studies have shown that even though *P. chrysosporium* was able to degrade 9-fluorenone in soils (8), other studies have indicated persistence of 9-fluorenone in polluted coastal sediments (10) and in model ecosystems (22). Our previous experiments with the fungus *C. elegans* have indicated that the fungal metabolic pathways are indeed detoxification pathways in the metabolism of polycyclic aromatic hydrocarbons (PAHs). In this study, the fungal metabolism of fluorene reported indicates that oxidation of both aliphatic and aromatic rings occurs in detoxification of fluorene by *C. elegans*, since hydroxylated intermediates of PAHs have generally been shown to be less toxic than the parent compounds (4, 21). The application of *C. elegans* to PAH-contaminated soils should be demonstrated in order to evaluate its potential to detoxify these persistent pollutants.

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