Fungal Oxidation of Benzo[a]pyrene and (±)-trans-7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene

EVIDENCE FOR THE FORMATION OF A BENZO[a]PYRENNE 7,8-DIOL-9,10-EPOXIDE

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The oxidation of benzo[a]pyrene by intact cells of the filamentous fungus Cunninghamella elegans resulted in the formation of a complex mixture of polar products which were detected by high pressure liquid chromatography. One of the products had a retention time on high pressure liquid chromatography identical with that given by (±)-7β,8α,9α,10β-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene. In addition the absorption and mass spectra given by the fungal metabolite were consistent with this structural assignment.

Incubation of (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene with cells of C. elegans led to the formation of the same tetrahydroxymetabolite. Two other products were formed that were identified as new phenolic derivatives of (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene. Experiments with (±)-trans-[G-3H]7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene showed that over a 12-h period 4.0% of the dihydrodiol was converted to ethyl acetate-soluble products and 7β,8α,9α,10β-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene accounted for 25% of these metabolites.

The results suggest that C. elegans can oxidize benzo[a]pyrene to either (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro[a]pyrene or one of the two possible enantiomers of this compound. Under the experimental conditions, no evidence was found to suggest the formation of (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydro[a]pyrene. Thus, C. elegans has the ability to form at least one of the two diastereomeric benzo[a]pyrene 7,8-diol-9,10-epoxides that have been implicated as the ultimate carcinogenic forms of benzo[a]pyrene in higher organisms.

Benzo[a]pyrene is an abundant environmental carcinogen that is formed by the pyrolysis of organic material (1-3). Considerable evidence is now available which indicates that metabolic activation is necessary for the initiation of carcinogenesis (4-7) and recent studies have implicated the diastereomeric benzo[a]pyrene 7,8-diol-9,10-epoxides as the probable ultimate carcinogenic benzo[a]pyrene metabolites (8-12). It is now known that benzo[a]pyrene is metabolized by rat liver microsomes (13-15) and rat liver nuclear enzymes (16) with high stereospecificity to (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (benzo[a]pyrene 7,8-diol). Further micromosomal oxidation leads to the formation of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (±-diol epoxide-2) as the major enantiomer (14, 15, 17). When (±)-benzo[a]pyrene 7,8-diol is metabolized by liver microsomes (+)-7α,8β-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (±-diol epoxide-1) is the major enantiomer that is formed (18, 19). (+)-Diol epoxide-2 and, to a lesser extent, (±)-diol epoxide-1 were shown to be bound to nucleic acid when bronchial explants were exposed to benzo[a]pyrene and also when benzo[a]pyrene was applied to mouse skin (20, 21). Of the four optically pure isomers of benzo[a]pyrene 7,8-diol, 9,10-epoxide, (+)-diol epoxide-2 is the most active in terms of mutagenic and tumorigenic activity (18). The metabolic pathway for the formation of (±)-diol epoxide-2 is shown in Fig. 1. Since diol epoxides-1 and -2 are unstable they are usually characterized by analysis of the tetrools that are formed by hydrolysis. The individual tetrools are easily separated by high pressure liquid chromatography (14, 15, 17, 22, 23), although the ratios of each that are formed depend on the pH of hydrolysis (24). The tetrools formed from the diastereomeric diol epoxides are shown in Fig. 2.

In contrast to the vast literature on the metabolism of benzo[a]pyrene by mammals little information is available that relates to the products formed from this substrate by microorganisms. Bacteria oxidize benzo[a]pyrene to a mixture of cis-benzo[a]pyrene 7,8- and 9,10-diols (25). Recently we reported that the fungus, Cunninghamella elegans oxidizes benzo[a]pyrene to form phenols, quinones, benzo[a]pyrene 7,8- and benzo[a]pyrene 9,10-diols (26). Sulfuric and glucuronic acid conjugates of these primary metabolites were also detected. We now report evidence that this organism oxidizes benzo[a]pyrene and (+)-benzo[a]pyrene 7,8-diol to diol epoxide-2.

The abbreviations used are: benzo[a]pyrene 7,8-diol, trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; benzo[a]pyrene 9,10-diol, trans-9,10-dihydroxy-9,10-dihydrobenzo[a]pyrene; diol epoxide-1, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; diol epoxide-2, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; cis-tetrol 1, (±)-7β,8α,9β,10β-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; cis-tetrol 2, (±)-7β,8α,9α,10α-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; trans-tetrol 2, (±)-7β,8α,9β,10β-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; trans-tetrol 1, (±)-7β,8α,9α,10α-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; HPLC, high pressure liquid chromatography.

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acetate. The organic layer was dried over anhydrous sodium sulfate, and the solvent removed in vacuo at 30°C in the dark. The residue was redissolved in 500 μl of methanol and 250 μl was applied to a preparative silica gel thin layer plate (20 × 20 cm; 250 μm thickness; Analtech). The chromatogram was developed in a solvent system consisting of benzene:ethanol (9:1). Benzo[a]pyrene metabolites were located on chromatograms by viewing under ultraviolet light (254 nm) and by comparing the chromatographic mobilities to synthetic benzo[a]pyrene derivatives. The area of the chromatogram that contained reaction products more polar than benzo[a]pyrene 9,10-diol was designated the pre-9,10-diol region. The areas corresponding to the pre-9,10-diol region were removed and extracted with methanol. After removal of the solvent the residue was stored at −20°C prior to analysis by high pressure liquid chromatography (HPLC). Analyses were conducted on a Waters model 440 high pressure liquid chromatograph fitted with two coupled Bondapak C18 columns (3.9 mm × 39 cm). The metabolites were separated by gradient elution. The initial solvent composition was 40% methanol and 60% water. The final solvent concentration was 95% methanol and 5% water. A linear gradient was employed (1 h) with an initial flow rate of 0.8 ml/min to 2000 p.s.i.g. In experiments with [14C]benzo[a]pyrene, fractions (0.4 ml) were collected at 10-min intervals and added to tubes containing 5.0 ml of Aquasol-2. The radioactivity present in each fraction was determined in a Beckman LS-250 liquid scintillation counter.

Ultraviolet and visible spectra were determined on a Beckman model 25 recording spectrophotometer. Mass-spectral analysis was performed on a Finnigan model 2100 mass spectrometer at 70 eV ionizing voltage with a solid probe. Samples were dissolved in 10 μl of tetrahydrofuran. Spectra were recorded at a probe temperature of 240°C and an ion source temperature of 325°C.

**RESULTS AND DISCUSSION**

When *C. elegans* was grown on Sabouraud dextrose broth in the presence of benzo[a]pyrene, several oxidation products were detected by thin layer chromatography. Metabolites more polar than benzo[a]pyrene 9,10-diol were further analyzed by HPLC. Fig. 3 shows that a complex mixture of products was detected. Our previous studies have shown that many of these compounds are sulfate conjugates of hydroxylated benzo[a]pyrene derivatives (26).

One product (Compound VI) gave an identical absorption spectrum to those given by the tetroals formed by the addition of water to diol epoxides-1 and -2 (Fig. 4). Although the absorption spectrum is only indicative of the presence of a pyrene ring it suggests that the fungal metabolite was reduced at positions 7,8,9, and 10 of the benzo[a]pyrene molecule. When [14C]benzo[a]pyrene was incubated with *C. elegans* and the polar metabolites analyzed as described above, Compound VI accounted for 1.3% of the total polar benzo[a]pyrene metabolites.

Diol epoxides-1 and -2 are readily hydrolyzed to tetroals (Fig. 2). Thus, diol epoxide-1 undergoes predominantly cis addition of water to the epoxide group by a reaction mechanism that is acid-catalyzed at low pH and spontaneous above
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FIG. 3. Separation of polar metabolites formed from benzo[a]pyrene by C. elegans. HPLC conditions are described under "Materials and Methods. Compounds I to IX were collected as they eluted from the column and their absorption spectra recorded on a Beckman model 25 recording spectrophotometer. Radioactive metabolites formed from [14C]benzo[a]pyrene were monitored by collecting 0.4-ml fractions at ½-min intervals and measured for radioactivity as described under "Materials and Methods."
the metabolite formed from benzo[a]pyrene by C. elegans is trans-tetraol 2. It is of interest to note that only trans-tetraol 2 has been identified as a benzo[a]pyrene metabolite. Hydrolysis of diol epoxide-2 would produce cis-tetraol 2 (see Fig. 2). Under the HPLC conditions used in the initial isolation experiment (Fig. 3) cis-tetraol 2 would elute with Compound VII. Fractions that eluted between 33 to 39 min were pooled and resolved into four components by the chromatographic conditions used to separate the diol epoxide hydrolysis products. None of the compounds in this fraction had spectral properties similar to those of cis-tetraol 2. It seems likely that this hydrolysis product is formed in amounts too low to be detected by the experimental conditions. If this supposition is correct it suggests that diol epoxide-2 formed by C. elegans may undergo an acid-catalyzed hydrolysis reaction inside of the cell. The pH of the culture medium changes from 5.8 to 6.4 during the experiment and these conditions would favor the formation of cis-tetraol 2. It is also of interest that cis-tetraol 1 was not detected as a product formed from either benzo[a]pyrene or benzo[a]pyrene 7,8-diol (see below). This would be the major tetraol formed from diol epoxide-1 irrespective of the pH of hydrolysis (24). These observations suggest that C. elegans may oxidize benzo[a]pyrene in a stereospecific manner also to benzo[a]pyrene 7,8-diol. If the (+)-enantiomer of benzo[a]pyrene 7,8-diol were formed one would expect, by analogy with mammalian systems, that epoxidation would yield mainly diol epoxide-1 and hence cis-tetraol 1 as a major hydrolysis product. However, diol epoxide-1 is extremely susceptible to nucleophilic attack due to anchimeric assistance by the proximate cis-hydroxyl group (22). Thus, if diol epoxide-1 is formed inside the cell it may react with cell nucleophiles and not be excreted into the culture medium. Future experiments with fungal microsomes should establish whether or not diol epoxide-1 is formed from benzo[a]pyrene and benzo[a]pyrene 7,8-diol. Although the results strongly indicate that C. elegans oxidizes benzo[a]pyrene to a diol epoxide it is not possible at this time to eliminate the alternative possibility that trans-tetraol 2 could be formed by the hydrolysis of the analogous benzo[a]pyrene 9,10-diol-7,8-epoxide. Results of studies on the fungal oxidation of benzo[a]pyrene 9,10-diol will be reported at a later date.

When 3H-labeled (±)-trans-7,8-diol was incubated for 12 h with intact cells of C. elegans 4% of the substrate was converted into metabolites. Analysis by HPLC gave the results shown in Fig. 7. Compound 1 had an identical retention time to that given by trans-tetraol 2. Calculations based on radioactivity measurements show that trans-tetraol 2 accounts for 25% of the trans-7,8-diol metabolites whereas Compounds III and IV accounted for 23 and 14%, respectively.

The experiment was repeated in the absence of radioactive benzo[a]pyrene 7,8-diol in order to isolate Compounds I, III, and IV for characterization purposes. Compound VII is unchanged benzo[a]pyrene 7,8-diol and no attempt was made to isolate Compounds II, V, and VI. The absorption and mass spectra of Compound I were identical with those given by trans-tetraol 2. Similar observations were reported for the oxidation of benzo[a]pyrene 7,8-diol by microsomes prepared from livers of control and phenobarbital-induced rats. However, these liver preparations also formed significant amounts of diol epoxide-1 from both (+)- and (±)-benzo[a]pyrene 7,8-diol. In contrast, liver microsomes from rats treated with 3-methylcholanthrene oxidized (−)- and (±)-benzo[a]pyrene 7,8-diol.

Mass spectral analysis of Compounds III and IV revealed that each compound gave a parent ion at m/e 302 and a base peak at m/e 284 (pH 40). These observations suggest that Compounds III and IV are dihydroyd derivatives of a benzo[a]pyrene phenol. Further evidence was obtained by observing the spectral changes that occurred when each compound was treated with acid (Fig. 8). The actual structures of Compounds III and IV which are presumed to be new mono-hydroxylated derivatives of trans-benzo[a]pyrene 7,8-diol have yet to be determined.

From the data presented we propose the metabolic sequence in Fig. 9 for the oxidation of benzo[a]pyrene and (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene by C. elegans. The possibility that a diol epoxide could also be formed from benzo[a]pyrene 9,10-diol is currently under investigation. However, the metabolism of benzo[a]pyrene 9,10-diol by liver microsomes does not lead to the formation of significant amounts of benzo[a]pyrene 9,10-diol-7,8-oxides (29, 30). It has been pointed out that the enzymes involved in the mammalian metabolism of benzo[a]pyrene make all of the wrong stereo-
chemical choices. Thus, at each step in the formation of the 7,8-diol-9,10-epoxides, a predominant amount of the more tumorigenic isomer is produced (18). It is conceivable that these stereochemical choices evolved in eukaryotic microorganisms and in this context it should be noted that Blumer and Youngblood (31) have suggested that the presence of polycyclic aromatic hydrocarbons in the environment may have contributed to the processes of natural selection, mutation, and to the evolution of species.

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