

Fungal Metabolism and Detoxification of the Nitropolycyclic Aromatic Hydrocarbon 1-Nitropyrene

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Nitropolycyclic aromatic hydrocarbons are ubiquitous environmental pollutants, many of which are potent mutagens in bacterial and mammalian cells and carcinogenic to rodents. In this study, we investigated the fungal metabolism of 1-nitropyrene and determined the mutagenic activity of the metabolites toward *Salmonella typhimurium* TA98, TA98NR, and TA100. *Cunninghamella elegans* metabolized 1-nitropyrene to form glucoside conjugates of 6-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene. The metabolites were isolated by reversed-phase high-pressure liquid chromatography and characterized by application of UV absorption, ¹H-nuclear magnetic resonance, and mass spectroscopy. Mutagenicity assays performed on samples extracted from incubations of *C. elegans* with 1-nitropyrene indicated that mutagenic activity decreased with time. Consistent with the loss in mutagenic activity, the glucoside conjugates of 6- and 8-hydroxy-1-nitropyrene were nonmutagenic in the *Salmonella* reversion assay. The results indicate that the fungus *C. elegans* metabolizes 1-nitropyrene to detoxified products.

Nitropolycyclic aromatic hydrocarbons are a widely distributed class of environmental contaminants which can be formed in the atmosphere by reaction of nitrogen oxides with polycyclic aromatic hydrocarbons (40, 41). Nitropolycyclic aromatic hydrocarbons occur in aquatic and terrestrial ecosystems and have also been identified in diesel engine exhaust, coal fly ash, and carbon black photocopier toners (25, 27, 29, 41-43). Their presence has also been suggested in cigarette smoke (32). Experimental evidence indicates that nitropolycyclic aromatic hydrocarbons are "direct-acting" mutagens in prokaryotic and eukaryotic cells (2, 5, 10, 22, 26, 27, 33, 37, 41, 43, 45), are carcinogenic in experimental animals (12, 16, 39), are metabolized to derivatives which bind to DNA and proteins (15, 17, 21; R. H. Heflich, E. K. Fifer, Z. Djuric, and F. A. Beland, *Environ. Health Perspect.*, in press), and induce unscheduled DNA synthesis (5), sister chromatid exchanges (31, 36), and DNA repair (5) and transformation (9, 19) in cultured eukaryotic cells. This has prompted considerable research on the sources, occurrence, metabolism, and disposition of these pollutants. It has been postulated that the biological activities exhibited by this class of compounds may be mediated by metabolic activation via oxidative and reductive pathways to form intermediates which react with critical cellular macromolecules (2, 7, 10, 13, 14, 17, 18, 20, 21, 24, 28, 35, 38, 44; Heflich et al., in press; M. W. Chou, R. H. Heflich, and P. P. Fu, *Carcinogenesis*, in press; Z. Djuric, E. K. Fifer, and F. A. Beland, *Carcinogenesis*, in press) (Fig. 1).

In contrast to the number of studies on the metabolism of nitropolycyclic aromatic hydrocarbons by intestinal microflora (4, 13, 18, 20, 23, 34), little has been reported about the metabolism of these compounds by microbial populations isolated from soil or aquatic environments. In this study, we describe the metabolism of 1-nitropyrene by the fungus *Cunninghamella elegans*. In addition, extracts of cultures grown in the presence of 1-nitropyrene were tested for mutagenicity in the *Salmonella typhimurium* reversion

assay to determine the effect of fungal metabolism on the mutagenicity of 1-nitropyrene.

MATERIALS AND METHODS

Chemicals. [4,5,9,10-³H]1-nitropyrene (specific activity, 117 mCi/mmol; radiochemical purity, >99%) was obtained from Robert W. Roth, Midwest Research Institute, Kansas City, Mo. 1-Nitropyrene was purchased from Aldrich Chemical Co., Milwaukee, Wis., and was purified as described previously (18). 6- and 8-hydroxy-1-nitropyrene were kindly provided by Frederick A. Beland at the National Center for Toxicological Research. High-pressure liquid chromatography (HPLC)-grade solvents were purchased from Fisher Chemical Co., Pittsburgh, Pa. All other chemicals were of reagent grade and in the highest purity available.

Microorganism and culture conditions. Stock cultures of the fungus *C. elegans* ATCC 36112 were maintained on Sabouraud dextrose agar slants and stored at 4°C. The spores, mycelia, or both from several slants were used to inoculate 125-ml Erlenmeyer flasks which contained 30 ml of Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.). After the flasks were incubated for 48 h at 25°C on a rotary shaker at 150 rpm, the mycelium was removed by aseptic filtration and transferred to 10 sterile 125-ml Erlenmeyer flasks containing 30 ml of Sabouraud dextrose broth. One milligram of 1-nitropyrene was dissolved in 0.4 ml of dimethyl sulfoxide and added to each flask. Sterile control experiments were prepared by autoclaving the culture at 121°C for 40 min before adding 1-nitropyrene. No metabolism was detected in the control flasks. All of the flasks were incubated as described above at 25°C for 48 h.

Kinetic experiments with [4,5,9,10-³H]1-nitropyrene were conducted as described above with the exception that [³H]1-nitropyrene (3.7 μM) was added to each flask. The flasks were incubated for various periods, and their contents were extracted and analyzed for metabolites as described below. The percent metabolism was quantified as described previously (18).

Isolation, detection, and identification of 1-nitropyrene me-

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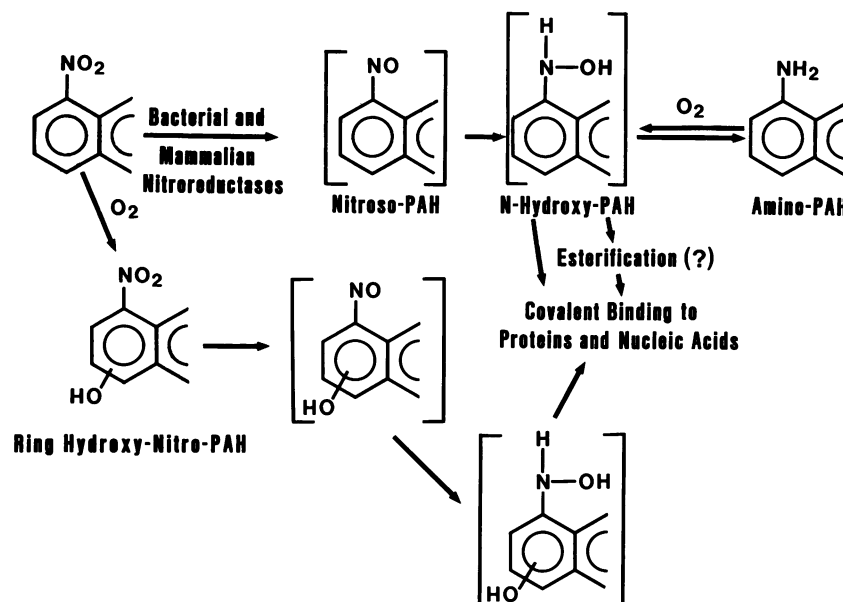


FIG. 1. Schematic representation of the metabolic activation pathways for nitropolycyclic aromatic hydrocarbons (PAH).

tabolites. After 48 h of incubation, the flask contents were pooled and filtered to separate the broth from the mycelia. The broth and the mycelia were then extracted with 6 equal volumes of ethyl acetate. The extracts were combined and dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure at 40°C in the dark. The residue was dissolved in methanol and analyzed by HPLC. Reversed-phase HPLC was performed with a Beckman system consisting of two model 100A pumps and a model 155-10 variable-wavelength absorbance detector adjusted to 254 nm. The 1-nitropyrene metabolites were separated on a C₁₈ μ Bondapak column (0.39 by 30 cm; Waters Associates, Milford, Mass.) by elution with a 30-min linear methanol-water gradient (50 to 80%, vol/vol) and then with 100% methanol for 10 min. The flow rate was 2 ml/min. Each compound was collected, and after repeated injections of the 1-nitropyrene extract, fractions of UV-absorbing material with similar HPLC retention times were pooled and concentrated in a Speed Vac Concentrator (Savant Instruments, Inc., Hicksville, N.Y.). In experiments with [4,5,9,10-³H]1-nitropyrene, 1.0-ml fractions were collected every 0.5 min and added to scintillation vials containing 5.0 ml of Aquasol-2. The radioactivity was determined in a Beckman LS-250 liquid scintillation counter. UV-visible absorption spectra of the metabolites were determined in methanol on a Beckman DU-7 spectrophotometer. Silica gel 60 F₂₅₄ glass plates (E. Merck AG, Darmstadt, West Germany) were used for thin-layer chromatographic analyses of the metabolism extracts and authentic standards. The solvent used for chromatography was benzene-ethanol (19:1). Mass spectral analyses were performed on a Finnigan model 4023 quadrupole mass spectrometer, using a Vacumetrics model DCI current programmer and a platinum wire probe (Vacumetrics Corp., Ventura, Calif.). The source temperature was 270°C, the electron energy was 70 VU, and the probe current ramp time was 120 s. For the negative ion chemical ionization (NICI) mass spectra, the source pressure was 0.2 torr for the reagent gas, which consisted of 10% ammonia and 90% nitrogen.

The ¹H-nuclear magnetic resonance (NMR) spectra were

recorded on a Bruker WM 500 spectrometer in acetone-*d*₆. The data were acquired under the following conditions: data size, 32,000; sweep width, 7,042 Hz; filter width, 7,800 Hz; temperature, 305 K; flip angle, 68°. The chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane. Assignments were made via homonuclear decoupling experiments and by consideration of substituent effects.

Mutagenicity assays. Mutagenicity assays were performed on both the crude extracts and purified metabolites formed during the fungal metabolism of 1-nitropyrene. The assays were conducted with and without an hepatic post-mitochondrial supernatant (S9) metabolizing system essentially as described by Maron and Ames (30). To assess

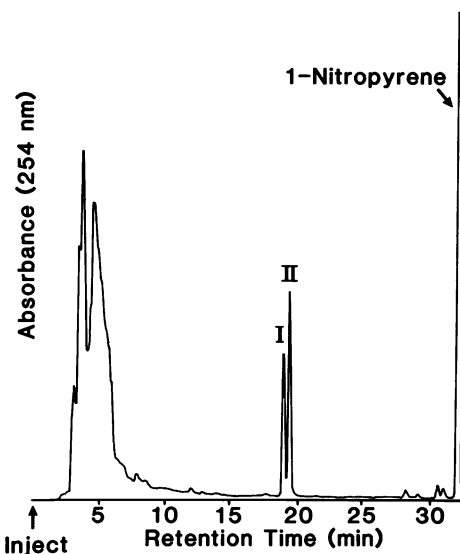


FIG. 2. HPLC elution profile of the metabolites formed from 1-nitropyrene by *C. elegans*.

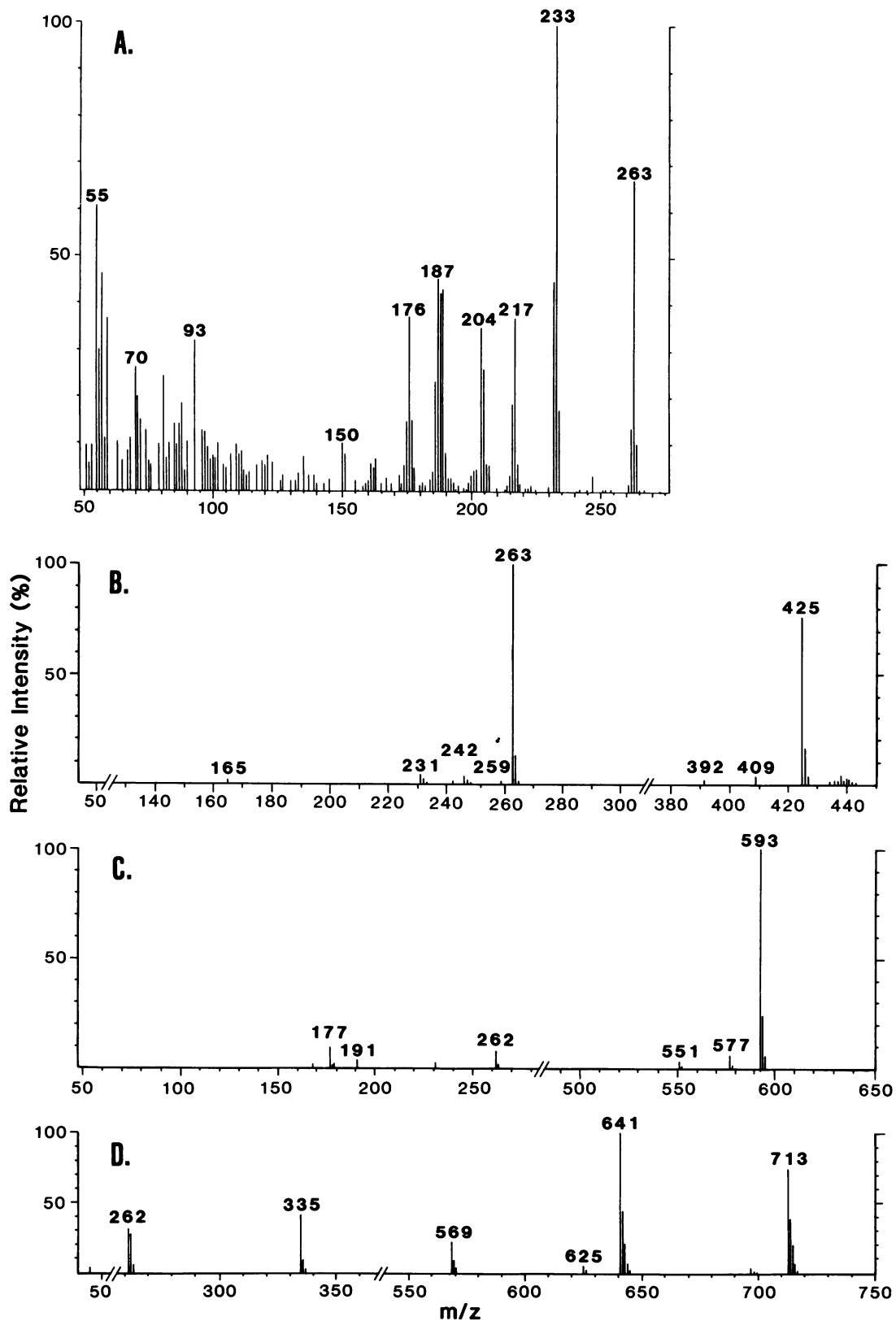


FIG. 3. Mass spectra of compound I formed from 1-nitropyrene by *C. elegans*. (A) Electron impact mass spectrum of compound I. (B) Ammonia NICI mass spectrum of compound I. (C) Ammonia NICI mass spectrum of acetylated derivative of compound I. Compound I (80 μg) was mixed with 0.5 ml of ethylacetate, 5 ml of pyridine, and 1 ml of acetic anhydride and incubated at 70°C for 5 h. The reaction mixture was evaporated under nitrogen and the residue was dissolved in methanol and analyzed by mass spectrometry. (D) Ammonia NICI mass spectra of trimethylsilylated derivative of compound I. Compound I (80 μg) was mixed with ethyl acetate-pyridine-*N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, Ill.) (87.5:7.5:5.0, by volume) and incubated at 70°C for 2 h. The reaction mixture was evaporated under nitrogen and the residue was dissolved in methanol and analyzed by mass spectrometry.

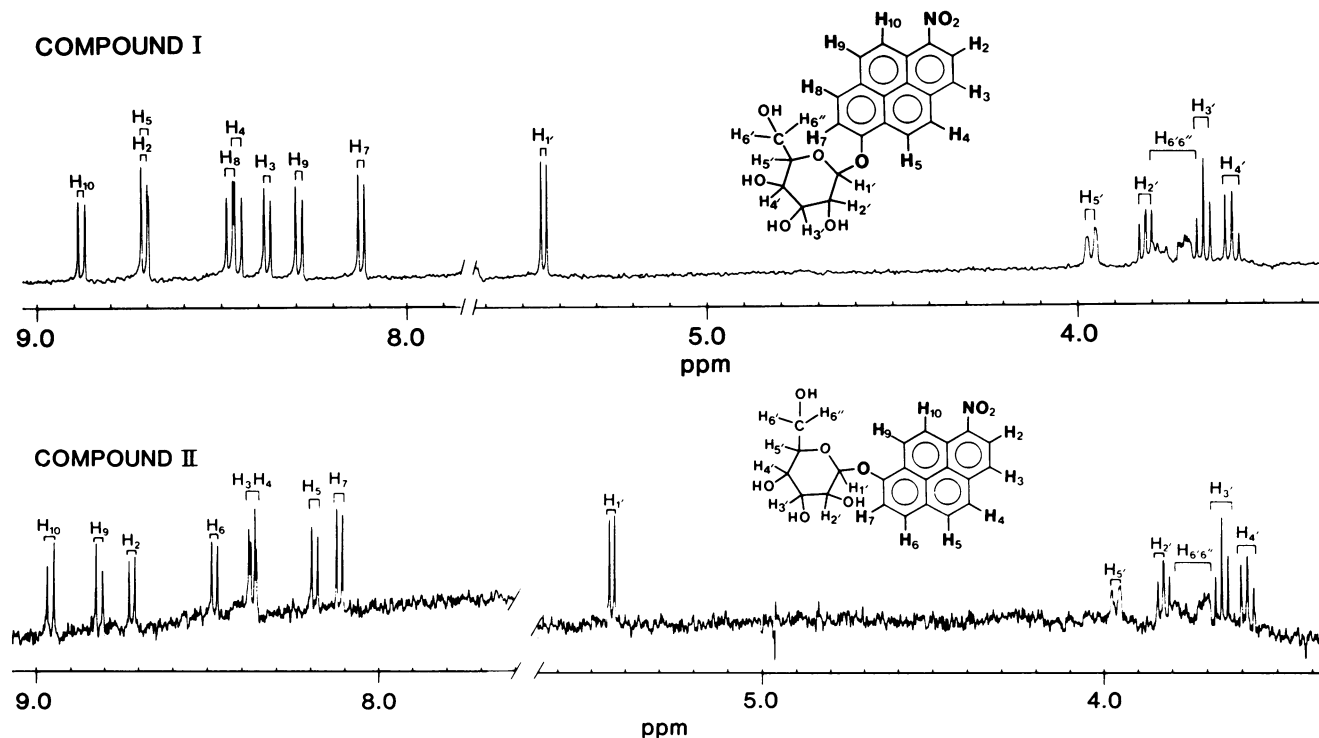


FIG. 4. Proton NMR spectra (500 MHz) of compounds I and II formed from 1-nitropyrene by *C. elegans*. The assignments for compound I are 8.12 (d, 1, $J_{7,8} = 9.0$ Hz, H₇), 8.29 (d, 1, $J_{9,10} = 9.5$ Hz, H₉), 8.38 (d, 1, $J_{2,3} = 8.6$ Hz, H₃), 8.45 (d, 1, $J_{4,5} = 9.5$ Hz, H₄), 8.48, (d, 1, H₈) 8.71 (d, 1, H₅), 8.71 (d, 1, H₂), 8.91 (d, 1, H₁₀), 5.44 (d, 1, $J_{1',2'} = 7.3$ Hz, H_{1'}), and 3.82 (dd, 1, H_{2'}) and the remainder of the glucoside resonances were tentatively assigned as 3.95 (apparent-d, 1, H_{5'}), 3.69 to 3.81 (m, 2, H_{6'} and H_{6''}), 3.66 (dd, 1, H_{3'}), and 3.59 (dd, 1, H_{4'}) ppm. The assignments for compound II are 8.11 (d, 1, $J_{6,7} = 8.2$ Hz, H₇), 8.19 (d, 1, $J_{4,5} = 9.0$ Hz, H₅ or H₄), 8.36 (d, 1, $J_{2,3} = 8.2$ Hz, H₃), 8.37 (d, 1, H₄ or H₅), 8.48 (d, 1, H₆), 8.72 (d, 1, H₂), 8.82 (d, 1, $J_{9,10} = 9.9$ Hz, H₉), 8.96 (d, 1, H₁₀), 5.44 (d, 1, $J_{1',2'} = 7.3$ Hz, H_{1'}), and 3.83 (dd, 1, H_{2'}), ppm, and the remainder of the glucoside moiety was tentatively assigned as 3.97 (apparent-d, 1, H_{5'}), 3.69 to 3.81 (m, 2, H_{6'} and H_{6''}), 3.66 (dd, 1, H_{3'}), and 3.59 (dd, 1, H_{4'}) ppm. Both glucosides contained a β -linkage.

"direct" mutagenic activity, 0.1 ml of the test substance dissolved in dimethyl sulfoxide (Burdick and Jackson, Muskegan, Mich.) was mixed with 0.1 ml of a 10-h culture of *S. typhimurium* tester strain TA98, TA98NR (deficient in nitroreductase activity), or TA100 and 2.5 ml of molten top agar (50 μ M L-histidine, 50 μ M biotin, 0.5% NaCl, 0.6% agar). The mixture was poured into petri plates containing Vogel minimum salts agar with glucose. To assess S9-mediated mutagenicity, 0.1 ml of the test substance, 0.1 ml of the tester strain, 0.5 ml of an S9 mix (0.15 ml of S9 plus 0.35 ml of 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 8 mM MgCl₂, 100 mM sodium phosphate buffer, pH 7.4), and 2.0 ml of molten top agar were mixed and poured into plates containing Vogel agar. The S9 was prepared from male Sprague-Dawley rats pretreated with Aroclor 1254 (1). All assays were performed in triplicate. The plates were incubated inverted at 37°C for 48 h, and the revertant colonies were counted visually.

RESULTS AND DISCUSSION

The HPLC elution profile of the ethyl acetate-extractable metabolites formed by incubation of 1-nitropyrene with *C. elegans* is shown in Fig. 2. 1-Nitropyrene eluted at 32.6 min. The material which eluted within 8 min were medium-extractable compounds and not 1-nitropyrene metabolites. Compounds I and II eluted at 18.6 and 19.4 min, respectively. Both compounds gave identical UV-visible (λ_{\max} 235, 285, 375, and 405 nm) and electron impact mass spectra (M^+ at m/z 263 and m/z 233 [M^+ -NO]; Fig. 3A). Based on

previous studies in this laboratory (18) and by El-Bayoumy and Hecht (10), the UV-visible and mass spectral data indicated that compounds I and II were phenolic derivatives of 1-nitropyrene (1-nitropyrenols). However, compounds I and II had thin-layer liquid chromatographic and HPLC properties different from those of authentic 1-nitropyrenols. Ammonia NICI mass spectra of compounds I and II gave a similar molecular ion at m/z 425 (M^-) and a base peak at m/z 263 [(M -glucose) $^-$] (Fig. 3B). These data suggested that compounds I and II were glucoside conjugates of 1-nitropyrenols. Acetylation and silylation of compounds I and II and subsequent ammonia NICI mass spectral analyses confirmed that these compounds were glucoside conjugates. Figure 3C is the mass spectrum of the peracetylated derivative of compound I. There is a molecular ion at m/z 593 (M^-) and minor fragments at m/z 551 [(M -acetyl) $^-$] and m/z 262 [(M -peracetylated glucose) $^-$]. The mass spectrum of the trimethylsilyl derivative of compound I (Fig. 3D) produced a molecular ion at m/z 713 (M^-) and fragments at m/z 641 [(M -TMS) $^-$], m/z 569 [(M -2TMS) $^-$], and m/z 262 (M -persilylated glucose $^-$). Similar results were found for compound II. The mass spectra produced by the acetylated and silylated derivatives of compounds I and II are typical of arylglucosides (6).

The structures of the glucoside conjugates (compounds I and II) were confirmed by 500-MHz NMR spectroscopy. The NMR spectra of compounds I and II are shown in Fig. 4. The structural assignments for these compounds were accomplished via standard homonuclear decoupling, integra-

TABLE 1. Reversion induction in *S. typhimurium* strains TA98, TA98NR, and TA100 by the glucoside conjugates of 6- and 8-hydroxy-1-nitropyrenes

Compound	$\mu\text{g/plate}$	Revertants per plate (\pm SD)					
		TA98		TA98NR		TA100	
		-S9	+S9	-S9	+S9	-S9	+S9
Solvent control		26 \pm 4	39 \pm 2	21 \pm 4	52 \pm 2	151 \pm 16	166 \pm 9
6-Hydroxyglucoside-1-nitropyrene	0.2	32 \pm 6	40 \pm 4	24 \pm 5	39 \pm 3	148 \pm 15	184 \pm 6
	0.4	21 \pm 4	46 \pm 4	22 \pm 3	48 \pm 7	154 \pm 4	187 \pm 17
	0.8	29 \pm 0	47 \pm 12	24 \pm 7	37 \pm 8	150 \pm 7	155 \pm 9
	1.7	26 \pm 5	45 \pm 5	27 \pm 2	33 \pm 5	170 \pm 3	187 \pm 19
Revertants induced per nmol		0 ^a	0	0	0	0	0
8-Hydroxyglucoside-1-nitropyrene	0.2	28 \pm 10	46 \pm 8	23 \pm 3	37 \pm 6	163 \pm 5	139 \pm 7
	0.4	28 \pm 5	43 \pm 9	26 \pm 5	38 \pm 1	169 \pm 10	169 \pm 11
	0.8	29 \pm 1	44 \pm 12	22 \pm 4	42 \pm 7	161 \pm 9	150 \pm 8
	1.7	28 \pm 4	43 \pm 3	28 \pm 3	44 \pm 6	153 \pm 15	145 \pm 6
Revertants induced per nmol		0	0	0	0	0	0
1-Nitropyrene	0.2	524 \pm 36	154 \pm 2	59 \pm 3	94 \pm 19	178 \pm 22	182 \pm 9
	0.4	929 \pm 9	282 \pm 45	99 \pm 7	141 \pm 10	172 \pm 12	174 \pm 3
Revertants induced per nmol		585	153	42	78	0	0
Benzo[a]pyrene	7	NT ^b	583 \pm 67	NT	598 \pm 61	NT	902 \pm 160

^a Solvent control values have been subtracted. Concentrations of the test chemicals which did not double the solvent control values were considered nonmutagenic and assigned a value of 0 induced revertants per nmol.

^b NT, Not tested.

tion experiments, and comparison of the aromatic resonances with the data reported for authentic 6- and 8-hydroxy-1-nitropyrenes (18). The aromatic resonances are all doublets for compounds I and II, which indicates substitution at C-6 or C-8 of 1-nitropyrene. The most upfield resonance for compounds I and II was assigned to H₇ (8.11 and 8.12 ppm, respectively) compared to H₇ (7.80 ppm) for 6- and 8-hydroxy-1-nitropyrenes (18). The anisotropic effect of the nitro group on a *peri* proton and the magnitude of the K-region coupling constants ($J_{9,10} = 9.5$ and 9.9 Hz) for compounds I and II, respectively, suggests C-6 and C-8 aromatic ring carbon substitution. The H₉ resonance for compound I at 8.29 ppm compared to 8.82 ppm for compound II and the *peri* effect of the oxygen-glucoside substituent further indicate that compound II is substituted at C-8 of 1-nitropyrene. The observation of H₅ for compound I at 8.71 ppm as compared to 8.19 or 8.37 ppm for compound II and the *peri* effect of the oxygen glucoside moiety indicate that compound I is substituted at C-6. The presence of a doublet resonance at 5.44 ppm ($J_{1,2} = 7.3$ Hz) for compounds I and II (Fig. 4) is consistent with an α proton (H_{1'}) of a glucoside. The other assigned chemical shifts and coupling constants are consistent with data previously reported for other glucosides and acetylated derivatives of β -D-glucopyranose (8).

The extent of 1-nitropyrene metabolism by *C. elegans* was determined at various time intervals, and the *Salmonella* reversion assay was used to determine the mutagenic activity of the extracts at each time point. Figure 5 indicates that, as the percent metabolism of 1-nitropyrene increased, there was a corresponding decrease in mutagenicity in *S.*

typhimurium TA98 without the rat liver S9 metabolic activation system. In addition, the metabolism extracts were not mutagenic in the presence of S9. Similar results were found when the extracts were assayed with *S. typhimurium* TA100, although the numbers of induced revertants per plate were much lower.

The mutagenicities of the glucoside conjugates of 6- and 8-hydroxy-1-nitropyrene formed from 1-nitropyrene by *C. elegans* were measured with *S. typhimurium* strains TA98, TA98NR, and TA100 in the absence and presence of S9 (Table 1). Benzo[a]pyrene and 1-nitropyrene were also assayed as positive controls. At the concentrations tested, the microbial metabolites of 1-nitropyrene showed no mutagenic activity in any of the *Salmonella* sp. tester strains with or without S9. Since previous studies have indicated that phenolic derivatives of 1-nitropyrene are highly mutagenic in the *Salmonella* reversion assay (2, 10), these results suggest that glucosylation of 1-nitropyrenols is a detoxification step in the metabolism of 1-nitropyrene.

Metabolism studies with rat and mouse liver cell homogenates, perfused rat livers and lungs, intact animals, and intestinal microflora have indicated that both ring oxidation and nitroreduction pathways contribute to the mutagenicity and possibly the carcinogenicity of 1-nitropyrene (3, 7, 10, 11, 13, 15, 17, 18, 20, 22, 23, 34, 35). Metabolites identified in those reports were 1-aminopyrene, *N*-acetyl-1-aminopyrene, 3-,5-,8-,10-hydroxy-1-nitropyrene, *trans*-4,5-dihydro-4,5-dihydroxy-1-nitropyrene, *trans*-9,10-dihydro-9,10-dihydroxy-1-nitropyrene, 1-hydroxypyrene, and hydroxyaminopyrenes. Sulfate and glucuronide conjugates of these primary metabolites have also been detected.

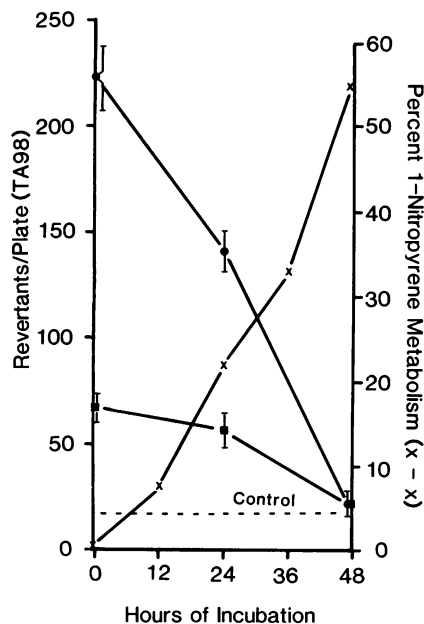


FIG. 5. Mutagenic activity of ethyl acetate extracts from cultures of *C. elegans* incubated with 1-nitropyrene for various time intervals. Mutagenic activity was assayed with *S. typhimurium* TA98 in the absence of S9 mix. Symbols: (■) 0.25 µg of extract per plate; (●) 1 µg of extract per plate; (x) percent 1-nitropyrene metabolized by *C. elegans*. No mutagenic activity was found in the presence of S9 mix.

There have been several studies on the microbial metabolism of nitropolycyclic aromatic hydrocarbons. Kinouchi et al. (23) showed a decrease in the mutagenicity when 1-nitropyrene was incubated with the feces of a healthy individual or with various intestinal microflora. The decrease in mutagenicity was attributed to the enzymatic reduction of 1-nitropyrene to 1-aminopyrene by intestinal microflora. Various *S. typhimurium* strains, bacterial suspensions, intestinal contents from human and rat feces, and pure cultures of anaerobic bacteria have been shown to be capable of reducing 1-nitropyrene, 1,8-dinitropyrene, and 6-nitrobenzo[a]pyrene to the corresponding amines (4, 18, 20, 23, 34). The present investigation indicates that the fungus *C. elegans* metabolized 1-nitropyrene by ring oxidation to form 6- and 8-hydroxy-1-nitropyrenes followed by glucoside conjugation. No nitroreduction products were detected. The two glucoside conjugates were not mutagenic toward *S. typhimurium* strain TA98, TA98NR, or TA100 with or without exogenous metabolic activation. Although both 6- and 8-hydroxy-1-nitropyrenes are mutagenic in *Salmonella* sp., none of the free nitropyrenols was detected as metabolites and the overall effect of incubating 1-nitropyrene with *C. elegans* was to reduce the mutagenicity associated with these cultures (Fig. 5). These results suggest that fungal metabolism could play an important role in detoxifying nitropolycyclic aromatic hydrocarbons in the environment.

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