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1-HYDROXYPYRENE GLUCURONIDE AS THE MAJOR AQUEOUS PYRENE METABOLITE IN TISSUE AND GUT FLUID FROM THE MARINE DEPOSIT-FEEDING POLYCHAETE *NEREIS DIVERSICOLOR*

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Abstract—Both 1-hydroxypyrene and 1-hydroxypyrene glucuronide are identified as the primary phase I and phase II metabolites of the four-ringed polycyclic aromatic hydrocarbon (PAH) pyrene in the marine deposit-feeding polychaete *Nereis diversicolor*. Identification of pyrene and primary metabolites was performed using high-pressure liquid chromatography (HPLC) with diode-array detection and fluorescence detection (HPLC/DAD/F) and an ion-trap mass spectrometer for positive identification of 1-hydroxypyrene glucuronide. Besides 1-hydroxypyrene and 1-hydroxypyrene glucuronide, the HPLC/F trace of tissue samples from pyrene-exposed worms showed three additional low-intensity peaks that may be related to pyrene metabolism based on similar excitation/emission wavelengths. The peaks were all too low in intensity to be positively identified. Of the total PAH in tissue, 1-hydroxypyrene glucuronide, 1-hydroxypyrene, and pyrene constituted 73%, 2%, and 25% respectively. Gut elimination of metabolic products is supported by the identification of 1-hydroxypyrene and 1-hydroxypyrene glucuronide in both gut fluid and defecation water. Being the only phase I metabolite of pyrene, 1-hydroxypyrene becomes a useful marker for PAH exposure, and it may serve as a valuable model compound for assessing species-specific PAH metabolic capabilities.

Keywords—Polycyclic aromatic hydrocarbon Bioaccumulation Metabolism Polychaetes 1-Hydroxypyrene glucuronide

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) constitute a broad class of environmentally persistent organic compounds that are ubiquitous in both marine and terrestrial environments [1]. Because several PAHs have been identified as potent procarcinogens, PAH analysis has been incorporated into environmental monitoring and risk-assessment programs. The PAHs themselves are relatively inert molecules, and it is generally accepted that toxic and carcinogenic effects of PAHs are caused by their metabolites rather than by the parent compounds [2,3]. Detection and quantification of PAH metabolites in conjunction with their parent compounds therefore might provide a better method for environmental risk assessment.

The ability of marine polychaetes to accumulate and metabolize PAHs has been documented for a few species (e.g., [4–6]). Among marine invertebrates in general, the ability to metabolize PAHs varies widely both within and among phyla, though at rates much lower than those observed in vertebrates [7]. Metabolism of PAH in marine invertebrates is apparently related to the cytochrome P450 enzyme suite, though the mechanism by which their genetic expression is regulated is still poorly understood [8]. The general function of P450 enzymes is to convert hydrophobic, lipid-soluble parent PAH to water-soluble metabolites in a two-phase process. The enzymes of phase I metabolism introduce or modify a functional group ($-$OH, $-$COOH, NO_2, etc.) into the PAH to which glucuronosyltransferase or other phase II enzymes attach a large polar moiety (glucuronic acid, glutathione, sulfate, amino acid, etc.), which is then readily excreted from the animal.

The present study investigates formation of phase I and II metabolites of pyrene in the deposit-feeding marine polychaete *Nereis diversicolor*, which has known PAH metabolic capacity [4,9,10]. However, direct identification of phase II biotransformation products in a marine polychaete has, to our knowledge, not yet been published. Most enzymes responsible for biotransformation of xenobiotics in mammals have also been identified in invertebrates. Phase I enzymes, like cytochrome P450, have been found or been indicated in annelids, crustaceans, echinoderms, and mollusks [11–14]. In the case of phase II metabolism, glucose and sulfate conjugation is indicated to predominate over glucuronic acid conjugation in marine invertebrates [15]. However, as for mammals and fish, large species-specific differences in phase II metabolism are observed among invertebrates. Consistent with this, Hryk et al. [16] found formation of glucoside but not glucuronide conjugates of phenol in the terrestrial snail *Capaea nemoralis*, and Michel et al. [17] found sulfate and glucuronide conjugates of benzo[*a*]pyrene (B*a*P) in the mollusk *Mytilus galloprovincialis*. This species-specific difference in phase II metabolism is also evident in metabolism of pentachlorophenol in an oyster (*Crassostrea gigas*) and two species of abalone (*Haliotis fulgens* and *H. rufescens*). All three species formed sulfated conjugates, whereas only the abalone formed glucoside conjugates of pentachlorophenol [18,19].

Pyrene is not known for its toxicity, so more attention has usually been given to B*a*P, which is an extremely potent carcinogen after metabolic activation [20]. However, measurement of pyrene metabolites, primarily 1-hydroxypyrene, in excretory products of animals and humans has gained consid-

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erable attention as a potential biomarker for PAH exposure [21]. 1-Hydroxypyrene was the only observed phase I metabolite of pyrene metabolism when pyrene was administered to pigs, rats, rabbits, and a terrestrial isopod [22–25], thereby limiting the number of potential phase II metabolites. Several reports have appeared concerning the use of 1-hydroxypyrene as a biomarker for PAH exposure in both marine (primarily in fish) and terrestrial environments. Stroomberg et al. [25] reported glucoside and sulfate conjugates of 1-hydroxypyrene in the hepatopancreas of a terrestrial isopod [25], and both glucuronide- and sulfate-conjugated 1-hydroxypyrene have been observed in fish [26].

The purpose of the present study was to investigate formation of pyrene metabolites in *N. diversicolor*, with special attention given to the detection and identification of phase II biotransformation products in both issue and gut fluid. The gut is assumed to the primary route of both uptake and elimination of organic contaminants in deposit-feeding polychaetes [27–29], and elimination of aqueous metabolites through gut fluid is expected as an aqueous intermediary necessary for excretion. Metabolites were studied using high-pressure liquid chromatography (HPLC) with fluorescence and ultraviolet (UV)/vis detection, and a liquid chromatography/mass spectrometry (LC/MS) system was used for structural identification of the dominant phase II metabolite.

MATERIALS AND METHODS

Pyrene (98%), 1-hydroxypyrene (98%), and ammonium acetate (>99%) were purchased from Aldrich Chemical (Milwaukee, WI, USA). Acetonitrile and methanol (ChromAR® HPLC grade) were purchased from Mallinckrodt (Paris, KY, USA). 100% Ethanol was obtained from Quantum Chemical (Tuscola, IL, USA). Glucuronidase-arylsulfatase (from *Helix pomatia*, EC 3.2.1.31 and EC 3.1.6.1., 30 and 60 U/ml, respectively) was supplied by ICN Biochemicals (Aurora, OH, USA). All chemicals were used as received. All water was Milli-Q® quality and was filtered through a 0.22-μm filter (Durapore polyvinylidene fluoride membrane; Millipore, Bedford, MA, USA) before use.

Test animals

The test animals used in the experiments, *N. diversicolor*, were collected on an intertidal sand flat in the Damariscotta River (South Bristol, ME, USA) in July 2000. Gravid females were avoided, and all animals were kept in culture in sieved sediment (<1 mm) with filtered (0.45 μm) seawater for a minimum of 7 d before use.

Sediment spiking

Sediment was collected at the same time the worms were sieved (<1 mm) and frozen (−20°C) before use. Thawed sediment was spiked by adding the desired amount of pyrene, dissolved in a minimum of acetone, to a slurry of sediment and filtered (0.45 μm) seawater to give a nominal concentration of 25 μg pyrene g^{-1} sediment (dry wt). Seawater used in the experiments was always filtered unless otherwise stated. The slurry was placed on a stand with a power drill fitted with a mud slinger and was mixed continuously for a minimum of 24 h. The slurry was then allowed to settle, the water decanted, and the sediment allowed to sit at 5°C for one week before use in microcosms.

Metabolite identification experiment

In a separate experiment, accumulation of pyrene and induction of pyrene metabolism in *N. diversicolor* was studied by exposing worms to 136 μg pyrene g^{-1} sediment (dry wt). Worms were sampled daily, and pyrene and 1-hydroxypyrene were quantified using synchronous fluorescence spectroscopy (A.M.B. Giessing et al., University of Maine, Walpole, ME, USA, unpublished data). Accumulation of pyrene and formation of 1-hydroxypyrene in *N. diversicolor* reached quasi-steady-state tissue concentrations after approximately 3 d of exposure. An exposure period of 5 d was chosen in later experiments.

Total metabolite pattern was studied by exposing worms to 25 μg pyrene g^{-1} sediment (dry wt) in a microcosm setup fitted with an airstone, changing the overlying water daily during the 5-d exposure period. On day 5, five worms were used for tissue analysis, and five were taken for gut fluid extraction. Unexposed worms were kept separate in an identical microcosm and sampled at the end of the experiment. Pyrene, 1-hydroxypyrene, and phase II metabolites were identified using HPLC with diode-array detection (HPLC/DAD) and fluorescence detection (HPLC/F). Fractions of aqueous phase II metabolites were collected, stored at −80°C, and reinjected on a liquid chromatograph coupled with a mass spectrometer at a later time.

Sample treatment

Worms were collected after the 5-d exposure period and allowed to purge their guts in individual Petri dishes filled with 10 ml of seawater for a maximum of 12 h before extraction—a time sufficient to empty their guts of sediment. Tissue extraction was performed by homogenizing whole worms in 1 ml of methanol using a Tissue-Tearor™ (Biospec Products, Bartlesville, OK, USA) at full speed for 1 min and, subsequently, sonicating samples for 10 min. Samples were then centrifuged at 420 *g* (IEC Centra MP4R [Needham Heights, MA, USA] centrifuge fitted with a 49119 rotor head) for 10 min to precipitate any debris, and 500 μl of supernatant were filtered through a 0.22-μm syringe filter and transferred directly to brown HLPC vials without any further sample preparation.

Gut fluid from *N. diversicolor* was removed under a microscope by carefully cutting open the body wall and inserting a small-gauge hypodermic needle mounted on a micromanipulator fitted with a syringe. Each worm yielded only several microliters of gut fluid; therefore, the gut fluid extracted from five worms was combined (total, ~30 μl) by rinsing the needle and syringe using the same 500 μl of methanol. The mixture was filtered through a 0.22-μm syringe filter and analyzed using HLPC/F.

Enzymatic hydrolysis

The presence of glucuronic acid and sulfate conjugates of 1-hydroxypyrene in tissue samples was studied by enzymatic hydrolysis of manually collected chromatographic peaks. Glucuronidase-arylsulfatase enzymes effectively hydrolyze the conjugates to give free 1-hydroxypyrene. Aliquots (100 μl) of a collected fraction were diluted (to 500 μl) with deionized water and then incubated for 2 h at 37°C with 10 μl of glucuronidase-arylsulfatase solution. After addition of 500 μl of ice-cold, 100% ethanol, the samples were mixed and centrifuged at 420 *g* for 5 min to precipitate denatured protein. The

supernatant was transferred to brown HPLC vials and analyzed using HLPC/F.

Chromatography

Total metabolite pattern was analyzed on a Hitachi D-7000 HPLC using a method optimized for pyrene metabolites provided by Gerard Stroomberg (AquaSense, Amsterdam, The Netherlands). A Supelcosil LC-PAH column (25 cm × 4.6 mm inner diameter; Supelco, Bellefonte, PA, USA) was used, and the acetonitrile:water (v/v) gradient profile went from 5:95 linearly to 90:10 over 40 min and then held at 90:10 for 10 min at a flow rate of 0.8 ml min^{-1}. Detection was by absorbance at 339 nm, and fluorescence at $\lambda_{EX/EM}$ (i.e., $\lambda_{excitation/emission}$) was 346/384 nm for metabolites/conjugates and 333/384 nm for parent compound. Injection volume was 100 μl, and column temperature was kept at 28°C. Peaks were identified with a Hitachi L-7450 DAD by collecting spectra in the 190- to 370-nm range.

LC/MS system

An Iontrap LCQ Deca (ThermoFinnigan, San Jose, CA, USA), fitted with an atmospheric pressure chemical ionization (APCI) probe connected to a HPLC/DAD system (TSP Spectra Systems, ASI, El Sobrante, CA, USA), was used to identify the collected fraction of aqueous metabolites. The method is described in detail by Giessing and Lund [30]. Briefly, the chromatographic method used was identical to the one described above. Interfacing an HPLC with an MS system requires the use of volatile, mobile-phase additives. Adding 10 mM ammonium acetate to the eluent and adjusting to pH 5 was chosen for this purpose. Because of the acidic characteristics of the supposed glucuronide conjugate, samples were analyzed using APCI in negative-ionization mode ($APCI^-$). The MS system used in this experiment was equipped with an ion trap that allows for isolation of specific product ions and subsequent performance of multiple MS experiments (MS^n) across a single chromatographic peak in real time.

RESULTS

An HPLC/UV absorption trace of a methanol extract of tissue from a single *N. diversicolor* exposed to 25 μg pyrene g^{-1} sediment (dry wt) for 5 d (Fig. 1, top) shows three peaks, marked I, II, and III, at 8.9, 32.9, and 42.2 minutes, respectively. These peaks were not present in the chromatogram of an unexposed worm (dotted line). The high absorption band in the first 5 min of the chromatogram is caused by polar endogenous compounds (i.e., protein and carbohydrates) from polychaete tissue and is not related to pyrene metabolism. The 339-nm HPLC/UV chromatograms of all tissue samples from exposed *N. diversicolor* were identical to the one shown in Figure 1. The corrected DAD UV absorption spectra of peaks I, II, and III are presented in the middle panel of Figure 1. Peaks II and III are readily identified by their retention times (32.8 and 42.2 min, respectively) and their DAD absorption spectra as 1-hydroxypyrene and pyrene, respectively. The more hydrophilic peak I, with a retention time of 8.9 min, has a UV spectrum similar to 1-hydroxypyrene and was collected manually for enzymatic hydrolysis.

Figure 2 shows the effect of glucuronidase-arylsulfatase treatment of peak I. The lower chromatogram is the fluorescence trace ($\lambda_{EX/EM}$ = 346/384 nm) of the manually collected fraction of peak I, and the upper chromatogram is the fluorescence trace of the same sample after glucuronidase-arylsulfatase hydrolysis. The original peak at 8.9 min has disappeared, and 1-hydroxypyrene is identified at 32.8 min, indicating either a glucuronide or sulfate conjugate. All fractions of peak I (n = 5) collected from *N. diversicolor* tissue samples that were treated with glucuronidase-arylsulfatase gave identical chromatograms after enzymatic hydrolysis. Complete enzymatic hydrolysis of peak I allows for the quantification of conjugated 1-hydroxypyrene in tissue samples by measuring the increase in 1-hydroxypyrene in the sample after glucuronidase-arylsulfatase treatment. Total PAH is defined as the sum of pyrene, 1-hydroxypyrene, and hydrolyzable conjugate in a

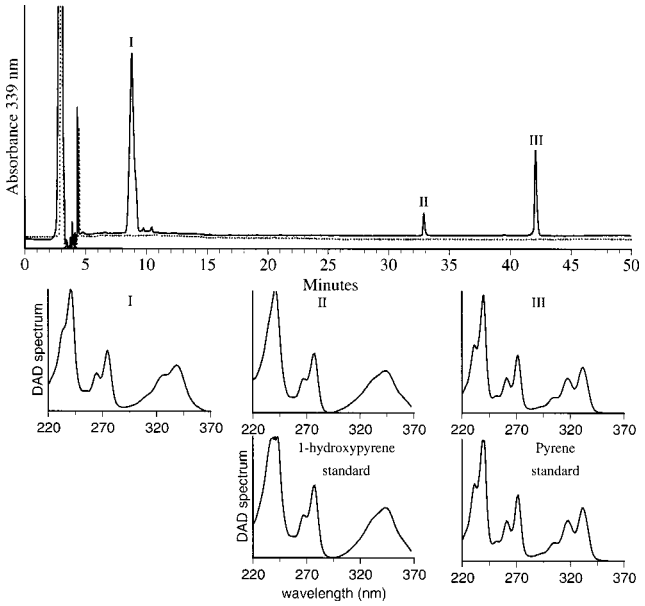

Fig. 1. High-pressure liquid chromatography. **Top.** A 339-nm ultraviolet (UV) chromatogram of a single *Nereis diversicolor* exposed to 25 μg pyrene g^{-1} sediment (dry wt) for 5 days. Peaks not present in the 339-nm UV trace from an unexposed worm are marked I, II, and III. **Middle** and **bottom.** Diode-array detector (DAD) absorption spectra of peak I, II, and III and spectra of 1-hydroxypyrene and pyrene standards, respectively.

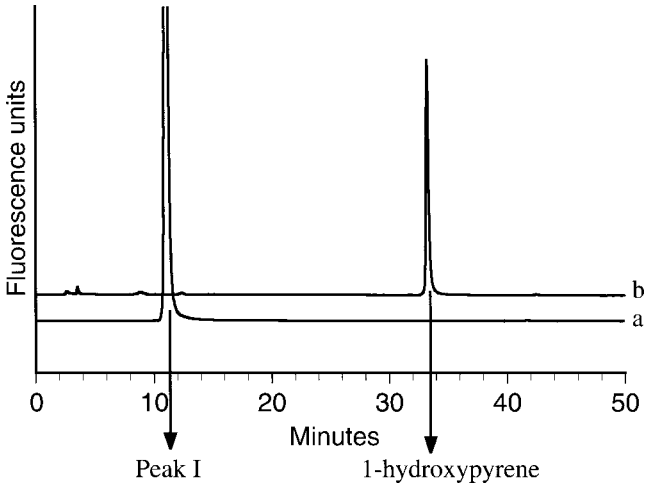

Fig. 2. High-pressure liquid chromatograms with fluorescence detection ($\lambda_{EX/EM}$ = 346/384 nm) showing the effect of glucuronidase-arylsulfatase treatment of peak I manually collected from the tissue sample presented in Figure 1. The lower chromatogram (a) shows the unhydrolyzed conjugate after fractionation, and the upper chromatogram (b) shows the same sample after hydrolysis, featuring 1-hydroxypyrene.

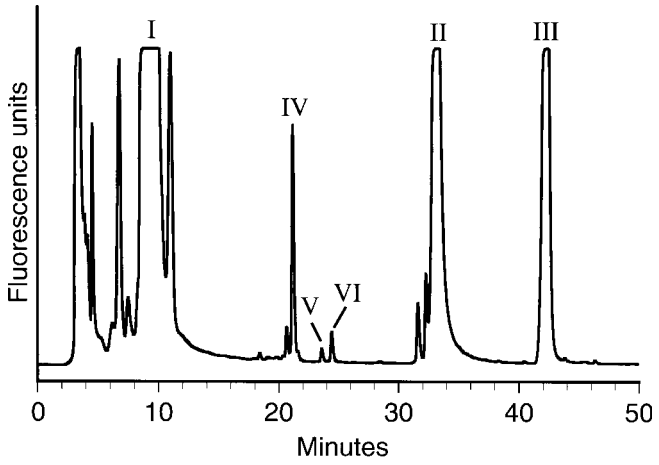

Fig. 3. High-pressure liquid chromatogram with fluorescence detection of the tissue sample presented in Figure 1. Besides peaks I–III, at least three additional unidentified peaks, marked IV–VI, are present in the fluorescence trace.

sample. Of the total PAH in tissue, 1-hydroxypyrene glucuronide, 1-hydroxypyrene, and pyrene constituted 73% ± 23%, 2% ± 1%, and 25% ± 23% (mean ± standard deviation, n = 5), respectively.

Figure 3 shows the fluorescence trace of the tissue extract of the same sample presented in Figure 1. Peaks I, II, and III in the fluorescence spectrum are readily identified by comparing retention times to the 339-nm UV trace in Figure 1. The fluorescence chromatogram shows three additional peaks, marked IV, V, and VI, between 20 and 25 min that are not present in the 339-nm UV trace (Fig. 1). These peaks could not be identified. The large peak at 21.5 min (Fig. 3, peak IV) was collected manually, and its fluorescence spectra (emission wavelength, 430 nm) was similar to the fluorescence spectra of peak I (data not shown), indicating a second 1-hydroxypyrene conjugate in the sample. The remaining peaks were present with varying intensity in all samples of pyrene-exposed worms, but their intensity in collected fractions was too low to allow identification.

The 339-nm UV trace of the diluted gut fluid sample taken from pyrene-exposed worms (Fig. 4A) shows the 1-hydroxypyrene conjugate, 1-hydroxypyrene, and pyrene at 8.7, 32.8, and 42.2 min, respectively, which were not present in gut fluid from unexposed worms. The DAD spectra of the three small peaks at 10 min had no spectroscopic features that could be related to pyrene or 1-hydroxypyrene (data not shown) and are presumably not related to pyrene metabolism. Of total PAH in gut fluid, 1-hydroxypyrene glucuronide, 1-hydroxypyrene, and pyrene constituted 90%, 9%, and 1%, respectively, based on a triplicate measurement of the pooled gut fluid sample. The relative distribution among chemical forms in gut fluid versus tissue was not significantly different (Student's t test, $p > 0.05$).

Before homogenization, all worms were placed in 10 ml of seawater and allowed to purge their guts for up to 12 h. A small sample (1 ml) of this water was passed through a 0.22-μm syringe filter and analyzed using HPLC/DAD (Fig. 4B). The DAD spectrum of the small peak at 8.7 min (Fig. 4B, insert) is identical to that of the 1-hydroxypyrene-conjugate peak in tissue (Fig. 1) and gut fluid chromatograms (Fig. 4A).

Atmospheric pressure chemical ionization MS analysis of peak I from Figure 1 is presented in Figure 5. Retention time

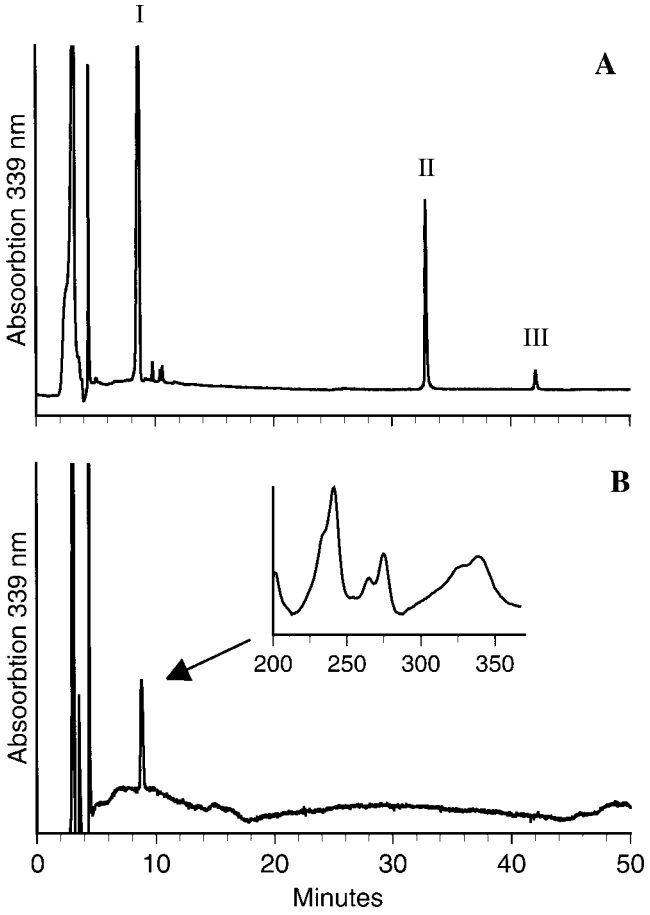

Fig. 4. High-pressure liquid chromatography (HPLC). **A.** HPLC 339-nm ultraviolet (UV) chromatogram of gut fluid taken from five exposed *Nereis diversicolor*. Peaks marked I, II, and III can be identified by comparing retention time and diode-array detector (DAD) spectrum as identical to those shown in Figure 1. **B.** HPLC 339-nm UV chromatogram and DAD (insert) of water taken from the defecation dish of an exposed worm. Retention time and DAD spectrum of the small peak are identical to peak I.

of the peak in the 339-nm chromatogram (Fig. 5, top) shifted to 16.5 min after the application of the ammonium acetate buffer. The dominating mass (m/z = 429) of the peak at 16.5 min corresponds to the molecular ion of 1-hydroxypyrene glucuronide plus eluent adducts, $[M - H + 2H_2O]^-$; m/z = 393 is the 1-hydroxypyrene glucuronide molecular ion, $[M - H]^-$; and m/z = 217 corresponds to the 1-hydroxypyrene fragment ion, [M − H − glucuronide]$^-$ (Fig. 5B). Ion-trap MS^n allows multiple MS experiments to be performed across an HPLC peak, thereby affording more detailed structural information. To understand fragmentation pathways and connect them back to the parent molecule, MS^2 and MS^3 experiments were conducted by isolating the dominating mass of each subsequent MS experiment in the ion trap and applying 30% (arbitrary units) of the available chemical ionization energy. The MS^2 experiments on m/z = 429 yielded a simple product ion spectrum with a dominant ion at m/z = 393, corresponding to the precursor ion with the loss of eluent adducts (Fig. 5C). The product ion spectrum collected in the MS^3 experiments (Fig. 5D) for the ion at m/z = 393 yielded a relatively simple spectrum with ions at m/z = 217, 175, and 113, corresponding to fragment ions of 1-hydroxypyrene (m/z = 217) and glucuronide (m/z = 175); m/z = 113 is a known fragment of m/z = 175 [31].

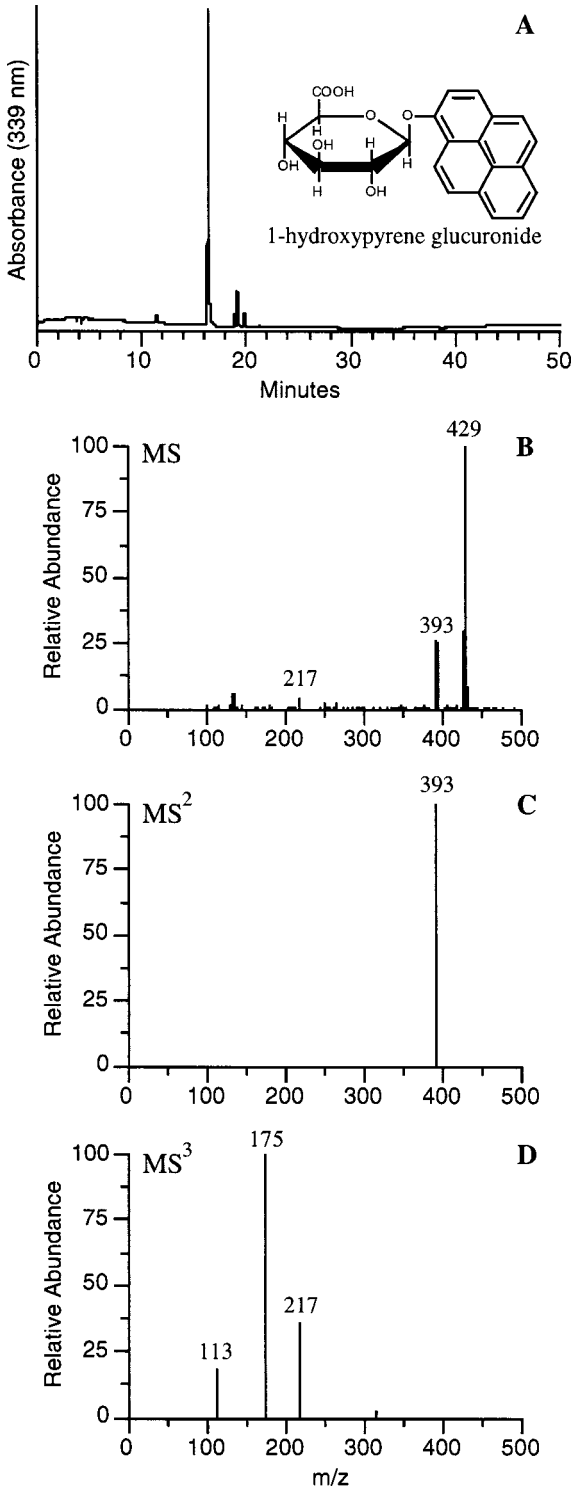

Fig. 5. **A.** A 339-nm liquid chromatography/mass spectrometry (MS) ultraviolet chromatogram. **B–D.** Atmospheric pressure chemical ionization multiple MS analysis (MS^n) for product ion spectra of peak I collected from the tissue sample presented in Figure 1. The peak is identified as 1-hydroxypyrene glucuronide (insert in **A**) by its molecular ion (m/z = 393) and its product ion spectra. See *Results*.

DISCUSSION

The purpose of the present study was to investigate formation of pyrene metabolites in the marine deposit-feeding polychaete *N. diversicolor*. Special attention was given to detection and identification of phase II biotransformation products and detection of 1-hydroxypyrene in gut fluid.

Besides pyrene, only two metabolites, 1-hydroxypyrene and a 1-hydroxypyrene glucuronide conjugate, were identified in the 339-nm UV trace from tissue extracts of *N. diversicolor* exposed to sediment-associated pyrene at 25 $\mu g\ g^{-1}$. To our knowledge, this identification of 1-hydroxypyrene glucuronide is the first direct identification of a phase II metabolic product in a marine deposit-feeding polychaete. Previous studies of PAH metabolism in marine polychaetes have been limited to separation into aqueous and polar metabolites based on three-phase extraction schemes and using ^{14}C-labeled parent compounds [4,5,9,10,32]. *Nereis diversicolor* has known PAH metabolic capability [4,9,10]. Christensen et al. [10] recently identified 1-hydroxypyrene in tissue from *N. diversicolor* exposed to sediment-associated pyrene for two weeks. Their metabolite identification used a three-phase extraction scheme separating parent PAH and water-soluble and -insoluble metabolites. However, the authors only reported HPLC/UV analysis of the water-insoluble fraction, thereby limiting their metabolite identification to 1-hydroxypyrene.

McElroy [32] found that a congener species, *N. virens*, rapidly accumulated and metabolized radiolabeled benz[*a*]anthracene (BA) and that most radioactivity was either in the form of water-soluble or unextractable, bound metabolites, with very little remaining as BA. Their study also showed that levels of water-soluble and bound fractions increased over time. Glucuronidase-arylsulfatase treatment of aqueous extracts could only convert 2 to 6% of radioactivity to nonpolar, solvent-extractable metabolites, indicating that the water-soluble fraction is not conjugated to glucuronic acid or sulfate but to some other endogenous compound such as glucose, which is not hydrolyzed by glucuronidase-arylsulfatase. In a recent study, McElroy et al. [5] surveyed in vivo metabolism of [^{3}H]B*a*P in 10 small marine invertebrates from three phyla: Annelida, Mollusca, and Arthropoda. Metabolite identification was done using a liquid:liquid extraction scheme of [^{3}H]B*a*P and HPLC radiochromatography of tissue extracts from a representative species of each phyla. High variability in metabolic capability within each phylum was observed, with polar metabolites ranging from 7 to 96% in annelids. The radiochromatograms presented indicated at least four identifiable phase I [^{3}H]B*a*P metabolites and two (possibly three) unidentified phase II metabolites in animal extracts from *N. succinea* [5]. In a study of PAH metabolism in the deposit-feeding polychaete *Capitella* sp. I., Forbes et al. [6] tentatively identified at least two hydroxylated, phase I metabolites of fluoranthene.

Chemicals with phenolic structure are substrates for both carbohydrate and sulfate conjugation. In vertebrates, the preferred carbohydrate cosubstrate is uridine 5′-diphosphoglucuronic acid (UDP-glucuronic acid), whereas in marine invertebrates, uridine 5′-diphosphoglucose is indicated to predominate over UDP-glucuronic acid as the preferred carbohydrate cosubstrate [15]. However, foreign compounds are rarely metabolized in the same manner by even closely related species [15], and identification of 1-hydroxypyrene glucuronide as the major phase II metabolite in *N. diversicolor* further emphasizes this commonly observed trend.

The fluorescence trace (Fig. 3) revealed at least three additional peaks not detected in the UV trace (Fig. 1) of the same tissue sample. Fluorescence spectra (emission wavelength, 430 nm) measured from the fractionated peak IV from a single

tissue sample resembled that of 1-hydroxypyrene glucuronide (data not shown). This similarity suggests that a second conjugate is present in the sample. However, the intensity of the peak was low, and fractions from more samples would need to be pooled to determine the exact identity of this conjugate. The two additional peaks, marked V and VI, in the fluorescence spectrum appeared with varying intensity in all the examined worms. They were too low in intensity to be reliably identified by the current method and most likely were insignificant in the mass balance. Giessing and Lund [30] presented 339-nm UV evidence for two additional aqueous metabolites in tissue extracts from *N. diversicolor* exposed to 50 μg pyrene g^{-1} sediment (dry wt). The peaks were low in intensity and could not be identified by their MS spectra because of ion suppression by very abundant coeluting worm tissue impurities. Deconjugation with glucuronidase-arylsulfatase enzymes effectively hydrolyzes one of the two peaks to 1-hydroxypyrene, indicating the presence of a sulfate conjugate (A.M.B. Giessing et al., unpublished data). As mentioned earlier, even closely related animal species vary considerably in the extent to which they metabolize foreign compounds. Most species have a preferred route of conjugation, but other routes are still available and utilized. Furthermore, the balance between different phase II pathways can depend on the concentration of phase I product delivered [33]. No increase in the number of pyrene conjugates formed was observed when terrestrial isopods were fed a single high dose of pyrene compared to continuous exposure of pyrene for 14 d in the food; however, the relative levels of pyrene metabolites did change [25]. Thus, identification of a sulfate conjugate at the higher pyrene exposure concentration seems to suggest either an induction of phase I enzymes, resulting in increased production of 1-hydroxypyrene, or saturation of the glucuronidation pathway at faster rates of 1-hydroxypyrene generation, leading to accumulation of unconjugated 1-hydroxypyrene and, thereby, increasing sulfate and other conjugation pathways.

Of the total pyrene in worm tissue, roughly 73% was in the form of 1-hydroxypyrene glucuronide, and only 2% was in the form of 1-hydroxypyrene, indicating an efficient turnover of 1-hydroxypyrene in *N. diversicolor* tissue. Christensen et al. [10] reported up to 50% of total body burden as aqueous metabolites in *N. diversicolor* after 42 d of exposure to sediment-associated pyrene. The results for *N. diversicolor* in the present study agree with those seen for B*a*P and BA in nereid polychaetes. In both *N. diversicolor* (75%) and *N. virens* (58%), most B*a*P was recovered as aqueous metabolite, and in *N. virens*, ≤10% of total body burden was recovered as BA [4,34]. In another deposit-feeding marine polychaete, *Arenicola marina*, more than 80% of total body burden remained as pyrene in both a short-term (11-d) and a long-term (52-d) experiment [10], and Kane Driscoll and McElroy [4] reported less than 20% of total body burden as polar and aqueous B*a*P metabolites in a screening experiment of seven species of marine polychaetes.

A similar mass balance made for gut fluid yielded 90% as 1-hydroxypyrene glucuronide and 9% as 1-hydroxypyrene. Though based on a triplicate measure of the pooled gut fluid sample, the mass balance for gut fluid presented here seems only to suggest that the relative distribution of pyrene-derived compounds in gut fluid resembles that of tissue. In a study of 9-OH-B*a*P, a highly lipophilic primary metabolite of B*a*P, in American lobster (*Homarus americanus*), Li and James [35] found that 9-OH-B*a*P was readily conjugated to sulfate and glucose and that, despite its high lipophilicity, 9-OH-B*a*P was excreted from the lobster hemolymph and tissue much more rapidly than B*a*P. More studies concerning the pharmacokinetics of hydroxylated PAH and their metabolic conjugates in individual animals are needed to identify if this holds true in tissue and gut fluid of deposit-feeding marine invertebrates.

Fecal elimination is assumed to be the major route for removal of contaminants in marine polychaetes [9,28,36]. Unlike other marine invertebrates such as mollusks and crustaceans, annelid worms do not have an easily separable digestive gland (commonly used in biotransformation studies). Annelids have specialized tissue associated with the alimentary tract (chloragogen), the function of which is comparable to the mussel's digestive gland as well as the vertebrate liver. Assuming that chloragogen tissue is the site with the highest biotransformation capacity in marine polychaetes, elimination of aqueous metabolites through the gut would be expected. Elimination of metabolic products through the gut is supported by the identification of 1-hydroxypyrene and 1-hydroxypyrene glucuronide in both gut fluid and defecation water from *N. diversicolor* (Fig. 4). Goerke and Ernst [36] found that fecal matter from *N. virens* contained from 7.5 to 41% of the accumulated dose of PCB, of which 72 to 94% were polar metabolites. Polar and aqueous metabolites of PAHs eliminated in fecal matter accounted for up to 50% of accumulated dose in the two deposit-feeding polychaetes, *Capitella* sp. I. and *N. diversicolor* [9,28]. Whether the 1-hydroxypyrene glucuronide identified in defecation water is eliminated directly to the aqueous phase or is desorbed from fecal matter after defecation remains to be investigated.

The 1-hydroxypyrene might be of either biotic and abiotic origin. The pyrene-contaminated sediment used in the experiment was allowed to sit in the lab for a week before worm addition, and the containers were exposed to day/night cycles of artificial light throughout the acclimation and experimental period. The 1-hydroxypyrene has been identified as a product of photochemical oxidation, although this process yields primarily 1,6- and 1,8-pyrenequinones [37]. However, sediment samples taken at the time of worm addition and at the end of the experiment showed no detectable traces of 1-hydroxypyrene (A.M.B. Giessing and L.M. Mayer, unpublished data), and it seems likely that abiotic processes did not contribute significantly to the measured 1-hydroxypyrene. It is also well known that PAH can be degraded aerobically by microorganisms [38]. Low-molecular-weight PAHs degrade readily in sediments, whereas high-molecular-weight PAHs appear more resistant to microbial attack, even in aquatic ecosystems, which are chronically exposed to petrogenic hydrocarbons [39]. Microorganisms associated with *N. diversicolor* gut or body surfaces could be responsible for the observed biotransformation of pyrene. However, prokaryotic microorganisms in general utilize dioxygenase enzymes to incorporate both atoms of molecular oxygen into the aromatic nucleus to form *cis*-dihydrodiols, and the proposed microbial catabolic pathway for pyrene does not produce 1-hydroxypyrene [38]. Because 1-hydroxypyrene is the only measured phase I metabolite, it seems likely that the measured pyrene-derived compounds are neither abiotic nor microbial in origin and are, instead, the result of worm metabolism.

Using pyrene as a model compound for PAH metabolism in marine invertebrates has a clear advantage over, for example, B*a*P [5] and fluoranthene [6] by having only one phase I metabolite. Detection of 1-hydroxypyrene provides insight into

the accumulation of pyrene, integrated over all uptake routes and modulated by bioavailability. The 1-hydroxypyrene is only detected if pyrene is accumulated and metabolized by the animal. Pyrene usually co-occurs with the more toxic PAHs, and the limited number of total phase I and II metabolites makes pyrene an attractive model compound with which to assess both PAH exposure and species-specific biotransformation capacity of marine invertebrates.

Limitations in the analytical procedure used to study biotransformation products should always be considered carefully even when dealing with simple biotransformation pathways like the one presented for pyrene in the present study. It is possible that other polar metabolites do not exhibit UV absorption at 339 nm or at the chosen excitation/emission fluorescence wavelengths in the samples of *N. diversicolor*. Although the wavelength of 254 nm is better for PAH measurements in complex mixtures, higher-molecular-weight PAHs like pyrene have absorption maxima at higher wavelengths that can be utilized for more selective analysis [40]. Loss of aromaticity of one of the four rings with the formation of phenanthrene-like compounds would, therefore, not be detected by the present method. The software associated with the HPLC used in the present study can extract multiple-wavelength chromatograms from recorded DAD data, and no extracted 254-nm chromatograms had additional peaks besides the three presented in Figure 1. Lack of additional peaks at 254 nm does not account for the possibility of pyrene-derived compounds falling outside the analytical window (i.e., coeluting with tissue components during the first 5 min of the solvent gradient program). The presence of phenanthrene-like compounds originating from *N. diversicolor* metabolism of pyrene could be resolved by redoing the total metabolite experiment using radiolabeled pyrene. Stroomberg et al. [25] reported a pyrene metabolite not showing UV absorbance at 339 nm in the hepatopancreas of a terrestrial isopod exposed to [^{14}C]pyrene. The metabolite never exceeded 5% by area of total radioactivity found in the sample and could not be conclusively identified as pyrene-derived according to its DAD spectrum. Based on these arguments and the present data, it therefore seems likely that 1-hydroxypyrene and 1-hydroxypyrene glucuronide are the primary phase I and phase II metabolites of pyrene in *N. diversicolor*.

The liquid:liquid extraction scheme of radiolabeled PAH commonly applied in biotransformation studies yields little information regarding chemical speciation of the separated metabolites. Subsequent analysis of separated fractions, primarily using HPLC/UV, has been limited to phase I biotransformation products with commercially available standards (e.g., [5,6,10]). The HPLC method presented here is fast, is less labor-intensive than liquid:liquid extraction schemes, and incorporates both phase I and phase II metabolites in the same chromatogram. Furthermore, it emphasizes the use of HPLC with fluorescent detection in studies of PAH biotransformation products. Fluorescence detection is usually two to three orders of magnitude more sensitive than UV detection, and by choosing excitation/emission wavelengths that are specific to the assumed PAH metabolites, low detection limits can be obtained [41]. The present study also shows that LC/MS is a powerful analytical tool for identification of phase II biotransformation products (Fig. 5), even without standards and previous knowledge of the chemical speciation of metabolites. Modern ion-trap LC/MS^n allows multiple MS experiments to be performed across an HPLC peak, affording more detailed structural data, and recent developments in ionization technology will expand the application of LC/MS to include the otherwise undetectable, nonpolar, parent PAHs [42].

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