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# Screening of Human Enteric Microorganisms for Potential Biotransformation of Polycyclic Aromatic Hydrocarbons

**C. M. Hurdzan · N. T. Basta · P. G. Hatcher · O. H. Tuovinen**

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**Abstract** This study examined the potential for metabolism of select polycyclic aromatic hydrocarbons (PAH) by human enteric microorganisms. Experiments were performed under anaerobic conditions with various combinations of enteric microbial suspensions, PAH concentrations, nutrient mixtures, and time courses. No PAH metabolites were detected upon GC–TOF–MS analysis of samples digested by tetramethylammonium hydroxide thermochemolysis. No mineralization of $^{14}$C-labeled phenanthrene was observed. These results suggest the lack of partial or complete metabolism of PAHs by enteric microorganisms and therefore the absence of major bioactivation pathways that would expose intestinal lining to potentially carcinogenic PAH metabolites.

C. M. Hurdzan · N. T. Basta
Soil Science Program, School of Environment and Natural Resources, Ohio State University, 2101 Coffey Road, Columbus, OH 43210, USA

P. G. Hatcher
Department of Chemistry, Ohio State University, 100 W. 18th Avenue, Columbus, OH 43210, USA

O. H. Tuovinen (✉)
Department of Microbiology, Ohio State University, 484 West 12th Avenue, Columbus, OH 43210, USA
e-mail: tuovinen.1@osu.edu

C. M. Hurdzan
Environmental Science Graduate Program, Ohio State University, Columbus, OH 43210, USA

P. G. Hatcher
Department of Chemistry and Biochemistry, Old Dominion University, 4541 Hampton Boulevard, Norfolk, VA 23529, USA

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The human digestive tract can be exposed to pro-toxins through the ingestion of foodstuff. When these pro-toxins reach the large intestine they may interact with enteric microorganisms shown to metabolize a variety of substrates (Guyton 1991; Boron and Boulpaep 2003; Eckburg et al. 2005). However, under normal circumstances pro-toxin metabolism takes place through the cytochrome P450 mediated pathways in the liver.

Van de Wiele et al. (2005) reported the formation of 1-hydroxypyrene (4.4 μg/L) and 7-hydroxybenzo[*a*]pyrene (1.9 μg/L) following exposure of parent pyrene and benzo[*a*]pyrene to human enteric microorganisms. The toxicological significance of these findings is unclear given the trace conversion (< 1%) of parent PAH. No other reports have been published in the literature on PAH metabolism by human enteric microbial populations. Microbial transformation of PAHs under anaerobic conditions is fundamentally different from mono- or dioxygenase-initiated biodegradation under aerobic conditions. Studies on anaerobic mineralization of naphthalene by mixed cultures of sulfate-reducing bacteria have confirmed the formation of carboxylated and methylated intermediates (Zhang and Young 1997; Annweiler et al. 2002; Safinowski and Meckenstock 2006). In view of the known anaerobic intermediates of microbial metabolism of PAHs, the monohydroxylated pyrene and benzo[*a*]pyrene derivatives reported by Van de Wiele et al. (2005) may represent only one of many intermediate or even terminal metabolites formed during the anaerobic biodegradation of the PAHs with four or five aromatic rings.

It was hypothesized in this study that human enteric microorganisms can modify PAH structures through

hydroxylation or other substitution reactions, thereby confirming the previous findings of the monohydroxylated PAH derivatives. The purpose of the present study was to seek evidence for active metabolism of select PAHs by human enteric microorganisms. Related to this study is our recent work (Hurdzan et al. 2007), showing that the bioaccessibility of phenanthrene is significantly influenced by the availability of nonpolar binding sites in both the gastrointestinal system and sorbent matrix. Microbial transformations of PAHs should increase PAH bioaccessibility as a result of, for example, OH-substitution. Confirmation of this hypothesis would provide yet another example of the link between environmental contamination and human toxicology and prompt environmental risk assessment to consider exposure of the human intestinal lining to bioactivated and subsequently carcinogenic (e.g., DNA adduction) hydroxy-PAHs in addition to those produced in the liver through conventional cytochrome P450 mediated pathways.

## Materials and Methods

Phenanthrene, pyrene, benzo[*a*]pyrene, 1OH-pyrene, and 7OH-benzo[*a*]pyrene were obtained from Sigma-Aldrich (St. Louis, MO). The working solutions were prepared in methanol and stored in the dark at 22 ± 3°C in stoppered glass vials. [C9-$^{14}$C]-phenanthrene (>99% purity; 8.5 mCi/mmol) and [U-$^{14}$C]-glucose (>98% purity; 6.0 mCi/mmol) were obtained from Sigma-Aldrich. The working solutions were prepared in methanol and stored in the dark at −20 ± 3°C in stoppered vials.

Suspensions of enteric microorganisms were prepared from stools of a 24-year-old non-medicated male subject over a period of 8 months. Stool samples were chilled on ice and prepared within 24 h. Stools were weighed (wet wt.) and diluted in distilled water to produce a 20% (w/w) suspension. Each suspension was passed through a bilayer cheesecloth filter and distributed into screw cap centrifuge tubes in 40 mL aliquots. Samples were stored in the dark at −20 ± 2°C.

Food transit time through the human colon is variable and presented in the literature as a range of values between 24 and 72 h (Daugherty and Mrnsy 1999). Therefore, this study used 24, 48 and 72 h incubation times. All anaerobic PAH experiments were conducted using enteric microorganisms (10 mL) in argon-flushed, septum-sealed glass serum bottles (50 mL) in triplicate. Suspensions received 20 mg/L phenanthrene, pyrene or benzo[*a*]pyrene. Select experiments were amended with a nutrient mixture composed of arabinogalactan, xylan, pectin, starch, and glucose (Molly et al. 1994). This polysaccharide mixture, in conjunction with a trace element and vitamin solution, was shown by Molly et al. (1994) to sustain the metabolic activity of human enteric microorganisms. The serum bottles were incubated at 37 ± 2°C in a water bath during the experiments. A control without PAH was used as a baseline reference for GC–TOF–MS analyses. At 24, 48 or 72 h, samples were transferred into 50 mL round bottom centrifuge tubes centrifuged at 7,000×*g* for 5 min to produce a pellet and supernatant fraction. The supernatant was decanted into a 20 mL glass scintillation vial while the pellet was re-suspended twice in 5.0 mL distilled water and then transferred. The supernatant and pellet sample were bubbled with argon (1 L/min) for 20 s. Supernatant and pellet sample were stored in the dark at 0 ± 1°C until analyzed.

Biometers (50 mL glass serum bottles) were dosed in duplicate with 80 mg (0.44 mmol; 2.64 mCi) of $^{14}$C-glucose or 400 μg (2.24 μmol; 19.1 μCi) of $^{14}$C-phenanthrene and 20 mL fecal inoculum and flushed with argon. An open top vial filled with 1.0 mL of 0.5 M KOH solution to trap $CO_2$ was suspended from the septum cap. The biometers were incubated at 37 ± 2°C. At intervals of 24 h, the 1.0 mL KOH trapping solutions were replaced with fresh KOH. The trapping solutions were mixed with 10 mL Scintiverse (Fisher Scientific, Fairlawn, NJ) fluid, and the vials were shaken and capped for liquid scintillation. Scintillation analyses were performed using a Beckman 7500 microprocessor controlled scintillation detector (Beckman Instruments, Fullerton, CA). Extract recoveries for $^{14}$C-phenanthrene and $^{14}$C-glucose ranged from 60% to 80%.

The PAH test compounds were anticipated to adduct to non-polar constituents of fecal inoculum including the microbial fraction. Previously, to address the possibility of PAH adduction to constituents to gastrointestinal matrix, Van de Wiele et al. (2005) treated each sample with deconjugation enzymes (*β*-glucuronidase and aryl sulfatase) prior to analysis. Deconjugation increased the concentration of 1-OH-pyrene from 2.5 to 4.4 μg/L, suggesting PAH adduction. In this study, samples were digested with tetramethylammonium hydroxide (TMAH) using a thermochemolytic technique (Briggs et al. 2003), which has been shown to cleave ether and ester linkages through methylation.

Supernatants were concentrated 4-fold under a nitrogen stream before an aliquot (200 μL) was added to a glass ampoule. Stock pellet samples were air dried for 24 h in the dark on a watch glass. Dry pellet sample (5.0 mg) was added to a glass ampoule. Each ampoule (supernatant or pellet) received 200 μL TMAH (Sigma-Aldrich) (25% v/v in methanol) and was vortexed for 10 s. Ampoule contents were dried under nitrogen prior to being evacuated for 1 h, flame sealed and baked at 250°C for 30 min. Ampoules were split open, 50 μL of an internal standard N-tetracosane (107 ng/μL) was added, ampoule halves were washed with ethyl acetate (5 × 200 μL), all washings were

combined and filtered through pre-combusted (550°C for 30 min) glass wool into 2.0 mL amber gas chromatography vials. The sample was concentrated (from 1,050 μL) under nitrogen to an approximate volume of 250 μL, transferred to an identical 2.0 mL amber glass chromatography vial with insert and immediately analyzed.

Samples were analyzed by capillary GC–TOF–MS on a Hewlett-Packard (Palo Alto, CA) 6890 series GC system interfaced with a Pegasus II time-of-flight mass spectrometer (LECO, St. Joseph, MI). The GC separation column was a 30 m × 0.25 mm (i.d.) fused silica capillary column with a 5% methylsilicone bonded phase and a film thickness of 0.25 μm (Supelco DB-5; Supelco, Bellefonte, PA). Sample volumes of 1.0 μL were drawn into a split/splitless injector operating in splitless mode. The carrier gas was helium (1.5 mL/min) and the column temperature was ramped from an initial 50 to 200°C at 15°C/min, and then to 300°C at 25°C/min whereupon the temperature was held for 3 min before recycling. The ionization mode on the mass spectrometer was electron impact at 70 eV. Data acquisition and analysis were performed using LECO Pegasus II software (version 1.33).

Extract recoveries for phenanthrene, pyrene and benzo[*a*]pyrene supernatant fraction ranged from 90% to 105% for 1OH-pyrene and 7OH-benzo[*a*]pyrene. Extract recoveries for phenanthrene, pyrene and benzo[*a*]pyrene pellet fraction ranged from 75% to 95% and from 80% to 100% for 1OH-pyrene and 7OH-benzo[*a*]pyrene. The detection limit was 310 and 300 μg/L for the supernatant and pellet fraction, respectively.

## Results and Discussion

To investigate potential anaerobic PAH transformation, enteric microbial suspensions (live, autoclaved) and the supernatant fraction were spiked with 20 mg/L of phenanthrene, pyrene or benzo[*a*]pyrene and incubated for 24 and 48 h. Metabolites were not detected under any of the conditions at either incubation length (24 and 48 h). The experiment was repeated using an extended incubation of 72 h, but no metabolites were detected.

These data did not exclude the possibility that PAHs were inhibitory to enteric microorganisms and thus to PAH biotransformation. To investigate this possibility, enteric microbial suspensions were dosed with phenanthrene to a final concentration of 1, 10, 20 and 40 mg/L. No metabolites were detected after a 48 h incubation.

To examine the influence of nutrient mixture, fecal suspensions were supplemented with 8.0%, 16% or 24% (w/v) each and dosed with 10 mg phenanthrene/L. No metabolites were detected after 48 h. The experiment was repeated under aerobic conditions. No metabolites were detected.

**Table 1** Glucose mineralization by enteric microorganisms in the presence of pyrene (n = 2)

| Time (h) | $^{14}$C-glucose mineralization (%) | | | |
|---|---|---|---|---|
| | 1.0 mg/L pyrene | 10 mg/L pyrene | 20 mg/L pyrene | 40 mg/L pyrene |
| 24 | 10.9 | 7.80 | 13.1 | 10.7 |
| 48 | 11.5 | 8.55 | 14.5 | 12.0 |
| 72 | 11.9 | 8.84 | 15.3 | 12.5 |

Substrate-induced respiration was used to assess the metabolic activity of enteric microorganisms in this study. Enteric microorganisms were incubated with [U-$^{14}$C]-glucose (4.0 g/L) in the presence of 1.0, 10, 20 and 40 mg pyrene/L. Glucose respiration was unaffected by the increasing pyrene concentration, indicating that pyrene was not inhibitory and that enteric microorganisms used in this study were metabolically active (Table 1).

To investigate $^{14}$C-phenanthrene mineralization, enteric microbial suspensions were spiked with 20 mg of $^{14}$C-phenanthrene/L. Biometer trapping solutions were sampled for $^{14}CO_2$ every four days over a 20 days period. No mineralization was detected. The experiment was repeated under aerobic conditions. No mineralization was detected.

These results indicate a consistent lack of partial or complete biodegradation of the test PAH compounds under anaerobic and aerobic conditions, but do not exclude the possibility, as already reported by Van de Wiele et al. (2005), that intermediate hydroxylated metabolites were formed below parent PAH to metabolite conversion ratio detectable in this study. However, the present study suggests that PAH metabolism mediated by enteric microorganisms is not a major process in the human large intestine.

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