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METABOLISM OF NITROPOLYCYCLIC AROMATIC HYDROCARBONS BY HUMAN INTESTINAL MICROFLORA

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SUMMARY: Anaerobic bacterial suspensions from human and rat feces and intestinal contents, and pure cultures of anaerobic bacteria metabolized 1-nitropyrene and 6-nitrobenzo[a]pyrene to 1-aminopyrene and 6-aminobenzo[a]pyrene, respectively. The metabolites were isolated by reversed-phase high performance liquid chromatography and identified by comparison of their chromatographic and mass spectral properties with those of authentic compounds. The results suggest that anaerobic intestinal bacteria could play a significant role in the metabolism of potentially carcinogenic nitropolycyclic aromatic hydrocarbons.

Nitropolycyclic aromatic hydrocarbons are ubiquitous environmental pollutants which can be formed by nitration of polycyclic aromatic hydrocarbons and have been found in diesel exhaust particulates, fly ash, cigarette smoke, photocopy toners and various combustion processes (1-7). There has been recent concern regarding possible adverse human health effects due to exposure to nitropolycyclic aromatic hydrocarbons since they have been shown to be extremely potent direct-acting mutagens in the Ames Salmonella mutagenesis assay (1, 4-6, 8-11). Furthermore, these compounds induce gene mutations, sister chromatid exchanges, unscheduled DNA synthesis in mammalian cells and cause cancer in male rodents (11-15). It has been postulated that the biological activity of this class of compounds may be due to the enzymatic reduction of the nitro group via nitroso intermediates to form aryl hydroxylamines

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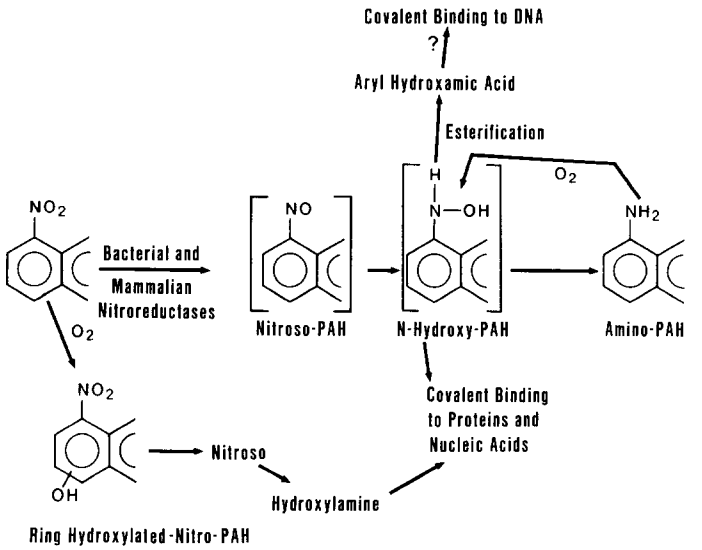

Figure 1. The proposed metabolic activation pathways of nitropolycyclic aromatic hydrocarbons.

with further conversion to aminopyrenes (Figure 1). Both bacterial and mammalian nitroreductases have been implicated in the metabolic activation of nitropolycyclic aromatic hydrocarbons (16-22). Aryl hydroxylamines could undergo esterification to form highly electrophilic hydroxamic acid esters which are capable of reacting with DNA. In addition to nitro-reduction, ring hydroxylation of nitropolycyclic aromatic hydrocarbons has recently been demonstrated and suggested to be important in the metabolic activation of nitropolycyclic aromatic hydrocarbons (23-25). This paper describes the metabolism of 1-nitropyrene and 6-nitrobenzo[a]pyrene by human intestinal microflora.

MATERIALS AND METHODS

Chemicals. [4,5,9,10-^{3}H]1-Nitropyrene (sp. act. 117 mCi/mmole; radiochemical purity >99%) was obtained from Robert W. Roth, Midwest Research Institute, Kansas City, MO. 1-Nitropyrene and 1-aminopyrene were purchased from Aldrich Chemical Co., Milwaukee, WI., and were purified as described previously (18). [G-^{3}H]6-Nitrobenzo[a]pyrene (sp. act. 93.2 mCi/mmol radiochemical purity >99%) (26), 6-nitrobenzo[a]pyrene (27) and 6-aminobenzo[a]pyrene (27) were synthesized and purified as described previously. Bacterial growth media were obtained from Scott Laboratories, Fiskville, RI.

TABLE 1. METABOLISM OF [^{3}H]6-NITROBENZO[a]PYRENE BY ANAEROBIC BACTERIA

Organism (ATCC)		% Conversion To 6-Aminobenzo[a]pyrene[a]
Bacteroides thetaiotaomicron	29148	> 95
Bifidobacterium infantis	15697	65
Citrobacter sp.	25405	45
Clostridium perfringens	3626	> 95
Lactobacillus acidophilus	332	22
Peptococcus anaerobius	14955	> 95
Clostridium sp.		> 95
Peptostreptococcus productus	27340	> 95
Escherichia coli	25992	15
Rat intestinal microflora		> 95
Human intestinal microflora		> 95

[a] Microorganisms were incubated with [G-^{3}H]6-nitrobenzo[a]pyrene for 8 hr under anaerobic conditions. The contents in the incubation bottles were extracted with ethyl acetate and the metabolites separated by hplc and quantified as described in Materials and Methods.

Source of Microorganisms. Human intestinal microflora were isolated from freshly voided fecal samples from healthy volunteers at the National Center for Toxicological Research (NCTR). The samples were collected in polyethylene bags which were thoroughly flushed with oxygen-free nitrogen. A 5-ml aliquot of a 1:20 suspension of feces in brain heart infusion supplemented broth was used as the inoculum after removal of coarse debris by aseptic filtration. All samples were processed within 10 min of defecation. Rat intestinal microflora used in the incubations were obtained from 90-120 day old female CD rats of strain NCTR:S23 (SD) from colonies maintained at the NCTR. The animals were anesthetized with ether and the small and large intestine were removed through a mid-ventral incision. Fecal pellets were than aseptically removed and rapidly transferred anaerobically to 50 ml of chopped meat glucose broth.

The organisms listed in Table 1 were obtained from the American Type Culture Collection (ATCC), Rockville, MD. All of the bacterial strains were subcultured at 7-day intervals and maintained at 4°C on chopped meat agar slants. All cultures were incubated at 37°C for 24 hr without agitation except for Clostridium perfringens ATCC 3626 which was incubated at 45°C.

Culture and Nitropolycyclic Aromatic Hydrocarbon Biotransformation Conditions. All the media used in this study were pre-reduced and transfers were made anaerobically under 100% CO_2 with an oxygen-free cannula. Cultures were suspended in 0.5 ml of chopped meat glucose medium and a 0.4-ml aliquot from each culture was used to inoculate 5 ml of brain heart infusion supplemented broth. The cultures were incubated at 37°C for 24 hr. Clostridium perfringens ATCC 3626 was incubated at 45°C. After 24 hours of incubation, a 10-ml aliquot (1 x 10^9 cell/ml) was aseptically removed from each incubation bottle and transferred anaerobically to 45 ml of brain heart infusion broth. [4,5,9,10-^{3}H]1-Nitropyrene (3.7 µM) or [G-^{3}H]6-nitrobenzo[a]pyrene (2.6 µM) was then added into the media and the cultures were incubated as described above. After various periods of incubation, 3-ml samples were aseptically removed and centrifuged at 10,000 x g for 20 min. Supernatants and cell

pellets were analyzed for metabolites as described below. Two control experiments were conducted; one contained sterile brain heart infusion supplemented broth plus nitropolycyclic aromatic hydrocarbon without microorganisms and the other consisted of sterile medium alone. Bacterial growth was measured spectrophotometrically (A_{425}) using a Beckman Model 25 spectrophotometer.

Analysis of nitropolycyclic aromatic hydrocarbon metabolites formed by intestinal microflora. At various periods of incubation, cultures were centrifuged at 10,000 x g for 20 min and the cell-free supernatants and cell pellets were extracted with three equal volumes of ethyl acetate. The ethyl acetate extracts were dried with anhydrous sodium sulfate and evaporated under reduced pressure at 30°C. Each residue was dissolved in methanol and analyzed by high-performance liquid chromatography (hplc). Reversed-phase hplc was performed with a Beckman system (Houston, TX) consisting of two model 100A pumps and a model 155-10 variable wavelength absorbance detector adjusted to 280 nm. The 1-nitropyrene metabolites were separated on a 0.39 x 30 cm 10 C_{18} µ Bondapak column (Waters Associated, Milford, MA) by elution with a linear 36 min gradient of 50-80% methanol, and then with 100% methanol for 10 min. The flow rate was 2 ml/min. A 5 µC_{18} Ultrasphere ODS column (4.6 mm X 25 cm) [Altex Scientific, Berkeley, CA] was used to separate 6-nitrobenzo-[a]pyrene metabolites. The separation was achieved with a linear methanol/water gradient of 55% to 100% methanol in 45 min with a flow rate of 1 ml/min. In experiments with [^{3}H]1-nitropyrene or [^{3}H]6-nitrobenzo[a]pyrene, fractions were collected at 0.5 min intervals in scintillation vials, and 7 ml of Scintisol (Isolabs, Akron, OH) was added to each vial. The radioactivity present in each fraction was measured with a Searle Mark III liquid scintillation spectrometer. Mass spectra were obtained with a Finnigan model 4023 mass spectrometer (Finnigan, Corp., San Jose,CA) operated at 70eV with a solid probe.

RESULTS AND DISCUSSION

1-Nitropyrene and 6-nitrobenzo[a]pyrene could not be utilized as sole source of carbon and energy as indicated by the lack of growth by any bacterial strains or mixed intestinal microflora cultures examined in this study. However after 2 hr incubation, the brain heart infusion supplemented broths containing either human or rat intestinal anaerobic bacterial suspensions grown in the presence of [^{3}H]1-nitropyrene and [^{3}H]6-nitrobenzo[a]pyrene were able to transform these nitropolycyclic aromatic hydrocarbons to the corresponding amines. Hplc analysis of the anaerobic broth culture extracts of human intestinal microflora incubated with [^{3}H]1-nitropyrene or [^{3}H]6-nitrobenzo[a]pyrene revealed one major peak in each chromatogram which coeluted with authentic 1-aminopyrene (Figure 2A, retention time = 18.7 min) and 6-aminobenzo[a]pyrene

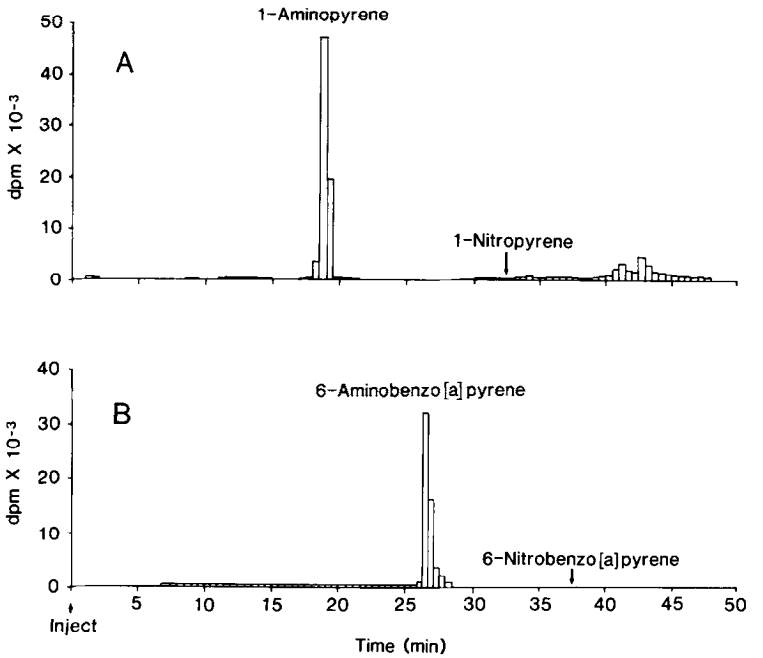

Figure 2. Hplc elution profiles of metabolites obtained from the incubation of: A. [^{3}H]1-nitropyrene and B. [^{3}H]6-nitrobenzo[a]pyrene with human intestinal anaerobic bacteria.

(Figure 2B, retention time = 27.0 min), respectively. Rat intestinal microflora incubated with [^{3}H]1-nitropyrene and [^{3}H] 6-nitrobenzo[a]pyrene gave similar results. Amino-derivatives of 1-nitropyrene and 6-nitrobenzo[a]pyrene were not detected in the sterile control samples. Mass spectral analyses (Figure 3) of the compounds which eluted at 18.7 min. (Figure 2A) and 27.0 min (Figure 2B) confirmed the identification of these compounds as 1-aminopyrene and 6-aminobenzo[a]pyrene, respectively. The mass spectral fragmentation patterns for 1-aminopyrene (Figure 3A) [M^+ at m/z 217, m/z 202 (M^+-15; NH loss) m/z 189 (M^+-28, HCNH loss)] and 6-aminobenzo-[a]pyrene (Figure 3B [M^+ at m/z 267, m/z 239 (M^+-28, HCNH loss)], formed from 1-nitropyrene and 6-nitrobenzo[a]pyrene by human intestinal microflora were identical to those of authentic samples. The time course for the reduction of [^{3}H]1-nitropyrene and [^{3}H]6-nitrobenzo[a]pyrene to the corresponding amines by human

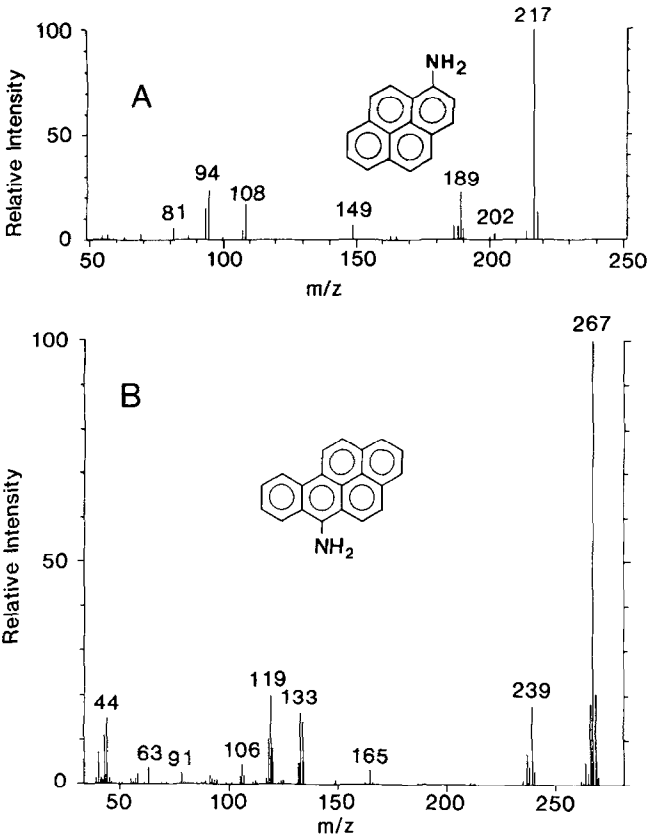

Figure 3. Electron impact mass spectra of A. 1-Aminopyrene formed from 1-nitropyrene by human intestinal anaerobic bacteria. B. 6-Aminobenzo[a]pyrene formed from 6-nitrobenzo[a]pyrene by human intestinal anaerobic bacteria.

intestinal microflora was monitored at various time intervals by hplc. Figure 4A and B shows that there is a rapid and quantitative conversion of these nitropolycyclic aromatic hydrocarbons to amino derivatives. Within 1 hr of incubation, 85 to 95% of 1-nitropyrene and 6-nitrobenzo[a]pyrene added to the culture was converted to 1-aminopyrene and 6-aminobenzo[a]pyrene, respectively. Since the predominant bacterial genera identified in the human fecal samples were Bacteroides, Lactobacillus, Bifidobacterium, Eubacteriuim and Clostridium, we screened pure bacterial stains for their capability to metabolize [^{3}H]6-nitrobenzo[a]pyrene. The results in Table 1 indicate that all of the cultures tested reduced 6-nitrobenzo[a]-

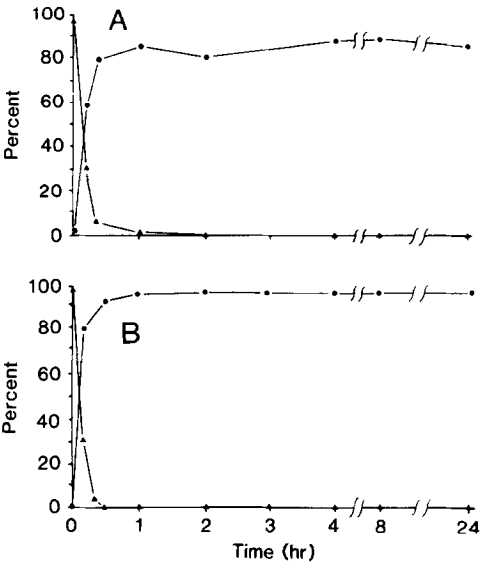

Figure 4. Kinetics of metabolism of: A. [^{3}H]1-nitropyrene (▲--▲) to 1-aminopyrene (● --●) B. [^{3}H]6-Nitrobenzo[a]pyrene (▲ -- ▲) to 6-aminobenzo[a]pyrene (● -- ●) by human intestinal microflora.

pyrene to 6-aminobenzo[a]pyrene to varying extent. Similar results were previously reported for the metabolism of 1-nitropyrene by intestinal bacteria (18).

Inhalation exposure of rodents to particulates containing nitropolycyclic aromatic hydrocarbons has indicated that these nitropolycyclic aromatic hydrocarbons containing particles are readily deposited in the lungs, and that some of these particles clear rapidly from the lungs into the gastrointestinal tract (28). In this investigation we showed that a wide spectrum of pure anaerobic bacterial cultures and mixed human and rat fecal cultures are capable of transforming 1-nitropyrene and 6-nitrobenzo[a]pyrene to the corresponding amines. Nitroreduction of nitropolycyclic aromatic hydrocarbons by intestinal microflora could be of toxicological significance if these compounds do pass through the gastrointestinal tract and interact with the gut microflora. The aminopolycyclic aromatic hydrocarbons formed may be returned to the liver

where further metabolism by mammalian oxidative enzymes can take place (Figure 1). Until recently, there was a paucity of information about the nature of the bacterial enzymes responsible for reduction of nitropolycyclic aromatic hydrocarbons. Kinouchi and Ohnishi (19) have purified and characterized four nitroreductases from Bacteroides fragilis. Nitroreductase I catalyzed the binding of 1-nitropyrene to E. coli DNA which suggested that nitroreduction was a necessary step in the binding of 1-nitropyrene to DNA. El-Bayoumy and coworkers (16) recently reported a study on the metabolism of 1-nitropyrene in germfree and conventional rats which indicated that intestinal microflora are involved in the in vivo nitroreduction of 1-nitropyrene. Our results confirm and extend these observations by showing that human intestinal microflora are capable of metabolizing nitropolycyclic aromatic hydrocarbons to the corresponding amine.

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