Ring Opening of Benzo[*a*]pyrene in the Germ-Free Rat Is a Novel Pathway for Formation of Potentially Genotoxic Metabolites†

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ABSTRACT: The metabolism of benzo[*a*]pyrene (BP) is known to lead to a large number of oxygenated compounds, some of which can bind covalently to DNA. We have studied the integrated metabolism of BP in vivo in germ-free rats given ^{14}C-labeled BP. Urinary metabolites were separated into groups according to acidity using lipophilic ion exchangers. The groups were analyzed by mass spectrometry and were further fractionated by high-performance liquid chromatography. The fraction of urinary metabolites previously shown to contain *N*-acetylcysteine and glucuronic acid conjugates was found to contain derivatives of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid as major components. These compounds, which were identified by mass spectrometry and NMR, accounted for about 30% of the total metabolites in urine, demonstrating that, surprisingly, ring opening is a major pathway for metabolism of BP in the germ-free rat. The dicarboxylic acid may be excreted in urine as an ester glucuronide. By using the single cell gel electrophoresis or COMET assay, we were able to demonstrate that the anhydride of 7-oxo-benz-[*d*]anthracene-3,4-dicarboxylic acid was an efficient inducer of DNA damage. Taken together, these results indicate that the novel ring opening metabolic pathway may provide alternative mechanisms for the toxicity of BP.

Polycyclic aromatic hydrocarbons constitute a large class of chemicals with widespread occurrence in the environment. It is essentially impossible to avoid exposure to these substances on a daily basis. Benzo[*a*]pyrene (BP)[1] is a powerful mutagenic and carcinogenic agent in various experimental systems and is also suspected to be a significant health risk to humans. The major mechanisms for metabolic activation of BP include formation of bay-region diolepoxides and one-electron oxidation with formation of a radical cation (*1*, *2*). Products of these reactions may subsequently bind covalently to DNA and lead to carcinogenesis.

To date, most studies to characterize BP metabolites have been performed with cells, tissues, and microsomal fractions. The metabolism of BP in whole organisms may be quite different from that in isolated cells. In vivo, metabolites of BP are conjugated with glucuronic acid, sulfuric acid, or glutathione (phase II) to form more water-soluble metabolites that can be readily excreted. Analysis of these conjugates may reveal metabolic pathways involving formation of toxic compounds. However, the analysis of the integral metabolism of BP in the whole animal has been hampered by methodological difficulties in isolating and identifying the myriad of partly labile metabolites occurring at low levels in complex matrixes.

We have developed isolation and group separation methods (*3*) that permit analysis of unconjugated metabolites of BP by gas chromatography−mass spectrometry (GC/MS) (*4*) and conjugated metabolites by capillary column HPLC and electrospray ionization mass spectrometry (ESMS) (*5*). While several novel BP conjugates were identified, when these methods were applied to the analysis of urine from germ-free rats given BP, the major metabolites were found to consist of compounds not previously described to be formed from this carcinogen (*6*). The present paper describes the identification of these compounds as derivatives of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid (Chart 1). Our results indicate that oxidative opening of the ring in BP containing carbons 1−3 with elimination of carbon 2 is a major metabolic pathway for BP. The genotoxic potential of one

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[1] Abbreviations: BP, benzo[*a*]pyrene; BPDE, (±)*r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene (anti); ES, electrospray; NOE, nuclear Overhauser enhancement; DQF-COSY, double-quantum-filtered two-dimensional correlated spectroscopy; HSQC, heteronuclear single-quantum coherence; HMBC, heteronuclear multiple-bond correlation; Fpg, *Escherichia coli* formamidopyrimidine-DNA glycosylase.

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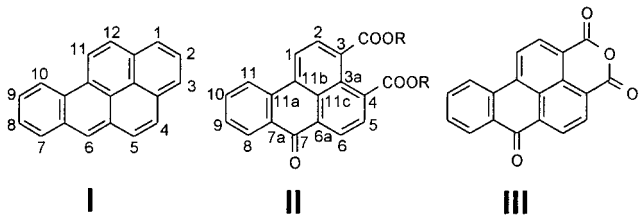
Chart 1: Metabolites of Benzo[*a*]pyrene Formed by Ring Opening. **I**: Benzo[*a*]pyrene; **II**: 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic Acid and Derivatives (R = H or CH_3); **III**: Anhydride of the Dicarboxylic Acid, **II**



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of these derivatives, the anhydride of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid, has been studied using the single cell gel electrophoresis or COMET assay. This assay is a sensitive and rapid technique enabling the detection of DNA strand breaks and alkali labile sites in individual cells (*7*, *8*). The observation that this derivative could induce DNA damage and the knowledge regarding the facile formation of the anhydride from the dicarboxylic acid suggests that this novel metabolic pathway may represent a possible alternative mechanism for BP toxicity.

MATERIALS AND METHODS

Caution: Benzo[*a*]pyrene and its derivatives are hazardous. All laboratory procedures involving these chemicals should be performed using safety gloves and where possible in a well-ventilated fume hood.

Chemicals. All chemicals and solvents were of analytical grade, and solvents were redistilled prior to use. (±)*r*-7,*t*-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene (anti-BPDE) was purchased from Midwest Research Institute (Kansas City, MO). Water was obtained from a Milli-Q water system (Millipore, Molsheim, France). Sep-Pak C_{18} cartridges (Waters-Millipore, Milford, MA) were washed with methanol and water prior to use. Ion-exchange gels were washed and converted to the proper forms as described previously (*3*).

Synthesis of BP Metabolites. The anhydride of 7-oxo-benz-[*d*]anthracene-3,4-dicarboxylic acid (**III**) was synthesized by ozonization of BP as described by Moriconi et al. (*9*). The IR data of **III**, 1790 (w), 1773(s), 1728 (s), 1652, 1595, 1511-(w), 1458(w), 1344, 1284(s), 1231, 1153, 1122, 1025(s), 769-(s) cm^{-1}, agreed nicely with the absorptions reported by Moriconi et al. (*9*).

Animals and Isolation of BP Metabolites from Urine. Five adult male germ-free rats of the AGUS strain were dosed intraperitoneally with 10 μCi of [7,10-^{14}C]-BP (59 mCi/mmol, 98.1% radiochemical purity by HPLC, Amersham, Bucks, UK) mixed with 10 mg unlabeled BP (Sigma) as described previously (*6*). Urine was collected 24 h before and every 24 h for 7 days after the administration. Lipophilic substances including BP metabolites were extracted onto a Sep-Pak C_{18} cartridge and recovered in methanol. Twenty-four hour batches of urine (3−5 mL) collected during days 2−3 after the administration were usually used for the isolation of metabolites. The extracts were separated by ion-exchange chromatography into groups of neutral and acidic (conjugated) compounds (*6*). The fractions containing conjugated metabolites were analyzed by LC/ESMS. One fraction (fraction II), representing about 50% of the total urinary radioactivity and assumed to consist of glucuronides and mercapturates, was found to contain three components not compatible with any known types of BP metabolites (*6*). In the present study, this fraction was subfractionated by HPLC (*6*) and analyzed by capillary LC/ESMS. Samples of pooled HPLC peaks were analyzed by NMR.

ESMS and Capillary Column LC/ESMS. ES spectra were recorded on a VG AutoSpec-TOFFPD hybrid magnetic sector-orthogonal time-of-flight tandem mass spectrometer equipped with a nano-ES interface (Micromass Ltd, Manchester, UK). The latter has two probes: one for continuous infusion of liquids at low flow rates and the other to hold a gold-coated borosilicate capillary (Protana A/S, Odense, Denmark). Samples were sprayed from the latter in 50% aq methanol (1−5 μL at a concentration of about 1 pmol/μL). A fused silica capillary column (0.1 × 100 mm) packed with 5-μm particles of Kromasil C_{18} (*5*) was connected to the other probe. The solvents were A: 20% methanol/water and B: methanol, both containing 30 mM ammonium acetate, pH 7.2. Samples were injected in 2−20 μL of solvent A, and elution was started with 0% B, increased to 80% B in 30 min and then to 100% B in 5 min, where it was maintained for 10 min with a flow rate of less than 1 μL/min. ES spectra of the HPLC effluent were recorded by repetitive scanning of *m*/*z* 1000−300 at a rate of 2 s/decade. The accelerating voltage was −4 kV and the resolution 1500 (5% valley definition). For determination of accurate mass, high resolution (5000) spectra were recorded by voltage scanning at a rate of 15 s/decade. Sample injections were bracketed between calibrant injections (PEG 200−1500) using the borosilicate glass capillaries. Product ion spectra were generated by collision-induced dissociation, using xenon as collision gas, and were recorded on the orthogonal time-of-flight analyzer (*5*).

GC/MS. GC/MS was performed essentially as described in the previous study (*4*). A fused silica capillary column (0.32 mm i.d. × 30 m) coated with 5% cross-linked phenyl methyl silicone was employed. The components were separated by temperature programming on a Hewlett-Packard 5890A gas chromatograph and emerged into the ion source of an HP 5972 Series Mass Selective detector. Spectra were recorded by repetitive scanning for about 40 min.

NMR Spectroscopy. NMR spectra were recorded with 7 μg (^{1}H NMR) or 40 μg (^{13}C NMR) of sample in 180 μL of deuteriochloroform solution at 15 °C using a 5-mm Shigemi sample tube on a Bruker DMX 600 NMR spectrometer. 1D NOE spectra (*10*) were recorded with mixing times of 500 ms. Two-dimensional spectra recorded included DQF-COSY (*11*, *12*), sensitivity-enhanced ^{13}C-HSQC (*13*), and ^{13}C-HMBC with a 90° purge pulse to suppress one-bond ^{13}C-^{1}H correlations (*14*, *15*). Pulsed-field gradients were used for all ^{13}C-correlation spectra. Three ^{13}C-HMBC spectra were recorded with coupling evolution delays for the generation of multiple-bond correlations set to 45.4, 62.5, and 142 ms, respectively, using t_{1max} = 13 ms, t_{2max} = 511 ms and a total recording time of 20 h/spectrum. Window multiplication before Fourier transformation was important to obtain adequate sensitivity. The ^{13}C-HMBC spectrum for the detection of correlations with aromatic carbons was generated by multiplying the data sets recorded with 45.4 and 62.5 ms coupling evolution delay by exponential functions corresponding to 3 Hz line broadening in both dimensions,

followed by calculation of magnitude spectra and summation of both data sets. The ^{13}C-HMBC spectrum for the detection of correlations with carbonyl carbons was derived from the data set recorded with 142 ms coupling evolution delay. The data were multiplied by an unshifted sine-bell window function in the t_2 dimension, using only data up to t_{2max} = 255 ms, and a magnitude spectrum was calculated.

Cell Culture. A human colon adenocarcinoma cell line, HT-29, was obtained from ATCC, Rockville, MD. The cells were grown as monolayers in Dulbecco's modified eagle medium: 4500 mg/L glucose, DMEM (Gibco, Life Technologies Ltd, UK) supplemented with 10% fetal calf serum, 2 mmol L-glutamine/L, 1×10^5 U penicillin/L, and 100 mg streptomycin/L in a humidified incubator at 37 °C with 5% CO_2.

COMET Assay. The method was basically that of Klaude et al. (*16*) with minor modifications. Clear microscope slides (Mentzel super frost) were pretreated with 40 μL of 0.3% low melting point (LMP) agarose (Sigma, type VII) and allowed to air-dry. The HT-29 cells were incubated with the test substance for 2 h at 37 °C in a 5% CO_2 atmosphere. Ten microliters of cell suspension [$(0.5-5) \times 10^6$ cells/mL] was mixed with 150 μL of LMP agarose [0.75% in PBS (phosphate buffered saline) kept at 37 °C]. A Flexi-Strip spatula was used to distribute the mixture on the precoated slides that were thereafter left to sit on an ice tray. After the samples solidified, the slides were treated in one of two ways. For low alkali conditions, the slides were immersed in room temperature lysing solution (0.03 M NaOH, 1 M NaCl, 1 mM EDTA, and 0.5% sodium *N*-lauroylsarcosinate) in darkness for 1 h. The slides were then rinsed with 0.03 M NaOH and 1 mM EDTA for 45 min. Electrophoresis was performed at room temperature, in darkness, in a Bio-Rad subcell GT unit containing the same solution, for 15 min using 20 V (0.67 V/cm). For high alkali conditions, lysis was performed in darkness for 1 h with an ice-cold freshly prepared solution containing 2 M NaCl, 25 mM EDTA, 20 mM Tris, and 0.5% Triton X-100, pH 10. The slides were then placed in an electrophoresis solution (0.3 M NaOH and 1 mM EDTA) in darkness at room temperature for 45 min. Electrophoresis was then carried out at room temperature, for 30 min at 0.67 V/cm. After electrophoresis, the slides were neutralized in 0.4 M Tris, pH 7.4, air-dried, fixed in methanol, and stored in a dry and dust-free box until analysis. The DNA was stained with ethidium bromide [10 μg/mL in TAE (Tris acetate EDTA)] for 5 min followed by destaining for 5 min in TAE. The comets were examined in a fluorescence microscope (Olympus BH2 with a 20 × apochromatic oil immersion objective), using the program Comet Assay II from Perceptive Instruments (Liverpool, UK). Images of 50 randomly selected cells were analyzed from each sample, and tail moment was determined according to Olive (*17*). Straight lines were fitted to the linear part of the dose response curves.

Experiments were also conducted using the *Escherichia coli* formamidopyrimidine-DNA glycosylase (Fpg, Trevigen Inc., Gaithersburg, MD). Teflon-coated slides with 3 × 14 mm Teflon-free areas were precoated with 20 μL/area of 0.3% LMP agarose and left to air-dry. The HT-29 cells were incubated with the test substances as described earlier. Thirty microliters of 0.75% LMP made in PBS and kept at 37 °C was mixed with 4 μL of cell suspension [$(0.5-5) \times 10^6$ cells/mL] and distributed on the pretreated slides. The slides were left to solidify on an ice tray before immersing them for an hour in an ice-cold freshly made lysing solution (2 M NaCl, 25 mM EDTA, 20 mM Tris, pH 10, and 0.5% Triton X-100). The slides were thereafter placed in a solution containing 25 mM EDTA in PBS overnight at 4 °C. The slides were transferred for 1 h to an ice-cold enzyme buffer, 20 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5, and 0.1 mg/mL bovine serum albumin. Twenty microliters of Fpg (final concentration: 1 μg/mL) or enzyme buffer was distributed to the slides, which were then put on a chilled plate for 30 min and thereafter in a humidified chamber at 37 °C for 30 min. The slides were transferred to an electrophoresis chamber containing an electrophoresis solution (0.03 M NaOH and 1 mM EDTA) for an hour before performing electrophoresis for 15 min using 20 V (0.67 V/cm), and the slides were thereafter treated as described above. The slides were protected from light during the experiment, as far as possible.

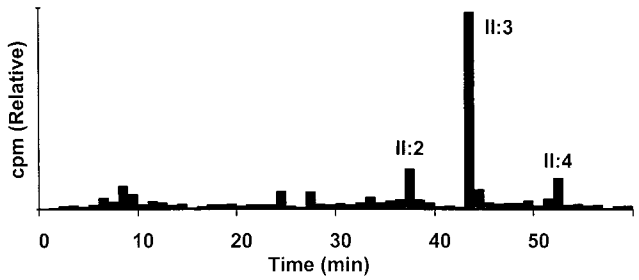

FIGURE 1: HPLC separation of metabolites of BP in fraction II.

RESULTS

HPLC Separation. About 90% of the radioactivity was excreted in feces and urine during 7 days following the administration of the labeled BP. About 7−10% of this appeared in the urine. Almost half of the urinary BP metabolites were eluted in that fraction from the anion exchanger (fraction II), which is the focus of the present paper (*6*). An example of the HPLC analysis of this fraction is shown in Figure 1. The retention time of peak II:4 at 53 min, which was the major peak in some analyses (*6*), indicated a highly nonpolar compound. Upon C_{18} extraction and rechromatography, peak II:4 decreased dramatically, and peaks appeared at the retention times of the major peak II:3 (44 min) and the minor peak II:2 (38 min). When reanalyzed, most of peaks II:2 and II:3 was retained by the anion exchanger, showing that these compounds were acids. Separate C_{18} extraction of peaks II:2 and II:3 using acetonitrile instead of methanol for elution, yielded peak II:4 in both cases. This suggested that peaks II:2, 3, and 4 were structurally related and interconvertible. Together, these compounds constituted on average about 30% of the total urinary metabolites as calculated from the radioactivity.

Acetylation of the mixture of compounds using acetic anhydride/pyridine did not affect the HPLC retention times, while methylation with diazomethane converted 90% of the radioactivity in the mixture into a new peak at retention time 54 min. Alkaline hydrolysis of the mixture yielded predominantly peak II:2.

GC/MS Analysis. Peak II:4 and a freshly methylated fraction II containing predominantly peak II:3 (see Figure

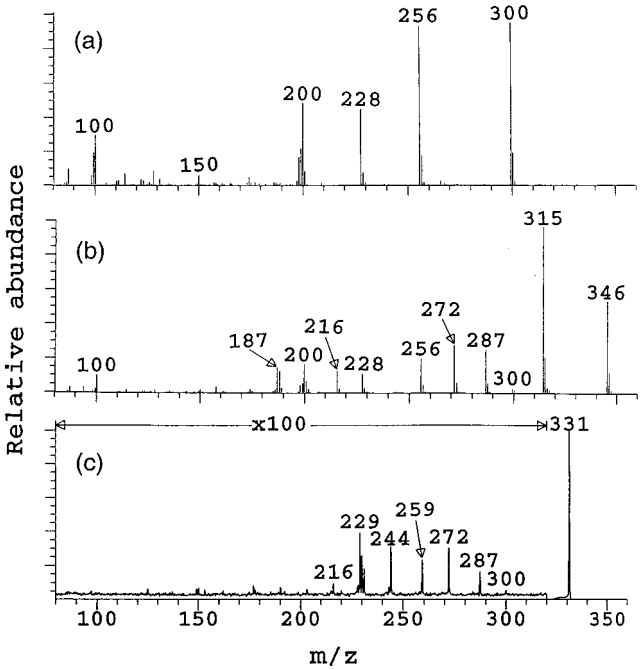

FIGURE 2: Mass spectra of the major metabolites of BP in fraction II. (a) Electron impact spectrum of the anhydride of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid (peak II:4); (B) Electron impact spectrum of the dimethyl ester of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid formed by treatment of peak II:3 with diazomethane; (c) Product ion spectrum obtained by collision-induced dissociation of the mixture of deprotonated monomethyl esters of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid, *m*/*z* 331, generated by electrospray ionization.

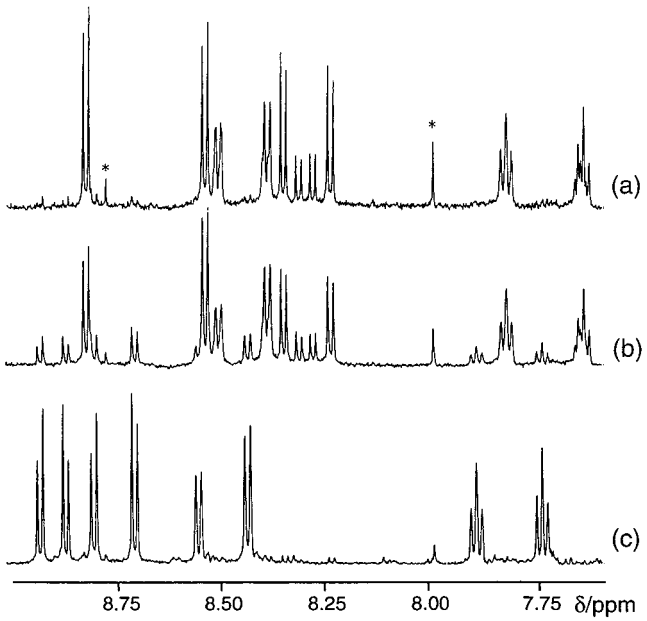

FIGURE 3: ^{1}H NMR spectra of the major metabolites in fraction II. (a) Mixture of isomeric monomethyl esters of 7-oxo-benz[*d*]-anthracene-3,4-dicarboxylic acid (peak II:3); (b) Same sample as panel a, after 2 days' storage in deuteriochloroform at 4 °C; (c) Anhydride of the 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid (peak II:4). Asterisks identify peaks from impurities in the deuteriochloroform solvent used.

1) were subjected to GC/MS analyses. The former gave a peak with a GC retention time of 29.4 min whose mass spectrum is shown in Figure 2, panel a. A molecular ion at *m*/*z* 300 gave the most intense peak. Other abundant ions were present at *m*/*z* 256, corresponding to loss of 44 Da from the ion at *m*/*z* 300 and at *m*/*z* 228 and 200, representing successive losses of 28 Da from the ion at *m*/*z* 256. Corresponding doubly charged ions were seen at *m*/*z* 150, 128, 114, and 100, respectively. The absence of other low mass ions indicated an aromatic character of the compound.

The methylated sample gave a major peak with a GC retention time of 28.9 min. Figure 2, panel b, shows the mass spectrum of this peak. The most abundant ion was at *m*/*z* 315, corresponding to loss of 31 Da (OCH_3) from the molecular ion at *m*/*z* 346. A fragment ion at *m*/*z* 287 (M-59) and ions at *m*/*z* 256 (M-59-31) and 228 (M-59-59) indicated the possible presence of two methylated carboxyl groups. Doubly charged ions corresponding to the singly charged ions at *m*/*z* 346, 315, 300, 256, 228, and 200 were present. The spectrum was similar to spectra of unidentified metabolites previously found in the neutral fraction (*4*).

ESMS Characterization. Fraction II was analyzed by nano-ESMS and capillary column LC/ESMS. The most intense peak in the negative-ion spectrum appeared at *m*/*z* 331. A minor peak was present at *m*/*z* 317 and several other peaks were due to conjugated metabolites of BP (*6*). The reconstructed ion chromatogram of *m*/*z* 331 in the LC/ESMS analysis showed one intense peak at a retention time of 30.5 min (see Figure 2 in ref *6*). A peak at the same retention time was present in the chromatogram of *m*/*z* 663, suggesting a deprotonated dimer of a compound of mass 332 Da. A small peak of a more polar compound was seen in the chromatogram of *m*/*z* 317. When a solution of fraction II in deuteriomethanol/deuterium oxide was analyzed by ESMS, the deprotonated ion at *m*/*z* 331 did not shift while the mass of the protonated ion in the positive-ion spectrum was increased by 2 Da, indicating the presence of one acidic group in the molecule.

High-resolution accurate mass measurements gave a relative molecular mass of 331.0613, compatible with the elemental composition $C_{20}H_{11}O_5$ (theoretical value, 331.0606).

The product ion spectrum of the ion at *m*/*z* 331 obtained by collision with xenon at 400 eV is shown in Figure 2, panel c. Fragment ions at *m*/*z* 300 (M-H-31) and 272 (M-H-59) indicated the presence of a methylated carboxyl group. A peak at *m*/*z* 287 corresponded to loss of CO_2. These fragment ions suggested the possible presence of one free and one methylated carboxyl group. The absence of major ions below *m*/*z* 200 indicated a polyaromatic structure.

NMR Identification. To obtain sufficient amounts of a reasonably pure compound for NMR, repeated HPLC purifications of fraction II were performed, and peak II:3 was collected under conditions minimizing formation of peak II:4 (see above). Part of this material was then converted into peak II:4 (C_{18} extraction using acetonitrile instead of methanol for elution). Both compounds were analyzed by 2D ^{1}H NMR, and peak II:4 was also analyzed by ^{13}C NMR spectroscopy.

The ^{1}H NMR spectrum of peak II:3 revealed the presence of two compounds with similar structures at a 2:1 ratio (Figure 3, panel a). After standing at 4 °C for 2 days, a third compound appeared (Figure 3, panel b) that was identical to the compound of peak II:4 (Figure 3, panel c**)**. A DQF-COSY spectrum of the sample of Figure 3, panel a, revealed a linear 4-spin system and two 2-spin systems in the aromatic

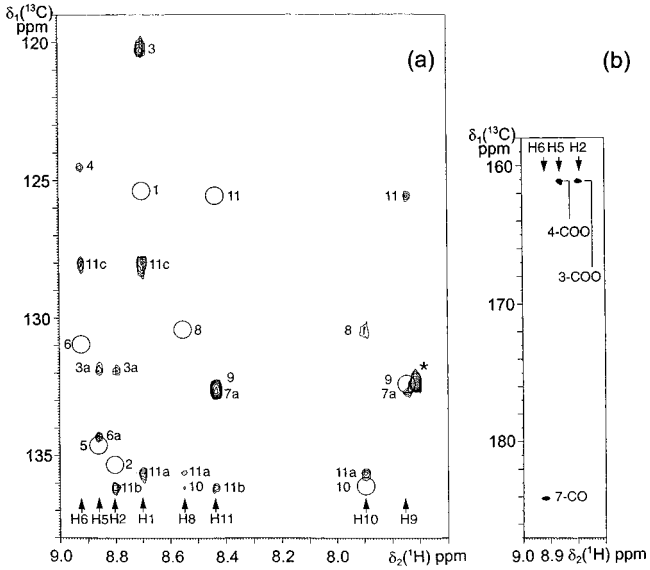

FIGURE 4: ^{13}C-HMBC spectra of the anhydride of 7-oxo-benz[*d*]-anthracene-3,4-dicarboxylic acid. (a) Connectivities in the aromatic region of the spectrum. Circles identify the positions of one-bond correlation peaks observed in a ^{13}C-HSQC experiment. Vertical arrows identify the ^{1}H resonance positions in the ^{1}H NMR spectrum, using the numbering shown in Chart 1. The cross-peaks are labeled according to the ^{13}C spins involved. An asterisk marks the cross-peak of an impurity (probably a phthalic acid derivative). (b) Connectivities with carbonyl carbons. Labeling as in panel a.

region of the spectrum for each of the two compounds. A 4-spin and two 2-spin systems are also apparent in the spectrum of peak II:4 (Figure 3, panel c). Furthermore, each of the compounds contained in peak II:3 displayed a methyl group signal at about 4 ppm that was absent in the compound of peak II:4 (data not shown). Together with the chemical information and mass spectrometric determination of the atomic composition, the compounds of peak II:3 were identified in a preliminary way as the two monomethyl esters of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid and the compound of peak II:4 as the anhydride of the same acid. This hypothesis was confirmed by complete ^{13}C resonance assignment of the anhydride. In particular, all predicted correlations through $^{3}J_{HC}$ couplings were observed in the ^{13}C-HMBC spectra but no correlation through $^{2}J_{HC}$ couplings because of their expected small size (Figure 4). (The cross-peak between C7 and H8 is absent from Figure 4 but was observed below the top level of white noise.) Finally, 1D NOE experiments established the proximity of H1 and H11. Table 1 summarizes the resonance assignments of the derivatives of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid analyzed.

The NMR (^{1}H and ^{13}C) data of the synthetic **III** were identical to those of the urinary metabolite (data not shown), as was the ES mass spectrum of the monomethyl ester of **II** obtained from synthetic **III**.

DNA Strand Break Induction. Induction of DNA damage by the anhydride of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid (**III**), dissolved in dimethyl sulfoxide, in HT-29 cells was analyzed using the COMET assay. The experiments were conducted with 0.03 M NaOH alkali electrophoresis buffer and mainly showed direct strand breaks. After treatment, a dose-dependent increase in DNA damage was observed (Figure 5). Dimethyl sulfoxide was used as control in all experiments and had no effect on tail moment.

Table 1: NMR Resonance Assignments of the Derivatives of 7-Oxo-benz[*d*]anthracene-3,4-dicarboxylic Acid (in ppm)[a]

position	3-methyl ester	4-methyl ester	anhydride	
	^{1}H	^{1}H	^{1}H	^{13}C
1	8.53	8.53	8.70	125.7
2	8.24	8.32	8.80	135.2
3				120.5
3a				131.9
4				124.5
5	8.35	8.28	8.86	134.5
6	8.81	8.81	8.92	131.0
6a				134.3
7				184.0
7a				132.7
8	8.50	8.51	8.55	130.6
9	7.65	7.66	7.74	132.6
10	7.83	7.83	7.90	136.2
11	8.39	8.39	8.44	125.9
11a				135.5
11b				136.0
11c				128.1
3-COO	3.99 (CH_3)			161.2
4-COO		4.00 (CH_3)		161.2

[a] The numbering is shown in Chart 1. Chemical shifts are referenced to tetra-methylsilane. The resonance assignments of the methyl ester compounds are based on scalar coupling constants $^{3}J_{H1H2}$ and $^{3}J_{H5H6}$ of 7.8 and 7.3 Hz, respectively, measured for all three compounds, and the expectation that the protons H2 and H5 experience larger chemical shift changes upon substitution of the carboxyl groups than the protons H1 and H6. The chemical shifts of the ortho protons H2 and H5 were used to identify the position of the methyl ester group.

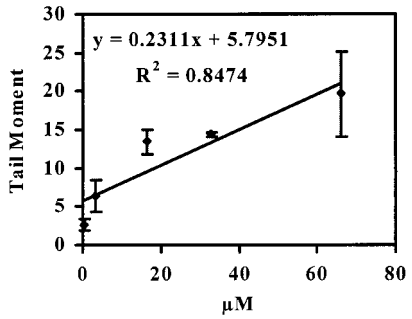

FIGURE 5: Effect of the anhydride of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid on induction of DNA strand breaks in HT-29 cells subjected to the comet assay. Values are means of three experiments ± standard error of the mean.

Strand break induction by the anhydride was estimated to be about 90 strand breaks per micromolar, calculated via calibration with gamma radiation assuming 1000 single strand breaks per cell per Gy (*18*), while induction by BPDE (a known genotoxic metabolite of BP, dissolved in tetrahydrofuran) was estimated to be 540 strand breaks per micromolar (Table 2). It should be noted that we cannot distinguish between breaks induced by the chemical itself or from transient breaks caused by excision repair of damaged bases.

Use of the 0.3 M NaOH alkali electrophoresis solution also detects alkali labile sites, e.g., damaged bases, and makes it possible to calculate the ratio between strand breaks (0.03 M NaOH) and strand breaks + alkali labile sites (0.3 M NaOH) for the substances tested (Table 2). The lower the ratio value, the higher the relative amount of alkali labile base damage. The ratio values are above one since the high alkali electrophoresis conditions give a lower tail moment than low alkali electrophoresis conditions, at a certain number

Table 2: Induction of DNA Strand Breaks (SB), Analyzed Using the COMET Assay, under Low and High Alkali Electrophoresis Conditions and in the Presence or Absence of *E. coli* Formamidopyrimidine-DNA Glycosylase (Fpg)

agent	SB μM^{-1} (low alkali)	low alkali/ high alkali	Fpg sensitive sites (μM^{-1})
anhydride	90	2.2	198
BPDE	540	1.7	nd[a]
Gamma rays	1000/Gy (18)	2.6	nd[a]

[a] nd, not determined.

of strand breaks. The results shown in Table 2 are consistent with BPDE and to a slightly lesser degree the anhydride inducing a relatively high number of base adducts, as compared to gamma rays.

Results obtained by treating the cells after lysis with an *E. coli* formamidopyrimidine-DNA glycosylase, which is specific for the cleavage of 8-oxoguanine and other oxidized purines (*19*), indicate that a large part of the damage, induced by the anhydride, is oxidative damage (Table 2). Once again dimethyl sulfoxide, with and without treatment with Fpg, had no effect on tail moment.

DISCUSSION

This study demonstrates a new pathway in the metabolism of BP in the rat that involves an opening of the aromatic ring system. On the basis of the elemental composition of the compound ($C_{20}H_{12}O_5$) determined by mass spectrometry, the configuration of the new metabolites was established by NMR spectroscopy. Seven micrograms of a mixture of two chemically closely related metabolites was sufficient for ^1H NMR spectroscopy and led to a correct guess of the structures (Chart 1). Definite proof was obtained by ^{13}C NMR resonance assignment using 40 μg of the pure anhydride isolated from urine. Furthermore, supporting the identification were the identical NMR data for the synthetic anhydride and the mass spectral data for the synthetic monomethyl ester.

The compounds were isolated from urine using a series of solid-phase extractions and ion exchange chromatographies. It was therefore surprising that a neutral compound, the anhydride, was a major component in a fraction that was expected to contain only glucuronide and mercapturic acid derivatives of BP metabolites (*6*). This may be explained by the previous finding that the anhydride is spontaneously formed from the dicarboxylic acid during the synthesis of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid by ozonolysis of BP (*9*). The same process may occur in urine or during sample isolation, and it is notable that the spectrum of the anhydride was previously recorded in the GC/MS analyses of metabolites of BP from the same urine (*4*). It was then suggested to be a trioxo-BP, which has the same molecular mass but a different elemental composition that was not determined in the previous study. When the anhydride is treated with methanol on a C_{18} solid-phase material, it undergoes methanolysis to the two isomeric monomethyl esters of the dicarboxylic acid. These are reconverted to the anhydride when eluted with acetonitrile instead of methanol from the C_{18} sorbent.

In all likelihood, the dicarboxylic acid is the original metabolite of BP. Whether it is excreted in this form in urine or as a conjugate that is hydrolyzed during the sample isolation is not known. So far we have not been able to obtain mass spectrometric evidence for a conjugate. However, ester glucuronides of carboxylic acids are known to be unstable and prone to methanolysis or hydrolysis during sample preparation (*20*, *21*).

The 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid was formed in the germ-free rat. Thus, the metabolic reactions were catalyzed by mammalian enzymes and did not require participation of enzymes in the intestinal microflora. We are not aware of any previous reports regarding ring opening in the metabolism of BP or other polycyclic aromatic hydrocarbons, although it has been demonstrated in the metabolism of benzene in vivo. *Trans-trans*-muconic acid formed via muconaldehyde is the ultimate metabolite in this pathway (*22*, *23*) and has been used as a biomarker of benzene exposure in humans (*24*). These reactions together with the formation of phenolic metabolites of benzene by peroxidases in bone marrow have been postulated to play a role in the haematopoietic toxicity of benzene (*25*).

It is reasonable to assume that the formation of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid proceeds via 1,6- or 3,6-quinones of BP. Experimentally, the latter quinones were formed by ozonolysis of BP with an equimolar amount of ozone (*9*). 7-Oxo-benz[*d*]anthracene-3,4-dicarboxylic acid was formed by ozonolysis of BP and the two quinones using a 2-fold excess and equimolar amounts of ozone, respectively, followed by alkaline hydrogen peroxide oxidation (*9*). It has also been demonstrated that BP quinones are formed metabolically via an initial one-electron oxidation of BP, catalyzed by cytochrome P450, and that 1,6 and 3,6-quinones of BP are autoxidative products of 6-hydroxy-BP (*26*). We have demonstrated the presence of these two latter quinones in urine from germ-free rats, administered BP (*4*). On the basis of these results, it appears likely that the enzymatic formation of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid in the rat involves initial oxidation of BP at C6, further oxidation at C1 and C3 and finally ring opening with loss of C2.

The metabolic activation of BP is considered to occur mainly via two independent pathways. One leads to the formation of bay-region diolepoxides, i.e., 7,8-dihydrodiol-9,10-epoxide as the ultimate carcinogenic form of the molecule (*1*). The other starts with an oxidation at C6 leading to formation of a radical cation that can react with DNA (*2*). The metabolic pathway demonstrated by our study may provide yet additional mechanisms for BP toxicity. This would be the case if intermediates in the ring-opening reactions, as well as the anhydride, reacted with cellular macromolecules. It has earlier been demonstrated that the BP 1,6 and 3,6-quinone are cytotoxic to Syrian hamster embryo fibroblasts, BP6T cell line, and that they inhibit cellular DNA and RNA synthesis (*27*). Incubation of T7 bacteriophage DNA in aqueous buffer-ethanol solution with the quinones has also been shown to result in the production of single-strand breaks in DNA (*28*). In the present study, our initial studies, using the COMET assay, clearly demonstrate that the anhydride is an efficient inducer of DNA strand breaks in colonic cells. Also of interest was the similarity of the anhydride with the extensively studied genotoxic BPDE, regarding induction of DNA base adducts. In addition, in studies using the Fpg protein that nicks the DNA at sites of oxidative damage (*19*), we were able to show that

this type of damage was important in the case of the anhydride. Future studies will focus on the mechanisms of genotoxicity of this compound. The basic reasoning lying behind the use of colonic cells in the genotoxicity assay is similar to that lying behind the use of germ-free animals in the present study, i.e., to study the role of the intestinal microflora in colon cancer. It is also interesting to note that the type of genotoxic activity, detected by the COMET assay, precedes tumor formation in rats, treated with chemical carcinogens (*29*), and that components of human fecal water fractions have been shown to induce this type of toxicity in colonic cells (*30*).

The potential formation of ester glucuronide(s) of intermediates in the ring-opening reactions is also of interest since drug metabolites conjugated in this way have been shown to react with sulfhydryl and amino groups (*31*−*34*). In conclusion, since the formation of 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid appears to be a quantitatively important metabolic pathway for BP in the rat, it is now important to initiate more extensive studies to investigate the contribution of this compound and its potential precursors/derivatives to the toxicity of BP in mammals.

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