Metabolism of Benzo(a)pyrene by the Isolated Perfused Rat Testis

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ABSTRACT

Benzo(a)pyrene (BP) metabolism was studied in control and tetrachlorodibenzo-p-dioxin (TCDD)-induced rat testes using the isolated perfusion technique. The testicular artery was perfused with [7,10-14C]BP (specific activity, 60.7 mCi/mmol) dissolved in a Krebs-Ringer bicarbonate buffer fortified with 3% bovine serum albumin (Fraction V) and equilibrated with 95% O₂ and 5% CO₂ for 20 min at 32°C. The BP concentration was 2.8 × 10⁻⁷ M, and the rate of perfusion was 20 ml/hr/g testis. BP metabolites were extracted from both the perfusate and the testicular tissue and subjected to high-pressure liquid chromatographic analysis. Comparison of BP metabolites in control and TCDD-induced testes showed that the total amounts of BP metabolites recovered during the 60-min perfusion were 18.3 and 26.2% of total radioactivity, respectively. The pattern of BP metabolites in the control testis demonstrated that the major metabolites in the organic extractable phase were 9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene (52.1%) and 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene (16.3%). Smaller amounts of 3-hydroxybenzo(a)pyrene (9.2%), quinones (8.6%), and 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene (4.4%) were also present. This pattern of BP metabolites contrasts with that observed in rat livers or lungs, where phenols and quinones are the primary metabolites. TCDD increased BP metabolism with a concomitant increase in the testicular aryl hydrocarbon hydroxylase activity. The magnitude of various BP metabolites in TCDD-treated rats ranged from 1.5 to 2.8 times control, except 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene which was not altered by TCDD treatment. Thus, mixed-function oxidase(s) in rat testis preferentially epoxidate the 9,10 and 7,8 positions of BP. Since 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene is the precursor of the potent carcinogen, 9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene, the TCDD-induced shift in metabolic products could be significant in BP-induced mutagenic and/or carcinogenic effects.

INTRODUCTION

Current experimental evidence suggests that both cancer and mutation induced by PAH's are due to a metabolically activated arene oxide which reacts with the nucleophilic sites of DNA, RNA, and protein (8, 10–13, 17, 18, 24, 31). Therefore, the relative specific activities of PAH-metabolizing enzyme system(s) such as AHH (EC 1.14.14.2), EH (EC 4.4.9.7), and GSHT (EC 2.5.1.18) in both the liver and extrahaepatic tissues play an important role in both mutagenic and carcinogenic processes.

Previous studies of the activation and detoxification of foreign compounds have been focused primarily on the hepatic microsomal mixed monoxygenase system. However, there is little evidence that arene oxide(s) activated in the liver can escape the organ in sufficient quantities to affect extrahaepatic organs. Therefore, specific organ toxicity might be directly related to chemical activation and detoxification systems within the organs themselves. With respect to PAH-induced male gonadal toxicity, it has been shown that the treatment of male mice and rats with BP and dimethylnaphthanthracene produces a significant increase in early embryonic death in mice and premeiotic spermatogenic cell damage in rats (1, 5, 6). Genetic lesions which have been induced by BP treatment in male mice and which are significantly different among various mouse strains have been attributed to differences in DNA repair activity in germ cells (9).

We have reported previously that the transport characteristics of various chemicals and drugs across the blood-testis barrier resemble those across the blood-brain barrier and that transport rates correlated well with partition coefficients and molecular size (25). From these data, the transport rate constant for highly lipid-soluble BP is predicted to be 0.1 min⁻¹. We have also studied mixed-function oxidase(s) and cytochrome P-450 in male rat testis and reported the presence of AHH, EH, and GSHT which are involved in the activation and the detoxification of PAH's (23). Thus, locally activated electrophiles may exert significant toxic and mutagenic effects within both the interstitial and germ cell compartments. In vitro cell-free preparations, of course, do not accurately reflect the intact organs within the living organism. Therefore, in the present studies, the isolated perfused rat testis has been used to evaluate testicular metabolism of BP and to quantitate the spectrum of metabolites in control and TCDD-treated rats.

MATERIALS AND METHODS

Chemicals. [7,10-14C]BP (specific activity, 60.7 mCi/mmol; radiochemical purity, >99%) was obtained from Amersham/Searle Corp., Arlington Heights, Ill., and nonlabeled BP was purchased from Eastman Organic Chemicals, Rochester, N.Y. [7,10-14C]BP was purified by HPLC; nonlabeled BP was purified using alumina column chromatography followed by recrystallization in methanol. The purity of BP was greater than 99%. Chemically pure BP metabolites, 9,10-diol, 7,8-diol, 4,5-diol, 3,6-diol, 1,6-quione, benzo(a)pyrene 1,6-dione, 3,6-quinone, benzo(a)pyrene 3,6-dione, 6,12-quinone, benz(a)pyrene 6,12-dione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ACD, actinomycin D; CHD, cycloheximide.

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tone, ethyl acetate, and methanol) were purchased from Waters Associates, Milford, Mass. and Preiser Scientific, Durham, N.C. TCDD was obtained from the National Institute of Environmental Health Sciences Environmental Chemistry and Biology Branch. HEPES, ACD, CHD, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type XI), NADP+, and vitamin E were purchased from Sigma Chemical Company, St. Louis, Mo. Other chemicals were reagent grade and obtained from standard commercial sources.

Animals. Adult male CD Sprague-Dawley rats weighing 250 to 300 g were purchased from the Charles River Breeding Laboratories, Inc., Wilmington, Mass. Animals were maintained on NIH 31 sterilizable rodent diet with free access to water. TCDD Treatment. To study the effects of TCDD on BP metabolism, rats were pretreated by gavage with TCDD (10 μg/kg) 72 hr prior to sacrifice. Control animals received corn oil (0.01 ml/10 g body weight).

Preparation of Microsomes and Cytosolic Fractions. Animals were sacrificed by cervical dislocation. Testes were immediately excised, weighed, and homogenized with a Potter-Elvehjem homogenizer fitted with a Teflon pestle in 4 volumes of ice-cold 0.1 M KCl in 0.02 M HEPES (KCl:HEPES), pH 7.4, at 4°C. The homogenate was centrifuged for 15 min at 9,000 × g at 4°C. The supernatant was removed and centrifuged at 176,000 × g for 45 min to obtain microsomal and supernatant fractions. The microsomal pellet was resuspended in KCl:HEPES and centrifuged again. The washed microsomes were then suspended in KCl:HEPES using a small Potter-Elvehjem homogenizer. The protein concentration of microsomes and supernatant fractions was determined according to the method of Lowry et al. (21) using a bovine serum albumin (Fraction V) standard.

AHH Assay. The pH optimum for testicular AHH in vitro assay is broad between 7.2 and 7.8. The rate of 3-OH formation is linear up to 30 min of incubation time and 3 mg of testicular microsomal protein. AHH activity was assayed by measuring the fluorescence of alkali-extracted BP phenols with an Aminco-Bowman spectrofluorometer (32) following incubations in the dark for 30 min at 37°C in a Dubonff metabolic shaker. Enzyme activity was calculated using a standard curve of 3-OH. The fluorescence of 3-OH extracted from organic phase in 1.0 ml of 1 N NaOH was measured.

EH and GSHT Assays. The specific activities of EH and GSHT were determined in 176,000 × g microsomal and supernatant fractions of the testes. Both EH and GSHT activities were assayed using [4,5-oxide-3H]BP as a substrate; it had a specific activity of 10 mCi/mmol; radiochemical purity was greater than 99%. 4,5-Diol and GSHT-conjugated products were quantitated radiometrically (16, 23).

ACD and CHD Experiments. Animals were pretreated i.p. with ACD (1.5 mg/kg) or CHD (3 mg/kg) 30 min prior to TCDD treatment. Sixteen hr after treatment, the animals were sacrificed by cervical dislocation, and the specific activities of AHH, EH, and GSHT, as well as cytochrome P-450 content, were determined in each tissue. Preparation of Testis for Perfusion Studies. Adult rats weighing 250 to 300 g were sacrificed by cervical dislocation. The testes were immediately removed and placed in a pre-chilled beaker containing 0.15 M phosphate-buffered saline (pH 7.4). This procedure was necessary to prevent thrombi and to minimize cellular damage. The testis was placed under a stereomicroscope, and the testicular artery was cannulated through a small incision with a 80-μm-tip glass capillary which was secured with a silk suture. This procedure took approximately 5 min for each testis. The isolated testis was immediately placed in a perfusion chamber with a constant temperature of 32 ± 0.5°C (S.D.) which was designed and constructed at the National Institute of Environmental Health Sciences. The testis was perfused with an oxygenated Krebs-Ringer bicarbonate solution containing bovine serum albumin Fraction V (3%) and glucose (0.1%) at the rate of 20 ml/hr/g testis (an average blood flow rate for intact CD rats), using an Ismatec GJ-4, 13-channel precision pump. This procedure cleared blood from the testis and prevented thrombi formation without the use of anticoagulants. The perfusion pressure was maintained at 60 to 70 mm Hg, and the pH of the perfusion medium was controlled at 7.4 by exposing the medium to a gas mixture (95% O2 and 5% CO2). For the BP metabolism studies, the BP concentration ranged from 2.8 × 10−7 M to 3.9 × 10−8 M (ψmax).

In each experiment, 6 to 8 testes were perfused simultaneously in the dark, and the effluent was collected for 60 min from the testicular vein. In order to determine the spontaneous breakdown of [7, 10-14C]BP during perfusion of the isolated testis, the perfusate not passing through the testes was simultaneously collected for 60 min and subjected to HPLC analysis. The background radioactivities were associated with an unknown Peak D between 9,10- and 4,5-diol (200 dpm) and between 1,6-, 3,6-, and 6,12-quinone (1,600 dpm) (Chart 3). The background radioactivity profile obtained by HPLC was quantified and subtracted as background.

Isolation of Metabolites Contained in the Perfusion Medium. A stock solution of 80 mM vitamin E in 100% methanol was prepared weekly; the concentration of vitamin E was determined using a molar extinction coefficient of 3.06 × 103 M−1 cm−1 at 294 nm. The effluent in each fraction was added to 2 volumes of ethyl acetate:acetone (2:1, v/v) followed by 0.8 mwa vitamin E and 3.5 mM NaCl. The addition of vitamin E during the extraction procedure minimized spontaneous formation of quinones and did not interfere with HPLC analysis. BP metabolites were extracted 3 times through vigorous shaking with an organic solvent for 20 min for each extraction. The organic and water phases were separated by centrifugation at 10,000 × g for 10 min; the water phase was subjected to further extraction. The organic extracts were combined and dried over anhydrous Na2SO4. Pooled extracts were subsequently dried under a stream of nitrogen gas. The sample residue was dissolved in 0.1 ml of ethyl acetate for HPLC analysis.

Isolation of Metabolites Contained in the Testicular Tissue. The testicular tissue was first homogenized with a Polytron homogenizer for 5 min at 0° to 2°C in 4 volumes of ice-cold 0.15 M KCl in 0.02 M HEPES buffer (pH 7.4). Two volumes of ethyl acetate:acetone (2:1, v/v) were added to the homogenates and extracted 3 times with vigorous shaking. The organic and water phases were separated by centrifugation. The organic extracts were combined, dried over anhydrous Na2SO4, and transferred to a 50-ml conical centrifuge tube where the organic layer was further processed as described above.

HPLC. Chemically pure BP metabolites, 9,10-diol, 4,5-diol, 7,8-diol, 3-OH, 9-OH, 1,6-quinone, 3,6-quinone, 6,12-quinone, and BP dissolved in HPLC grade methanol were used to
establish their HPLC retention times (60 to 100% methanol gradient). The injection volume of both reference standards and samples was 30 μl. Analysis was performed on a Waters Associates Model ALC/GPC 204 liquid chromatograph with a 254-nm UV detector. Metabolites were separated into diol, quinone, and phenol fractions on a ODS-2 column (Whatman Partisol-10; particle size, 10 μm; column size, 25 cm x 4.5 mm) with a flow rate of 0.5 ml/min. The effluent was collected directly into scintillation vials using an Instrumentation Specialties Co. fraction collector with a stop-flow attachment. Each vial contained 10 ml of Aquasol. Radioactivity was determined with a Packard Model 2660 liquid scintillation spectrometer with an external standard for quench correction.

**Rate of Glucose Consumption and Testicular ATP Concentrations.** Aliquots of effluent collected at 15-min intervals for 90 min were used for enzymatic determination of glucose, and testicular ATP concentrations were measured to assess the metabolic integrity of the isolated perfused testis of control and TCDD-treated rats. The enzymatic assays were performed according to the methods of Bergmeyer (2).

**Statistical Methods.** Regression analysis and significant differences between control and treatment groups were determined using MINITAB II statistical computer program and PDP 11-70 and RSX-11M operating system.

**RESULTS**

**Chromatographic Properties of BP Reference Standards.** BP metabolite reference standards were separated by HPLC and detected by UV absorption. BP metabolites are identified by retention times of the reference standards. The HPLC procedure used is highly efficient for separation and quantitation of dihydrodiols, phenols, and quinones. Chart 1 shows the profile of BP metabolite reference standards separated by HPLC. The pattern demonstrates that 9,10-diol, 4,5-diol, 7,8-diol, benzo(a)pyrene, 4,5-quinone, 9-OH, 3-OH, and 1,6-quinone are readily separated; however, 3,6- and 6,12-quinones overlap. Unreacted BP is eluted last. Retention times of the reference standards were determined.

**In Vitro BP Metabolism by the Isolated Perfused Testis.** The rate of release of BP metabolite, 3-OH, into the effluent medium at varying times during the constant perfusion of 9.75 x 10^-7 M, 19.5 x 10^-7 M, and 39.0 x 10^-7 M BP is demonstrated in Chart 2. The rate of release of 3-OH metabolite into the effluent medium of 3 different concentrations of BP increases with time; thus, it indicates that the oxidative metabolism of BP is linear for a 60-min perfusion time period. From the slopes, the rates of 3-OH synthesis over the 60-min perfusion periods are estimated to be 1.4, 3.11, and 4.4 pmol/min/g testis, respectively. Analysis of the slopes (intercepts and regression analysis) of BP metabolism indicates that the approximate Km for the testicular mixed-function oxidase system in the intact testis is 2.7 x 10^-8 M. Times between the onset of perfusion and the release of 3-OH metabolites into the effluent perfusate were determined. The lower the concentration, the longer is the latent period. At BP concentrations of 9.57 x 10^-7 M, 19.5 x 10^-7 M, and 39 x 10^-7 M, the latent periods are approximately 9, 3, and 1 min, respectively. The lag period between the start of infusion and the appearance of 3-OH metabolite in the perfusate may be related to the time required for metabolite activation and transfer from the site of activation to the effluent medium.

**Comparison of BP Metabolites in the Perfusate and Testicular Tissues.** Chart 3 presents typical BP metabolite patterns in the perfusate and testicular tissues at 60 min. These BP metabolite profiles are very reproducible. The peaks of radioactivity are identified by cochromatography with synthetic BP standard metabolites. The initial peak (A) prior to elution of 9,10-diol has not been characterized. Four unidentified peaks (A to D) are shown in Chart 3. A and D peaks are found only in the perfusate, while unknown Peaks B and C are found in testicular tissues. All of the unknown peaks are not found in
Comparison of BP Metabolism in Control and TCDD-induced Rat Testes. Table 1 summarizes the pattern of BP metabolites in control and TCDD-induced testes. Five individual rat testes from control and TCDD-induced animals were perfused simultaneously. The patterns of BP metabolites in the control testes show that the major metabolites in the organic phase are 9,10-diol (52.1%) and 7,8-diol (16.3%) with less 3-OH (9.3%), 9-OH (9.2%), quinones (8.6%), and 4,5-diol (4.4%). The percentage calculation is based on the total metabolite concentration found in the organic phase being 100%. However, in TCDD-induced testes, the BP metabolite patterns were significantly different from controls. With the exception of 4,5-diol, the biosynthesis of each BP metabolite identified was significantly increased in TCDD-induced animals when compared to controls. The magnitude of increase was about 1.4- to 2.8-fold and is statistically significant in every case. Quinones, 3-OH, and 7,8-diol in TCDD-induced testes demonstrate 2.8-, 2.1-, and 1.8-fold increases, respectively. Thus, these data suggest that TCDD preferentially induces enzyme systems which form quinones, 3-OH derivatives, and 7,8-diols. The 9-OH and 9,10-diol metabolites are increased less. The 4,5-diol product is synthesized in low concentration (0.2% of the total radioactivity recovered at the end of 60 min of perfusion) and is unaffected by TCDD treatment.

As shown in Table 1, total BP metabolites formed in TCDD-induced testes are 19.3% of total (the total radioactivity recovered at the end of 60 min of perfusion) in the aqueous phase and 6.9% in the organic phase. In contrast, in the control testes, BP metabolites are 13.9% in the aqueous phase and 4.3% in the organic phase. Therefore, the percentage of total BP metabolites in TCDD-induced and control testes is 26.2 and 18.3%, respectively. Thus, TCDD-treated animals metabolized

Table 1

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>BP metabolites</th>
<th>TCDD-induced</th>
<th>Control</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/hr/g testis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Organic phase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-9,10-diol</td>
<td>1.1 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>(0.01)</td>
<td>2.2</td>
</tr>
<tr>
<td>9,10-diol</td>
<td>92.9 ± 3.6</td>
<td>66.9 ± 5.7</td>
<td>(2.3)</td>
<td>1.4</td>
</tr>
<tr>
<td>4,5-diol</td>
<td>6.4 ± 1.5</td>
<td>5.6 ± 0.7</td>
<td>(0.2)</td>
<td>1.1</td>
</tr>
<tr>
<td>7,8-diol</td>
<td>36.9 ± 3.7</td>
<td>21.0 ± 4.7</td>
<td>(0.7)</td>
<td>1.8</td>
</tr>
<tr>
<td>9-OH</td>
<td>17.5 ± 2.6</td>
<td>11.9 ± 2.7</td>
<td>(0.3)</td>
<td>1.5</td>
</tr>
<tr>
<td>3-OH</td>
<td>24.5 ± 5.1</td>
<td>12.0 ± 3.6</td>
<td>(0.4)</td>
<td>2.1</td>
</tr>
<tr>
<td>1,6-quinone</td>
<td>31.5 ± 2.3</td>
<td>11.1 ± 0.8</td>
<td>(0.3)</td>
<td>2.8</td>
</tr>
<tr>
<td>3,6-quinone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total metabolites</td>
<td>209.7 ± 18.9</td>
<td>128.3 ± 18.4</td>
<td>(4.3)</td>
<td>1.7</td>
</tr>
<tr>
<td>Unmetabolized BP</td>
<td>2,249.6 ± 286.2</td>
<td>2,415.7 ± 225.9</td>
<td>(81.7)</td>
<td>0.9</td>
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<tr>
<td><strong>Aqueous phase</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Total BP metabolites</td>
<td>587.3 ± 41.0</td>
<td>411.9 ± 44.1</td>
<td>(13.9)</td>
<td>1.4</td>
</tr>
<tr>
<td>Total radioactivity (recovered at end of 60 min of perfusion)</td>
<td>3,046.0 ± 356.0</td>
<td>2,955.5 ± 288.4</td>
<td>(100)</td>
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<table>
<thead>
<tr>
<th>Specific activity</th>
<th>pmol/min/mg microsomal protein</th>
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<tbody>
<tr>
<td>AHH</td>
<td>1.52 ± 0.23</td>
<td>1.03 ± 0.11</td>
</tr>
<tr>
<td>EH</td>
<td>880 ± 86</td>
<td>980 ± 85</td>
</tr>
<tr>
<td>GSH-T</td>
<td>18,500 ± 1,200</td>
<td>20,400 ± 1,600</td>
</tr>
</tbody>
</table>

a Mean ± S.D. from 5 separate experimental values with the exception of 4,5-diol (N = 3) combined from both the tissue and the effluent medium.
b Numbers in parentheses, percentage of total radioactivity recovered at the end of 60 min of perfusion.
c Significantly greater than control values (p < 0.05).
d Mean ± S.D. of 4 to 6 experiments.
1.5 times more BP than did control animals. Increased BP metabolism in TCDD-induced testes correlated well with the extent of AHH induction in rat testes (Table 1). BP metabolites in the aqueous and organic phases of TCDD-induced testes were 1.4 and 1.7 times greater than control. The extent of uncharacterized BP metabolites in the aqueous phase is about 3 times greater than that present in the organic phase in either induced or noninduced testes. The presence of BP metabolites in the aqueous phase is saved for later analysis. AHH activity in the induced testes was 1.5 times more than control, while EH and GSHT activities were unaltered by TCDD treatment.

Effects of ACD and CHD on TCDD-Induced AHH. To delineate whether TCDD induction of testicular AHH activity is attributed to de novo synthesis of AHH, testicular metabolism was examined after pretreatment with ACD and CHD. ACD is known to act at the transcriptional level to inhibit translation of mRNA, while CHD is understood to inhibit ribosomal protein synthesis in mammalian cells. ACD and CHD each blocked a significant induction of AHH (Table 2).

Comparison of BP Metabolites in the Effluent and Testicular Tissue of Control and TCDD-Treated Rats. Table 3 demonstrates the distribution of metabolites between the effluent perfusate and testicular tissues of both control and TCDD-induced testes. In the effluent of control testes, the BP metabolites, ranging from the highest to the lowest concentrations, are 9,10-diol, 7,8-diol, 9,10-diol, 9-OH, 3-OH, 9-OH, and 4,5-diol, respectively. In contrast, BP metabolites in testicular tissues from the highest to the lowest concentration are 9,10-diol, 7,8-diol, 9-OH, 3-OH, quinones, and 4,5-diol. These differences between BP metabolites in the effluent and in the testicular tissue compartment may reflect permeability differences in BP metabolites. In the effluent of TCDD-induced testes, all BP metabolites are significantly increased, with the exception of 4,5-diol. Quinones, 3-OH, 9-OH, 7,8-diol, and 9,10-diol are significantly increased and are 2.9, 2.8, 2.2, 1.9, and 1.4 times controls, respectively. However, in the tissue of TCDD-treated testes, only the formation of quinones and 9,10-diol was increased significantly. Although 7,8-diol, 9,10-diol, and 3-OH also show a 1.4-fold increase, these values are not statistically significant due to the greater standard deviation.

**DISCUSSION**

This paper reports the first application of the isolated perfused testis preparation for the study of testicular biotransformation of exogenous chemicals. The present data demonstrate that the isolated perfused testis is metabolically active for at least 60 min as judged by glucose uptake, cellular maintenance

Table 2
Effects of ACD and CHD on TCDD induction of testicular AHH
Animals were pretreated i.p. with ACD (1.5 mg/kg body weight) or CHD (3 mg/kg body weight) 30 min prior to TCDD treatment (10 μg/kg body weight p.o.). Sixteen hr after TCDD treatment, the animals were sacrificed, and AHH activity was determined as described in "Materials and Methods."

<table>
<thead>
<tr>
<th></th>
<th>Specific testicular AHH activity (pmol/min/mg microsomal protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.06 ± 0.11</td>
</tr>
<tr>
<td>ACD</td>
<td>0.88 ± 0.07</td>
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<tr>
<td>ACD + TCDD</td>
<td>0.91 ± 0.17</td>
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<tr>
<td>CHD</td>
<td>0.94 ± 0.04</td>
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<tr>
<td>CHD + TCDD</td>
<td>0.91 ± 0.09</td>
</tr>
<tr>
<td>TCDD</td>
<td>1.54 ± 0.17</td>
</tr>
</tbody>
</table>

* Mean ± S.D. from 4 to 5 experiments.

Table 3
Comparison of BP metabolites in the perfusate and the testicular tissue of control and TCDD-treated rats

<table>
<thead>
<tr>
<th>BP metabolites</th>
<th>TCDD</th>
<th>Control</th>
<th>Ratio</th>
<th>TCDD</th>
<th>Control</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>9,10-diol</td>
<td>53.6 ± 5.6</td>
<td>39.4 ± 0.7</td>
<td>1.4</td>
<td>37.4 ± 3.8</td>
<td>26.9 ± 4.5</td>
<td>1.4</td>
</tr>
<tr>
<td>4,5-diol</td>
<td>3.9 ± 0.4</td>
<td>3.1 ± 1.0</td>
<td>1.3</td>
<td>2.4 ± 1.1</td>
<td>2.3 ± 0.7</td>
<td>1.0</td>
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<tr>
<td>7,8-diol</td>
<td>17.2 ± 4.6</td>
<td>9.3 ± 3.4</td>
<td>1.9</td>
<td>18.3 ± 4.1</td>
<td>12.7 ± 4.1</td>
<td>1.4</td>
</tr>
<tr>
<td>9-OH</td>
<td>8.5 ± 1.6</td>
<td>3.9 ± 1.5</td>
<td>2.2</td>
<td>8.4 ± 2.2</td>
<td>7.5 ± 2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>3-OH</td>
<td>13.2 ± 3.8</td>
<td>4.7 ± 2.3</td>
<td>2.6</td>
<td>10.1 ± 1.9</td>
<td>7.1 ± 1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>1,6-quinone</td>
<td>23.1 ± 3.5</td>
<td>8.0 ± 1.9</td>
<td>2.9</td>
<td>8.1 ± 2.6</td>
<td>3.8 ± 0.6</td>
<td>2.1</td>
</tr>
<tr>
<td>3,6-quinone</td>
<td>21.7 ± 6.0</td>
<td>6.8 ± 10.8</td>
<td>2.2</td>
<td>84.6 ± 15.3</td>
<td>60.3 ± 14.1</td>
<td>1.4</td>
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<tr>
<td>Total metabolites</td>
<td>119.7 ± 19.5</td>
<td>68.3 ± 10.8</td>
<td>1.8</td>
<td>253.5 ± 45.1</td>
<td>217.4 ± 28.8</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Mean ± S.D. from 5 separate experimental values with the exception of 4,5-diol (N = 3).

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of testicular ATP levels, perfusate flow rate, and subcellular morphological studies. In addition, the isolated perfused testis actively metabolizes exogenous hydrocarbons and offers a convenient method for studying testicular absorption, distribution, biotransformation of chemicals, and factors modifying these processes. Perfusion of rabbit testis, either with whole blood or with Krebs-Ringer bicarbonate perfusate containing bovine serum albumin, for 60 min did not significantly alter testosterone secretion (7). Thus, short-term perfusion of the testis with an artificial medium does not appear to affect significantly testicular function.

The present study characterized the primary BP metabolites extractable in the organic phases of control and TCDD-induced rat testes using an isolated organ perfusion technique. In the isolated perfused rat testis, BP was metabolized at an overall rate of 0.54 ± 0.06 nmol/hr/g testis, which is approximately equivalent to 0.9 pmol total BP metabolites per min per mg testicular microsomal protein, considering that 1 g testis contains about 10 mg microsomal protein. This represents 18.3% of the total radioactivity recovered at the end of a 60-min perfusion in both the organic and the aqueous phases as well as in testicular tissue. In contrast, in the TCDD-induced testes, BP was metabolized at an overall rate of 0.8 ± 0.06 nmol/hr/g testis (approximately equivalent to 2.0 pmol/min/mg testicular microsomal protein) and represents 26.2% of total radioactivity recovered at the end of the 60-min perfusion. The rate of phenol formation in the isolated perfused testis was approximately 0.04 pmol/min/mg microsomal protein, in contrast to that in the cell-free testicular microsomes which was 1.5 pmol/min/mg microsomal protein. Thus, the phenol concentration in the organic phase of the isolated perfused testis was about 38 times lower than that of the cell-free system. These differences can probably be attributed to the presence and absence of conjugating enzymes in the intact testis and microsomal fractions, respectively.

The major testicular BP metabolites in the organic extractable phase of isolated perfused testis were 9,10- and 7,8-diol, followed by 3-OH and the quinones. Thus, in the CD rat testis, the 9,10 and 7,8 positions are preferentially epoxidated in contrast to the 4,5 position. Epoxidation reaction apparently exceeds that of other metabolic oxidation pathways. Similar preferential synthesis of 9,10- and 7,8-diol has been observed in the rat liver only after 3-methylcholanthrene treatment (34). In contrast to BP metabolism in the isolated testis, perfusion studies with isolated rat and rabbit lungs demonstrated that the major BP metabolites were phenolic and quinone derivatives of BP (27, 29, 30). Furthermore, the perfusion of isolated lungs from rats pretreated with 3-methylcholanthrene demonstrated that phenolic metabolites increased 7-fold while 7,8-diol production was unchanged (30).

When the BP pattern of metabolites formed by the TCDD-induced testes was compared to that of the control testes, TCDD-induced testes synthesized all BP metabolites at a higher rate with exception of 4,5-diol. Furthermore, several differences in the rate of BP metabolite formation were observed in the TCDD-induced testes. TCDD-induced testes synthesized quinones (15%), 3-OH (12%), and 7,8-diol (18%) at significantly higher rates, while 9,10-diol synthesis is decreased (from 52 to 44%).

The pre-9,10-diol and post-9,10-diol peaks have not been further characterized in the present study. However, because the pre-9,10-diol and post-9,10-diol peaks were absent in the perfusate simultaneously collected without passing through the testis, the unknown peaks are not likely spontaneous breakdown products of BP but instead probably represent metabolites. Furthermore, the pre-9,10-diol peak increased significantly following TCDD treatment, which further supports the hypothesis that pre-9,10-diol is metabolically formed.

Although the present data suggest that TCDD treatment preferentially induced enzyme systems following quinones, phenols, and 7,8-diol in rat testis, water-soluble metabolites in the aqueous phase should be characterized to determine the actual rate of BP metabolite synthesis in both control and TCDD-induced testes. Since 7,8-diol is the precursor for a most potent carcinogen, r,7-t-8-dihydroxy-r-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (14, 26, 28, 33), preferential synthesis of 7,8-diol in either noninduced or TCDD-induced testes might be important in BP-induced mutagenic and/or carcinogenic testicular effects.

Distribution pattern of BP metabolites between the perfusate effluent and the testicular tissue compartment of control and TCDD-induced testes suggests that quinones and 9,10-diol are lower in the tissues. Therefore, the data suggest that 9,10-diol and quinones move readily from the testis to the perfusate effluent as compared to the movement of 4,5-diol, 7,8-diol, 3-OH, and 9-OH. If the metabolic pathway leading to the formation of the diol-epoxide is the most important factor of PAH mutagenesis (3, 14, 19, 20, 22), male gonads may be highly susceptible to PAH-induced mutagenesis. Furthermore, if the venous return travels via the unique venous network of pampiniform plexus surrounding the internal spermatic artery and high levels of BP metabolites especially 7,8-diol-BP occur, then this may result in reabsorption of BP and BP metabolites into the internal spermatic artery. BP and its metabolites could continue to recycle between the testis and the pampiniform plexus, thus making germ cells even more vulnerable to carcinogenic BP metabolites.

In the present study, we have not determined whether TCDD affects other mixed-function oxidase-associated enzymes in the testis (e.g., 17α-hydroxylase and C17-20 lyase) for androgen...
biosynthesis which is vital for male germ cell differentiation. Since it has been suggested that interstitial cell tumors of testis are due to the differential presence of steroidogenic enzymes associated with mixed-function oxidase systems in Leydig cells of both rodents (15) and humans (4), it is tempting to speculate that environmental factors affecting differential activities of the testicular mixed-function oxidase system in male gonads may play an important role in genetic toxicity.

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