SUMMARY

Benzo(a)pyrene and five isomeric phenols of benzo(a)pyrene were tested for carcinogenicity by the topical application of 0.4 μg mole of each compound to the backs of C57BL/6J mice once every 2 weeks for 60 weeks. 1-, 3-, and 12-hydroxybenzo(a)pyrene were noncarcinogenic; 11-hydroxybenzo(a)pyrene was weakly carcinogenic, and 2-hydroxybenzo(a)pyrene and benzo(a)pyrene were highly carcinogenic. All of the mice treated with benzo(a)pyrene or 2-hydroxybenzo(a)pyrene had tumors after 53 weeks of treatment, whereas only 13% of the mice treated with 11-hydroxybenzo(a)pyrene had tumors after 60 weeks of treatment. The time required for 50% of the animals to develop tumors was 39 to 41 weeks for both benzo(a)pyrene and 2-hydroxybenzo(a)pyrene. Most of the tumors observed were squamous cell carcinomas. Since 4-, 5-, 6-, 7-, 8-, 9-, and 10-hydroxybenzo(a)pyrene were previously shown to be noncarcinogenic on mouse skin, our present results indicate that, among the 12 possible isomeric phenols of benzo(a)pyrene, only 2-hydroxybenzo(a)pyrene is a strong carcinogen and 11-hydroxybenzo(a)pyrene is weakly active. 2-Hydroxybenzo(a)pyrene is the first example of a phenolic derivative of a polycyclic aromatic hydrocarbon that possesses strong carcinogenic activity. Application of benzo(a)pyrene 11,12-oxide (a K-region oxide) to mouse skin under the same conditions as described above did not cause tumors. Previous studies have shown that benzo(a)pyrene 4,5-oxide (a K-region oxide) was weakly carcinogenic, benzo(a)pyrene 7,8-oxide was moderately carcinogenic, and benzo(a)pyrene 9,10-oxide was inactive.

INTRODUCTION

BP² (Chart 1) is a widespread, environmental pollutant and a potent carcinogen (5, 7) that is metabolized to a large number of metabolites by enzymes of the endoplasmic reticulum of various tissues. Some of these metabolites are arene oxides, phenols, quinones, dihydrodiols, dihydrodiol phenols, tetrads, and diol epoxides, but not all of the metabolites have been identified (6, 12, 13, 18, 28–31, 33, 34, 42). We have undertaken the testing of known and potential metabolites of BP for carcinogenicity and have previously reported on the strong carcinogenicity of (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene (15, 21), the moderate carcinogenicity of BP 7,8-oxide, the weak carcinogenicity of BP 4,5-oxide, and the noncarcinogenicity of BP 9,10-oxide (20) and 4-, 5-, 6-, 7-, 8-, 9-, and 10-HOBP (16) when applied chronically to mouse skin. More recently, other laboratories have reported on the activity of several of these hydrocarbons as initiators in a 2-stage mouse skin tumor model (4, 32). In the present study on the remaining 5 isomeric BP phenols and BP 11,12-oxide, we have found that 2-HOBP and BP are strong carcinogens and that 11-HOBP is weakly carcinogenic, whereas BP 11,12-oxide and 1-, 3-, and 12-HOBP are not carcinogenic under our test conditions.

MATERIALS AND METHODS

Chemicals. BP was purchased from Sigma Chemical Co., St. Louis, Mo. Procedures for the synthesis of 1-, 2-, 3-, 11-, and 12-HOBP (41) and BP 11,12-oxide³ have been described. All compounds used in the study were of analytical purity and were stored, handled, and checked for stability as previously described (16).

Animals. Female C57BL/6J mice, 6 weeks old, were obtained from The Jackson Laboratory, Bar Harbor, Maine. The mice were fed Purina laboratory chow (Ralston-Purina Co., St. Louis, Mo.) and water ad libitum. At 9 weeks of age and 24 hr before application of the chemicals, the mice were placed under light ether anesthesia, and their backs were shaved with electric clippers. Thereafter, the mice were shaved only when necessary. In 1 study, BP and BP phenols were dissolved in acetone at a concentration of 0.4 μg/mole/25 μl. In a 2nd study, BP and BP 11,12-oxide were dissolved in acetone:NH₄OH (1000:1) at a concentration of 0.1 and 0.4 μg/mole/25 μl. Twenty-five μl of the solutions were applied topically to the mice every 2 weeks for 60 weeks. Control mice were treated with 25 μl of acetone. The chemicals were applied under subdued light, and the mice were kept in the dark for 24 hr after each application.

Thirty mice were used in each treatment group. The presence of skin tumors was recorded every 3 weeks beginning with the 29th week. Tumors greater than 2 mm in diameter...
that persisted for 3 weeks or more were included in the cumulative total. During the course of the experiment, selected animals with large tumors were killed, and their skin and internal organs were subjected to histopathological examination. The study was terminated 60 weeks after its initiation, and the remaining animals were killed for histopathological examination.

**RESULTS**

2-HOBP was equipotent to BP in causing skin tumors, 11-HOBP was weakly active, and 1-, 3-, and 12-HOBP were inactive when 0.4 μmole of each compound was applied to mouse skin once every 2 weeks for 60 weeks (Table 1; Charts 2 and 3). All of the mice treated with BP or 2-HOBP had tumors after 53 weeks of treatment, whereas only 13% of the mice treated with 11-HOBP had tumors after 60 weeks of treatment. The time required for 50% of the animals to develop tumors was 39 to 41 weeks for both the BP- and 2-HOBP-treated animals. The 1st tumors in the BP and 2-HOBP groups appeared at 29 and 32 weeks, respectively. The total numbers of skin tumors observed in mice treated with BP, 2-HOBP, and 11-HOBP for 60 weeks were 32, 37, and 4, respectively (Table 1). Histological examination of

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**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose* (μmole)</th>
<th>Total no. of animals† alive at 60 wk</th>
<th>No. of animals with skin tumors</th>
<th>Total no. of skin tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.4</td>
<td>28</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>BP</td>
<td>0.4</td>
<td>25</td>
<td>13</td>
<td>25</td>
</tr>
<tr>
<td>1-HOBP</td>
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<td>25</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>2-HOBP</td>
<td>0.4</td>
<td>29</td>
<td>11</td>
<td>29</td>
</tr>
<tr>
<td>3-HOBP</td>
<td>0.4</td>
<td>29</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>11-HOBP</td>
<td>0.4</td>
<td>28</td>
<td>28</td>
<td>4</td>
</tr>
<tr>
<td>12-HOBP</td>
<td>0.4</td>
<td>23</td>
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<td>0</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP</td>
<td>0.1</td>
<td>27</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>BP</td>
<td>0.4</td>
<td>30</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>BP 11,12-oxide</td>
<td>0.1</td>
<td>27</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>BP 11,12-oxide</td>
<td>0.4</td>
<td>28</td>
<td>28</td>
<td>0</td>
</tr>
</tbody>
</table>

* The solvent in Experiment 1 was acetone, whereas that in Experiment 2 was acetone:NH₄OH (1000:1).
* The dose was applied to the skin once every 2 weeks for 60 weeks.
* Total number of mice alive at the end of the study plus mice with skin tumors that died earlier or were killed for autopsy.

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Chart 2. Tumor incidence of C57BL/6J mice treated topically with 0.4 μmole of BP, 2-HOBP, or 11-HOBP in 25 μl of acetone once every 2 weeks for 60 weeks. The percentage of animals with tumors was calculated from surviving nontumorous and tumor-bearing mice and from tumor-bearing mice that died during the 60 weeks of treatment.

Chart 3. Cumulative tumor development in C57BL/6J mice treated topically with BP, 2-HOBP, or 11-HOBP as described in the legend to Chart 2. Data are expressed as cumulative number of tumors observed in tumor-bearing mice.
the tumors revealed that the majority of the tumors present at 60 weeks were squamous cell carcinomas; this included 14 of 20 histologically examined tumors in the 2-HOBP-treated group and 3 of 4 tumors in the 11-HOBP-treated group.

In another study, BP 11,12-oxide was inactive when 0.1 or 0.4 µmole of this compound was applied to mouse skin once every 2 weeks for 60 weeks (Table 1). In this experiment 97 to 100% of the animals treated with 0.1 or 0.4 µmole of BP every 2 weeks had tumors at 60 weeks. Since the lack of carcinogenicity of BP 11,12-oxide could be caused by the instability of this compound, we examined the stability of tritiated BP 11,12-oxide in a solution containing 3% acetone in 0.1 M potassium phosphate buffer (pH 7.4) at 37°. The concentration of BP 11,12-oxide was 0.2 µmole/ml. A 100-µl aliquot of the incubation mixture was extracted with 100 µl of ethyl acetate and chromatographed on Gelman ITLC-SA sheets (Gelman Instrument Co., Ann Arbor, Mich.) with 5% triethylamine in ethyl acetate:chloroform:benzene (1:1:1) as the developing solvent. The half-life determined by the disappearance of BP 11,12-oxide was 15 min. Under similar incubation conditions, BP 7,8-oxide, which is moderately carcinogenic, had a half-life of 30 min, and BP 4,5-oxide, which is only weakly carcinogenic, had a half-life of greater than 24 hr (20). Therefore, the noncarcinogenicity of BP 11,12-oxide does not appear to be attributable to its short half-life.

**DISCUSSION**

This study indicates that 2-HOBP is highly carcinogenic to mouse skin and that 11-HOBP is a very weak carcinogen. In contrast, all of the other 10 monophenols of BP were noncarcinogenic on mouse skin (Ref. 16; Table 1) when tested at a dose level that caused a 100% tumor incidence with BP as the carcinogen. Although the data in Charts 2 and 3 indicate that BP and 2-HOBP have equal carcinogenic activity, a high dose (0.4 µmole/application) was used for each compound (a study with lower doses of each compound is underway to evaluate the comparative carcinogenic activities of BP and 2-HOBP). 2-HOBP is the 1st example of a phenolic polycyclic aromatic hydrocarbon with strong carcinogenic activity. Application of 2-HOBP or 9-HOBP to mouse skin was recently found to cause marked epidermal hyperplasia that resembled that caused by tumor promoters, but the other 10 isomeric BP phenols were either less active or completely inactive (3). BP was less active than 2-HOBP or 9-HOBP in causing epidermal hyperplasia (3). The ability of 2-HOBP to cause marked skin hyperplasia may have contributed to the strong carcinogenicity of this phenol. The inactivity of 9-HOBP as a skin carcinogen and its strong promoter-like activity suggest that 9-HOBP lacks initiating activity. In other studies 3-HOBP was weakly, if at all, carcinogenic when applied topically to or injected s.c. into the mouse (8), and 11-HOBP was inactive on s.c. injection (10).

Earlier studies (20) from our laboratories indicated that BP 7,8-oxide is moderately carcinogenic to mouse skin, whereas BP 4,5-oxide (a K-region oxide) is a poor carcinogen and BP 9,10-oxide is not carcinogenic. The present study indicates that BP 11,12-oxide (a K-region oxide) is noncarcinogenic on mouse skin at a dose level that causes a 100% tumor incidence with BP as the carcinogen (Table 1). Therefore, benzo(a)pyrene 1,2-oxide and benzo(a)pyrene 2,3-oxide remain as the only possible metabolic arene oxides of BP that have not been tested for carcinogenicity. Although these 2 arene oxides are likely precursors for 1- and 3-HOBP, direct evidence for their formation has not been forthcoming. The high instability expected for these arene oxides may make such evidence difficult to obtain.

Earlier studies (36) on the intrinsic mutagenicity of 2-HOBP and 11-HOBP in Salmonella typhimurium and in Chinese hamster V-79 cells indicated that both compounds were inactive. Incubation of 2-HOBP with a highly purified hepatic cytochrome P-448 monooxygenase system from 3-methylcholanthrene-treated rats indicated metabolism of 2-HOBP to compound(s) that were mutagenic to S. typhimurium strain TA 98 (39). With similar incubation conditions, BP was metabolically activated to a greater extent than was 2-HOBP, whereas 11-HOBP was not metabolized to mutagens (39). With procedures described by Ames et al. (1), the metabolic activation of 2-HOBP by a postmitochondrial liver supernatant fraction or by liver microsomes from Aroclor-pretreated rats revealed that 2-HOBP was metabolized to mutagens more effectively than was BP (unpublished observations). Under these incubation conditions, 11-HOBP did not undergo metabolic activation.

The results obtained from in vitro and in vivo binding (2, 9, 11, 14, 17, 23, 24, 27, 31, 35), mutagenicity (13, 22, 26, 37, 38, 39, 40), metabolism (13, 33, 34, 42), and carcinogenicity studies (15, 21) have led to the conclusion that 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene are ultimate carcinogenic metabolites of BP. Interestingly, hydroxyl groups at the 2- or 11-positions of either or both stereoisomers of trans-9,10-dihydroxy-7,8-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrenes would serve to enhance chemical reactivity of the oxirane ring. However, it cannot be assumed that diol-epoxide derivatives of 2- and 11-HOBP are the ultimate carcinogenic forms of 2- and 11-HOBP. A BP phenol would be expected to have different substrate properties with respect to monooxygenase enzymes when compared to BP. Also, oxidative metabolites of a BP phenol would have different chemical properties (reactivity toward nucleophiles, etc.). Studies on the metabolism of BP by liver indicate the formation of 1-, 3-, 6-, 7-, and 9-HOBP (6, 19, 25, 28), but 2-HOBP has not been detected. In addition, the identification of the phenols formed in skin by the metabolism of BP has not been attempted. The results of our studies that indicate that 2-HOBP is a strong carcinogen suggest a need for additional research to determine whether or not 2-HOBP is formed from BP in skin or in other tissues and whether or not 2-HOBP contributes to the carcinogenic action of BP.

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High Carcinogenicity of 2-Hydroxybenzo(a)pyrene on Mouse Skin


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