The Role of Aryl Hydrocarbon Hydroxylase in 7,12-Dimethylbenz(a)anthracene Skin Tumorigenesis: On the Mechanism of 7,8-Benzoflavone Inhibition of Tumorigenesis

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SUMMARY

Aryl hydrocarbon hydroxylase of mouse skin is inducible by benz(a)anthracene or 7,12-dimethylbenz(a)anthracene (DMBA) and is inhibited by 7,8-benzoflavone. 7,8-Benzoflavone inhibits the formation of covalently bound complexes of DMBA with DNA, RNA, and protein and also inhibits tumor formation caused either by a single application of DMBA, followed by croton oil treatment, or by the repeated application of DMBA. These results indicate that DMBA requires metabolic activation by aryl hydrocarbon hydroxylase for its carcinogenic activity. The inhibitory effect of 7,8-benzoflavone is limited to its application within 12 hr of administration of the DMBA, suggesting that the metabolic activation of DMBA is completed within 12 hr. The level of aryl hydrocarbon hydroxylase and DMBA tumorigenesis varies markedly in different mouse strains, indicating either that there are genetic differences in the profile of DMBA metabolite formation or that metabolic activation is necessary but not sufficient for DMBA tumorigenesis.

INTRODUCTION

Polycyclic hydrocarbons are metabolized by mixed-function oxidases localized in the microsomes of mammalian cells. The microsomal complex also metabolizes steroids and chemicals of exogenous origin, such as drugs, pesticides, and preservatives (10, 17, 23). The enzyme system AHH1 is part of the microsomal complex and converts polycyclic hydrocarbons to phenols, dihydrodiols, and epoxides (4, 5, 10, 30, 31, 47-50). The enzyme system is present and inducible in vivo in most of the tissues of several mammalian species (11, 44, 52) and in a variety of cells grown in culture (42—44). Although the enzyme complex clearly functions in many cases as a detoxification system (10, 17, 41), we have suggested that this enzyme system is also responsible for polycyclic hydrocarbon activation to toxic and carcinogenic metabolites (18-22). This hypothesis is supported by the following evidence. The amount of enzyme in a variety of mammalian cells is positively correlated with their susceptibility to BP cytotoxicity (1, 14, 20). The enzyme system catalyzes the formation of covalently bound complexes of hydrocarbon with DNA (18, 19, 24) and protein (24). A powerful inhibitor of the enzyme 7,8-BF (59) prevents polycyclic hydrocarbon cytotoxicity (13) and inhibits DMBA tumorigenesis in mouse skin (22); the microsomal complex catalyzes the formation of epoxides of naphthalene (31) and dibenz(a,c)anthracene (47), the latter epoxide being a more potent transforming agent in vitro than the parent hydrocarbon (25).

In this paper, we explore the role of the enzyme system in DMBA skin tumorigenesis by examining the mechanism of tumorigenesis inhibition by 7,8-BF. We have studied the effects of 7,8-BF on (a) DMBA- and 7-OHMe-12-MBA-induced skin tumor formation, (b) mouse skin AHH, (c) the disappearance of DMBA from mouse skin, and (d) the binding of DMBA to the macromolecules of mouse skin epidermis. We have also studied the time required for the metabolic activation stage of DMBA tumorigenesis and the relationship between enzyme level and tumor susceptibility in different mouse strains.

MATERIALS AND METHODS

Animals. Random-bred NIH general purpose Swiss male mice weighing 18 to 22 g were most often used. In specific experiments, certain inbred strains were used, as indicated.

Chemicals. DMBA and BP were obtained from Eastman Organic Chemicals, Rochester, N. Y.; 7,8-BF and the other flavones, 5,6-BF, apigenin, and nobiletin, were purchased from Aldrich Chemical Co., Inc., Cedar Knolls, N.J. BA was obtained from K and K Laboratories, Inc., Plainview, N. Y. 7-OHMe-12-MBA was a generous gift from Dr. Charles Huggins and Dr. John Pataki. Each compound was recrystallized from 95% ethanol before use. DMBA-14C (6.53 mCi/mmole) and BA-3H (750 mCi/mmole) were obtained from Amer sham/Searle Corp., Arlington Heights, 111. 7,8-BF-3 H (815 mCi/mmole) was prepared by Nuclear-Chicago Corp., Des Plaines, Ill., by tritium exchange. All radiochemicals were purified by thin-layer chromatography.

Tumorigenesis Experiments. Male mice weighing 18 to 22 g were shaved with electric clippers 1 day before the chemicals were applied. Thirty mice were used in each group in the tumorigenesis experiments. DMBA, generally 100 m/moles in 0.2 ml of acetone, was applied to the backs of the cleanly shaven mice. The other compounds were dissolved either in
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the DMBA solution or in 0.2 ml acetone and were applied at the times indicated. Two different types of tumorigenesis experiments were performed. In 1 type, the carcinogen, with or without the inhibitor, was applied just 1 time. This single treatment was followed by a weekly application of 1% croton oil. In the 2nd type of experiment, the carcinogen and inhibitor were applied 2 times/week for the duration of the experiment. Papilloma formation was recorded weekly and is shown for the indicated times.

The Disappearance of Radioactive DMBA and 7,8-BF from Mouse Skin. Three mice were used in each experiment. The isotope-labeled chemical was applied in a manner identical to that used in the tumorigenesis experiment. The mice were sacrificed at Hr 0, 2, 4, 8, 16, and 24 after the single application of the chemical. The skin was removed and placed in cold 0.25 M sucrose:0.05 M Tris buffer, pH 7.5, and the epidermis and dermis containing the hair follicles and sebaceous glands were scraped from the whole skin with a surgical scalpel. The material scraped from 3 mice was homogenized in 1.5 ml sucrose:Tris buffer with 30 strokes, in a Potter-Elvehjem glass-glass homogenizer. One hundred μl of the homogenate were placed in 1 ml of NCS (Amersham/Searle) for 1 hr at room temperature, and radioactivity was measured in 10 ml of toluene solution of LiquiFlour scintillator (New England Nuclear, Boston, Mass.). The protein content of the homogenate was measured by a modification of the method of Lowry et al. (38), with RNase A as standard.

Enzyme Assay. The skin homogenate was obtained as described above and was the enzyme source for the AHH assay (42). The reaction mixture, in a total volume of 1 ml, contained 50 μmoles of Tris-chloride buffer, pH 7.5; 0.5 mg of NADPH; 3 μmoles of MgCl₂; 0.20 ml of skin homogenate (usually containing 1 to 3 mg of protein); and 100 μmole of BP (added in 0.040 ml of methanol just prior to incubation). The mixture was incubated with gentle shaking at 37° for 20 min in air. The incubation mixture was extracted with 4 ml of a mixture of hexane:acetone (3:1) by shaking at 37° for 10 min. A 1.0-ml aliquot of the organic phase was extracted with 2.0 ml of 1.0 N NaOH. The concentration of extracted phenolic derivatives in the alkali phase was determined spectrophotometrically with activation at 396 nm and fluorescence at 522 nm in an Aminco-Bowman spectrophotometer. The amount of enzyme is defined as the pmole of phenolic product equivalent to 3-hydroxybenzo(a)pyrene formed during the incubation. Enzyme activity was determined in duplicate and compared with a blank control flask to which acetone had been added prior to incubation.

Preparation of DNA, RNA, and Protein from Mouse Skin. Isolation and purification of nucleic acids and protein was carried out according to the procedures of Diamond et al. (12) and of Colburn and Boutwell (9), which were modifications of the methods of Kirby et al. (33, 35-37). The skins of 5 mice were scraped as described above, combined, and homogenized with 30 strokes in 5 ml of 6% sodium p-aminosalicylic acid in a tight-fitting Potter-Elvehjem glass-glass homogenizer. A 10% sodium dodecyl sulfate solution (0.5 ml) was added, and the mixture was shaken for 1 hr. The resulting highly viscous solution was extracted with an equal volume of a phenol:m-cresol:8-hydroxyquinoline:water mixture (500:70:0.5:55, by weight).

DNA was precipitated from the aqueous layer by the addition of an equal volume of cold 2-ethoxyethanol. DNA was removed on a glass rod, and 2 volumes of cold ethanol were added to the remaining aqueous phase to precipitate RNA. The phenol layer was extracted once again with 6% sodium p-aminosalicylic acid and then was added to excess methanol, which precipitated the protein. The DNA was dissolved in 2 ml of 0.001 M K₂HPO₄ buffer (pH 7.0) and incubated at 37° for 15 min with RNase, again extracted with the phenol reagent, and precipitated as before with 2-ethoxyethanol. Carbohydrates were removed by the extraction of DNA dissolved in 1.25 M K₂HPO₄ with 2-methoxyethanol. The DNA was precipitated from the upper layer by the addition of 0.5 volume of 1% cetyltrimethylammonium bromide and was converted back to the sodium salt with 70% aqueous ethanol containing 2% sodium acetate. The DNA was washed with ethanol, then washed with ether, and dried. The RNA was freed of carbohydrates by the same procedure used for the DNA. The protein was washed 4 times with large volumes of methanol and 1 time with acetone, and then was washed with ether and dried.

Radioactivity Assays. The concentration of nucleic acid dissolved in 0.001 M K₂HPO₄ (pH 7.0) was calculated from the absorbance at 260 nm, by assuming that, at this wavelength, \( E^{1%}_{\text{em}} = 200 \) for DNA and 250 for RNA. The protein was dissolved in 200 μl of 0.5 N NaOH at 90° for 15 min, and 20 μl of the solution were to measure the concentration. A Beckman Model LS-100 liquid scintillation system was used for measuring radioactivity. One hundred to 150 μl of aqueous solution of sample were treated with 1 ml of NCS, and the clear solution obtained was counted in 10 ml of toluene solution of LiquiFlour scintillator. Quenching was corrected by use of automatic external standard ratios.

The extent of binding of DMBA was calculated from the specific activity of the isolated protein and nucleic acids. We derived the concentrations of nucleic acid in the solutions assayed for radioactivity from their absorbances by taking a value of \( E_p \) (extinction/mole phosphorus) = 6900 for DNA and \( E_p = 8600 \) for RNA.

RESULTS

Kinetics of AHH Induction in Mouse Skin Epidermis. Chart 1 shows the kinetics of AHH induction after the topical application of 100 nmoles each of BA, DMBA, and 7,8-BF to the backs of mice. BA showed the greatest inducing activity, DMBA exhibited somewhat less activity, and 7,8-BF was not an inducer. BA caused a slight rise in enzyme activity at 2 hr, and the level of enzyme at 4 and 8 hr was 5- and 8-fold greater, respectively, than in the controls. At 16 and 24 hr, the enzyme level had dropped considerably. The rapid decrease in enzyme level is probably due to a relatively short half-life of the enzyme and to the low level of inducer applied. In other studies in tissue cultures, we reported the half-life of the enzyme to be approximately 4 hr (43). In different experiments with BA applied at much higher concentrations,
The enzyme level was maintained at a high level for longer periods of time. The DMBA also exhibited inducing activity but was not as effective as the BA. At higher concentrations of DMBA, cytotoxicity was observed, and the relatively weaker inducing activity of DMBA may be due in part to its cytotoxic effects. This may also be the explanation for the lower inducing activity of BA in combination with DMBA. With the concentrations used, 7,8-BF shows no inducing activity. In other experiments at relatively higher doses of 7,8-BF given i.p., some induction was observed (22). 7,8-BF had little effect on the induction of the enzyme by DMBA.

Chart 1 shows the level of total enzyme induced in the tissue by topical application of several compounds. The in vitro assay, however, does not indicate to what extent each compound may alter the activity of a given amount of enzyme in vivo. Rather, the assay reflects the maximum amount of enzyme present. This is due to the fact that the AHH assay is performed in the presence of a large excess of BP, the enzyme substrate. Under these conditions, the small residual amounts of BA or 7,8-BF that may be extracted with the tissue probably will not significantly reduce or alter enzyme activity (Table 1).

Residual Concentration of 7,8-BF-3H in Assay Mixture after Topical Application to Mouse Skin. Table 1 shows the amount of 7,8-BF or of its metabolites that was present in the assay mixture of skin homogenates prepared at various times after the application of 100 nmoles of 7,8-BF-3H. A comparison with data shown in Table 2 indicates that the residual concentration is considerably lower than that necessary to interfere significantly with the assay of AHH in mouse skin, i.e., when BP is present at 10^{-4} M, as in our assay. 7,8-BF is an effective inhibitor only at 10^{-8} M (or greater) concentration.

Thus, values given in Chart 1 indicate the level of enzyme present in vivo but do not reflect any inhibitory effect of 7,8-BF that occurs in vivo.

The Inhibitory Effect of 7,8-BF on AHH in Vitro. In previous studies (22, 59), we reported that 7,8-BF is a potent inhibitor of AHH. The inhibitory effect of various concentrations of 7,8-BF and BA on control and DMBA-induced mouse skin aryl hydrocarbon hydroxylase activity in vitro is shown in Table 2. 7,8-BF has a marked inhibitory activity on the enzyme preparation derived from skin of either control mice or BA-pretreated mice with high induced levels of AHH. Thus, 7,8-BF at equivalent concentrations to the substrate inhibits hydroxylation by 73 to 88%. 7,8-BF at one-tenth the substrate concentration inhibits hydroxylation by 33 to 53%. BA, on the other hand, was relatively inactive as an inhibitor of the enzyme. In the tumor experiments reported later, the DMBA and 7,8-BF were
applied at equimolar concentrations. It is reasonable to conclude from the above data that, in the tumor experiments, the 7,8-BF was an effective inhibitor of AHH in vivo. This conclusion assumes that the transport of 7,8-BF and DMBA and their concentration at the enzyme site are not too dissimilar. This assumption is supported by the disappearance curves of the 2 compounds reported below.

The Disappearance of Radioactive DMBA, 7,8-BF, and BA from Mouse Skin. One possible mechanism of inhibition of DMBA tumorigenesis by 7,8-BF may be related to a more rapid rate of removal of the carcinogen from the skin. Thus, a reduced period of exposure of the tissue to the carcinogen might cause an apparent inhibition of tumorigenesis. This, however, is not the case, as is shown in Chart 2. Both BA (Chart 2B) and 7,8-BF (Chart 2A) inhibit rather than accelerate the removal of DMBA-14C from the skin. Both 7,8-BF and BA exert their greatest inhibitory effect on DMBA disappearance at Hr 2 and 4 after their simultaneous application with DMBA-14C. This may be due to a competition for receptor sites only at the higher concentrations of compounds present at Hr 0, 2, and 4, a competition that is diminished at lower concentrations of DMBA. Chart 2 also shows that DMBA has an inhibitory effect on the disappearance of either BA (Chart 2B) or 7,8-BF (Chart 2A). Thus, the data suggest that both BA and 7,8-BF may compete with DMBA for the enzyme site or other cellular binding sites. Since BA and 7,8-BF have similar effects on DMBA disappearance, this parameter is apparently irrelevant in the inhibition of tumorigenesis by 7,8-BF.

Kinetics of the Binding of DMBA to Skin Proteins and Nucleic Acids. The formation of covalently bound hydrocarbons with proteins (27, 28, 39) and nucleic acids (7) is reported to be implicated in the carcinogenic process. Chart 3 shows the kinetics of binding of DMBA to mouse skin protein, DNA, and RNA. With application of the low dose of DMBA, the maximum binding of DMBA to protein was observed at Hr 6 and the maximum binding of DMBA to DNA and RNA was observed at Hr 12. At Hr 24, the extent of binding was about one-half that observed at Hr 12. The kinetics of disappearance of the bound carcinogen also suggests a possible biphasic type of loss, with a fast rate of disappearance at Hr 12 to 24, followed by a slower phase at Hr 48 to 72.

The Effect of 7,8-BF and BA on the Binding of DMBA to DNA, RNA, and Protein of Mouse Skin (Table 3). The simultaneous application of 7,8-BF with DMBA caused a diminution in the amount of DMBA bound to all 3 macromolecule species measured 8 and 24 hr later. At Hr 8, the inhibition of binding by 7,8-BF was 71, 52, and 51%, respectively, to DNA, RNA, and protein. At Hr 24, the inhibition was about 50% for protein and 65% for DNA and RNA. Eight to 24 hr after the simultaneous administration of BA with DMBA, there was an approximate 30% decrease in the amount of DMBA bound to DNA; a 50 to 60% decrease in

Chart 2. A, disappearance of DMBA-14C and 7,8 BF-3H from mouse skin. One hundred nmoles of each compound were applied topically to the backs of mice. Animals were sacrificed at the times indicated. The experimental procedure is described in "Materials and Methods." 100%, radioactivity/mg of protein found in homogenates of skin at 0 time after application of the labeled compounds. The recovery of labeled compounds found in whole homogenate of skin at 0 time is 5.4 ± 1.8 nmoles. •, DMBA-14C in the absence of 7,8-BF; o, DMBA-14C in the presence of 7,8-BF; •, 7,8-BF-3H in the absence of DMBA. B, disappearance of DMBA-14C and BA-3H from mouse skin. •, DMBA-14C; o, DMBA-14C in the presence of BA•, BA-3H; o, BA-3H in the presence of DMBA.
RNA bound DMBA, and very little effect on the amount of DMBA bound to protein. These data suggest that 7,8-BF may exert its tumorigenesis-inhibitory action by reducing the amount of DMBA bound to macromolecules. These results and those in Table 3 showing the inhibitory effect of 7,8-BF on hydroxylase activity suggest that the formation of covalently linked DMBA with protein, DNA, or RNA depends on the activation of DMBA by the AHH system.

The Effect of 7,8-BF and BA on DMBA-initiated Mouse Skin Tumorigenesis. Table 4 shows 2 representative experiments in which we measured the effect of BA and 7,8-BF on tumorigenesis initiated by a single application of DMBA followed by weekly croton oil treatment. When DMBA was applied 1 time only, both BA and 7,8-BF inhibited the appearance of tumors, as seen by the tumor count at Week 10. Thus, at this earlier period of the experiment, the 7,8-BF suppressed tumor formation by 80%, while BA caused a 66% suppression of tumor formation. At Week 16, however, the 7,8-BF treated mice continued to show suppressed tumor formation, and this inhibition by 7,8-BF was observed throughout the lifetime of the mice or at the time the experiment was terminated. The simultaneous application of BA with DMBA, however, did not significantly effect tumor incidence at Week 16. Table 4 (Experiment 3) shows that, in a similar experiment in which the tumor count is shown at 20 weeks, the 7,8-BF-inhibited DMBA initiated tumor formation by 74%. In this experiment, BA as well as the flavone analogs, apigenin and nobiletin, failed to inhibit tumor formation. These analogs of 7,8-BF have moderate hydroxylase inhibitory activity in vitro (unpublished observations) but are not as effective as 7,8-BF. Their failure to inhibit tumorigenesis may be a function of an inability to be transported to the enzyme site in the tumorigenesis experiment. Although BA has been reported to be noncarcinogenic in the mouse skin system (26), others found that it shows weak tumor-initiating activity (51). In our system, BA could increase the tumor yield by acting as an initiator. However, Table 4, Experiment 3, shows that this was not the case. At a dose 20 times that used in the tumorigenesis experiments, the BA activity as an initiator was insignificant with respect to the interpretation of our experiments. The same was true with 7,8-BF. Possible inhibitor-induced alterations in systemic DMBA metabolism can also be ruled out as a factor in these experiments, since the small dose of DMBA that we applied topically, when given i.p., produced no skin tumors which indicated that its action was a direct result of its topical application and that the carcinogen was not systemically recirculated to the skin to any significant degree.

The Effect of 7,8-BF and BA on Tumorigenesis Induced by the Repeated Application of DMBA (Table 5). 7,8-BF is also an effective and potent inhibitor of tumorigenesis, induced by the repeated application of DMBA. Thus, Table 5 shows that tumor formation was suppressed 74% by 7,8-BF. The 7,8-BF

Table 3

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Hr 8 DNA (µmol/mole P)</th>
<th>RNA (µmol/mole P)</th>
<th>Protein (µmol/100 g)</th>
<th>Hr 24 DNA (µmol/mole P)</th>
<th>RNA (µmol/mole P)</th>
<th>Protein (µmol/100 g)</th>
</tr>
</thead>
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<tr>
<td>DMBA</td>
<td>4.1 (100)</td>
<td>4.2 (100)</td>
<td>7.7 (100)</td>
<td>3.9 (100)</td>
<td>2.5 (100)</td>
<td>4.7 (100)</td>
</tr>
<tr>
<td>DMBA + 7,8-BF</td>
<td>1.2 (29)</td>
<td>2.0 (48)</td>
<td>3.8 (49)</td>
<td>1.3 (33)</td>
<td>0.9 (36)</td>
<td>2.2 (46)</td>
</tr>
<tr>
<td>DMBA + BA</td>
<td>2.8 (68)</td>
<td>1.6 (38)</td>
<td>6.8 (83)</td>
<td>2.8 (71)</td>
<td>1.3 (52)</td>
<td>5.3 (113)</td>
</tr>
</tbody>
</table>

a One hundred nmoles of each compound were topically applied.

b No. in parentheses, percentage of values found in the group treated with DMBA alone.
Table 4

The effect of 7,8-BF, BA, and other flavones on DMBA-initiated and croton oil-promoted tumorigenesis in mouse skin

<table>
<thead>
<tr>
<th>Experiment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Compounds</th>
<th>Survivors</th>
<th>Mice with tumors</th>
<th>Total tumors</th>
<th>Tumors/mouse</th>
<th>Inhibition (%)</th>
<th>Survivors</th>
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<th>Tumors/mouse</th>
<th>Inhibition (%)</th>
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<td>1</td>
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<td>144</td>
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<td>28</td>
<td>337</td>
<td>11.6</td>
<td>81</td>
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<td>DMBA + 7,8-BF</td>
<td>28</td>
<td>8</td>
<td>15</td>
<td>0.5</td>
<td>90</td>
<td>22</td>
<td>17</td>
<td>48</td>
<td>2.2</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>DMBA + BA</td>
<td>29</td>
<td>19</td>
<td>48</td>
<td>1.7</td>
<td>66</td>
<td>28</td>
<td>27</td>
<td>296</td>
<td>10.6</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>DMBA</td>
<td>30</td>
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<td>24</td>
<td>0.8</td>
<td>87</td>
<td>30</td>
<td>28</td>
<td>245</td>
<td>8.2</td>
<td>87</td>
</tr>
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<td>29</td>
<td>3</td>
<td>4</td>
<td>0.1</td>
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<td>29</td>
<td>12</td>
<td>33</td>
<td>1.1</td>
<td>87</td>
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<td></td>
<td>DMBA + BA</td>
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<td>6</td>
<td>9</td>
<td>0.3</td>
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<td>26</td>
<td>26</td>
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<td>16&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>24</td>
<td>291</td>
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<td>16</td>
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<td></td>
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<td>28</td>
<td>28</td>
<td>369</td>
<td>13.2</td>
<td>4</td>
<td>22</td>
<td>6</td>
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<tr>
<td>4</td>
<td>7,8-BF</td>
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<td></td>
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<td>3</td>
<td></td>
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<tr>
<td></td>
<td>BA</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>22</td>
<td>6</td>
<td>7</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> In each experiment, groups of 30 mice were treated by the single application of 100 nmoles of each of the compounds indicated. When 2 compounds were applied, they were applied simultaneously in the same solution or immediately following one another. This single treatment was followed by the weekly application of 1% croton oil in acetone.

<sup>b</sup> Percentage of stimulation.
DMBA Tumorigenesis: 7,8 Benzoxyflavone Inhibition

**Table 5**
The effect of 7,8-BF, BA and other flavones on tumorigenesis induced by the repeated applications of DMBA

Twenty µg of DMBA were applied twice weekly; an equimolar concentration of inhibitor was applied simultaneously.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Survivors</th>
<th>Mice with tumors</th>
<th>Total tumors</th>
<th>Tumors/mouse</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA</td>
<td>29</td>
<td>29</td>
<td>531</td>
<td>18.3</td>
<td>74</td>
</tr>
<tr>
<td>DMBA + BF</td>
<td>21</td>
<td>19</td>
<td>99</td>
<td>4.7</td>
<td>33</td>
</tr>
<tr>
<td>DMBA + 5,6-BF</td>
<td>24</td>
<td>24</td>
<td>295</td>
<td>12.3</td>
<td>5</td>
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<tr>
<td>DMBA + apigenin</td>
<td>27</td>
<td>27</td>
<td>433</td>
<td>16.0</td>
<td>13</td>
</tr>
<tr>
<td>DMBA + nobiletin</td>
<td>29</td>
<td>29</td>
<td>501</td>
<td>17.3</td>
<td>5</td>
</tr>
<tr>
<td>DMBA + BA</td>
<td>22</td>
<td>22</td>
<td>320</td>
<td>14.5</td>
<td>21</td>
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<tr>
<td>7,8-BF</td>
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<td>BA</td>
<td>20</td>
<td>2</td>
<td>2</td>
<td>0.1</td>
<td>0</td>
</tr>
</tbody>
</table>

a 16 weeks.

**Table 6**
The effect of 7,8-BF and BA on skin tumorigenesis induced by DMBA or 7-OHMe-12-MBA

In Experiment 1, 100 nmoles of the indicated compounds were applied once to the backs of 30 mice. The BA and 7,8-BF were applied simultaneously with the DMBA. In Experiment 2, the above treatment was repeated 3 days later. All mice were subsequently treated once weekly with 1% croton oil.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Compounds</th>
<th>Survivors</th>
<th>Mice with tumors</th>
<th>Total tumors</th>
<th>Tumors/mouse</th>
<th>Inhibition (%)</th>
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<td>348</td>
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<td>100</td>
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<tr>
<td></td>
<td>DMBA + 7,8-BF</td>
<td>29</td>
<td>18</td>
<td>69</td>
<td>2.4</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>DMBA + BA</td>
<td>22</td>
<td>19</td>
<td>149</td>
<td>6.8</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>7-OHMe-12-MBA</td>
<td>26</td>
<td>11</td>
<td>16</td>
<td>0.6</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>7-OHMe-12-MBA + 7,8-BF</td>
<td>28</td>
<td>13</td>
<td>17</td>
<td>0.6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>7-OHMe-12-MBA + BA</td>
<td>24</td>
<td>10</td>
<td>29</td>
<td>1.2</td>
<td>95a</td>
</tr>
<tr>
<td>2</td>
<td>DMBA</td>
<td>24</td>
<td>22</td>
<td>289</td>
<td>12.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>DMBA + 7,8-BF</td>
<td>25</td>
<td>22</td>
<td>190</td>
<td>7.6</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>DMBA + BA</td>
<td>26</td>
<td>25</td>
<td>241</td>
<td>9.3</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>7-OHMe-12-MBA</td>
<td>27</td>
<td>15</td>
<td>49</td>
<td>1.8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>7-OHMe-12-MBA + 7,8-BF</td>
<td>25</td>
<td>12</td>
<td>27</td>
<td>1.1</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>7-OHMe-12-MBA + BA</td>
<td>26</td>
<td>11</td>
<td>25</td>
<td>1.0</td>
<td>44</td>
</tr>
</tbody>
</table>

a Percentage of stimulation.

analog, 5,6-BF, which is a good inducer of the enzyme (54–56) but is a weaker inhibitor of the enzyme, reduced tumorigenesis only 33%. The other flavones, apigenin and nobiletin, were inactive as inhibitors of tumorigenesis, and BA, which is a strong enzyme inducer, had relatively little effect on tumor formation. BF and BA applied individually had insignificant activity as carcinogens, in relation to the interpretation of their effects on DMBA tumorigenesis. This experiment demonstrates that 7,8-BF is a strong inhibitor of DMBA as a full carcinogen, as well as an inhibitor of its activity as a tumor initiator.

The Effect of 7,8-BF and BA on DMBA and 7-OHMe-12-MBA Tumorigenesis (Table 6). It has been suggested that 7-OHMe-12-MBA is a metabolite of DMBA which is possibly more closely related to the reactive carcinogenic species (6, 16, 45). In the 2-stage skin tumorigenesis system we used, 7-OHMe-12-MBA shows considerably weaker activity than DMBA. In Experiment 1, when the 7-OHMe-12-MBA was applied only once, only 16 tumors appeared in 26 mice, compared with 348 tumors in 28 mice initiated by DMBA. In this experiment, the simultaneous application of BF did not change the tumor yield initiated by 7-OHMe-12-MBA. However, this yield was so low that the activity of BF as an inhibitor was probably not adequately measured. The increased yield of tumors observed when BA was applied with the 7-OHMe-12-MBA is probably due to the weak initiating activity of BA (shown above). In Experiment 2, the 7-OHMe-12-MBA tumor yield was increased by applying the initiator twice instead of once. In this experiment, both the 7,8-BF and BA reduced the tumor yield by almost 50%, suggesting that 7-OHMe-12-MBA also requires metabolic activation by the enzyme system. Alteration of this system by 7,8-BF or BA reduces tumor formation. The 7,8-BF may inhibit the enzyme system. The BA inhibits the early
appearance of tumors but has relatively little effect at later times (Table 4). Inhibition was also observed at later times (Table 6, Experiment 1). The BA is also a substrate for AHH, and this inhibition can be due to relatively weak competitive effects that either cause a delay or, in some experiments, prevent the activation of DMBA or 7-OHMe-12-MBA.

**Time Interval Required for DMBA Activation (Table 7).** In order to determine the time required for the metabolic activation of DMBA as well as the duration of 7,8-BF action, we applied the 7,8-BF at various times before and after administration of DMBA and observed the effect on subsequent tumor formation. When 7,8-BF was applied 12 hr prior to or 12 hr after the administration of DMBA, essentially no inhibition of tumor formation was observed. When the inhibitor was applied 6 hr prior to or after the DMBA, a moderate inhibitory effect was observed. The maximum inhibition of 84% was observed when the BF was applied simultaneously with the DMBA. These data indicate that the part of the initiation stage of tumorigenesis that relates to the enzymatic activation of DMBA is essentially completed within 12 hr after the application of DMBA. It also indicates that the 7,8-BF remains bound to the active enzyme site for a period up to about 6 hr. These data are consistent with the disappearance curves of both DMBA and 7,8-BF, described above.

### Table 7

**Dependency of inhibition of tumorigenesis on the times of application of 7,8-BF**

The amount of DMBA and 7,8-BF administered and the conditions of the experiment are identical to those described in Table 4, except that the 7,8-BF was applied at the indicated times, relative to DMBA application. All groups received DMBA at zero time.

<table>
<thead>
<tr>
<th>Time of 7,8-BF application</th>
<th>Survivors</th>
<th>Mice with tumors</th>
<th>Total tumors</th>
<th>Tumors/mouse</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No 7,8-BF</td>
<td>25</td>
<td>23</td>
<td>158</td>
<td>6.3</td>
<td>10</td>
</tr>
<tr>
<td>−12 hr</td>
<td>25</td>
<td>20</td>
<td>143</td>
<td>5.7</td>
<td>18</td>
</tr>
<tr>
<td>−6 hr</td>
<td>24</td>
<td>14</td>
<td>75</td>
<td>3.1</td>
<td>36</td>
</tr>
<tr>
<td>Zero time</td>
<td>23</td>
<td>10</td>
<td>23</td>
<td>1.0</td>
<td>84</td>
</tr>
<tr>
<td>+6 hr</td>
<td>21</td>
<td>8</td>
<td>31</td>
<td>1.5</td>
<td>76</td>
</tr>
<tr>
<td>+12 hr</td>
<td>25</td>
<td>23</td>
<td>139</td>
<td>5.6</td>
<td>11</td>
</tr>
</tbody>
</table>

### Table 8

**AHH and tumor incidence in different strains of mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control Control</th>
<th>Hr 14</th>
<th>Hr 16</th>
<th>Tumors/mouse at Week 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swiss (NIH)</td>
<td>105</td>
<td>256</td>
<td>458</td>
<td>7.32</td>
</tr>
<tr>
<td>C57</td>
<td>86</td>
<td>153</td>
<td>380</td>
<td>1.17</td>
</tr>
<tr>
<td>C3H</td>
<td>86</td>
<td>182</td>
<td>337</td>
<td>0.09</td>
</tr>
<tr>
<td>DBA</td>
<td>35</td>
<td>177</td>
<td>143</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*See “Materials and Methods.”

The control values are those of untreated mice. Induced mice are those treated by the topical application of 1 mg of BA at Hr 4 or 16 prior to the assay.

The average no. of tumors/mouse. Each group consisted of 20 to 25 male mice weighing 18 to 22 g. A single application of 100 nmoles of DMBA was applied and was followed by weekly croton oil treatment as described in the “Materials and Methods.”
rate-limiting step in DMBA tumorigenesis. The data also clearly indicate that the presence of the enzyme system in C3H and DBA strains does not necessarily confer susceptibility to DMBA tumorigenesis. Since metabolic activation of carcinogen is an early stage in the long, multistaged process of carcinogenesis, genetic and epigenetic determinants of subsequent stages would be expected to play key roles in tumorigenesis susceptibility. Another possibility is that the profile of metabolites formed from DMBA is different in the various mouse strains. This has not been investigated.

DISCUSSION

The microsomal enzyme complex is clearly responsible for the detoxification of a variety of exogenous compounds, which include drugs, pesticides, and carcinogens (10, 17, 23). The detoxification often results in a reduced carcinogenicity of administered carcinogens. This reduction of carcinogenicity is observed with aromatic amines (40), as well as polycyclic hydrocarbons (29, 53, 54). Huggins et al. (29) observed that pretreatment of rats with small amounts of polycyclic hydrocarbons decreases DMBA-induced tumor formation in the mammary gland. Wattenberg and Leong (54) reported similarly that i.p. injection of 5,6-BF inhibits DMBA-induced tumorigenesis in the lung and mammary gland of rodents. They also demonstrated that 5,6-BF has a strong inducing activity on AHH in the liver, lung, and small intestine of rats and mice (55, 56). It is possible that in these tumorigenesis experiments the protective effects of pretreatment with inducers are due to increased enzyme levels in the target tissue. The increased level of enzyme can effect either a more rapid elimination of the carcinogen or cause a preferential conversion to a less carcinogenic form. This could be specific for the induced enzyme. Treatment of animals with inducers also causes an increase in enzyme activity in the liver, the major site of metabolism, and this in turn could effectively lower the concentration of the carcinogen in the target tissue. Wheatley (57) suggested accordingly that the increase in DMBA-induced tumorigenesis in mammary glands by treatment with an inhibitor of the microsomal enzyme system, 2-diethylaminoethyl-2,2-diphenylvalerate-HCl is due to an increase in the effective dose of the carcinogen in the target tissue.

The microsomal enzyme system, in addition to its detoxification function, is also responsible for the metabolic conversion of certain carcinogens to their active forms. Thus, a number of aromatic amines and other nitrogen-containing compounds are activated to carcinogenic forms by the microsomal enzyme complex (41). It now appears that some polycyclic hydrocarbons also require enzymatic activation for carcinogenic activity. One of the observations that supports this view is that the potent inhibitor of the aryl hydrocarbon hydroxylase system 7,8-BF is also a strong inhibitor of DMBA-induced skin tumorigenesis (22).

An evaluation of the role of AHH in polycyclic hydrocarbon tumorigenesis requires a biological system in which the enzyme activity can be determined in the target tissue for the carcinogen and in which the systemic metabolism of the carcinogen is kept to a minimum. Also, it is desirable that the system should yield multiple tumors after a single small dose of carcinogen. The 2-stage system of skin tumorigenesis meets these requirements (2, 3). Topical application of small amounts of a carcinogen to mouse skin minimizes the influence of systemic carcinogen metabolism and initiates multiple tumor formation. With this tumorigenesis system, our results indicate that the inhibition of DMBA-initiated tumor formation parallels the inhibition of AHH rather than its induction, when the modifying substances, 7,8-BF and BA, are applied simultaneously with the carcinogen. At the concentration used, 7,8-BF exhibited no activity as an inducer of the enzyme system. Thus, the tumor-inhibitory effects observed are not the result of induced levels of enzyme metabolizing the DMBA more effectively. Furthermore, in the experimental system we used, the presence of a hydrocarbon inducer such as BA has relatively little effect on DMBA tumorigenesis. 7,8-BF, however, is a potent inhibitor of the AHH enzyme system in cell culture (13), in various tissues in vivo (59), and inhibits both the metabolism of BP and DMBA in homogenates of mouse skin (unpublished observations, and Ref. 59). The 7,8-BF does not cause a more rapid elimination of the DMBA; in fact, it slows the disappearance of the DMBA somewhat. This effect is probably due to an inhibition of DMBA metabolism.

The formation of covalently bound polycyclic hydrocarbons to macromolecules of mouse skin has been observed during hydrocarbon-induced skin tumorigenesis (7, 27, 28, 39). Although the relevancy of these bound forms to carcinogenesis remains to be clarified, it has been reasonably postulated that these interactions may be integral to DMBA carcinogenesis. Binding of hydrocarbons to DNA may induce genetic changes, while binding to RNA or proteins may cause epigenetic changes leading to an altered gene expression which can characterize the tumor state (17, 46).

We have observed that 7,8-BF applied at low doses equimolar with DMBA causes a large inhibition of the binding of DMBA to macromolecules of the skin. The 7,8-BF inhibition of the binding of DMBA to DNA, RNA, and protein is consistent with the findings that the microsomal enzyme system catalyzes the formation of metabolites which bind to macromolecules (18, 19, 24) in vitro and that the 7,8-BF is a strong inhibitor of this enzyme system. The finding that inhibition of tumorigenesis is accompanied by a reduction in the formation of carcinogen-macromolecule interaction is consistent with the view of the importance of these interactions in carcinogenesis. The reactive form of the hydrocarbon has been postulated to be a radical cation (58) or carbonium ion (15). Cavaliere and Calvin (8) postulated that the hydroxylating enzymes may generate nucleophile centers in DMBA and that these may be the reactive centers. Jerina et al. (31) demonstrated an epoxide intermediate in the microsomal hydroxylation of naphthalene, and Selkirk et al. (47) found that 1,2,5,6-dibenzanthracene undergoes epoxidation by the microsomal enzyme system. Recently, Grover et al. (25) showed that epoxides as well as some dihydrodiols of BA and dibenz(a,h)anthracene exhibit transforming activity on cells grown in culture. The epoxides are generally more potent transforming agents than either the parent hydrocarbons or diols. Although the epoxide may be a reactive intermediate with certain hydrocarbons, it is not clear...
whether this is a general phenomenon for all hydrocarbons or whether there are other reactive intermediates which are generated by the aryl hydrocarbon hydroxylase system. The assay which we use measures phenol formation from BP. Jerina et al. (32) have indicated that arene oxides are intermediates in the microsomal formation of some phenols of simpler aromatic compounds. In other studies, we have found that several other microsomal enzyme activities are inhibited by 7,8-BF. Thus, it is likely that microsomal epoxide formation is inhibited by 7,8-BF.

In the case of DMBA, the production of the reactive intermediate is clearly suppressed by the inhibitor of the AHH, 7,8-BF.

Our results indicate that 7,8-BF inhibits effectively only when applied within 12 hr of the administration of the carcinogen DMBA. This suggests that the inhibitor is operating at a very early time in the tumorigenic process. We suggest that this early stage is related to the early metabolism of the carcinogen, since the disappearance curve of the DMBA indicates that more than 95% of the DMBA is no longer present in the tissue 24 hr after its application. The lack of inhibitory effect by 7,8-BF, when applied 12 hr after DMBA, defines the metabolic activation stage of DMBA tumorigenesis as occurring within the 1st 12 hr after its application.

Since the enzyme system of our concern is also clearly one of detoxification, we do not know the specificity of its role of activation vis-à-vis detoxification for each hydrocarbon. In other studies with BP as the carcinogenic agent, we have found that equimolar levels of 7,8-BF either have no effect or they enhance BP-induced tumorigenesis (34). Thus, it appears that the enzyme may have a unique role in the metabolism of each polycyclic hydrocarbon. In some cases, it may be the major instrument of detoxification and, in other cases, it may be instrumental in carcinogen activation. Its role relative to each of these processes may be determined by a variety of factors, such as the amount and type of enzyme present, hydrocarbon concentration, cofactor or inhibitor concentration, or relative activity of enzymes related to the further metabolism of the products of AHH.

ACKNOWLEDGMENTS

We wish to acknowledge the valuable discussions with our colleagues, Dr. F. Wiebel and Dr. L. Diamond, and the valuable technical assistance of Mrs. Barbara Shears, Miss Janet Leutz, and Mr. Hayward Waters.

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The Role of Aryl Hydrocarbon Hydroxylase in 7,12-Dimethylbenz(a)anthracene Skin Tumorigenesis: On the Mechanism of 7,8-Benzoflavone Inhibition of Tumorigenesis

Nadao Kinoshita and Harry V. Gelboin