

Inhibition of the Tumor-initiating Ability of the Potent Carcinogen 7,12-Dimethylbenz(a)anthracene by the Weak Tumor Initiator 1,2,3,4-Dibenzanthracene¹

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SUMMARY

Aryl hydrocarbon hydroxylase (AHH) in mouse epidermis was inducible by topical application of several tumor-initiating polycyclic aromatic hydrocarbons. The weak tumor initiator 1,2,3,4-dibenzanthracene (1,2,3,4-DBA), at dose level of 200 nmoles, increased AHH activity more than 10-fold over that of the acetone controls at 12 hr after treatment. Administration of the same quantity of the potent initiator 7,12-dimethylbenz(a)anthracene (DMBA) increased AHH activity approximately 4-fold over that of the control at 12 hr after treatment. Simultaneous treatment with 200 or 100 nmoles of DMBA and 1,2,3,4-DBA resulted in AHH activity that was 546 and 732% that of the controls, respectively, 12 hr after treatment; this was less AHH activity than was observed when 1,2,3,4-DBA was administered alone. Doses of 20 nmoles or more of 1,2,3,4-DBA, when given at about the same time as DMBA, effectively inhibited DMBA initiation of skin tumors in a two-stage system of tumorigenesis. The results suggest that the weak initiator 1,2,3,4-DBA may program the epidermal AHH system to metabolize the strong carcinogen DMBA to noncarcinogenic intermediate(s).

INTRODUCTION

The AHH² enzyme complex has been found in most mammalian tissues, including mouse skin, in which it is highly inducible (6, 12, 41, 42). This complex may function as a detoxification system (8, 10) and as an activator of PAH to toxic and carcinogenic metabolites (11, 12, 41).

Current information suggests that at least some PAH are converted by AHH to ultimate carcinogens that may be reactive epoxides (13, 14, 26, 27, 33, 35). These electrophiles can then be converted to transdihydrodiols by the action of microsomal epoxide hydrase(s), rearranged spontaneously to phenols, and conjugated with glutathione or combined with cellular nucleophiles (5, 10, 17, 23, 28, 33,

34, 36-38). It has been proposed (9) that DMBA activation may proceed via the methyl group through a carbonium ion formation.

This report deals with the role of AHH in PAH carcinogenesis in a specific target tissue, the skin, for which a good dose-response relationship exists between the application of PAH and the formation of papillomas and carcinomas. Also, chemical carcinogenesis in mouse skin can be broken down into a rapid, irreversible initiation step brought about by a subthreshold dose of a carcinogen followed by a reversible promotion process requiring continued administration of a nontumorigenic promoter.

Various studies in our laboratory have used several agents that modify tumorigenesis, such as benzoflavones, trichloropropene oxide, and antiinflammatory agents, to determine the role AHH plays in skin carcinogenesis. In this report, the role of PAH with weak tumor initiation is presented to help determine this function.

MATERIALS AND METHODS

Animals. Female Charles River CD-1 mice were purchased from Charles River Mouse Farms, North Wilmington, Mass. Mice, 7 to 9 weeks old, were carefully shaved with surgical clippers 2 days before treatment, and only those mice in the resting phase of the hair cycle were used in the biochemical and tumor experiments. Mice were always killed between 8 a.m. and 12 p.m. to minimize the effects of diurnal variations.

Chemicals. MC was purchased from J. T. Baker, Phillipsburg, N. J., and DMBA and 1,2,3,4-DBA were purchased from Sigma Chemical Co., St. Louis, Mo. 7,8-Benzoflavone was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. [³H]DMBA, >99% pure (6.4 Ci/mole), was obtained from Amersham/Searle, Arlington Heights, Ill. Croton oil was obtained from S. B. Penick and Co., New York, N. Y. 12-O-tetradecanoylphorbol-13-acetate was prepared as previously described (3) and purified by preparative thin-layer chromatography.

Tumor Induction Experiments. Each experimental group contained 30 preshaven mice. 1,2,3,4-DBA and BA in 0.2 ml of acetone were applied topically 5 min before initiation with DMBA or MC. One week after initiation, mice received twice-weekly applications of 10 μg of 12-O-tetradecanoylphorbol-13-acetate in 0.2 ml of acetone, and promotion was continued for 30 weeks. The incidences of both papillomas

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² The abbreviations used are: AHH, aryl hydrocarbon hydroxylase; PAH, polycyclic aromatic hydrocarbons; DMBA, 7,12-dimethylbenz(a)anthracene; MC, 3-methylcholanthrene; 1,2,3,4-DBA, 1,2,3,4-dibenzanthracene; BA, benzanthracene.

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and carcinomas were observed weekly, and papillomas and carcinomas were removed at random for histological verification.

AHH Enzyme Assay. The epidermis was isolated as previously described (41), and the epidermal material from 3 mice was homogenized with a Polytron PT 10 homogenizer. This homogenate was the source of enzyme for the AHH assay, which was performed as described by Bowden *et al.* (4) with the modifications detailed in a previous report (41). The specific activity was expressed as pmoles of 3-hydroxybenzo(a)pyrene formed in 30 min of incubation per mg protein. In most cases the specific activities were expressed as a percentage of the acetone control groups, and each time point tested represented an average of 2 to 5 groups containing 3 mice each. The average S.D. for the control groups for each experiment was less than 20%.

Determination of Covalent Binding of DMBA to DNA. This assay system is based on the one originally described by Gelboin (11) and by Grover and Sims (15), with modifications as previously described (7). The specific activity of binding is expressed as pmoles of hydrocarbon bound per μg DNA per mg protein per 15 min of incubation. A zero-incubation value was subtracted from each specific activity, and each experiment was done in triplicate. In most cases, each specific activity represents the average of 2 to 3 experiments.

RESULTS

The weak tumor initiator 1,2,3,4-DBA was found to be a potent inhibitor of DMBA tumor initiation in a 2-stage system of tumorigenesis (Chart 1). Doses of either 200 or 100

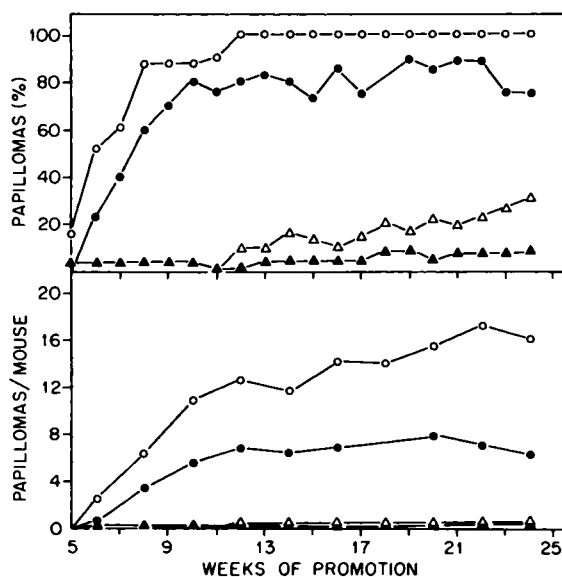


Chart 1. Effect of 1,2,3,4-DBA on tumor initiation by DMBA. Each group consisted of 30 mice, and all animals were initiated with either 200 or 100 nmoles of DMBA and promoted with $10 \mu\text{g}$ of 12-O-tetradecanoylphorbol-13-acetate twice weekly starting 1 week after initiation. *Top*, percentage of mice with papillomas as a function of weeks of tumor promotion; *bottom*, average papillomas per mouse as a function of weeks of tumor promotion. 1,2,3,4-DBA was given 5 min before DMBA initiation. Treatment (in nmoles): DMBA (200), \circ ; DMBA (100), \bullet ; DMBA (100) plus 1,2,3,4-DBA (100), Δ ; DMBA (200) plus 1,2,3,4-DBA (200), \blacktriangle .

nmoles of 1,2,3,4-DBA and DMBA resulted in 95 and 90% inhibition, respectively, of DMBA initiation of skin tumors (both papillomas and carcinomas) at 24 weeks after promotion was begun. 1,2,3,4-DBA, administered in a 200-nmole dose and followed 1 week later by twice-weekly applications of 12-O-tetradecanoylphorbol-13-acetate, did not cause any tumors after 40 weeks of promotion. Much higher doses of 1,2,3,4-DBA (2500 nmoles) have been reported to have initiating abilities (32).

Chart 2 depicts the effect of the administration of 20 nmoles each of 1,2,3,4-DBA and DMBA on the initiating ability of DMBA. As shown, such a dose of 1,2,3,4-DBA had much less effect on DMBA initiation than did the higher dose just discussed. There was also a slight inhibitory effect of BA on DMBA initiation when both compounds were given in 20-nmole doses. After the 20-nmole concentration was found to be less effective than 100 and 200 nmoles of 1,2,3,4-DBA, the effect of 5 nmoles each of 1,2,3,4-DBA and DMBA on DMBA tumor initiation was determined. Such a dose regimen had no effect on DMBA tumorigenic response (Chart 3).

Table 1 summarizes the effects of 1,2,3,4-DBA and BA on the initiation of skin tumors by DMBA. A dose of 20 nmoles or more of 1,2,3,4-DBA, given at approximately the same time as DMBA, effectively inhibited the DMBA tumor initiation (Table 1); 20 nmoles of BA were less effective than the same amount of 1,2,3,4-DBA in decreasing the initiating ability of DMBA. It is interesting to note that 200 nmoles of 1,2,3,4-DBA inhibited the initiating ability of DMBA quite effectively when 5 nmoles of DMBA were administered.

For comparison, the effect of the weak tumor initiator BA on tumor initiation by MC was investigated, and the results are shown in Chart 4. At the doses used, BA was without tumor-initiating ability in this 2-stage system. Treatment

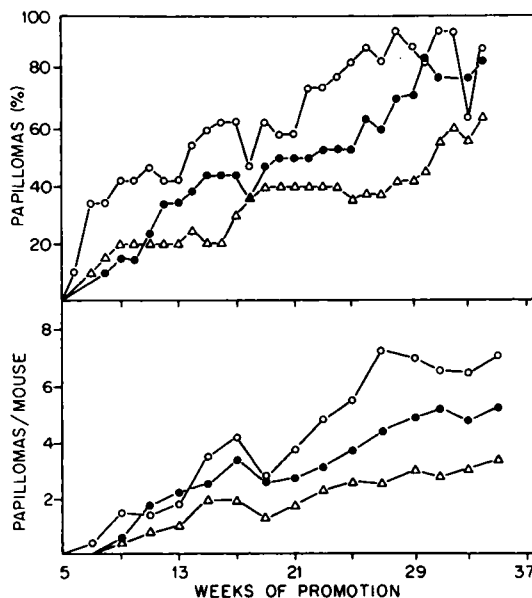


Chart 2. Effect of either 1,2,3,4-DBA or BA on tumor initiation by DMBA. Each group consisted of 30 mice, and all animals were initiated with 20 nmoles of DMBA and promoted with $10 \mu\text{g}$ of 12-O-tetradecanoylphorbol-13-acetate twice weekly starting 1 week after initiation. 1,2,3,4-DBA and BA were given 5 min before DMBA initiation. Treatment (in nmoles): DMBA (20), \circ ; DMBA (20) plus 1,2,3,4-DBA (20), \bullet ; DMBA (20) plus BA (20), Δ .

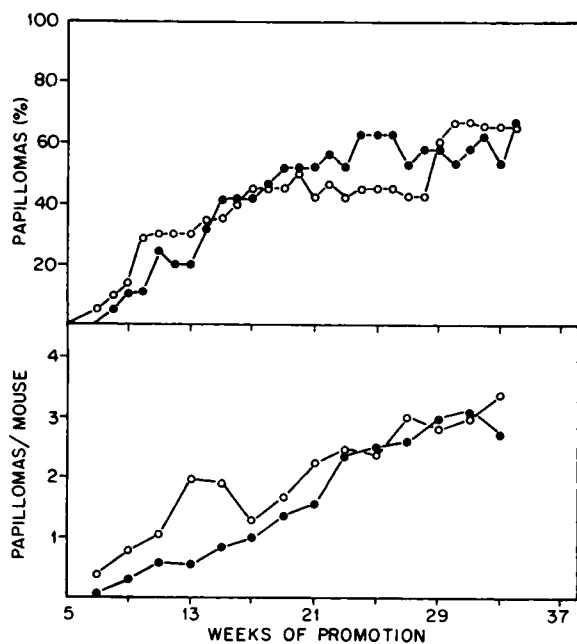


Chart 3. Effect of 1,2,3,4-DBA on tumor initiation by DMBA. Each group consisted of 30 mice, and all animals were initiated with 5 nmoles of DMBA and promoted with 10 μ g of 12-O-tetradecanoylphorbol-13-acetate twice weekly starting 1 week after initiation. 1,2,3,4-DBA was given 5 min before DMBA initiation. Treatment (in nmoles): DMBA (5), \circ ; DMBA (5) plus 1,2,3,4-DBA (5), \bullet .

Table 1

Effects of 1,2,3,4-DBA and BA on the initiation of skin tumors by DMBA

1,2,3,4-DBA and BA were applied 5 min before initiation with DMBA.

Pretreatment dose (nmoles)	DMBA initiation dose (nmoles)	Papillomas/mouse ^a (% of control)
1,2,3,4-DBA (200)	200	4.5
1,2,3,4-DBA (100)	100	10.5
1,2,3,4-DBA (20)	20	45.0
BA (20)	20	67.0
1,2,3,4-DBA (5)	5	103.0
1,2,3,4-DBA (200)	5	6.5

^a The average number of papillomas per mouse after 24 weeks of promotion is expressed as a percentage of the DMBA controls.

with either 50 or 100 nmoles of BA and MC had little influence on the initiating ability of MC. Even 10-fold higher doses of BA than MC resulted in only a slight inhibition.

To elucidate the mechanism of 1,2,3,4-DBA inhibition of DMBA tumor initiation, the effects of these agents on epidermal AHH activity were investigated. We reported recently (41) the inducibility of epidermal AHH activity by several PAH (1,2,3,4-DBA > 1,2,5,6-dibenzanthracene > BA \cong MC \gg DMBA). Table 2 summarizes a detailed investigation of the time course of induction of epidermal AHH by DMBA and 1,2,3,4-DBA. The induction of epidermal AHH by 200 nmoles of DMBA reached a peak around 12 hr after induction, whereas 200 nmoles of 1,2,3,4-DBA caused peak AHH induction at 6 and 36 hr after treatment. Table 3 shows the effects of various dose levels of either DMBA, 1,2,3,4-DBA, or both on epidermal AHH activity 12 hr after topical treatment. The best AHH-inducing dose of DMBA, as well as of 1,2,3,4-DBA, was 100 nmoles. The inducing ability of DMBA

was essentially titrated out at 5 nmoles, whereas 5 nmoles of 1,2,3,4-DBA induced a greater than 3-fold increase in AHH activity. Given simultaneously, 100 nmoles of DMBA and 1,2,3,4-DBA produced the greatest increase in AHH activity. Only at doses of 5 nmoles each were the effects of 1,2,3,4-DBA and DMBA additive (Table 3).

Table 4 shows the effects on epidermal AHH activity when various PAH were added directly to the *in vitro* assay. Both MC and 7,8-benzoflavone were found to be potent inhibitors of epidermal AHH activity at all doses investigated. DMBA and 1,2,3,4-DBA were found to be good inhibitors of the *in vitro* AHH activity only when given at 4 times the benzo(a)pyrene substrate concentration.

Table 5 shows the effects of a topical application of either 1,2,3,4-DBA, DMBA, or both on the *in vitro* epidermal-mediated covalent binding of radioactive DMBA to DNA. Pretreatment with 100 nmoles of unlabeled DMBA increased the *in vitro* binding of [³H]DMBA to DNA more than 2-fold

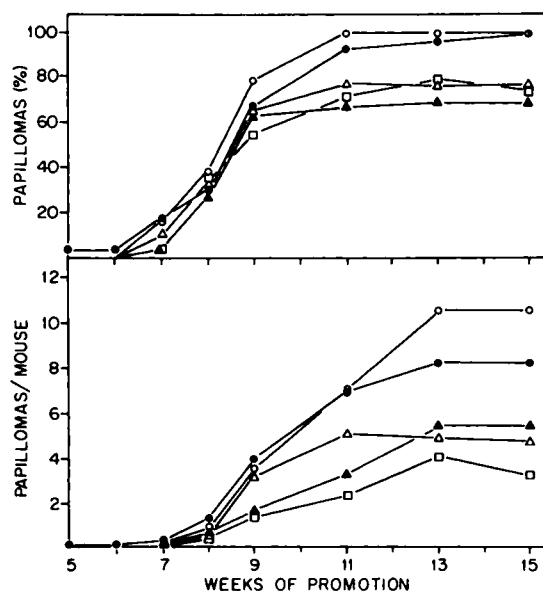


Chart 4. Effect of BA on tumor initiation by MC. Each group consisted of 30 mice, and all animals were initiated with either 50 or 100 nmoles and promoted with 10 μ g of 12-O-tetradecanoylphorbol-13-acetate twice weekly starting 1 week after initiation. BA was given 5 min before DMBA initiation. Treatment (in nmoles): MC (100), \circ ; MC (100) plus BA (100), \bullet ; MC (50), Δ ; MC (50) plus BA (50), \blacktriangle ; MC (50) plus BA (500), \square .

Table 2

Time course of AHH activity in mouse epidermis after a single topical application of 200 nmoles of DMBA or 1,2,3,4-DBA

Time after treatment (hr)	Specific activity (% of controls) ^a after application of:	
	DMBA	1,2,3,4-DBA
2	108	192
6	165	1557
12	411	1148
24	245	1187
36	148	2285

^a Specific activity is expressed as pmoles of 3-hydroxybenzo(a)pyrene formed per mg of protein; average for the controls was 38. Each value represents an average of 2 experiments, with duplicate determinations for each experiment. S.D. for each figure was less than 20%.

Table 3

Epidermal AHH activity 12 hr after a single topical application of various dose levels of DMBA, 1,2,3,4-DBA, or both

Treatment	Specific activity (% of controls) ^a for applied dose level (nmoles) of:				
	200	100	20	10	5
DMBA	418	503	327	168	138
1,2,3,4-DBA	1054	1149	992	528	334
1,2,3,4-DBA + DMBA	546	732	616	576	425

^a Specific activity is expressed as pmoles of 3-hydroxybenzo(a)pyrene formed per mg of protein. Each value represents an average of 2 experiments, with duplicate determinations for each experiment. S.D. for each figure was less than 20%.

Table 4

In vitro effect of various PAH on epidermal AHH activity

Mice were pretreated topically with 200 nmoles of BA 24 hr before they were killed. The substrate concentration for benzo(a)pyrene in the AHH assay was 10^{-4} M.

<i>In vitro</i> addition	Specific activity (% of controls) ^a for concentration (M) of:			
	10^{-5}	10^{-4}	2×10^{-4}	4×10^{-4}
MC	35	28	24	22
1,2,5,6-DBA	47	50	55	41
1,2,3,4-DBA	80	72	62	19
DMBA	58	73	69	32
1,2,3,4-DBA + DMBA		60	41	
7,8-Benzoflavone		9	6	

^a Specific activity is expressed as pmoles of 3-hydroxybenzo(a)pyrene formed per mg protein; average for the controls was 40. S.D. for each figure was less than 15%.

Table 5

Effect of a topical application of either 1,2,3,4-DBA, DMBA, or both on the *in vitro* covalent binding of [³H]DMBA to DNA by epidermal homogenates

Mice were treated topically with either 1,2,3,4-DBA, DMBA, or both 12 hr before they were killed.

Treatment	Dose (nmoles)	Specific activity ($\times 10^4$) ^a
Acetone control		4.1
DMBA	100	10.8
1,2,3,4-DBA	100	18.2
1,2,3,4-DBA	10	14.4
1,2,3,4-DBA + DMBA	100 ^b	6.5

^a Specific activity is expressed as pmoles bound per μ g of DNA per mg of protein per 15 min of incubation at 37° in the dark. Each value represents 2 experiments, with duplicate determinations per experiment. S.D. for each figure was less than 20%.

^b 1,2,3,4-DBA and DMBA were both given in 100-nmole doses.

over that of acetone controls, whereas 100-nmole doses of unlabeled 1,2,3,4-DBA increased the binding more than 4-fold. Somewhat better induction of a higher level of *in vitro* binding of DMBA to DNA resulted from pretreatment with 10 nmoles of 1,2,3,4-DBA instead of 100 nmoles of DMBA (Table 5). This corresponds to the AHH-inducing ability of 1,2,3,4-DBA and DMBA (Table 3), with 10 nmoles of 1,2,3,4-DBA being slightly more effective than 100 nmoles of DMBA. It should be noted that *in vitro* covalent binding of [³H]DMBA to DNA was substantially decreased when epidermal homogenates were isolated from mice pretreated with DMBA and 1,2,3,4-DBA rather than with DMBA only. Table 6 shows

Table 6

In vitro effect of several PAH and benzoflavones on the covalent binding of [³H]DMBA to DNA by epidermal homogenates

Mice were pretreated topically with 200 nmoles of unlabeled DMBA 18 hr before they were killed.

<i>In vitro</i> addition ^a	Concentration (-fold)	Specific activity (% of controls) ^b
1,2,3,4-DBA	1	63.4
	2	53.6
MC	1	85.2
	2	67.8
7,8-Benzoflavone	1	49.3
	2	28.1
5,6-Benzoflavone	1	47.5
	2	44.8

^a The compounds in acetone were added directly to the incubation tubes at concentrations either equal to or twice the DMBA substrate concentration (36 nmoles).

^b Specific activity is expressed as a percentage of the induced controls and defined as pmoles bound per μ g of DNA per mg of protein. Each value represents 2 experiments with duplicate determinations per experiment. S.D. for each figure was less than 17%.

the effects of adding PAH or benzoflavones directly to the *in vitro* [³H]DMBA binding assay. Both benzoflavones effectively inhibited the *in vitro* binding of [³H]DMBA to DNA by epidermal homogenates, whereas 1,2,3,4-DBA and MC only moderately counteracted it.

DISCUSSION

The enzyme system AHH is thought to be part of the microsomal mixed-function oxidases that convert PAH to ultimate carcinogens, possibly reactive epoxides. These electrophilic epoxides can then be converted to various metabolites or combined covalently with cellular nucleophiles (5, 10, 17, 23, 28, 33, 34, 36-38). However, in the case of the methylated PAH, DMBA metabolism to an ultimate carcinogen(s) may proceed via an epoxide intermediate and/or through an activated methyl group or other reactive intermediate.

Several chemical carcinogens have been shown to bind covalently to cellular nucleic acids and proteins (31), and it seems likely that the interaction with 1 or more of these macromolecules is essential for the carcinogenic process. Previous studies from this laboratory have demonstrated that a correlation exists between the tumor-initiating ability of several PAH and their capacity to bind covalently to DNA *in vitro* following incubation with epidermal homogenates and NADPH as the electrophile-generating system (7). A correlation has also been noted between the extent of *in vivo* binding of several PAH to mouse-skin DNA (5) and protein (17) and the carcinogenic activity of the hydrocarbons.

Many of the "K-region" epoxides have been shown to be potent carcinogens for cells in culture (16, 20, 29) and potent mutagens for different mutagen assay systems (1, 2, 19, 21, 22, 30, 43). The notable exception to the above is the K-region epoxide of DMBA, which poorly transforms cells in culture (29). An investigation of the metabolism of DMBA in

several different tissues showed only small quantities or no 7,12-dimethylbenz(a)anthracene-5,6-epoxide and its trans-dihydrodiol derivative (25, 40, and 44).³ Results in this report suggest that the nonmethylated PAH, 1,2,3,4-DBA, may program the epidermal cells to metabolize DMBA to a very weak carcinogenic intermediate(s). However, such is the case only when both 1,2,3,4-DBA and DMBA are applied to mice. [³H]DMBA was found to bind better to DNA when 1,2,3,4-DBA was applied topically to mice 12 hr before they were killed and the epidermal homogenates were used in *in vitro* experiments than when epidermal homogenates isolated from DMBA-pretreated mice were used (Table 5). Only when the tumor experiments were mimicked (1,2,3,4-DBA was administered 5 min before DMBA initiation) did a decrease in DMBA binding to DNA *in vitro* occur. An alternative explanation may be that some competition at the genome level may exist between metabolites of the weak carcinogen and those of the strong carcinogen.

Presently, the only explanation that can be offered is that some competition for metabolism or interaction with the genome takes place between DMBA and 1,2,3,4-DBA which results in a decreased DMBA tumor initiation. In general, pretreatment with 1,2,3,4-DBA, rather than with DMBA, more effectively induces the epidermal metabolizing system. The *in vitro* binding of [³H]DMBA to DNA by epidermal homogenates from mice pretreated with either 1,2,3,4-DBA or DMBA supports this thesis.

Several investigators have shown that metabolism of DMBA proceeds mainly via hydroxylation of the side-chain methyl groups (25, 40, 44).³ It is therefore of interest that Jellnick and Goudy (25) reported that pretreatment of rats with MC and 1,2,5,6-dibenzanthracene altered the metabolism of DMBA by hepatic tissue from side-chain to ring hydroxylation. Pretreatment with 1,2,3,4-DBA may inhibit DMBA tumor initiation by a similar mechanism. In a preliminary experiment, we have found 7,12-dimethylbenz(a)anthracene-5,6-epoxide to be a weak tumor initiator in mouse skin.

Induction of mammary cancer was inhibited when mice were repeatedly fed certain PAH with DMBA (24). Also, some investigators have shown that certain PAH protect against carcinogenesis caused by potent carcinogenic hydrocarbons such as DMBA (18, 39). A similar inhibitory mechanism may apply to these studies.

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