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Inhibition of Benzo(*a*)pyrene Metabolism Catalyzed by Mouse and Hamster Lung Microsomes[1]

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SUMMARY

Induced and constitutive microsomal enzymes of mouse and hamster lungs catalyze both the hydroxylation of benzo(*a*)pyrene and reactions that lead to its irreversible binding to macromolecules. For mouse and hamster, the induced lung hydroxylases have K_m values of 1.10 and 0.52 μM, respectively. The induced hydroxylases are strongly inhibited by 7,8-benzoflavone and are stimulated by cyclohexene oxide, an inhibitor of epoxide hydrase.

Formation of the macromolecular product by the induced "binding" enzyme follows Michaelis-Menten kinetics, except for substrate inhibition, and has K_m values of 0.52 and 0.25 μM for lung microsomes from mouse and hamster, respectively. These reactions are also inhibited by 7,8-benzoflavone.

The reaction catalyzed by the constitutive hydroxylase of mouse lungs is characterized by a brief lag period but proceeds in a linear fashion after the lag. The enzyme requires 60 μM benzo(*a*)pyrene to achieve maximum reaction velocity. Above this concentration, strong substrate inhibition is observed; accurate values for V_{max} and K_m cannot be derived. The constitutive hydroxylases are moderately inhibited by butylated hydroxytoluene, retinol, cyclohexene oxide, and 7,8-benzoflavone.

The product of the constitutive "binding" enzyme is formed in a reaction that follows Michaelis-Menten kinetics. The K_m value for enzymes from mouse and hamster lungs are 11.8 and 4.9 μM, respectively. Formation of this product is strongly inhibited by butylated hydroxytoluene and by retinol but not strongly by 7,8-benzoflavone or cyclohexene oxide. Since other evidence indicates that a constitutive enzyme may be involved in carcinogenesis by benzo(*a*)pyrene and since this reaction is inhibited by two known anticarcinogens, we suggest that it may be involved in this process.

INTRODUCTION

The elevated incidence for lung cancer in American men has been associated with increased exposure to cigarette smoke, which contains BP[2] along with other carcinogens (46). In order to exert their biological effect, polycyclic hydrocarbons such as BP must be metabolized by microsomal mixed-function oxidases to generate reactive compounds that bind with macromolecules, although some microsomal oxidations lead to noncarcinogenic products (5, 9, 15, 22, 23, 25, 27). Most of the detailed investigations of polycyclic hydrocarbon metabolism have been accomplished with liver preparations (19, 37, 38), although the liver is not a principal target organ for polycyclic hydrocarbon carcinogenesis. Polycyclic hydrocarbons induce tumors in the pulmonary tissues of experimental animals (20, 34), and it might be expected that a reduction in the formation of active products in these tissues would reduce the carcinogenicity of the compounds.

This report concerns the characterization of microsomal enzymes metabolizing BP in mouse and hamster lungs and their inhibition by various agents.

MATERIALS AND METHODS

[3,6-^{14}C]BP (21 μCi/μmole) was obtained from the Amersham/Searle Corporation, Arlington Heights, Ill. A sample of 3-hydroxy-BP was generously supplied by Dr. Hans Falk, National Institute of Environmental Sciences, Research Triangle Park, N. C. Retinyl acetate, methyl retinoate, 13-*cis*-retinoic acid, *N*-ethylretinamide, retinoic acid, *N*,*N*-diethylretinamide, and 9-(2-acetyl-5,5-dimethyl-1-cyclopenten-1-yl)-3,7-dimethylnona-2,4,6,8-tetraenoic acid were prepared by Hoffmann-La Roche, Inc., Nutley, N. J., and supplied to us by the Lung Cancer Segment of the National Cancer Institute. Retinol and retinal were purchased from the Sigma Chemical Company, St. Louis, Mo.

Animals used in this investigation were female DBA/2 mice, 2 months old and weighing 18 to 23 g, and female Syrian golden hamsters, 2 months old and weighing 60 to 70 g. Both were maintained on Wayne laboratory chow. For induction of BP hydroxylase activity in lung tissue, mice were given injections of 3 mg of benz(*a*)anthracene in 0.25 ml of corn oil 16 hr before they were killed (8). Hamsters, treated similarly, received 9 mg of benz(*a*)anthracene. Washed lung microsomes were prepared daily from the pooled tissues of 20 mice or 5 hamsters by the procedure of Gram (12). As used, 1 μl of microsomal preparation contained 7 to 9 μg of protein and was equivalent to 1 mg of lung tissue.

Table 1 contains reaction conditions for the various assays performed with mouse and hamster lung microsomes.

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[2] The abbreviation used is: BP, benzo(*a*)pyrene.

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Table 1

Reaction conditions for various assays with mouse and hamster microsomes

All reactions were accomplished at 37° in assay systems containing a total of 500 μl. All contained 1.0 mg of bovine serum albumin and 3.5 μmoles of buffer.

Tissue	Product	pH optimum	NADPH (nmoles)	$MgCl_2$ (nmoles)	Microsomes equivalent to mg of tissue (mg)	BP (nmoles)	Time of incubation (min)
Lung-I[a]	3-Hydroxy-BP	8.0 (Tris)	300	500	60	1.0	5
Lung-N	3-Hydroxy-BP	7.6 (Tris)	300	250	20	20	20
Lung-I	Bound BP	8.0 (Tris)	300	500	60	1.5	20
Lung-N	Bound BP	8.0 (Tris)	300	500	30	15	20[b]
Liver-N[c]	3-Hydroxy-BP	7.6 (PO_4)	400	500	40	7.7	10
Liver-N[c]	Bound BP	8.0 (Tris)	400	500	40	7.7	30

[a] I, induced; N, noninduced; PO_4, phosphate buffer; Tris, Tris chloride buffer.
[b] Thirty min for hamster lung microsomes.
[c] For DBA/2 mouse only.

For hydroxylation of BP by microsomes, reactions were stopped by adding 0.5 ml of acetone, and BP and soluble metabolites were extracted with 1.6 ml of hexane by shaking at 37° for 10 min. The hexane fraction was further extracted with 1.5 ml of 1 N NaOH, and the NaOH solution was assayed for hydroxy derivatives according to the spectrophotofluorimetric procedure of Nebert and Gelboin (30). With activation at 390 nm, the fluorescence spectrum for the product of BP hydroxylation, maximal at 520 nm, was identical to that for authentic 3-hydroxy-BP, and the heights of the fluorescence peaks were proportional to the concentration of 3-hydroxy-BP. The degree of quenching was considered in calculations for all inhibitors (19).

For oxidation of BP to products that bind to macromolecular reaction components, reactions were stopped by adding 0.5 ml of acetone and 1.6 ml of hexane; soluble metabolites were extracted into the hexane layer by shaking the tubes for 10 min at 37°. After the organic solvent was discarded, the procedure was repeated. To the remaining aqueous solutions, 1 ml of 10% trichloroacetic acid was added, and the preparations were centrifuged. The precipitates were suspended in 3 ml of 10% trichloroacetic acid and collected on GF/A glass fiber filters. The filters were washed sequentially with 10% trichloroacetic acid and acetone and placed in vials for scintillation counting.

For all reactions, BP and potential inhibitors were added in a total of 5 μl of dimethyl sulfoxide, an amount that had no detectable effects on the reactions. Unless otherwise indicated, the rates of reactions were linear with time over the period of incubation, and the pH, amounts of microsomes, $MgCl_2$, and NADPH were optimal. Systems lacking NADPH served as blanks for each assay, and no reaction occurred in the absence of microsomes. All assays were performed in duplicate; separate determinations were made with different microsome preparations. For reactions following Michaelis-Menten kinetics, initial velocities (v) and substrate concentrations ([S]) were fitted by an IBM 1130 computer to the equation, $v = V[S]/K_m + [S]$, where V is maximum velocity and K_m is the Michaelis constant. The FORTRAN program developed by Cleland (8) was used. For this procedure, values obviously influenced by substrate inhibition were deleted.

RESULTS

Induced Enzymes. For all substrate concentrations used, hydroxylase reactions catalyzed by the induced aryl hydrocarbon hydroxylases of mouse and hamster lungs were linear with time for a minimum of 5 min. Obtained within this time, the initial rates were used to determine kinetic constants for these reactions. For the mouse enzyme, the apparent Michaelis constant (K_m) was 1.10 ± 0.05 μM, and the apparent maximum velocity (V_{max}) was 120 ± 4 fmoles/min/mg lung tissue (Chart 1; Table 2). Reduction of the microsome concentration used in this assay by 50% did not change the K_m value (results not shown). The corresponding hamster enzyme gave values of 0.52 μM for the K_m and 90 fmoles/min/mg for the V_{max} (Table 2).

7,8-Benzoflavone at a concentration of 0.25 μM was a good inhibitor of the mouse hydroxylase (Chart 1). With 50 μM 7,8-benzoflavone, the reaction was inhibited by 95% (Table 2). The induced hydroxylases of both mouse and hamster lungs were not strongly inhibited by 50 μM retinol or any of its analogs or derivatives or by butylated hydroxytoluene but were stimulated 72 and 45%, respectively, by 2 mM cyclohexene oxide (Table 2).

Another reaction for BP occurring with lung microsomes from induced mice and hamsters involved the binding of BP to macromolecular reaction components, a reaction similar to that previously observed for a constitutive liver enzyme but not detected in earlier, less sensitive assays of lung tissue (19). In contrast to the liver enzyme that leads to binding of BP, the induced "binding" enzymes of lungs of mice and hamsters were inhibited strongly (91%) by 50 μM 7,8-benzoflavone but were only weakly inhibited (<21%) by vitamin A compounds and by butylated hydroxytoluene (Table 2). Binding was increased in the presence of 2 mM cyclohexene oxide. Apparent K_m values of 0.52 ± 0.04 μM for the mouse enzyme and 0.25 μM for the hamster enzyme (Chart 2) were derived. Substrate inhibition was evident at high BP concentrations. The maximum rates of reaction, derived by extrapolation, were about the same as those for the corresponding induced hydroxylases (Table 2).

Constitutive Enzymes. The constitutive aryl hydrocarbon hydroxylases of mouse and hamster lung require relatively

high BP concentrations to allow an appreciable rate of reaction (Chart 3). These hydroxylases are characterized by a 4-min lag period, after which the reaction proceeds in a linear fashion for more than 30 min (results not shown). Attempts to shorten the lag period by prior incubation of the reaction system at 37° for 10 min without either BP, NADPH, or microsomes and of the complete system at 0° were unsuccessful. Using only the linear portion of the reaction curves for the constitutive mouse lung hydroxylase, the substrate saturation curve in Chart 3 was derived. The reaction reached a maximum rate at 60 μM BP and was subject to substrate inhibition at higher concentrations. A Lineweaver-Burk plot of these points (not shown) revealed that substrate inhibition occurred at concentrations less than the anticipated K_m value. Both constitutive hydroxylases were moderately inhibited by cyclohexene oxide, butylated hydroxytoluene, and vitamin A compounds (Tables 2 and 3); but the constitutive mouse hydroxylase was less sensitive to 7,8-benzoflavone than the corresponding hamster enzyme. Of a series of vitamin A compounds and analogs, *N*,*N*-diethylretinamide was the most potent inhibitor (Table 3).

Lung microsomes from mice and hamsters not pretreated with benz(*a*)anthracene also had enzymatic activities leading to binding of BP to macromolecular reaction components. These reactions were linear with time for 20 to 30 min (see Table 1); no lag period was detected. The substrate saturation curves were hyperbolic with K_m values of 10.7 and 5.6 μM, respecitvely (Chart 4; Table 2). Substrate inhibition was evident at the highest BP concentration. Compared to the other enzymes, the maximum rates of reaction were low. These reactions were not inhibited by

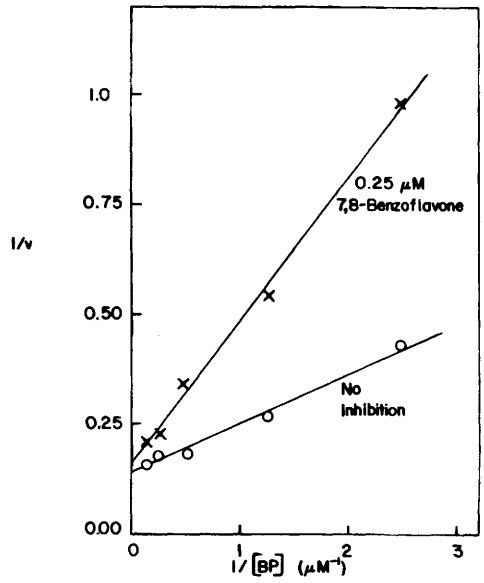

Chart 1. Lineweaver-Burk plot for the induced DBA/2 mouse aryl hydrocarbon hydroxylase showing inhibition by 7,8-benzoflavone. *Vertical axis*, reciprocal of pmoles of product formed per min.

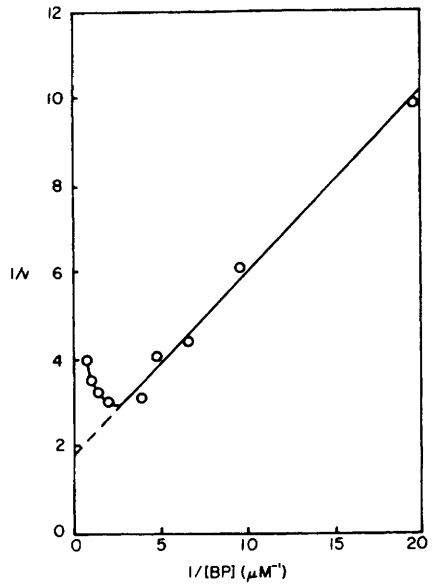

Chart 2. Lineweaver-Burk plot for the formation of bound BP by induced enzymes of hamster lung microsomes. *Vertical axis*, reciprocal of pmoles of BP bound per 20 min × 100.

Table 2

Kinetic values and effect of various agents on enzymes involved in BP metabolism

Species	Tissue	Product	V_{max} (fmoles/min/mg tissue)	K_m (μM)	% inhibition by: Cyclohexene oxide (2 mM)	Butylated hydroxytoluene (50 μM)	Retinol (50 μM)	7,8-Benzoflavone (50 μM)
DBA/2 mouse	Lung-I[a]	3-Hydroxy-BP	120 ± 4[b]	1.10 ± 0.05[b]	−67, −77	17, 24	−13, −7	98, 94
Hamster	Lung-I	3-Hydroxy-BP	95, 84	0.50, 0.53	−45	−24	−16	89
DBA/2 mouse	Lung-N	3-Hydroxy-BP	Not obtainable	Not obtainable	43 ± 3[b]	93 ± 1[b]	64 ± 6[b]	26 ± 2[b]
Hamster	Lung-N	3-Hydroxy-BP	Not determined	Not determined	74, 73	64, 67	68 ± 6[b]	60, 53
DBA/2 mouse	Lung-I	Bound BP	96 ± 22[b]	0.52 ± 0.04[b]	−6, −16	16, 11	9, 21	85, 98
Hamster	Lung-I	Bound BP	47, 59	0.24, 0.26	−30, −10	0, 7	7, 12	85, 99
DBA/2 mouse	Lung-N	Bound BP	13, 16	10.7, 13.0	−9	100[c]	98 ± 2[b]	8, 0
Hamster	Lung-N	Bound BP	6.0, 4.3	5.6, 4.2	9	100, 97	100, 100	45, 49
DBA/2 mouse	Liver-N	3-Hydroxy-BP	1485 ± 55[b]	8.5 ± 0.2[b]	10, 20	12, 13	−7, −7	10, 26
DBA/2 mouse	Liver-N	Bound BP	3333, 3125	10.0,[d] 11.1	−52 ± 10[d]	76 ± 9[d]	38 ± 6[d]	46 ± 9[d]

[a] I, induced; N, noninduced.
[b] Average ± S.D. for 3 separate determinations.
[c] The amount required for 50% inhibition is 4 μM.
[d] Previously reported (19).

7,8-benzoflavone or cyclohexene oxide but were blocked by retinol and some of its derivatives and by butylated hydroxytoluene (Tables 2 and 3). Retinol and retinyl acetate were more potent inhibitors than other vitamin A compounds and analogs and more strongly inhibited the reaction leading to bound BP than to 3-hydroxy-BP formation (Table 3).

DISCUSSION

The kinetic constants for various lung enzymes involved in BP metabolism are summarized in Table 2. Included in this list are values for the hydroxylase and "binding" enzyme of DBA/2 mouse liver. These liver enzymes, which are not inducible by polycyclic hydrocarbons (19, 24, 31), have relatively high K_m and V_{max} values. Reported K_m values for induced aryl hydrocarbon hydroxylases are lower than for constitutive hydroxylases, and the V_{max} values are higher (1, 18, 30). Our results are generally in accord with these reports. However, an unexpected observation is that the constitutive hydroxylase of mouse lungs has a maximum velocity of 85 fmoles/min/mg at a BP concentration of 60 μM (Chart 2). This rate is almost as large as the maximum for the induced enzyme (120 fmoles/min/mg). Instead of resulting in a large change in reaction velocity, induction decreases the amount of substrate required to achieve the

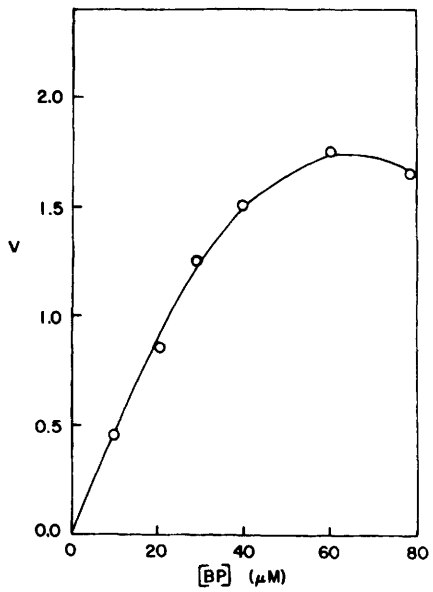

Chart 3. Substrate saturation curve for the constitutive aryl hydrocarbon hydroxylase of DBA/2 mice. *Vertical axis*, pmoles of product formed per min.

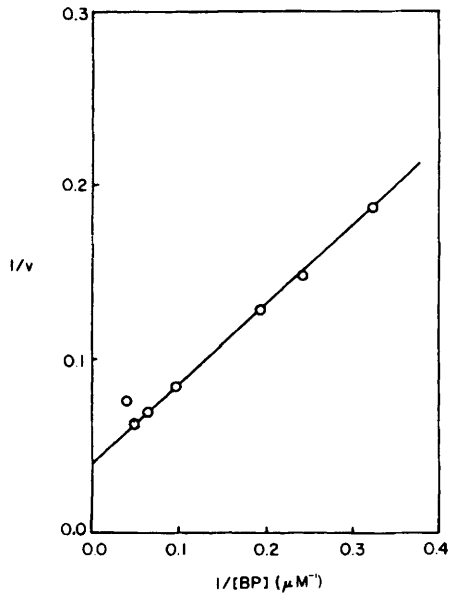

Chart 4. Lineweaver-Burk plot for the formation of bound BP by constitutive DBA/2 mouse microsomes. *Vertical axis*, reciprocal of pmoles of BP bound per 20 min.

Table 3

Inhibition of noninduced lung BP hydroxylase and bound BP by vitamin A compounds and analogs

Inhibitor	% inhibition			
	BP hydroxylase[a]		Bound BP formation[b]	
	DBA/2 mouse	Hamster	DBA/2 mouse	Hamster
None	0	0	0	0
Retinol	70	75	100[c]	100
Retinoate	14	25	46	40
Retinal	56	57	62	64
13-*cis*-Retinoate	31	7	−14	12
Methyl retinoate	31	22	10	22
Retinyl acetate	60	53	93	85
N-Ethylretinamide	83	73	41	51
N,*N*-Diethylretinamide	89	83	−5	26
9-(2-Acetyl-5,5-dimethyl-1-cyclopenten-1-yl)-3,7-dimethylnona-2,4,6,8-tetraenoic acid			10	18

[a] Assayed in the presence of 50 μM inhibitor.
[b] Assayed in the presence of 10 μM inhibitor.
[c] The amount required for 50% inhibition is 3 μM.

maximum reaction rate. Nevertheless, in the benz(*a*)-anthracene-treated mouse the low K_m value probably results in increased metabolism, for the BP concentrations (40 to 60 μM) necessary to achieve high-reaction velocities in lungs of noninduced mice are not likely to be reached. At BP concentrations of less than 10 μM, the reaction velocity would be much higher in mice treated with an inducer. The fact remains that, with assays accomplished with 60 μM BP, only a 30% difference is observed for induced and noninduced DBA/2 mouse lung hydroxylases. The constitutive mouse and hamster lung hydroxylases were not detected in earlier tests (19) because a low (3 μM) BP concentration and a pH (8.0) greater than optimum were used in the assays.

Since the lag period for the constitutive hydroxylase could not be reduced by various procedures involving prior incubation of components of the reaction system, it is possible that this enzyme must undergo a conformational change, in the presence of substrates, to allow the formation of products. A similar delay for *N*-hydroxylation of 2-acetylaminofluorene by mouse liver microsomes has been reported (2).

7,8-Benzoflavone may inhibit the induced hydroxylase reaction by inhibiting the enzyme "DT diaphorase," which is thought to be associated with the aryl hydrocarbon hydroxylase system in intact animals and the activity of which is blocked by 7,8-benzoflavone (26). In contrast, butylated hydroxytoluene binds to cytochrome P-450 and displays a "Type I" binding spectrum (48).

Soluble metabolites of BP produced by rat lung microsomes are BP-4,5-epoxide; the 4,5-, 7,8-, and 9,10-dihydrodihydroxy derivatives, each of which could be formed by the hydration of an epoxide; 9-hydroxy-BP, which could be formed by rearrangement of the appropriate epoxide; and 3-hydroxy-BP, for which a corresponding epoxide and dihydrodiol have not been found (14, 16, 35). The enzymatic products of BP that become tightly attached to macromolecules could be formed by the alkylating reactions of epoxides, which are also intermediates in the metabolism of several other polycyclic hydrocarbons (15, 21, 36). The 4,5-epoxide of BP can react with DNA without further activation (4, 41), and K-region epoxides of polycyclic hydrocarbons are potent transforming agents (17, 27).

The mechanism for the effects of cyclohexene oxide on microsomal oxidases is not clear. This compound stimulates the induced hydroxylases of mouse and hamster lungs but inhibits the constitutive lung hydroxylases (Table 2). Cyclohexene oxide is an *in vitro* inhibitor of epoxide hydrase (32, 47), and, with BP as the substrate for liver microsomal enzymes, it gives rise to increased yields of BP-4,5-epoxide (41) and to increased oxidase-catalyzed binding of BP to macromolecules (Table 2; Ref. 19). Another inhibitor of epoxide hydrase, 1,1,1-trichloro-2-propene oxide, also increases the NADPH-dependent binding of BP and 3-methylcholanthrene to DNA (6) and reduces the enzymatic hydration of 3-methylcholanthrene-11,12-oxide (39).

Although cyclohexene oxide may increase the production of 3-hydroxy-BP and bound BP in systems containing lung microsomes from induced animals by preventing the hydration of intermediate epoxides, it has other biological effects (32) and may stimulate 1 or both of the reactions by another mechanism. The lack of stimulation of the formation of 3-hydroxy-BP by liver microsomes (19) and of 3-hydroxy-BP and bound BP by lung microsomes from noninduced animals favors a mechanism independent of epoxide hydrase. Although epoxide hydrase activity is not detectable in the skin of DBA/2N mice, it is present in the livers of these animals (29) and may or may not be present in the lungs.

For microsomes from animals not pretreated with inducer, the potent inhibition of formation of bound BP by retinol and other vitamin A compounds and by butylated hydroxytoluene may be related to the anticarcinogenic effect of these agents. Vitamin A compounds have a prophylactic effect on premalignant and malignant lesions induced in the epithelial tissues of animals by polycyclic hydrocarbons (3, 7, 34), and vitamin A deficiency of hamsters enhances the binding of BP to DNA of epithelial cells of tracheas excised and maintained in organ culture for 3 hr (11). Butylated hydroxytoluene strongly inhibits the formation of mammary tumors induced by 7,12-dimethylbenz(*a*)anthracene (43) and cancer induction in rats treated with *N*-2-fluorenylacetamide or its *N*-hydroxy derivative (13, 40). Butylated hydroxyanisole, a related compound, inhibits the carcinogenic effect of p.o.-administered 7,12-dimethylbenz(*a*)anthracene and BP on mouse forestomach and adenoma formation in lungs (43, 44).

In contrast, 7,8-benzoflavone actually promotes the carcinogenicity of BP to mouse skin (23). This agent may reduce the formation of 3-hydroxy-BP and other inactive products more than the production of the active, carcinogenic metabolite. The constitutive BP hydroxylase of rat liver is not inhibited by 7,8-benzoflavone (45), but the induced hydroxylases of rat liver (45) and mouse skin (10) are blocked by this agent. 7,8-Benzoflavone inhibits the binding of BP to DNA of hamster tracheas in organ culture when the tracheas come from animals pretreated with BP but not the smaller amount of binding catalyzed by constitutive enzymes of tracheas from untreated animals (22). Our results are consistent in that they show strong inhibition by 7,8-benzoflavone of induced lung hydroxylases and "binding" enzymes and less inhibition of constitutive enzymes.

The report that induction of aryl hydrocarbon hydroxylase in experimental animals generally leads to a reduction in the carcinogenic action of polycyclic hydrocarbons (42) further suggests that a reaction catalyzed by a constitutive enzyme, such as that involved in formation of the metabolite which gives rise to bound BP, could be a critical event in lung carcinogenesis. All of the chemical carcinogens that have been thoroughly studied are known to bind to macromolecules of the target tissue (28). Although binding is necessary, it is not a sufficient criterion for carcinogenesis, because 7-bromomethyl-12-methylbenz(*a*)anthracene binds to mouse skin DNA to a smaller extent than its homolog, 7-bromomethylbenz(*a*)anthracene, and yet the latter compound is almost devoid of carcinogenic activity (33). Binding of BP by the induced enzyme may not be involved in carcinogenesis.

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