Dimethylbenzanthracene Tumorigenesis and Aryl Hydrocarbon Hydroxylase in Mouse Skin: Inhibition by 7,8-Benzoflavone

Abstract. *Mouse skin contains aryl hydrocarbon hydroxylase which is highly inducible. The enzyme system is inhibited when 7,8-benzoflavone is added to homogenates of skin epidermis. 7,8-Benzoflavone also inhibits mouse skin tumorigenesis caused by repeated treatment with 9,10-dimethylbenzanthracene or by a single treatment with this chemical followed by weekly treatment with croton oil. These findings suggest that this enzyme system may be responsible for the activation of 9,10-dimethylbenzanthracene to its carcinogenic form.*

Aryl hydrocarbon hydroxylase is an inducible enzyme system found in a variety of tissues of many mammalian species. It is part of the microsomal enzyme complex which is largely responsible for the metabolism of many exogenous chemicals including drugs, pesticides, and carcinogens (*1*). The microsomal aryl hydrocarbon hydroxylase system converts many polycyclic hydrocarbons to hydroxylated derivatives (*2*) and has been considered a "detoxification" system. Recent studies have suggested that the hydroxylase enzyme complex may also convert polycyclic hydrocarbons to a reactive form (*3*, *4*). The evidence for enzyme involvement in polycyclic hydrocarbon activation is: (i) the toxic effect of polycyclic hydrocarbons in a number of cell lines in culture is positively correlated with their level of aryl hydrocarbon hydroxylase in the cells (*3*); (ii) the inhibition of aryl hydrocarbon hydroxylase activity by 7,8-benzoflavone is paralleled by an inhibition of the toxic effect of the polycyclic hydrocarbon on cell growth (*5*); (iii) rat liver microsomal aryl hydrocarbon hydroxylase catalyzes the formation of covalent complexes of polycyclic hydrocarbon with DNA (*6*, *7*) and with protein (*7*). The latter processes may be involved in carcinogenesis.

The study of aryl hydrocarbon hydroxylase in a specific target tissue for the carcinogen, such as skin, should help to clarify the role of this enzyme in polycyclic hydrocarbon carcinogenesis. This paper presents evidence that aryl hydrocarbon hydroxylase is present and inducible in mouse skin, that the enzyme is inhibited by 7,8-benzoflavone, and that this compound inhibits skin tumorigenesis induced by 9,10-dimethylbenzanthracene (DMBA).

Both 7,8-benzoflavone and 5,6-benzoflavone have marked inhibitory activity on enzyme preparations derived from skin of either control mice or mice in which the enzyme is induced by prior treatment with 1,2-benz(*a*)anthracene (Table 1). When added to the enzyme preparations at concentrations equimolar with the substrate, the benzoflavones inhibit enzyme activity by 77 to 91 percent. When added at one-tenth of the substrate concentration, the inhibition ranges from 56 to 71 percent. These flavones similarly inhibit the enzyme derived from other tissues such as lung and kidney (*8*). As Table 1 shows, 1,2-benz(*a*)anthracene is relatively inactive as an inhibitor of the hydroxylase system although it is itself hydroxylated (*4*).

Table 2 shows the induction of aryl hydrocarbon hydroxylase in skin epidermis of mice after the intraperitoneal or topical application of various enzyme inducers. Wattenberg *et al.* (*9*) showed that 5,6-benzoflavone is a potent inducer of benzopyrene hydroxylase in lung, liver, and intestine. We found that 5,6-benzoflavone more effectively induces the enzyme in the epidermis than 7,8-benzoflavone or 1,2-benz(*a*)anthracene when administered intraperitoneally. However, when applied topically, 1,2-benz(*a*)anthracene is a more effective inducer than 5,6-benzoflavone. Sixteen hours after topical application, 5,6-benzoflavone caused a fourfold increase and 1,2-benz(*a*)anthracene caused a 16-fold increase in hydroxylase activity. The 7,8-benzoflavone fails to induce activity 4 or 16 hours after topical application, although it does increase activity when given intraperitoneally. Since 7,8-benzoflavone is a potent inhibitor of the enzyme when added to the enzyme assay, the low activity after topical application may be due to residual amounts of 7,8-benzoflavone in the skin that inhibits an induced level of enzyme. An inhibitory effect of residual 5,6-benzoflavone might similarly explain why this compound, given topically, is less effective than 1,2-benz(*a*)anthracene, in contrast to intraperitoneal application.

Table 2 (Experiment 2) also shows the enzyme-inducing effect of low levels of DMBA alone and in combination with 7,8-benzoflavone and 1,2-benz(*a*)anthracene. Four hours after treatment with DMBA there was an induction of enzyme activity. Simultaneous application of 7,8-benzoflavone resulted in a somewhat lesser induction of enzyme. This may be due to inhibition of the

Table 1. Effect of 7,8-benzoflavone, 5,6-benzoflavone, and 1,2-benz(*a*)anthracene on aryl hydrocarbon [3,4-benzo(*a*)pyrene] hydroxylase* in homogenates of mouse skin. Homogenates of skin epidermis were obtained from groups of NIH Swiss male mice (20 to 25 g) given 0.25 ml of corn oil only or 3 mg of 1,2-benzanthracene with 0.25 ml of corn oil intraperitoneally 16 hours earlier. The mice were killed by cervical dislocation. The skin was removed and placed in cold 0.25*M* sucrose–0.05*M* tris, *p*H 7.55. The epidermal layer was removed by scraping with a surgical scalpel and was homogenized with 30 passes in a Potter-Elvehjem homogenizer in the above-described buffer solution. The aryl hydrocarbon hydroxylase was assayed by the procedure of Nebert and Gelboin (*4*), a modification of Wattenberg *et al.* (*20*). The substrate was 3,4-benzopyrene at $10^{-4}M$. One unit of activity catalyzes the production, during a 20-minute incubation period, of an amount of fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo(*a*)pyrene. Protein concentration was 1 to 3 mg per flask and was determined by a slight modification of the method of Lowry *et al.* (*21*). The benzoflavones and benzanthracene in 0.010 ml of methanol or methanol alone were added to the incubation mixture.

Addition	Concentration (molarity)	Inhibition† (%)	
		Control enzyme	Induced enzyme
7,8-Benzoflavone	10^{-4}	83	91
7,8-Benzoflavone	10^{-5}	64	71
7,8-Benzoflavone	10^{-6}	10	21
5,6-Benzoflavone	10^{-4}	77	84
5,6-Benzoflavone	10^{-5}	56	65
5,6-Benzoflavone	10^{-6}	21	34
1,2-Benz(*a*)anthracene	10^{-4}	27	21
1,2-Benz(*a*)anthracene	10^{-5}	2	11
1,2-Benz(*a*)anthracene	10^{-6}	0	5

* The enzyme system hydroxylates a variety of other polycyclic hydrocarbon substrates (*4*) as well as benzopyrene and therefore is called aryl hydrocarbon hydroxylase. † With no additions the specific activity (units per milligram of protein) of enzyme from control mice was 44; from mice treated with 1,2-benz(*a*)anthracene, 447. The values shown are the percent decrease in specific activity.

Table 2. Induction of aryl hydrocarbon hydroxylase in mouse skin epidermis. Groups of three to four mice were used. Two-tenths of a milliliter of acetone or 0.2 ml of acetone containing the inducer was evenly applied on the shaven area. The mice treated intraperitoneally were inoculated with the compound in 0.2 ml of corn oil. The enzyme preparation and assay were as described in Table 1. 7,8-BF, 7,8-benzoflavone; 1,2-BA, 1,2-benz(*a*)anthracene.

Treatment (μg)	Mouse skin aryl hydrocarbon hydroxylase* (specific activity)		
	Topical application		Intraperitoneal application
	4 hours	16 hours	16 hours
	Experiment 1		
Control	54	30	34
7,8-Benzoflavone (50)	18	26	
7,8-Benzoflavone (300)	8	30	198
7,8-Benzoflavone (1000)	10	40	
1,2-Benz(*a*)anthracene (50)	199	278	
1,2-Benz(*a*)anthracene (300)	181	564	240
1,2-Benz(*a*)anthracene (1000)	249	478	
5,6-Benzoflavone (300)	71	165	560
	Experiment 2		
Control	39	29	
DMBA (25)	109	94	
DMBA (25) + 7,8-BF (27)	87	87	
DMBA (25) + 1,2-BA (23)	252	315	

* The values shown reflect the amount of enzyme activity measured in vitro after prior treatment of the mice in vivo. The enzyme activity shown may be lowered by residual amounts of the inhibitor present in the assay mixture. The assay does not contain added inhibitor and therefore does not reflect enzyme inhibition occurring in vivo because of the large excess of substrate present in the assay.

induced enzyme by residual amounts of 7,8-benzoflavone present in the assay mixture. 1,2-Benz(*a*)anthracene, applied with the DMBA, caused a relatively large induction of enzyme activity. Table 2 indicates that the flavones, 1,2-benz(*a*)anthracene, and DMBA exhibit inducing activity under certain conditions. Table 1, however, indicates that the flavones, in contrast to the 1,2-benz(*a*)anthracene, also exhibit enzyme inhibitory activity.

A single small dose of DMBA topically applied to mouse skin initiates tumorigenesis (*10, 11*). The initiated skin may then be promoted to the visible papilloma stage by the weekly application of a promoting agent such as croton oil (*11*). Tumorigenesis may also be induced by repeated doses of DMBA with no croton oil treatment (*12*). Table 3 shows that 7,8-benzoflavone inhibits mouse skin tumorigenesis induced by either repeated applications of DMBA or by a single DMBA application followed by croton oil treatment. In four experiments ranging from 14 to 20 weeks in duration, 7,8-benzoflavone inhibited papilloma formation by 39 to 90 percent. Benz(*a*)anthracene is essentially nontumorigenic in this skin tumorigenesis system (*12*). This compound when applied simultaneously with DMBA does not significantly affect tumor induction by DMBA (experiments 1 and 2).

These results show that 7,8-benzoflavone is effective both in inhibiting aryl hydrocarbon hydroxylase in skin and in inhibiting DMBA-induced tumor formation in the same tissue. Huggins *et al.* (*13*) have shown that treatment of rats with low levels of polycyclic hydrocarbons inhibits to a large extent the incidence of mammary cancer induced by DMBA. Wattenberg *et al.* (*9, 14*) have described the potent inducing effect of an isomer, 5,6-benzoflavone, on the enzyme in liver, lung, and intestine of rats and mice. They also found that 5,6-benzoflavone inhibits lung and mammary gland tumorigenesis induced by DMBA (*14*). In the latter experiments, the inhibitory effects may be due either to inhibition of the enzyme or to a high level of the induced hydroxylase which may be more effective than the control enzyme in converting the carcinogen to noncarcinogenic metabolites in the target tissue. Prior treatment of the animal with inducer, however, also increases the level of enzyme in the liver, and since this is the major site of metabolism, a high level of hydroxylase in the liver may reduce the effective dose of carcinogen in the lung or mammary gland. Wheatley (*15*) has also shown that an inhibitor of microsomal drug metabolism, β-diethylaminoethyldiphenyl-*n*-propyl acetate (SKF 525-A), enhances mammary tumorigenesis by DMBA. He suggested that inhibition of the enzyme system in liver could result in an increase in the effective dose of carcinogen in the mammary gland.

Tumor induction by topical application of small amounts of carcinogen is not likely to be affected by systemic metabolism of the carcinogen. Under such conditions inhibition of tumor formation is caused by 7,8-benzoflavone, a compound exhibiting strong enzyme inhibitory activity. 1,2-Benz(*a*)anthracene, a compound which does not inhibit the enzyme but rather induces the enzyme in the target tissue, has no effect on tumorigenesis when applied simultaneously with the carcinogen. The effect of preinduction of the enzyme in the target tissue on the tumorigenic response requires study.

Table 3. Effect of 7,8-benzoflavone (7,8-BF) and 1,2-benz(*a*)anthracene (BA) on tumor induction by DMBA only or DMBA and croton oil. In Experiments 1 and 3, 0.2 ml of acetone, 27 μg of 7,8-BF, or 23 μg of BA in 0.2 ml of acetone was applied topically to the backs of cleanly shaven mice. Five minutes later 25 μg of DMBA in 0.2 ml of acetone was applied. The mice in Experiments 1 and 3 received topical applications of 0.2 ml of 1 percent croton oil once a week starting 1 week after DMBA was given. In Experiments 2 and 4, 20 μg of DMBA in 0.2 ml of acetone was applied twice weekly. Either 21.2 μg of 7,8-BF or 17.8 μg of BA was applied in the same solution. Mice in Experiments 2 and 4 were treated for 10 weeks, at which time treatment stopped. At no time did they receive croton oil.

Group	Treatment	Survivors	Mice with tumors	Total tumors	Tumors per mouse	Inhibition (%)
	Experiment 1 (15 weeks)					
1	DMBA (once only)	29	28	373	12.9	
2	DMBA + 7,8-BF (once only)	24	21	81	3.4	74
3	DMBA + BA (once only)	29	27	317	10.9	16
	Experiment 2 (14 weeks)					
1	DMBA (twice weekly)	29	29	391	13.5	
2	DMBA + 7,8-BF (twice weekly)	26	10	36	1.4	90
3	DMBA + BA (twice weekly)	28	28	354	12.6	7
	Experiment 3 (20 weeks)					
1	DMBA (once only)	26	24	239	9.2	
2	DMBA + 7,8-BF (once only)	28	17	70	2.5	73
	Experiment 4 (20 weeks)					
1	DMBA (twice weekly)	22	21	305	13.9	
2	DMBA + 7,8-BF (twice weekly)	21	21	178	8.5	39

Our previous studies suggested that aryl hydrocarbon hydroxylase generates an active intermediate and is responsible for polycyclic hydrocarbon toxicity (*3*, *5–7*). Phenolic products of enzymatic hydroxylation are either of low or no carcinogenicity. Thus, the ultimate carcinogenic form is likely a reaction intermediate. In the case of DMBA this may be one of the hydroxymethyl derivatives which have been found to be carcinogenic (*16*). The carcinogenic forms may also be epoxides, carbonium ions, or radical cations (*17*). The mechanism of some microsomal aromatic hydrocarbon hydroxylations seems to involve an epoxide intermediate. Thus, naphthalene hydroxylation has been shown to proceed via naphthalene 1,2-epoxide (*18*). Epoxides may be metabolized to hydroxylated derivatives by an epoxide hydrase (*18*, *19*). We do not know whether the inhibitory effect of 7,8-benzoflavone is specific to DMBA tumorigenesis or whether this inhibitor is equally effective on tumorigenesis induced by other polycyclic hydrocarbons. This requires investigation.

Since 7,8-benzoflavone and 5,6-benzoflavone show biological affinity for the enzyme system as well as the induction receptor site for polycyclic hydrocarbons, it is possible that they have affinity for a hypothetical receptor site other than the enzyme at which the carcinogenic polycyclic hydrocarbons initiate tumorigenesis. Although this possibility is not eliminated, the data presented suggest that the tumorigenesis inhibitory activity of 7,8-benzoflavone is due to inhibition of the aryl hydrocarbon hydroxylase and that this enzyme system is responsible for activation of the administered DMBA to its carcinogenic form.

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22. We thank Dr. S. Rothberg for valuable advice concerning the isolation of skin epidermis and Miss Janet Leutz, Mr. Haywood Waters, and Mr. William Henderson for valuable technical assistance. We also thank Miss Britney Shell and Miss Jennifer Blake, Mt. Vernon College, for helpful experimental aid. One of us (L.D.) is supported by PHS Research grant CA-08936 from the National Cancer Institute.

20 May 1970; revised 8 July 1970 ■

Structure of RNA in Ribosomes

Abstract. *The 50S and 30S ribosomes and 23S and 16S RNA were hydrolyzed with ribonuclease A. The rate constants and number of fragments produced were determined for each reaction. The conformation of 23S RNA changes when the RNA is extracted from the ribosome. Specific regions of the RNA in 50S and 30S ribosomes are protected from hydrolysis by the ribosomal proteins.*

The role of ribosomal RNA (rRNA) in the structure and function of ribosomes has not been resolved. Optical rotatory dispersion and x-ray diffraction studies suggest that the average secondary structure is the same for protein-free rRNA and for rRNA in ribosomes (*1*, *2*). Most of the phosphate groups of rRNA, both protein-free and in ribosomes, bind dye molecules and magnesium ions, which suggests that the phosphates are available to the solvent (*1*, *3*). Ribonuclease hydrolyzes the RNA in ribosomes, which suggests that RNA may be a surface component of the ribosome (*4*). Examination of hydrolysis products of protein-free rRNA gives evidence that a definite tertiary structure exists for RNA in solution (*5*). Previous work has not shown differences in conformation for protein-free rRNA and RNA in ribosomes.

To determine whether specific regions of rRNA in ribosomes differ in conformation from the same regions of protein-free rRNA, we studied the initial stages of the ribonuclease-catalyzed hydrolysis reactions of 50*S* and 30*S* ribosomes and their 23*S* and 16*S* rRNA components by high resolution methods.

Ribosomes were extracted from *Escherichia coli* D10 (grown in tryptone medium) by standard methods (*6*). The 50*S* and 30*S* subunits were separated by zonal centrifugation in Beckman Ti14 rotor (*7*). Sucrose was removed with a diafiltration cell (Amicon). The 23*S* and 16*S* rRNA's were extracted from the 50*S* and 30*S* subunits, respectively, by the phenol–sodium dodecyl sulfate (SDS) method.

The hydrolysis reaction was carried out in a solution (50 μl) containing RNA (either free or in ribosomes, 0.5 mg/ml), magnesium acetate (0.005*M*), and tris(hydroxymethyl)aminomethane (0.005*M*, *p*H 7.4). Pancreatic ribonuclease (Worthington) was present at 0.01 μg/ml for 50*S*, 23*S*, and 16*S*, and at 0.4 μg/ml for 30*S*. The solutions were incubated at 0°C for the desired time. Addition of SDS to a concentration of 0.2 percent stopped hydrolysis and dissociated the ribosomal proteins from rRNA. Three small sucrose crystals were dissolved in each solution, which was then layered on a polyacrylamide gel. Electrophoresis resolved the undegraded RNA and the hydrolysis products (*8*).

Mild hydrolysis of the 50*S* ribosome produced two fragments of rRNA