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EFFECT OF PRETREATMENT WITH 7,8-BENZOFLAVONE AND DIETHYLSTILBESTROL ON THE HEPATIC METABOLISM OF DIETHYLSTILBESTROL IN THE MALE SYRIAN GOLDEN HAMSTER *IN VIVO*

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Abstract—Liver tumors are induced in male Syrian golden hamsters by the combined treatment with diethylstilbestrol (DES) and 7,8-benzoflavone (7,8-BF), but not with either substance alone. With the aim of clarifying whether metabolic activation of DES is involved in the mechanism of tumorigenesis in this animal model, we have studied the effect of pretreatment with 7,8-BF alone, DES alone, and 7,8-BF plus DES for 2, 8, 20 and 32 weeks on the hepatic *in vivo* metabolism of DES, using biliary metabolites collected from bile-duct cannulated male hamsters as probe. Formation of glucuronides and sulfates was not affected by treatment with 7,8-BF nor 7,8-BF plus DES. In contrast, animals pretreated with DES alone had a decreased amount of glucuronides and an increased proportion of unconjugated material in the bile. Oxidative metabolism of DES was not significantly altered in hamsters treated with 7,8-BF for up to 20 weeks, whereas pretreatment with DES alone and with 7,8-BF plus DES caused an enhancement of oxidative DES metabolism *in vivo*, leading mostly to highly polar, as yet unidentified products. From a consideration of various cytochrome P-450-associated enzyme activities, it is concluded that the observed effect on biliary DES metabolites is most likely to be due to an estrogen-induced intrahepatic cholestasis. Taken together, the data do not support a role for the metabolic activation of DES in this tumor model. Alternative mechanisms are proposed.

The synthetic estrogen diethylstilbestrol (DES)† induces kidney tumors in the male Syrian golden hamster with a 100% incidence after 8 months [1]. No liver tumors are observed under these conditions. However, combined treatment with DES and 7,8-benzoflavone (7,8-BF, α-naphthoflavone) leads to a marked reduction of the kidney tumor incidence and gives rise to hepatic tumors [2]. Administration of 7,8-BF alone does not cause tumors.

The reasons for the pronounced organotropism of DES carcinogenicity and for its shift by 7,8-BF are as yet unknown. Previous studies from our laboratory and from others have shown that DES is metabolically-activated and it has been proposed that the hormonal activity and the metabolic activation act in concert in the mechanism of DES carcinogenesis [3–5]. Thus, modulation of DES metabolism may alter DES carcinogenicity. For example, administration of vitamin C to DES-treated hamsters markedly lowered the incidence of kidney tumors, possibly by acting as a reducing agent for active DES metabolites [6]. Similarly, it is conceivable that 7,8-BF modulates DES metabolism, resulting in an increased metabolic activation of DES in the liver. Previous studies by Li and Li [4] have demonstrated that pretreatment with 7,8-BF for four months changes the pattern of cytochrome P-450 isoenzymes in the hamster liver, but it is as yet unknown whether this is associated with a change in DES metabolism. The present study was designed to clarify whether the hepatic *in vivo* metabolism of DES in the male Syrian golden hamster is affected by pretreatment with 7,8-BF. Biliary metabolites, collected from bile duct-cannulated animals, were assumed to represent hepatic metabolism. In addition to treatments with 7,8-BF alone, animals were pretreated with DES alone and with a combination of 7,8-BF and DES, and the effects of those pretreatments on the biliary excretion, on the conjugation pattern and on the oxidative metabolism of DES were investigated.

MATERIALS AND METHODS

Chemicals and reagents. (Monoethyl-2-^{14}C)-DES (spec. radioactivity 53 mCi/mmole, the Radiochemical Centre, Amersham, U.K.) was shown by HPLC to be of 95% radiochemical purity and to consist of 77% *E*- and 18% *Z*-isomer. Unlabelled E-DES and 4′-hydroxypropiophenone were purchased from Merck (Darmstadt, F.R.G.). *Z,Z*-Dienestrol (*Z,Z*-DIES) and 1-hydroxy-*Z,Z*-DIES were prepared in our laboratory as described previously [7]. 3′-Hydroxy-*E*-DES, 1-hydroxy-*E*-DES and 1-hydroxy-ψ-DES were kindly provided by Dr. J. A. McLachlan (National Institute of Environmental Health Sciences, Research Triangle Park, NC) and *Z*-DES by Dr. P. Murphy (Lilly Research Lab-

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† Abbreviations used: 7,8-BF, 7,8-benzoflavone; DES, diethylstilbestrol, 3,4-bis-(*p*-hydroxyphenyl)-hex-3-ene; ψ-DES, pseudo DES, 3,4-bis-(*p*-hydroxyphenyl)-hex-2-ene; DIES, dienestrol, 3,4-bis-(*p*-hydroxyphenyl)-hexa-2,4-diene; E_2, estradiol-17β; GC/MS, gas chromatography/mass spectrometry; HPLC, high pressure liquid chromatography.

oratories, Indianapolis, IN). Indenestrol A was synthesized according to Adler and Hägglund [8]. 7,8-BF and β-glucuronidase/arylsulfatase were from Serva GmbH (Heidelberg, F.R.G.). Liquid chromatography-grade methanol was from Promochem GmbH (Wesel, F.R.G.). All other chemicals and reagents used were of analytical grade.

Animals and pretreatment. Male Syrian golden hamsters (90–100 g body weight) were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, F.R.G.). The animals had access to standard pelleted lab chow (Altromin 1324, Altrogge, Lage/Lippe, F.R.G.) and tap water *ad libitum*. They were kept under controlled conditions of temperature and humidity on a 12-hr light: 12-hr dark cycle and acclimated at least 4 weeks prior to use.

For pretreatment with DES, a 20-mg DES pellet was implanted s.c. in the shoulder region. Every three months an additional pellet was implanted to maintain DES tissue levels. All pellets were removed 48–60 hr prior to the administration of ^{14}C-DES in order to clear unlabelled DES.

For pretreatment with 7,8-BF, a pelleted lab chow of Altromin 1324 containing 0.4% 7,8-BF was prepared by Altrogge (Lage/Lippe, F.R.G.) and fed to the animals *ad libitum*.

Bile duct fistula. The animals were anesthetized with Hypnorm® (Janssen GmbH, Neuß, F.R.G., dosed at 15 mg fluanisone/kg body weight and 0.3 mg fentanyl base/kg) and Valium® (Roche, Grenzach-Wyhlen, F.R.G., dosed at 5 mg diazepam/kg) and the bile duct was cannulated. 15 min after cannulation, a dose of 25 μmole ^{14}C-DES/kg body weight (14 μCi) dissolved in 1 ml propan-1,2-diol was administered by i.p. injection and the bile was collected continuously for 6 hours.

Separation and enzymatic hydrolysis of biliary metabolites. After saturation of the bile with ammonium sulfate DES metabolites were extracted with diethylether–ethanol (3:1, v/v) and subsequently separated into unconjugated metabolites and conjugates by chromatography on neutral alumina as previously described [9]. Briefly, the extract was applied to the column and eluted with 100 ml 95% ethanol (unconjugated metabolites), 100 ml water (sulfates) and 150 ml 40 mM phosphate–citrate buffer pH 6 (glucuronides). The conjugates were hydrolyzed with glucuronidase/arylsulfatase from *Helix pomatia* at 37° for 15 hr and the deconjugated metabolites extracted with diethylether. Previous work has shown that enzymatic hydrolysis of conjugates is virtually complete under these conditions [9].

Identification of DES metabolites. High performance liquid chromatography (HPLC) was carried out using a Waters instrument. A 10 cm × 4 mm i.d. column packed with RP-18 (Macherey–Nagel, Düren, F.R.G.) was operated at 20° with a flow rate of 1 ml/min and a linear gradient (solvent A: water–methanol 8:2, v/v; solvent B: methanol) changing from 45% B to 100% B in 30 min. The eluate was monitored continuously with a UV detector at 254 nm and collected in 0.3 ml fractions. Radioactivity was measured in a model Tri-Carb 4530 liquid scintillation counter with automatic external standardization (Packard Instruments, Frankfurt, F.R.G.). The HPLC fractions used for the analysis by gas chromatography/mass spectrometry (GC/MS) were evaporated to dryness under reduced pressure and derivatized with O,N-bis(trimethylsilyl)acetamide. GC/MS was performed on a Finnigan 4510 GC/MS as previously described [10].

RESULTS

Male Syrian golden hamsters were pretreated with 7,8-BF (0.4% in the diet) or DES (20 mg pellet s.c. implanted) or both 7,8-BF and DES for 2 weeks, 8 weeks, 20 weeks and 32 weeks. A single dose of ^{14}C-DES (25 μmole/kg) was then administered i.p. to the bile duct-cannulated animals, and bile was collected for 6 hr. Radioactivity was extracted from bile, separated into unconjugated material and the different conjugate fractions by column chromatography. The pattern of oxidative DES metabolites was determined in the various fractions by HPLC. In order to account for a conceivable age-dependent alteration in DES metabolism, animals of various ages up to 45 weeks were used as controls, but were all found to have very similar metabolism.

Effect of pretreatment on biliary excretion

In untreated male hamsters, 45 ± 7% (mean of 16 animals ± SD) of the administered radioactivity was excreted in the bile within 6 hours. Pretreatment with 7,8-BF, DES, or combined treatment had no effect on the amount of excreted biliary radioactivity, with the exception of pretreatment with DES for 32 weeks. These hamsters had a pronounced cholestasis with a decrease in bile flow from about 0.9 μl/min × g liver to 0.4 μl/min × g liver. Biliary excretion declined to 37 ± 6% of the administered dose. Although pronounced, this decrease was not statistically significant according to the Student's *t*-test.

Effect of pretreatment on conjugation

Chromatography on neutral alumina separated the extracted DES metabolites into unconjugated material, sulfates and glucuronides.

In untreated animals, unconjugated radioactivity represented less than 8% and sulfates 15–20%, whereas most of the radioactivity was found in the glucuronide fraction (Table 1). Pretreatment with 7,8-BF did not change the pattern of conjugates (Table 1). In contrast, pretreatment with DES led to an increase in the amount of unconjugated compounds with increasing time of pretreatment, mostly at the expense of the glucuronide fraction (Table 1). Animals pretreated with a combination of 7,8-BF and DES did not show this effect but exhibited a pattern of conjugates similar to that of control animals (Table 1).

Effect of pretreatment on oxidative DES metabolism

The sulfate and glucuronide fractions were enzymatically hydrolyzed and the deconjugated metabolites extracted and analyzed by HPLC. Typical radiochromatograms of the fractions (unconjugated material, sulfates and glucuronides) from untreated animals are given in Fig. 1. All three fractions contained the same compounds, although in different

Table 1. Pattern of unconjugated and conjugated DES metabolites in the bile from male hamsters pretreated for different time periods with DES, 7,8-BF and DES plus 7,8-BF

Pretreatment compound	Duration (weeks)	Number of animals	Unconjugated metabolites	Sulfates	Glucuronides
Controls		16	6.8 ± 3.1	18.0 ± 4.6	75.2 ± 6.0
7,8-BF	2	4	8.8 ± 3.9	14.8 ± 4.6	76.4 ± 3.5
	8	4	5.2 ± 1.4	15.4 ± 3.4	79.4 ± 4.7
	20	4	4.1 ± 0.8	22.2 ± 5.1	73.7 ± 5.4
	32	4	6.7 ± 2.4	24.5 ± 7.8	68.7 ± 9.3
DES	2	4	6.7 ± 2.5	30.7 ± 2.4	62.5 ± 4.6
	8	4	9.7 ± 2.1	16.0 ± 4.6	76.9 ± 5.3
	20	4	12.2 ± 0.6	21.5 ± 4.4	66.4 ± 4.4
	32	4	26.3 ± 6.4	13.0 ± 1.1	60.7 ± 6.8
DES plus 7,8-BF	2	4	8.1 ± 2.1	10.2 ± 4.4	84.0 ± 7.9
	8	5	6.1 ± 2.0	9.6 ± 6.9	84.2 ± 5.9
	20	4	13.3 ± 4.4	30.0 ± 9.1	56.7 ± 7.0
	32	4	10.0 ± 3.1	12.5 ± 0.8	77.5 ± 3.7

Data represent % of biliary radioactivity and are corrected for losses during alumina chromatography.

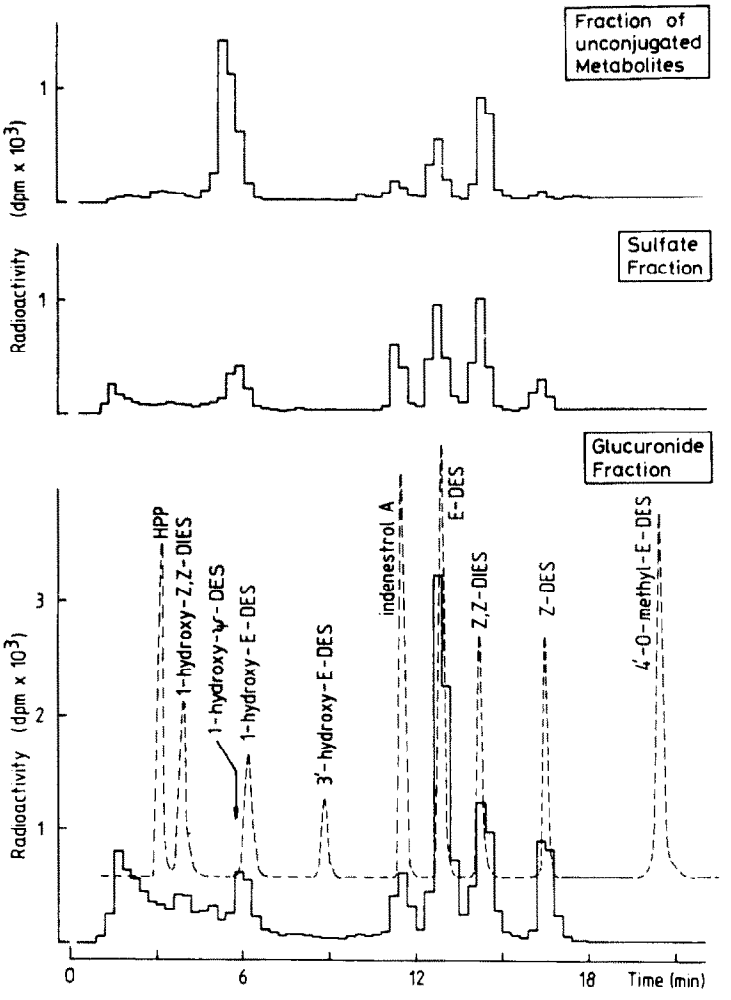

Fig. 1. HPLC-radiochromatogram of the biliary fractions from untreated male hamsters dosed with ^{14}C-DES. Sulfates and glucuronides were hydrolyzed prior to HPLC. The dotted peaks refer to the elution of authentic standards.

amounts. In addition to the parent compound *E*-DES and its *Z*-isomer, the metabolites *Z,Z*-dienestrol, indenestrol A and 1-hydroxy-ψ-DES were clearly detectable. These metabolites were identified by cochromatography with synthetic reference compounds and also by GLC/MS of the HPLC peaks of one glucuronide fraction as previously described [10].

The major oxidative metabolites in the biliary fractions of unconjugated material, sulfates and glucuronides were quantitated by HPLC. As an example, the data obtained after 20-week pretreatment are presented in Table 2. Similar data were obtained for the other pretreatment periods. In order to quantify the total amounts of the various oxidative metabolites formed under the different pretreatment regimens, the sum of the respective metabolites in the fractions of unconjugated material, sulfates and glucuronides was determined (Fig. 2). Pretreatment with 7,8-BF alone (Fig. 2, upper panel) did not significantly influence the amount of parent *E*-DES nor of any of the metabolites, except after 32 weeks, when an increase in polar metabolites was noted. The chemical nature of this polar material, which is eluted from the HPLC column with a retention time of 3–5 min (Fig. 1) is as yet unknown. Pretreatment with DES alone (Fig. 2, middle panel) and with 7,8-BF plus DES (Fig. 2, lower panel) led to a decrease in parent *E*-DES and to an enhanced formation of the polar material. The effect appears to increase with duration of pretreatment. Other metabolites were less affected, although the formation of 1-hydroxy-ψ-DES and indenestrol A seemed to be enhanced.

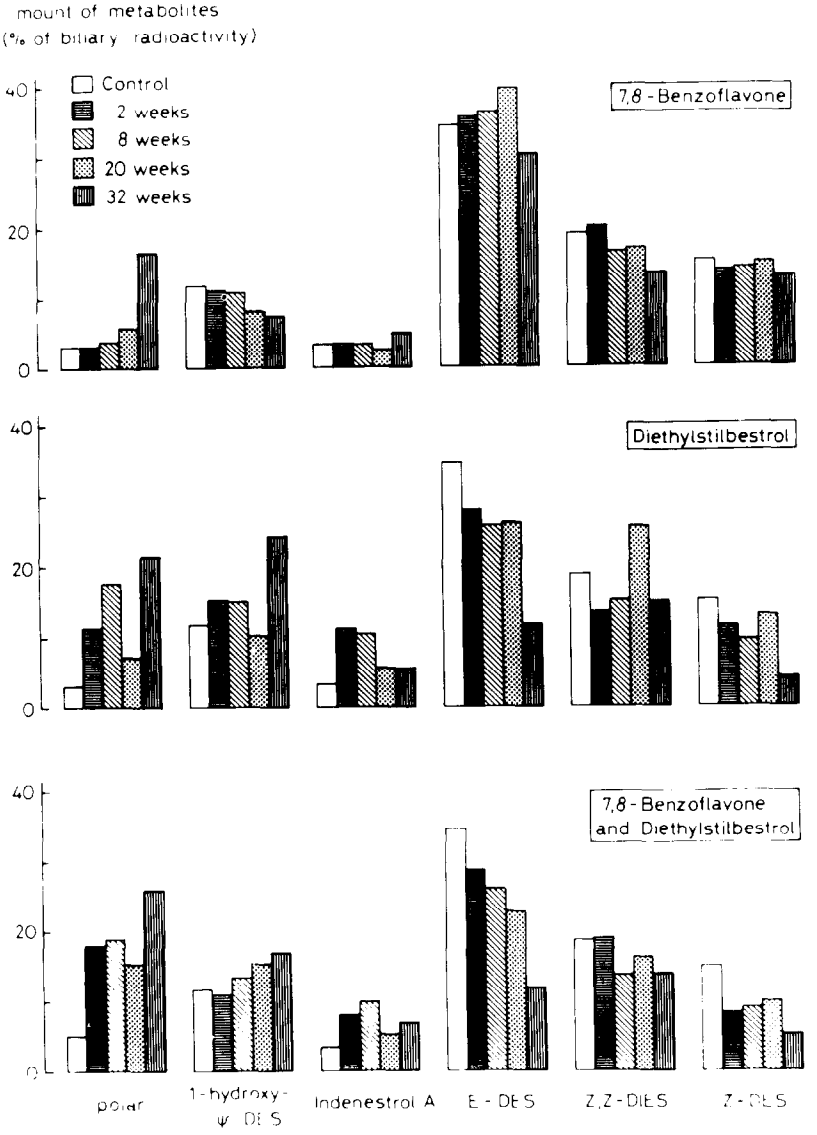

Fig. 2. Pattern of oxidative DES metabolites in hamster bile after various pretreatments. Data represent percent of biliary radioactivity and were obtained by adding the amounts of the individual metabolites present in the fractions of unconjugated material, sulfates and glucuronides. Values are corrected for losses during alumina chromatography, extraction after enzymatic hydrolysis, and HPLC analysis.

Table 2. Effect of pretreatment for 20 weeks on the pattern of oxidative DES metabolites in various fractions of hamster bile

Fraction	Pretreatment	Polar	1-hydroxy-ψ-DES	Indenestrol A	*E*-DES	*Z,Z*-DIES	*Z*-DES
Unconjugated metabolites	Control	≤0.1	2.7 ± 1.2	0.9 ± 0.2	0.3 ± 0.1	1.0 ± 0.2	≤0.1
	7,8-BF	≤0.1	1.0 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.8 ± 0.3	≤0.1
	DES	≤0.1	5.5 ± 0.5	2.4 ± 0.7	0.4 ± 0.1	2.6 ± 0.6	≤0.1
	DES + 7,8-BF	≤0.1	5.5 ± 1.8	2.1 ± 0.6	0.9 ± 0.3	3.0 ± 1.5	≤0.1
Sulfates	Control	0.9 ± 0.2	1.4 ± 0.3	0.2 ± 0.4	5.2 ± 2.2	1.3 ± 0.4	2.6 ± 1.0
	7,8-BF	1.8 ± 0.2	1.1 ± 0.2	0.3 ± 0.1	12.5 ± 4.3	1.1 ± 0.2	4.3 ± 1.0
	DES	2.7 ± 0.2	1.3 ± 0.4	0.8 ± 0.1	8.3 ± 0.3	1.9 ± 0.5	3.6 ± 0.3
	DES + 7,8-BF	5.0 ± 1.5	2.2 ± 0.5	0.9 ± 0.2	10.8 ± 5.0	2.5 ± 1.1	5.2 ± 2.5
Glucuronides	Control	2.1 ± 0.2	7.0 ± 1.4	2.3 ± 0.4	25.9 ± 4.2	15.6 ± 2.1	11.7 ± 2.0
	7,8-BF	3.9 ± 0.3	6.3 ± 0.7	1.6 ± 0.2	26.8 ± 3.8	14.5 ± 0.7	10.5 ± 1.2
	DES	4.7 ± 0.4	3.2 ± 0.1	2.4 ± 0.2	17.2 ± 3.5	21.1 ± 1.1	9.1 ± 1.5
	DES + 7,8-BF	10.3 ± 1.0	7.8 ± 1.4	2.3 ± 0.4	11.2 ± 2.6	10.7 ± 1.2	4.8 ± 0.9

After separation on alumina, the DES conjugates were enzymatically hydrolyzed and analyzed by HPLC. Data represent % of biliary radioactivity (mean of 4 animals ± SD) and are corrected for losses during alumina chromatography, extraction after enzymatic hydrolysis and HPLC analysis.

DISCUSSION

The experimental induction of liver tumors in male Syrian golden hamsters by certain estrogens in combination with 7,8-BF appears to be a unique animal model for studying the role of estrogen metabolism in the mechanism of estrogen carcinogenicity. If metabolic activation of the estrogen is required for its hepatotumorigenic effect, the pattern of biliary estrogen metabolites might be expected to be different under conditions of tumor induction as compared to treatments not leading to liver tumors. Therefore, the oxidative and conjugative metabolites of DES in the hamster bile were determined in this study in animals pretreated for various time periods with 7,8-BF alone, with DES alone, and with 7,8-BF plus DES in combination.

Previously studies have analyzed the urinary and fecal metabolites of DES in the hamster, indicating that DES is extensively oxidized and conjugated *in vivo*, and predominantly excreted with the feces [9]. The present study of the biliary DES metabolites of the male hamster is in accordance with these findings. Excretion into the bile was very efficient, and biliary metabolites consisted predominantly of glucuronides with only very little unconjugated material. Of the excreted material, over 50% represented oxidative DES metabolites, predominantly *Z,Z*-dienestrol and 1-hydroxy-ψ-DES.

The effects of the various pretreatments were studied on the level of biliary excretion, pattern of conjugates and pattern of oxidative metabolites of DES. With 7,8-BF alone, none of these factors was changed even after prolonged pretreatment. In contrast, pretreatment with DES alone or in combination with 7,8-BF led to a significant shift in the oxidative metabolism of DES. The amount of unchanged DES (both *E*- and *Z*-isomer) decreased and the amount of polar, unidentified products increased with increasing time of pretreatment, whereas the identified metabolites, *Z,Z*-dienestrol and 1-hydroxy-ψ-DES, did not change in quantity.

The finding that pretreatment with DES and DES plus 7,8-BF, but not with 7,8-BF alone affects the pattern of oxidative DES metabolites raises the question whether the observed effect is due to a change in enzyme activities in the liver or to other factors. For example, it is known that prolonged estrogen administration leads to intrahepatic cholestasis [11]. The reduced bile flow in estrogen-pretreated animals may cause the DES to stay longer in the liver, thus leading to a more extensive degradation.

In order to discriminate between these alternatives, a discussion of the enzyme activities in the hamster liver should be helpful. We have recently reported that pretreatment with 7,8-BF leads to a marked increase in the amount of cytochrome P-450 and cytochrome b_5 in male hamster liver microsomal preparations [12]. The activities of microsomal 7-ethoxycoumarin-*O*-deethylase and 7-ethoxyresorufin-*O*-deethylase were increased by a factor of 2 and 5 respectively, whereas aryl hydrocarbon hydroxylase activity was only marginally enhanced. However, the formation of all oxidative DES metabolites was significantly decreased in microsomes from 7,8-BF-pretreated hamster liver, suggesting that the 7,8-BF-inducible cytochrome P-450 isozymes are not involved in DES metabolism [12]. Preliminary data with hepatic microsomes of hamsters pretreated with DES alone or in combination with 7,8-BF are also not indicative of a stimulating effect on DES metabolism (G. Blaich, unpublished observations). Taken together, the *in vitro* data suggest that the observed shift in biliary DES metabolites *in vivo* is more likely estrogen-mediated, e.g. through cholestasis, than due to increased enzyme activities.

Whatever the underlying mechanism is, the fact that the same effect is observed in animals pretreated with DES alone and with a combination of 7,8-BF and DES suggests that this metabolic shift does not play a role in the induction of liver tumors, as these are only obtained after the combined pretreatment.

The findings reported so far raise doubts as to whether the metabolic activation of estrogens is a

prerequisite for hepatocarcinogenesis in this tumor model. Instead, it is conceivable that 7,8-BF rather than the estrogen acts as an initiating agent in the male hamster liver. Two recent observations appear to support this notion. By using the ^{32}P-postlabelling technique to detect DNA adducts, two adducts were found in hamster liver after pretreatment for 4 or 9 months with 7,8-BF alone and with 7,8-BF plus estradiol-17β (E_2), but not with E_2 alone nor in untreated control animals [13]. These results imply that 7,8-BF can bind to DNA whereas E_2 cannot. The ability of 7,8-BF to form DNA adducts and to act as a clastogen have recently been demonstrated in Chinese hamster ovary cells in the presence of rat liver microsomes induced with 2,3,7,8-tetrachlorodibenzo(*p*)dioxin [14, 15]. In the hamster liver, neither 7,8-BF alone nor DES alone gave rise to tumor formation [2]. Under the conditions of combined treatment, 7,8-BF may act as an initiator and DES as a promotor. Present studies in our laboratory aim to identify the DNA-binding metabolite(s) of 7,8-BF.

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