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PYRROLIZIDINE ALKALOID-INDUCED ALTERATIONS IN BENZO[*a*]PYRENE METABOLISM AND BINDING OF BENZO[*a*]PYRENE METABOLITES TO DEOXYRIBONUCLEIC ACID

DAVID E. WILLIAMS,* CRISTOBAL L. MIRANDA and DONALD R. BUHLER†
Department of Agricultural Chemistry and Environmental Health Sciences Center, Oregon State University, Corvallis, OR 97331, U.S.A.

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Abstract—Pretreatment of rats by oral administration of jacobine, a pyrrolizidine alkaloid and inducer of epoxide hydrolase, produced a marked shift in hepatic microsomal metabolism *in vitro* of benzo[*a*]pyrene. The formation of 9-hydroxybenzo[*a*]pyrene and 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene was decreased whereas the formation of 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrene was increased following jacobine treatment. This shift in the ratio of benzo[*a*]pyrene metabolites was accompanied by a significant reduction in DNA binding. Addition of purified epoxide hydrolase to control or jacobine microsomes produced a similar decrease in total DNA binding. Chromatography of benzo[*a*]pyrene metabolite–DNA nucleoside adducts showed a marked reduction in four peaks and the elimination of one peak with microsomes from jacobine-treated rats.

Benzo[*a*]pyrene (BP) is converted to various products, including phenols, quinones and dihydrodiols, through the formation of reactive epoxides catalyzed by the cytochrome P-450-containing monooxygenase system [1–3]. The metabolite profile obtained varies with the animal species and strain and within tissues of the same animal [3–6]. The metabolite pattern of BP in a particular animal, however, can be altered by pretreatment with chemicals such as 1,2-epoxy-3,3,3-trichloropropane (TCPO) [7], ethoxyquin [8], or *trans*-stilbene oxide [9]. The changes in metabolite profile brought about by these chemicals appear to be related to their abilities to inhibit (for TCPO) or induce epoxide hydrolase which catalyzes the hydration of epoxides to dihydrodiols [1, 10]. Since the various metabolites of BP exhibit differential biological activity [11], alteration of the metabolite profile of BP in a given animal species by pretreatment with chemicals may result in decreased or increased carcinogenicity and mutagenicity of BP. The present report deals with the effect of the pyrrolizidine alkaloid (PA) jacobine, an inducer of epoxide hydrolase [12], on the metabolite profile of BP and the binding of BP metabolites to DNA. Jacobine, found in plants such as *Senecio jacobaea* [13], unlike *trans*-stilbene oxide, inhibits aryl hydrocarbon hydroxylase activity (formation of 3- and 9-phenols) while inducing epoxide hydrolase activity [12].

MATERIALS AND METHODS

[^{14}C]BP (21.6 mCi/mmole), [^{3}H]BP (17.4 Ci/ obtained from the Amersham Corp., Arlington Heights, IL. BP metabolite standards were provided by Donald M. Jerina of The National Institutes of Health. Calf thymus DNA, DNase I (Type I, from bovine pancreas), phosphodiesterase I (Type VII, from *Crotalus atrox*), and alkaline phosphatase (Type III, from *Escherichia coli*) were purchased from the Sigma Chemical Co., St. Louis, MO. Jacobine was isolated from *S. jacobaea* in our laboratory using the procedure of Mattocks [14] and was recrystallized twice from methanol–water to yield a product with a purity of 98% as determined by high pressure liquid chromatography (HPLC) [15].

Male Sprague–Dawley rats (180–200 g) were dosed with jacobine (40 mg/kg) by gastric intubation for 3 consecutive days. This dose was found previously to cause approximately a 4-fold increase in hepatic epoxide hydrolase activity [12]. Control animals were given saline. Twenty-four hours after saline or jacobine administration, the animals were killed by CO_2 inhalation and livers were removed for the preparation of microsomes [12]. To determine the effect of jacobine pretreatment on the metabolite profile of BP, 1-ml reaction mixtures were prepared [16] and incubated for 20 min. The BP metabolites were identified and quantified by reverse phase HPLC using appropriate standards according to Thakker *et al.* [17]. Pyrrolic metabolites of the PAs bound to microsomes were estimated by the colorimetric method of Mattocks and White [18].

Epoxide hydrolase was purified from liver microsomes of polychlorinated biphenyl (PCB)-treated rats to a specific activity of 442 units (nmoles of [^{3}H]styrene oxide hydrated/min)/mg protein using the method of Guengerich and Martin [19]. This

* Present address: Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, U.S.A.
† To whom reprint requests should be addressed.

preparation was homogeneous by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and had a molecular weight of 49,000.

In the DNA binding experiment, the reaction mixture used above was modified slightly by adding DNA (1 mg/ml) and using [^{3}H]BP instead of [^{14}C]BP with a total volume of 2 ml. After a 20-min incubation, the DNA was isolated from the incubation mixture [20] and subjected to enzymatic hydrolysis [21]. The BP metabolite–DNA nucleoside adducts were then separated by LH 20 column chromatography [22].

RESULTS

The effects of jacobine administration on the metabolite profile of BP are given in Table 1. The total of the metabolites was decreased significantly after jacobine treatment (297 pmoles per min per mg protein vs 403 pmoles per min per mg protein for controls). This is consistent with the observation that jacobine inhibits AHH activity [12]. Formation of 9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene (BP-9,10-diol), 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BP-7,8-diol) and 9-hydroxybenzo[*a*]pyrene (BP-9-OH) was decreased significantly, while formation of the 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrene (BP-4,5-diol) was increased significantly. The levels of 3-hydroxybenzo[*a*]pyrene (BP-3-OH) and total quinones were also reduced by jacobine treatment, but not to the same extent. The ratio of non-K region dihydrodiol metabolites (BP-7,8-diol and BP-9,10-diol) to K region metabolites (BP-4,5-diol) was found to be considerably lower in jacobine-treated animals (0.88 vs 2.61 of controls). There was also a slight difference in the ratio of total phenols to total dihydrodiols between the two groups, 0.82 for the jacobine group and 1.13 for controls. The effect of jacobine on the entire BP metabolite profile is shown in Fig. 1.

Aside from the observed difference in the absolute amounts of metabolites between the two groups, there also was a change in the proportion of metabolites formed (Table 1). In the jacobine group, the relative amount of BP-9-OH was reduced significantly (7.4% of total metabolites compared to 10.1% for controls) while the percentage of BP-4,5-diol was increased significantly (16.9% of total metabolites in jacobine but only 8.3% in controls).

It would be of interest to compare these results with those obtained by Oesch [9] who used *trans*-stilbene oxide for pretreatment of the animals. A drastic reduction in the formation of BP-7,8-diol and BP-9,10-diol and a marked increase in BP-4,5-diol were obtained in both studies, but was not as marked in our present study. As with the findings of Oesch, the proportion of BP-3-OH and quinones was not altered appreciably by jacobine treatment.

The effects of jacobine pretreatment and the addition of purified epoxide hydrolase on the total binding of BP metabolites to DNA are seen in Table 2. Consistent with the shift in the BP metabolite pattern to a higher ratio of deactivated (K region) to activated (non-K region) metabolites, jacobine pretreatment significantly reduced total covalent DNA binding by almost 50%. Addition of excess amounts (300 units) of epoxide hydrolase produced about the same decrease in DNA binding as did jacobine pretreatment. Addition of epoxide hydrolase to microsomes from jacobine-pretreated rats produced an even further decrease in total DNA binding to levels of about 28% of control microsomes.

After enzymatic hydrolysis, [^{3}H]BP–DNA adducts were resolved by LH 20 chromatography (Fig. 2). Without microsomes in the incubation mixture, no radioactive peaks were observed after chromatography of the DNA hydrolysate. In the presence of control microsomes, two major peaks (peaks a and b) appeared before fraction 20 and three minor peaks (peaks c, d and e) after fraction 40. When microsomes from the jacobine-treated group were used in the incubation, a similar elution profile (Fig. 2) was obtained, but the peaks were reduced in size (peaks a, b and d) or disappeared (peak e). Although it is not possible to assign identities to any of these adducts, comparisons with previous reports [23–25] suggest that c, d and e were derived from BP-7,8-diol-9,10-epoxide, BP-4,5-epoxide and BP-9-OH-4,5-epoxide respectively. We repeated the enzymatic

Table 1. Effect of jacobine administration on *in vitro* metabolism of BP by rat liver microsomes*

	Control rats		Jacobine-treated rats		
Metabolites	pmoles/min/mg protein	% of metabolites	pmoles/min/mg protein	% of metabolites	Ratio of jacobine/control
BP-9,10-diol	58 ± 10	14.4 ± 1.1	38 ± 5†	13.0 ± 1.5	0.66
BP-4,5-diol	33 ± 4	8.3 ± 1.1	51 ± 12‡	16.9 ± 3.2†	1.55
BP-7,8-diol	28 ± 12	6.9 ± 2.8	13 ± 3‡	4.4 ± 1.0	0.46
Quinones (3,6; 1,6 and 6,12)	150 ± 46	37.1 ± 9.4	111 ± 28	37.1 ± 6.8	0.74
BP-9-OH	41 ± 8	10.1 ± 1.5	22 ± 6†	7.4 ± 1.5‡	0.54
BP-3-OH	93 ± 27	23.2 ± 6.8	62 ± 22	21.2 ± 9.0	0.67
Total	403 ± 50	100	297 ± 32†	100	0.74

* Mixtures (1 ml) containing 0.8 mg microsomes and 60 μM [^{14}C]BP were incubated at 37° for 20 min. Values represent means ± S.D. of six determinations.

† Significantly different from controls ($P < 0.01$).

‡ Significantly different from controls ($P < 0.05$).

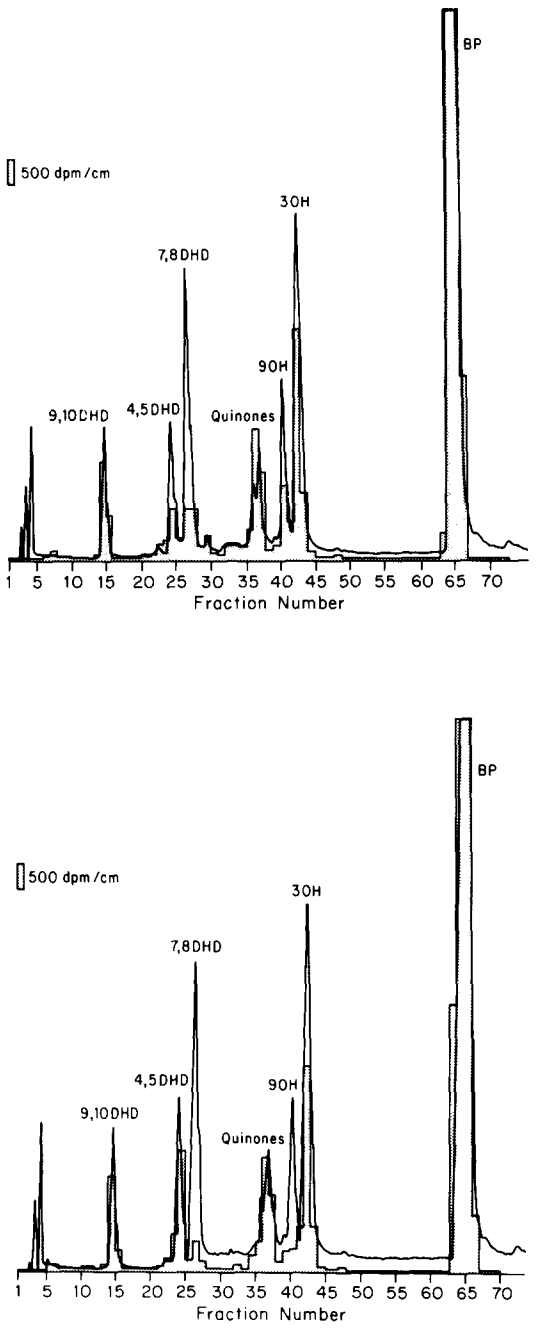

Fig. 1. HPLC metabolite profiles of [^{14}C]BP following incubation with microsomes from control (top panel) or jacobine-pretreated (bottom panel) rats. Incubations were performed as described in Materials and Methods. The reaction was terminated with methylene chloride–methanol (2:1), and the substrate and products were extracted twice into the organic phase. Each sample was then spiked with unlabeled BP metabolite standards. Following evaporation under N_2, the residue was redissolved in 50 μl of methanol and injected onto a Zorbax ODS (4.6 mm i.d. × 25 cm) column, and the metabolites were eluted using a linear gradient of acetonitrile (40–85% in 35 min) in water. The column temperature was 40°, and the flow rate was 2 ml/min. Total metabolites (labeled and unlabeled) were monitored at 254 nm (solid line). Radioactivity (histograms) was determined by collecting 0.5-min fractions directly into scintillation vials, adding 10 ml of scintillant, and counting.

digestion of the isolation DNA using longer periods of digestion (48 hr each for DNase, phosphodiesterase and alkaline phosphatase) but the size of the peaks remained unchanged.

DISCUSSION

Pretreatment of rats with the pyrrolizidine alkaloid jacobine altered the *in vitro* BP metabolite profile in a manner similar to that of another epoxide hydrolase inducer, *trans*-stilbene oxide [9]. Unlike *trans*-stilbene oxide, jacobine significantly inhibited total BP metabolism. The increase in the ratio of K to non-K region metabolites following jacobine treatment correlated with a significant reduction in total microsome catalyzed binding of BP to DNA. To determine if this decrease in DNA binding was due to jacobine-induced epoxide hydrolase, we re-examined BP–DNA binding in the presence of high levels of purified epoxide hydrolase. Adding exogenous epoxide hydrolase to control microsomes decreased BP–DNA binding to about the same extent as jacobine pretreatment. Interestingly, addition of epoxide hydrolase to jacobine microsomes further decreased BP–DNA binding by about 50%. The observation that the effects of exogeneous epoxide hydrolase and jacobine pretreatment were additive suggests the possibility that they may act by different mechanisms. Evidence has been presented that epoxide hydrolase exists as multiple forms [26]. Perhaps the jacobine-induced form(s) differs from the form purified from PCB-treated rats, which was used in this study. Alternatively, pyrrole metabolites remaining in the microsomes from jacobine-treated rats might compete with BP metabolites for binding to DNA. However, examination of the washed microsomes from the present study by the colorimetric method of Mattocks and White [18] failed to show significant quantities of PA pyrroles. Moreover, the pyrrole metabolites dehydroretronecine [27–29] and dehydroheliotridine [30] do not bind readily to DNA except in an acidic environment and dehydroretronecine binds preferentially to proteins over nucleic acids [27].

Microsomes from control and jacobine-treated rats were incubated with [^{3}H]BP and DNA, the DNA was hydrolyzed, and the [^{3}H]BP metabolite–DNA adducts were separated on LH 20. Five radioactive peaks could be resolved with control microsomes (a–e). The relative amounts of all peaks, except c, were reduced markedly (a, b and d) or eliminated (e) when BP metabolite–DNA adducts from jacobine microsomes were analyzed. The overall reduction in the size of the peaks in the jacobine-treated groups is consistent with the BP metabolite profile and total BP metabolite–DNA binding results. Furthermore, if peaks c, d and e do correspond to adducts of BP-7,8-diol-9,10-epoxide, BP-4,5-epoxide and BP-9-OH-4,5-epoxide, respectively, the relationship to the metabolite profile is clearer. The reduction in peak d (BP-4,5-epoxide–DNA) in the jacobine group would be due to the marked increase in conversion of BP-4,5-epoxide to the diol. The complete elimination of peak e would be due to the combined effects of inhibition of BP-9-OH formation and the increased conversion of BP-4,5-epoxide to the diol.

Table 2. Effect of jacobine pretreatment and epoxide hydrolase on total BP–DNA binding catalyzed by rat liver microsomes

Microsomes*	[^{3}H]BP† (pmoles bound/20 min/mg protein/mg DNA)
Controls (N = 7)	38.8 ± 9.5
Controls + EH (N = 4)	20.8
Jacobine (N = 7)	19.6 ± 6.1‡
Jacobine + EH (N = 4)	10.9

* Pooled microsomes from four or seven animals.

† Mixtures (2 ml) containing 1 mg pooled microsomes, 2 mg DNA and 60 μM [^{3}H]BP were incubated at 37° for 20 min, in the presence or absence of 300 units of epoxide hydrolase. The values shown for microsomes alone represent the means ± S.D. of four determinations, while the values in the presence of epoxide hydrolase are from a single determination.

‡ Significantly different from controls ($P < 0.05$).

Peak c, corresponding to BP-7,8-diol-9,10-epoxide, was reduced only slightly in the jacobine group. The BP metabolite profile with jacobine microsomes showed a slight decrease in BP-7,8-diol and BP-9,10-diol formation; the former would be expected to decrease c and the latter to increase c, the combined result being little change. Such an interpretation would be consistent with the results obtained by Alexandrov *et al.* [25]. In their study, addition of 300 units of epoxide hydrolase to microsomes from 3-methylcholanthrene-treated rats completely eliminated the BP-4,5-epoxide adduct and markedly reduced the BP-9-OH-4,5-epoxide adduct while the BP-7,8-diol-9,10-epoxide adduct was actually increased.

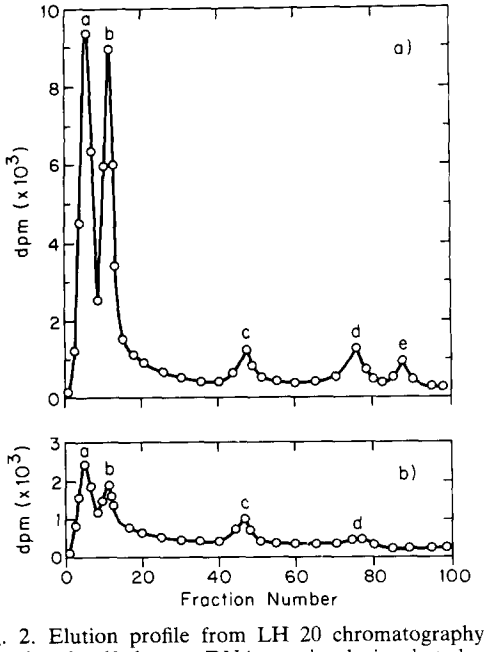

Fig. 2. Elution profile from LH 20 chromatography of hydrolyzed calf thymus DNA previously incubated with [^{3}H]benzo[*a*]pyrene and liver microsomes from control (a) or jacobine-treated rats (b). A Sephadex LH 20 column (20 × 1.2 cm) was equilibrated with 45% aqueous methanol. The DNA hydrolysate was loaded onto the column and eluted with a linear gradient of 45–75% methanol (150 ml). Fractions (1.5 ml) were collected, and a portion was counted using 10 ml of ACS scintillant.

Before conclusions can be drawn with regard to the effect of jacobine on BP-induced mutagenesis or carcinogenesis, it should be kept in mind that the profiles of BP metabolite–DNA adducts obtained *in vitro* and *in vivo* can differ [31]. It appears that the BP-9-OH-4,5-epoxide adduct is the main *in vitro* adduct, while *in vivo* the relative amount of the BP-7,8-diol-9,10-epoxide adduct is higher, and it is felt that this latter species is more closely related to BP-induced mutagenesis and carcinogenesis. In addition, the relationship between epoxide hydrolase, BP metabolite–DNA binding and mutagenesis may not be clear. Purified rat liver epoxide hydrolase markedly inhibited mutagenesis catalyzed by purified rat cytochrome P-448 when BP or BP-4,5-epoxide was the substrate, but it had no effect on the mutagenic activity of BP-7,8-diol [32]. Conversely, the addition of rat epoxide hydrolase to purified rabbit cytochrome LM_4 markedly reduced total DNA binding when BP-7,8-diol was the substrate [33]. Thus, the ability of added epoxide hydrolase to deactivate BP-7,8-diol-9,10-epoxide by forming the tetrol is uncertain. However, it does seem clear that epoxide hydrolase greatly decreases the mutagenicity of BP-4,5-epoxide but markedly increases the mutagenicity of BP-7,8-epoxide [32].

The results with jacobine indicate that, like *trans*-stilbene oxide, jacobine exhibits a 2-fold mechanism for lowering mutagenesis or covalent binding of BP metabolites to DNA. The first is an inhibition of primary P-450 catalyzed oxygenation at the non-K region (9-OH) of BP. The second involves stimulation of secondary oxygenation at the K region (to BP-4,5-diol) through induction of epoxide hydrolase.

Our results suggesting decreased formation of DNA adducts following jacobine administration are not in agreement with those obtained after ethoxyquin treatment. Ethoxyquin, like jacobine,

inhibits AHH activity (formation of 3- and 9-phenols), while inducing epoxide hydrolase [34], but was actually found to increase the formation of the BP-7,8-diol-9,10-epoxide–DNA adduct [8]. It appears that two chemicals with similar effects on AHH and epoxide hydrolase activities can vary in their abilities to generate reactive BP metabolites actually bound to DNA.

In conclusion, our findings suggest that ingestion of PA-containing plants by animals or humans may have the potential to alter the ability of metabolizing polycyclic aromatic hydrocarbons (PAHs). Whether PA ingestion actually modifies the carcinogenic effects of PAHs, however, remains to be established. Our results with the DNA binding study suggest this possibility, but it should be borne in mind that jacobine itself may be a potential carcinogen [34]. Moreover, the major metabolite of jacobine, dehydroretronecine, is also considered to be a potent carcinogen [29–36].

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