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INDUCTION OF HEPATIC MICROSOMAL CYTOCHROME P-448-MEDIATED OXIDASES BY 3,3′-DICHLOROBENZIDINE IN THE RAT*

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Abstract—Intraperitoneal administration of the hepatocarcinogen 3,3′-dichlorobenzidine (4,4′-diamino, 3,3′-dichlorobiphenyl) to adult male rats caused the induction of hepatic microsomal ethoxycoumarin *O*-deethylase and *p*-nitrophenetole *O*-deethylase activities comparable in magnitudes to those induced by 3-methylcholanthrene; neither aniline hydroxylase nor aminopyrine *N*-demethylase activity was affected by the pretreatment. The induction was not accompanied by a significant increase in content of hepatic microsomal cytochrome P-450; however, a shift in the absorption maximum of the reduced + CO spectrum of the cytochrome to 448 nm and an increase in the ratio of the 455 nm : 430 nm peaks of the reduced + ethylisocyanide spectrum of the hemoprotein was effected. Arylhydrocarbon hydroxylase activity was stimulated 5-fold by dichlorobenzidine pretreatment in comparison with a 12-fold stimulation following 3-methylcholanthrene pretreatment. However, enzymically mediated covalent binding of benzo[*a*]pyrene to microsomal protein was greater in microsomes from dichlorobenzidine-pretreated rats than in those from methylcholanthrene-pretreated rats. All of the dichlorobenzidine-induced enzymic activities were inhibited by α-naphthoflavone but not by SKF-525A. Hepatic microsomes from dichlorobenzidine-pretreated rats appeared to have a higher capacity for metabolizing dichlorobenzidine than those from untreated animals; both sets of microsomes elicited the Type II spectral change on combination with the compound, albeit with different binding affinities and capacities. The results show that dichlorobenzidine, although only a dihalogenated biphenyl derivative, is a potent inducer of cytochrome P-448.

Induction of enzymes of the hepatic endoplasmic reticulum, of which the cytochrome P-450-mediated class of oxidases is a major component, is of prime importance in the toxicity and carcinogenicity of xenobiotics. These oxidases catalyze the formation of toxic and carcinogenic derivatives from chemicals, in addition to their role in the detoxification of a variety of compounds. Cytochrome P-450 mediated mixed function oxidases can be broadly categorized into two major groups depending on whether they are induced by a class of chemicals whose prototype is phenobarbital or by chemicals exemplified by polycyclic aromatic hydrocarbons.§ Either group of hemoprotein, depending on the chemical involved, can catalyze the formation of reactive derivatives of the chemical which may cause toxicity [1–3]. It has been speculated that the carcinogenicity of some chemicals may, in part, be related to their reactive derivatives formed from the catalytic action of the polycyclic aromatic hydrocarbon-inducible class of hemoproteins [4–7].

The class of hemoprotein inducible by polycyclic aromatic hydrocarbons is conveniently characterized by, among other parameters, an absorbance maximum at 448 nm when its reduced form combines with carbon monoxide [8]. This is in contrast to the absorbance maximum at 450 nm elicited by the species induced by phenobarbital and the one in "untreated" animals. The polycyclic aromatic hydrocarbon-inducible species may be collectively designated cytochrome P-448 [8–11]. Phenobarbital and polycyclic aromatic hydrocarbons were the first identified inducers of cytochrome P-450; subsequently identified inducers have been categorized as either phenobarbital-like or methylcholanthrene-like, depending on the pattern of metabolism and species of P-450 they induce [12]. Compounds structurally unrelated to phenobarbital can induce cytochrome P-450 that is spectrally and catalytically similar to the species induced by phenobarbital; similarly, cytochrome P-448 can be induced by chemicals structurally unrelated to polycyclic aromatic

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§ Abbreviations: Cytochromes "P-450" and "P-448" as used in the present studies refer to the two broad categories of the hemoprotein which, on reduction and combination with CO, exhibit absorption maxima at 450 nm and 448 nm, respectively; no distinction is made between the several hitherto identified species of the hemoprotein which differ with respect to substrate specificity, immunochemical properties, etc.; AHH, arylhydrocarbon hydroxylase; DCB, 3,3′-dichlorobenzidine; EIC, ethylisocyanide; HPLC, high performance liquid chromatography; MC, 3-methylcholanthrene; α-NF, 7,8- or α-naphthoflavone; PCB, polychlorobiphenyl; SKF-525A, β-diethylaminoethyl diphenylpropylacetate; and Type II spectral change, a difference spectrum characterized in the present studies by an absorption maximum and minimum of 395 nm and 424 nm respectively.

hydrocarbons. One such group of chemicals which is of current major toxicological concern comprises the polychlorinated biphenyls (PCBs). With respect to P-448 induction, exhaustive studies including structure–activity relationships have been carried out on PCBs by various investigators [13–17]; these studies have contributed to the understanding of the mechanisms of toxicity of these agents. Data from studies on hetero-substituted chlorobiphenyls, i.e. biphenyls containing substituents in addition to chlorine atoms, however, are unavailable.

3,3′-Dichlorobenzidine (DCB) is a hetero-substituted lesser chlorinated biphenyl that has not received the environmental notoriety accorded the highly chlorinated biphenyls and is relatively unstudied. However, its important uses in the manufacture of azo dye pigments and in the curing of polyurethane foams place not only an occupational group but also the general population at risk from exposure to the chemical. The compound has a high carcinogenic potential in both humans and laboratory animals [18–20]. A survey of the literature seems to indicate that, with only a few exceptions, hitherto identified inducers of cytochrome P-448 tend to pose some carcinogenic or toxicological risks. The present studies were undertaken to assess the ability of the potent hepatocarcinogen DCB—a lesser chlorinated, amine substituted biphenyl—to induce cytochrome P-450-mediated monooxygenase in the liver. Such studies would contribute to the understanding of not only the biochemical factors associated with the toxicity and carcinogenicity of DCB but also the toxicity of substituted biphenyls as a chemical class.

METHODS

Handling of animals. Male Sprague–Dawley rats were obtained from Taconic Farms, Germantown, NY, and weighed 175–210 g at the beginning of the experiments. They were housed two per cage in stainless steel hanging-type cages with perforated bottoms, in a room with a 12-hr light–dark cycle and 50% humidity; the animals were fed water and Purina laboratory rodent chow *ad lib*. Dichlorobenzidine (DCB), 3-methylcholanthrene (MC) or benzidine (20 mg/ml in corn oil, unless indicated otherwise) was administered at the dose and frequently indicated in Results. Phenobarbital sodium (40 mg/ml in 0.9% NaCl) was administered for 3 consecutive days at a daily dose of 80 mg/kg. All drug administrations were intraperitoneal and were carried out between 8:00 and 10:00 a.m. Control animals received either corn oil or saline at a dose of 1 ml/kg.

Preparation of hepatic microsomal fractions. Animals were decapitated 48 hr after the last injection, and their livers were perfused *in situ* through the portal vein with ice-cold 0.1 M sodium/potassium phosphate buffer, pH 7.4, hereafter designated "buffer". The liver was then excised, minced with scissors, and homogenized on ice in 2 vol. of ice-cold buffer with a Dounce homogenizer, using a glass pestle with a loose clearance and twenty strokes. Washed microsomes from the total liver homogenate were prepared by differential ultracentrifugation as described previously [21] and used for metabolic and spectral studies within 5 hr of isolation. Carcasses and wastes from animals pretreated with MC, DCB or benzidine were stored frozen in plastic bags until picked up for disposal by the animal and hazardous waste disposal service.

Enzyme and chemical assays. All spectra were recorded with an Aminco DW2-A recording spectrophotometer equipped with a beam scrambler and calibrated for wavelength accuracy with holmium oxide. The contents of microsomal cytochromes P-450 and b_5 were determined as described by Omura and Sato [22]. Ethylisocyanide (EIC) difference spectra of reduced cytochrome P-450 were recorded as described by Sladek and Mannering [8].

Substrate-induced difference spectra in microsomes were determined as described by Schenkman [23]. The substrate DCB [in a 1:1:1 (by vol.) vehicle mixture of acetone–methanol–water] was added to the sample cuvette in 2 μl aliquots, and the reference cuvette was balanced with an appropriate aliquot of the vehicle. Spectral constants were determined from the Hofstee–Eadie plots of the successive spectral changes. Linear regression analysis of the spectral data, based on the method of least squares, was performed on a Hewlett–Packard model 9820 table computer.

NADPH–cytochrome *c* reductase activity was determined according to the procedure of Masters *et al.* [24]. Protein was determined by the method of Lowry *et al.* [25], using bovine serum albumin as the standard. *N*-Demethylation of aminopyrine, *p*-hydroxylation of aniline, and 3-hydroxylation of benzo[*a*]pyrene were determined as described by Sladek and Mannering [26], Kato and Gillette [27], and Nebert and Gelboin [28] respectively. *O*-Deethylation of *p*-nitrophenetole and ethoxycoumarin was determined as described by Shigematsu *et al.* [29] and Greenlee and Poland [30] respectively.

For the metabolism of dichlorobenzidine, the reaction mixture in a 25 ml Erlenmeyer flask containing 20 μmoles glucose-6-phosphate, 2 μmoles NADP, 15 μmoles $MgCl_2$, 2 units of glucose-6-phosphate dehydrogenase, and DCB at a final concentration of 50 μM [added in a 0.025 ml acetone–methanol–water mixture (1:1:1, by vol.)], in a total volume of 4.5 ml of 0.1 M sodium/potassium phosphate buffer, pH 7.4, was preincubated at 37° with shaking for 3 min. The reaction was initiated by adding 3 mg of microsomal protein in 0.5 ml of buffer. The blank incubation mixture was the same as described above except that the microsomal protein was heated at 70° for 15 min prior to addition to the incubation flask. The reaction mixtures were shielded from ambient light with aluminium foil wrapped around the incubation flasks. All subsequent procedures were performed in the dark or in vessels shielded from light to avoid the photodegradation of DCB and/or its metabolites. At the end of 20 min, the reaction was terminated by the addition of 5 ml ethyl acetate and further incubated for 30 min at 37° with vigorous shaking (150 oscillations/min). A 3 ml aliquot of the organic layer was removed into a test tube, dried under a stream of nitrogen, and dissolved in 0.5 ml methanol–water–acetonitrile (60:20:20, by vol.). A 50 μl aliquot of the freshly dissolved extract was then ana-

lyzed by a Varian model 5000 liquid chromatograph fitted with a Dupont ODS column and equipped with a Schoeffel GM 770 monochromator (detector set at 280 nm). Elution was with a reverse-phase solvent gradient consisting of methanol–acetonitrile–water (60:20:20, by vol.). For the radiometric analysis of DCB metabolites, ^{14}C-labeled DCB (16.3 mCi/mmole) was the substrate.

For the analysis of the binding of [^{14}C]benzo[*a*]pyrene to microsomal protein, the reaction mixture in a 10 ml Erlenmeyer flask was placed in an ice-bath and contained 16 μmoles glucose-6-phosphate, 1.6 μmoles NADP, 12 μmoles $MgCl_2$, 1.6 units of glucose-6-phosphate dehydrogenase and 10 nmoles of [^{14}C]benzo[*a*]pyrene, sp. act. 16.5 mCi/mmole, all in a total volume of 4 ml of buffer. The final microsomal protein concentration was 0.2 mg/ml. The flask was then incubated in the dark at 37° with shaking for 15 min; the reaction was stopped by chilling the flask in an ice-bath. A 1 ml aliquot of 20% (w/v) trichloroacetic acid was added to the flask to precipitate the protein. After sitting in the ice-bath for 20 min, the entire content of the flask was centrifuged for 20 min at 5000 *g* to sediment the precipitated protein. The resulting precipitate was resuspended in a 5 ml aliquot of 0.5 N NaOH and centrifuged refrigerated at 5000 *g* for 20 min. The latter procedure was repeated once; the resulting precipitate was extracted twice with 5 ml chloroform–methanol (1:2, v/v), air-dried, and layered with 2 ml of 1 N NaOH and heated in a boiling water bath for 45 min to solubilize the protein. After cooling, an aliquot of the protein solution was set aside for protein determination and the remainder was diluted to 5 ml with distilled water, brought to neutral pH with 1 N HCl, and prepared for scintillation counting by adding a 10 ml aliquot of Hydrofluor scintillation mixture.

Chemicals. 3,3′-Dichlorobenzidine was a gift from Dr. Dennis Chesney of Upjohn Fine Chemicals; [^{14}C(7,10)]benzo[*a*]pyrene of specific activity and radiochemical purity, 16.5 mCi/mmole and 98%, respectively, was purchased from California Binuclear (Sun Valley, CA). [^{14}C-(U)]DCB·2HCl with a specific activity of 31.7 mCi/mmole and purity >98% was purchased from the New England Nuclear Corp. (Boston, MA). All three chemicals were further purified by HPLC prior to use. 3-OH-Benzo[*a*]pyrene was supplied by Dr. James Keith, NCI Chemical Repository, I.I.T., Chicago; *p*-nitrophenetole, *p*-nitrophenol, 7,8-naphthoflavone, benzo[*a*]pyrene and 3-methylcholanthrene were purchased from Eastman Kodak (Rochester, NY), and ethoxycoumarin and 7-hydroxycoumarin from the Aldrich Chemical Co. (Milwaukee, WI). All the chemicals except 3-methylcholanthrene were purified by recrystallization from appropriate solvents prior to use. Aminopyrine, glucose-6-phosphate, NADP, and NADPH were obtained from the Sigma Chemical Co. (St. Louis, MO). Aniline hydrochloride was obtained from Mallinckrodt (St. Louis, MO). Glucose-6-phosphate dehydrogenase was from Boehringer-Mannheim (Indianapolis, IN). Phenobarbital sodium and SKF-525A were provided by Dr. Gilbert Mannering of the University of Minnesota. Hydrofluor was purchased from National Diagnostics (Somerville, NJ).

Handling of chemicals. All handling (opening of containers, weighings, preparation of suspensions for injections or solutions for metabolism studies) of DCB, MC or benzo[*a*]pyrene was done in a Labconco Chemical Carcinogen Glove Box. Durable latex gloves were used when procedures (such as injections, killing of animals and extractions) had to be performed outside the glove box. All unused solutions or suspensions of the chemicals, as well as

Table 1. Effects of monooxygenase inducers on some hepatic variables

	Pretreatment			
	Control*	MC†‡	DCB*‡§	Phenobarbital‖
Cytochrome P-450 Content (nmoles/mg microsomal protein)	0.82 ± 0.18	1.02 ± 0.10	1.08 ± 0.14	1.96 ± 0.20¶
A_{max} of reduced + CO difference spectrum†**	450.0	448.0	448.2	450.8
Ratio of 455:430 nm peaks of ethylisocyanide difference spectrum, pH 7.4**	0.37	1.21	1.43	0.41
NADPH–cytochrome *c* reductase activity (nmoles cytochrome *c* reduced/mg microsomal protein/min)	285 ± 20	260 ± 17	448 ± 52††	255 ± 24¶
Liver weight (g)	9.8 ± 1.1	10.2 ± 1.0	11.0 ± 1.5	14.0 ± 1.2
% Body weight	4.9 ± 0.6	5.1 ± 0.5	5.5 ± 0.8	7.0 ± 0.8

* Controls received saline for phenobarbital and corn oil for MC or DCB.
† Forty mg/kg, administered once.
‡ The 455 nm peak of the EIC spectrum was shifted to 453 nm in these preparations.
§ Forty mg/kg administered for 2 consecutive days.
‖ Eighty mg/kg administered for 3 consecutive days.
¶ Significantly different from the corresponding control ($P < 0.02$).
** Values are the averages of two experiments. Each of the other values represents the mean (± S.D.) of a minimum of three determinations in one experiment.
†† Significantly different from the corresponding control ($P < 0.05$).

wastes from metabolism studies, were diluted with ethanol and stored in sealed vials in a restricted fume hood until picked up for disposal by the hazardous chemical disposal service.

RESULTS

Effect of DCB pretreatment on the spectral characteristics of hepatic microsomal cytochrome P-450. Intraperitoneal administration of DCB (40 mg/kg) to rats caused a 2 nm hypsochromic shift in the maximum of the difference absorption spectrum of the CO complex of reduced hepatic microsomal cytochrome P-450 (Table 1). This change is similar to that caused in microsomes from animals pretreated with polycyclic aromatic hydrocarbons [8, 9, 31] and certain congeners of polyhalogenated biphenyls [13–17]. Similar to the changes caused by these two classes of compounds [8, 17], DCB pretreatment increased the peak height ratio of the 455 nm : 430 nm peaks of the difference spectrum of the EIC complex of reduced hepatic microsomal cytochrome P-450 and shifted the 455 nm peak of the latter spectrum to 453 nm (Table 1). The shift in the 455 nm of the EIC spectrum has also been reported to occur with MC pretreatment [32].

Comparative inductive effects of DCB, MC, and phenobarbital. The profile of the inductive effects of phenobarbital is different from that of MC; for example, phenobarbital pretreatment causes increases in liver weight, microsomal P-450 content and NADPH–cytochrome (P-450) *c* reductase activity [33, 34]. These variables are not changed significantly by MC pretreatment [8, 9]. The absorbance maximum of the CO-difference spectrum and the peak height ratio of the 455 nm : 430 nm peaks of the EIC difference spectrum of reduced cytochrome P-450 are shifted by MC but not by phenobarbital pretreatment [8]. These differences have been criteria for categorization of inducing agents as either phenobarbital- or MC-like. A comparison of the effects of pretreating rats with MC, DCB, or phenobarbital, on various hepatic variables is also shown in Table 1. Phenobarbital pretreatment caused a 2.4-fold increase in microsomal cytochrome P-450 content. Increases in the hemoprotein following MC and DCB pretreatment were slight (only 1.3- and 1.2-fold respectively). Similar to MC pretreatment, but in contrast to phenobarbital pretreatment, DCB pretreatment shifted the absorption maximum of the CO-difference spectrum of reduced microsomes to 448 nm from 450 nm and increased the peak-height ratio of the EIC spectrum of reduced microsomes to a value greater than unity. Microsomal NADPH–cytochrome (P-450) *c* reductase activity and liver weight were increased significantly only in phenobarbital-pretreated rats. The foregoing results suggest that the inductive effects of DCB on hepatic microsomal activities are akin to those of MC and similar compounds.

DCB pretreatment significantly induced ethoxycoumarin *O*-deethylase, *p*-nitrophenetole *O*-deethylase and arylhydrocarbon hydroxylase activities (5-, 6- and 5-fold respectively) (Table 2). Table 2 also shows that MC pretreatment significantly induced the activities of only these three enzymes.

Table 2. Effects of inducers on rat hepatic monooxygenase activities*

Pretreatment	Aniline hydroxylase†	Aminopyrine *N*-demethylase‡	Ethoxycoumarin *O*-deethylase§	*p*-Nitrophenetole *O*-deethylase‖	Benzo[*a*]pyrene hydroxylase¶
None	300 ± 20	9.0 ± 0.5	5.1 ± 0.8	2.9 ± 0.2	140 ± 12
Phenobarbital	610 ± 43**	26 ± 1.6**	7.1 ± 1.0	10.5 ± 0.6**	360 ± 20**
MC	270 ± 25	10 ± 0.5	29 ± 2**	21.6 ± 1.4**	1680 ± 75**
DCB	325 ± 30	11 ± 0.8	27 ± 1.6**	19 ± 1.2**	650 ± 75**
DCB + MC††	265 ± 30	8.5 ± 0.6	25 ± 1.7**	16 ± 1.4**	ND‡‡
DCB + phenobarbital††	450 ± 20**	21.4 ± 1.3**	31 ± 2.2**	23 ± 1.4**	ND

* Treatment regimens were as described in Table 1. Each value is the mean ± S.D. of a minimum of four replicate experiments.
† Expressed in pmoles *p*-aminophenol formed/mg protein/min.
‡ Expressed in nmoles formaldehyde formed/mg protein/min.
§ Expressed in nmoles 7-OH-coumarin formed/mg protein/min.
‖ Expressed in nmoles *p*-aminophenol formed/mg protein/min.
¶ Expressed in pmoles 3-OH-benzo[*a*]pyrene formed/mg protein/min.
** Significantly different from the corresponding control ($P < 0.02$).
†† Agents were administered simultaneously.
‡‡ Not determined.

Phenobarbital pretreatment, on the other hand, caused increases in all of the enzymic activities studied except ethoxycoumarin *O*-deethylation; also, the magnitudes of phenobarbital induction of AHH and *p*-nitrophenetole *O*-deethylase activities (2.6- and 3.6-fold respectively) were lower than those induced by DCB or MC (Table 2). Qualitatively, the pattern of activities induced by DCB resembled that of MC more than that of PB (Table 2). However, a 12-fold increase in AHH activity was observed with MC pretreatment in comparison with a 5-fold increase with DCB pretreatment (Table 2), suggesting, perhaps, important differences between the hepatic microsomes from animals pretreated with the two agents despite their apparent similar spectral characteristics (Table 1).

Effects of dose on the inductive action of DCB. A single i.p. administration of 40 mg/kg (172 μmoles/kg) of DCB to rats caused only a 6% increase in cytochrome P-450 content, a 1 nm hypsochromic shift in the CO difference spectrum of the hemoprotein, and a slight increase in the 455 nm : 430 nm peak-height ratio for the EIC spectrum (data not shown). The single dose also caused a 4.7-fold induction of *p*-nitrophenetole *O*-deethylase activity. The optimum inductive dose for the latter activity was found to be 40 mg/kg given on 2 consecutive days or 120 mg/kg (516 μmoles/kg) given as a single dose. The latter dose was the maximum that could be administered without causing death in the animals. The optimum inductive i.p. dose for the chemical is comparable to that of other P-448-inducing agents [14–17].

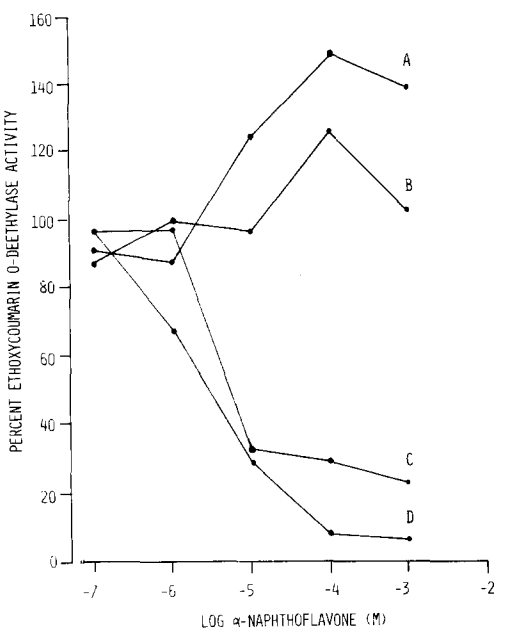

Fig. 1. Effect of α-naphthoflavone on ethoxycoumarin *O*-deethylase activity in hepatic microsomes from (A) phenobarbital-pretreated (80 mg/kg × 3 days) rats; (B) control rats; (C) 3-methylcholanthrene-pretreated (40 mg/kg × 1 day) rats; and (D) DCB-pretreated (40 mg/kg × 2 days) rats. One hundred percent activities (nmoles 7-OH-coumarin formed per mg protein per min) were: (A) 7.9; (B) 5.2; (C) 26.5; and (D) 28.0.

Table 3. Effects of inhibitors on rat hepatic monooxygenase activities*

	Pretreatment					
	None	Phenobarbital	MC	DCB	DCB + MC†	DCB + phenobarbital†
	+ α-Naphthoflavone (10^{-5} M)					
Monooxygenase activity						
Ethoxycoumarin *O*-deethylase (nmoles 7-OH-coumarin formed/mg protein/min)	7.0 ± 0.9‡ (+38)	9.6 ± 1.2‡ (+35)	8.7 ± 0.7§ (−70)	8.1 ± 0.5§ (−70)	ND‖	21.7 ± 4.1‡ (−30)
Benzo[*a*]pyrene hydroxylase (pmoles 3-OH-benzo[*a*]pyrene formed/mg protein/min)	172.4 ± 22 (+23)	659 ± 82§ (+83)	286 ± 38§ (−83)	137 ± 20§ (−79)	ND	ND
			+ SKF-525A (10^{-5} M)			
Ethoxycoumarin *O*-deethylase (nmoles 7-OH-coumarin formed/mg protein/min)	2.6 ± 0.4§ (−50)	2.8 ± 0.3§ (−60)	29.6 ± 4 (+2)	23 ± 3.1 (−16)	ND	15.5 ± 2.8§ (−50)

* Treatment regimens were as described in Table 1. Each value is the mean (±S.D.) of three experiments, each done in duplicate. Each value in parentheses is the percentage change from the corresponding value in the absence of inhibitor.
† Agents were administered simultaneously.
‡ Significantly different from the corresponding value obtained without inhibitor ($P < 0.05$).
§ Significantly different from the corresponding value obtained without inhibitor ($P < 0.02$).
‖ Not determined.

Effects of inhibitors on hepatic microsomal monooxygenase activities. The similarities between the DCB- and MC-induced hepatic monooxygenase activities were further examined by testing the effects of α-naphthoflavone (α-NF), an inhibitor of MC-inducible hepatic microsomal monooxygenase activity [35, 36], on ethoxycoumarin *O*-deethylation. Ethoxycoumarin was chosen as the test substrate because, unlike such substrates of the AHH system as benzo[*a*]pyrene, it is oxidized at only a single position in microsomal incubations: via *O*-deethylation [37]. At an α-NF concentration equal to that of the substrate (5×10^{-4} M), *O*-deethylation was inhibited 74% in microsomes from MC-pretreated rats and 92% in those from DCB-pretreated rats but stimulated 16 and 44% in microsomes from untreated and phenobarbital-treated rats respectively (Fig. 1). SKF-525A, in contrast, was inhibitory in microsomes from untreated or phenobarbital-pretreated rats but had no effects in microsomes from MC- or DCB-pretreated rats (Table 3). A profile of inhibition by α-NF similar to the above has been reported for AHH activity [35, 36].

The effects of α-NF on arylhydrocarbon hydroxylase activity were similar to those on ethoxycoumarin *O*-deethylase activity. Inhibition was observed in microsomes from MC- or DCB-pretreated rats in contrast to a stimulation in microsomes from control or phenobarbital-pretreated rats (Table 3). The differential sensitivity of ethoxycoumarin *O*-deethylation in microsomes from DCB- and MC-pretreated rats to low concentrations (10^{-6} M or less) of α-NF (Fig. 1), coupled with the quantitatively different induction of AHH by MC and DCB (Table 2), suggested differences in some characteristics of the microsomes from animals treated with the two agents.

AHH-mediated covalent binding of [^{14}C]benzo[a]pyrene to hepatic microsomal protein. Covalent binding of benzo[*a*]pyrene *in vitro* parallels the AHH activity of the particular microsomal preparation [38, 39]. Similarities between the AHH activities of hepatic microsomes from MC- and DCB-treated rats were further examined by comparing the binding of [^{14}C]benzo[*a*]pyrene to microsomal protein *in vitro*. In the presence of NADPH, benzo[*a*]pyrene was covalently bound to microsomal protein, with the binding in microsomes from DCB-pretreated rats being 2.0-fold that in microsomes from MC-pretreated rats; the levels of binding in microsomes from DCB- and MC-pretreated rats were 9.6- and 4.7-fold, respectively, of the binding in microsomes from untreated rats (Table 4). [^{14}C]Benzo[*a*]pyrene also bound to denatured microsomes incubated with or without NADPH but the extent of this "non-enzymic" binding did not differ among microsomes from control, DCB-, and MC-treated rats, and it was lower than the binding to the protein in enzymically active microsomes. The "non-enzymic" binding could have been due to the known ability of benzo[*a*]pyrene to autooxidize to reactive derivatives [40]. α-NF totally abolished the enzymically mediated binding in microsomes from MC- and DCB-pretreated rats but stimulated the binding in microsomes from control rats (Table 4); this pattern of inhibition suggests the involvement, specifically, of the "inducible" AHH [35, 36] in the formation of the reaction benzo[*a*]pyrene metabolites. An inference from the present studies is that microsomes from DCB-pretreated rats catalyzed the formation of less hydrophilic, but more reactive, metabolites of benzo[*a*]pyrene than microsomes from MC-pretreated rats. This is additional evidence that the microsomal AHH systems induced by the two agents may be different despite the similar spectral characteristics of their cytochrome P-450.

Spectral perturbations induced by DCB in microsomes. DCB elicited a Type II spectral shift in microsomal preparations from untreated, MC-, DCB- or phenobarbital-pretreated rats; in microsomes from untreated or phenobarbital-pretreated rats, however, a Type R-I-like spectral change (with a maximum at 420 nm and a minimum at 395 nm) predominated at concentrations of DCB $< 10\ \mu$M. A Hofstee–Eadie analysis of the spectral data, in which all the data points are weighted equally as a test of linearity [41], revealed quantitative similarities between microsomes from DCB- and MC-treated rats (Fig. 2); the plots were biphasic such that two pairs of spectral constants were observed in each microsomal preparation: a high affinity (K_{s1}), low capacity (A_{max1}) pair and a low affinity (K_{s2}), high capacity (A_{max2}) pair (Fig. 2). The binding affinities (K_{s1}, K_{s2}) were substantially increased while the binding capacities (A_{max1}, A_{max2}) were decreased by both MC and DCB pretreatments (Fig. 2). Phenobarbital pretreatment also increased the binding affinities but not to the same magnitudes effected by DCB or MC pretreatment.

Microsomal metabolism of DCB. Incubation of DCB with microsomes from control rats in the presence of NADPH caused the formation of one major

Table 4. Microsomal enzyme-catalyzed covalent binding of [^{14}C]benzo[*a*]pyrene to hepatic microsomal protein*

Microsomes from:	[^{14}C]Benzo[*a*]pyrene bound (pmole equivalents/mg microsomal protein)	Arylhydrocarbon hydroxylase activity†
Control rats	23.4	140.0
+ α-NF	25.7 (+8.6)‡	173.3 (+24)
MC-pretreated rats	110	1,680
+ α-NF	0 (−100)	286.7 (−83)
DCB-pretreated rats	224	653.3
+ α-NF	0 (−100)	133.3 (−80)

* Each value is the mean from three experiments, each determined in duplicate.
† Expressed in pmoles 3-OH-benzo[*a*]pyrene formed/mg protein/min; values are based on the data in Table 2.
‡ Percent change by α-NF.

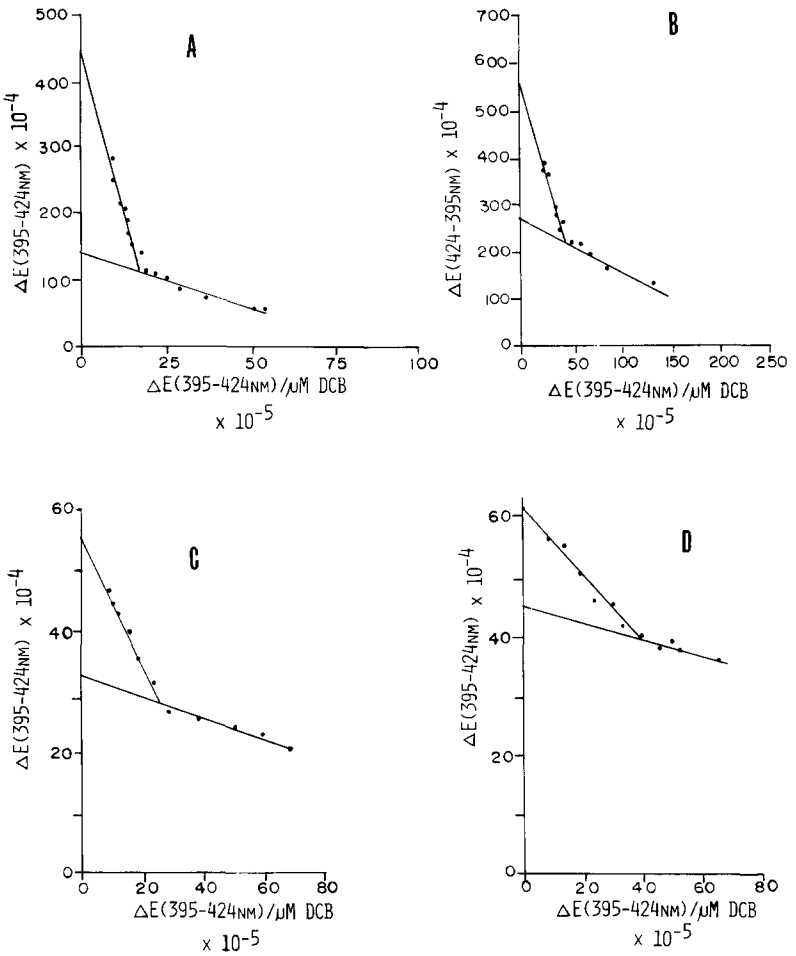

Fig. 2. Hofstee–Eadie plots of spectral changes induced in rat hepatic microsomes. The computed apparent spectral constants for each microsomal preparation were as follows: panel A (microsomes from control rats), lower line (r = correlation coefficient for the line = 0.952): A_{max1} (y intercept) = 0.0140, K_{s1} (slope) = 17.1; upper line (r = 0.973): A_{max2} = 0.0450, K_{s2} = 196.3. Panel B (microsomes from phenobarbital-pretreated rats), lower line (r = 0.985): A_{max1} = 0.0270, K_{s1} = 10.9; upper line (r = 0.970): A_{max2} = 0.0530, K_{s2} = 75.0. Panel C (microsomes from MC-pretreated rats), lower line (r = 0.946): A_{max1} = 0.0034, K_{s1} = 1.7; upper line (r = 0.991): A_{max2} = 0.0057, K_{s2} = 11.8. Panel D (microsomes from DCB-pretreated rats), lower line (r = 0.900): A_{max1} = 0.0046, K_{s1} = 2.5; upper line (r = 0.991): A_{max2} = 0.0062, K_{s2} = 6.3.

ethyl acetate-extractible compound having a retention time of 18.5 min in the high performance liquid chromatogram (Fig. 3, panel 1A). In microsomal incubations from DCB-pretreated rats, an additional compound having a retention time of 20.5 min was observed (Fig. 3, panel 1B). In the presence of SKF-525A, the major peak in the chromatogram from control microsomes was decreased (Fig. 3, panel 2A) but, in microsomes from DCB-pretreated rats, it was enhanced (Fig. 3, panel 2B). In the latter microsomal incubation, the compound with a retention time of 20.5 min disappeared and an additional major compound with a retention time of 13.5 min was also observed in the presence of SKF-525A (Fig. 3, panel 2B). In the presence of α-NF, only the compound with a retention time of 18.5 min was observed in microsomal extracts from control and DCB-pretreated rats (Fig. 3, panels 3A and 3B). With the exception of the peak with a retention time of 22 min, which represented the parent compound, DCB (Fig. 3, panels 1A–3B), none of the other peaks was observed when NADPH was omitted from, or when heat- or ethyl acetate-denatured microsomes were used in, any of the incubation mixtures; the peaks were also not observed when DCB was omitted from the incubation mixtures (data not shown).

In a preliminary experiment, radiochromatograms obtained under each of the experimental conditions in Fig. 3, using [^{14}C]DCB as the substrate, confirmed that, in all of the panels, all the peaks preceding the parent compound (retention time 22 min), except the peak at 2.5 min, represented derivatives of DCB. The quantitation and characterization of these DCB metabolites are underway.

DISCUSSION

The results demonstrate that 3,3′-dichlorobenzidine is a potent inducer of hepatic microsomal

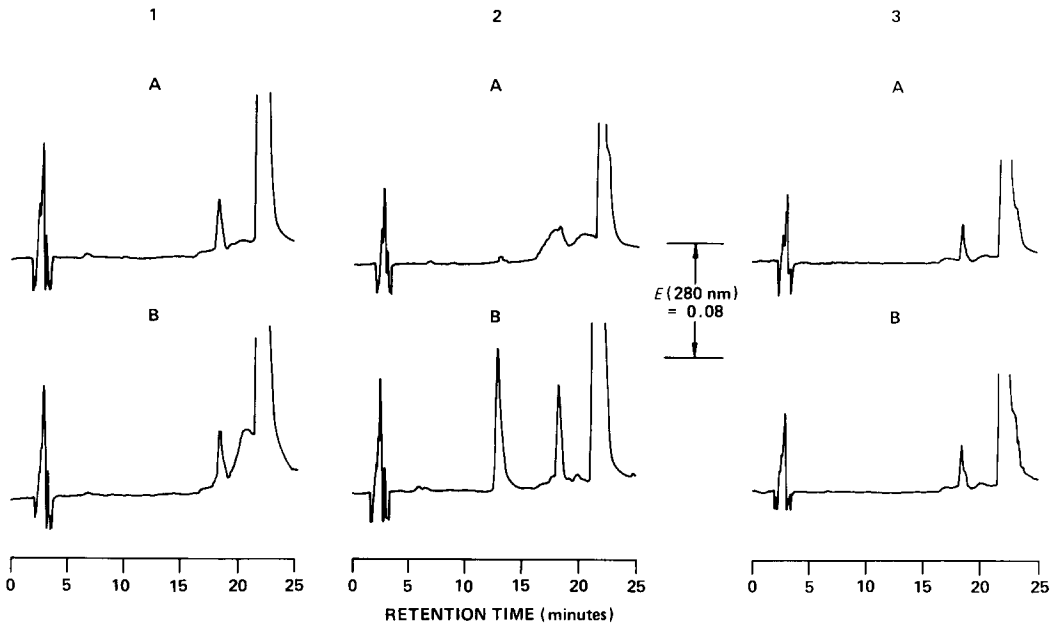

Fig. 3. High pressure liquid chromatography pattern of dried down derivatives formed from DCB incubated with NADPH in microsomes from (A) control rats; and (B) rats pretreated with DCB. Panels 1, 2 and 3 represent, respectively, microsomal incubations without inhibitor, in the presence of SKF-525A, and in the presence of α-NF. The major peak with a retention time of 22 min represents the parent compound. Retention times for the major DCB derivatives were 13.5 min, 18.5 min and 20.5 min.

enzymic activities mediated by cytochrome P-448. It has been suggested that some of the toxic properties as well as the carcinogenicity of compounds such as the polycyclic aromatic hydrocarbons and the polyhalogenated aromatics may be related to their abilities to induce cytochrome P-448-mediated monooxygenase activities; the hepatocarcinogenicity of DCB [19, 20] may thus be due, at least in part, to the induction of hepatic cytochrome P-448.

Hepatic cytochrome P-450 induction by chlorobiphenyls: structure–activity considerations. The ability of chlorinated biphenyls to induce hepatic cytochrome P-450 is well documented [13–17]. Induction studies have dealt mostly with highly substituted biphenyls bearing a minimum of four chlorine atoms as the only substituents. The present study is the first, to our knowledge, to report the induction of the hemoprotein by a biphenyl bearing only one chlorine substituent and an amine on each phenyl group. Depending on the congener, a polychlorinated biphenyl can induce either cytochrome P-450 or cytochrome P-448 along with the associated enzymic activities [14–16]. Data from detailed structure–activity relationship studies [14, 16, 17] have indicated that chlorination at both the meta and para (3,4,3′,4′) positions of both aromatic rings of the biphenyl moiety is the basic prerequisite for a chlorobiphenyl to induce cytochrome P-448 and that lower chlorinated biphenyls have no induction potential while additional substitution at the ortho position leads to induction of cytochrome P-450 rather than P-448.

Biphenyls containing fewer than four chloro substituents distributed between the 3 and 4 positions of each ring of the biphenyl molecule did not induce cytochrome P-448 and related activities; the chlorobiphenyl isomers 3,3′-dichlorobiphenyl and 4,4′-dichlorobiphenyl induced, albeit at very high doses, patterns of hepatic monooxygenase activities similar to those induced by phenobarbital [14, 42]. Benzidine or 4,4′-diaminobiphenyl, which differs from 4,4′-dichlorobiphenyl in having amino rather than chloro substituents, has absolutely no induction potential for hepatic monooxygenase activities.* As stated above, halogen substitution at the 3 and 4 positions of each ring of the molecule is the minimum prerequisite for a biphenyl to induce cytochrome P-448 [14, 16, 17]. Substitutions at these positions are also the minimum conditions for maintaining planarity of the molecule, and planarity is a structural requirement for a compound to induce cytochrome P-448 [13, 43]. The induction of cytochrome P-448 by DCB suggests that other suitable, non-halogen substituents capable of conferring planarity on the biphenyl molecule may cause a biphenyl to induce cytochrome P-448. As previously pointed out [14], and as evidenced by results of the present study, the degree of chlorination may be superceded by other considerations in the ability of a biphenyl to induce monooxygenase activities. Furthermore, the present studies indicate that the pharmacological and toxicological properties of the biphenyls as a chemical class may not be predicted by studying biphenyl

* M. M. Iba, unpublished observation.

congeners having halogens as the sole substituents. The latter have been, so far, the most, if not the only, studied members of the biphenyls. It is highly unlikely that the inductive effects of DCB were due to metabolites for two main reasons: (i) metabolism generally decreases tissue residues of chemicals and the induction potential of a chemical tends to parallel its retainability in the tissue [44]; and (ii) oxidized derivatives of certain PCB congeners have weak, if any, inducing potential when compared with the parent compounds [14].

Metabolism of DCB by microsomes. As shown in Fig. 3, chromatograms of extracts of microsomal suspensions incubated with DCB in the presence of NADPH had peaks representing metabolically derived products of DCB. Evidence that cytochrome P-450 or perhaps, more specifically, cytochrome P-448 was involved in the formation of these as yet uncharacterized products is the following: (i) SKF-525A, an inhibitor specific for reactions catalyzed by microsomes from control and phenobarbital-pretreated animals [45], inhibited DCB metabolism in microsomal incubations from control rats, but stimulated it in microsomal incubations from DCB-pretreated rats; and (ii) α-NF, a specific inhibitor of cytochrome P-448, was more inhibitory to DCB metabolism in microsomes from DCB-pretreated rats; in such microsomes, DCB metabolism was associated with the formation of an additional product which was not observed in microsomes from untreated rats. Thus, DCB was metabolized by cytochrome P-450 even though it elicited a Type II binding spectrum with the hemoprotein. The extent and rate of metabolism of the compound are yet to be determined. As evidenced by the data in Fig. 3, induction of microsomal enzymes by DCB caused quantitative as well as qualitative alterations in the pattern of DCB metabolism *in vitro*.

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Note added in proof—Further studies conducted since the submission of this paper have shown the spectral change induced in microsomes by DCB to be Type R-I.