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Structure-Activity Relationships in the Interactions of Alkoxymethylenedioxybenzene Derivatives with Rat Hepatic Microsomal Mixed-Function Oxidases *in Vivo*

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SUMMARY

Following *in vivo* administration to rats of equimolar amounts of a series of 4-*n*-alkoxymethylenedioxybenzene (AMDB) derivatives, hepatic microsomal aryl hydrocarbon hydroxylase (AHH) activities, total cytochrome P-450 levels, and AMDB metabolite-cytochrome P-450 spectral complex (455 nm) formation were well correlated in parabolic relationships with π, the hydrophobic constant of the *n*-alkoxy substituent. Each of these parameters increased progressively over control values with increasing carbon chain length of the alkoxy substituent, passed through an optimal value in compounds containing five or six carbon atoms, and subsequently decreased with the higher homologues. AHH activity was highly correlated in linear relationships with total (complexed plus uncomplexed) cytochrome P-450 content and intensity of the 455-nm spectral complex. Aminopyrine *N*-demethylase activities in microsomes from AMDB-treated rats were not well correlated with cytochrome P-450 levels or spectral complex formation. AMDB metabolite-ferricytochrome P-450 complexes varied considerably in their relative ease of displacement following treatment with 2-*n*-heptylbenzimidazole, those derived from the *n*-butoxy to *n*-hexoxy derivatives being particularly stable toward the displacer. The results are discussed in relation to the possible mechanisms involved in the interactions of methylenedioxyphenyl compounds with cytochrome P-450 and drug oxidation.

INTRODUCTION

MDP[1] compounds are both inhibitors (1–4) and inducers (5–8) of hepatic microsomal cytochrome P-450-mediated MFO activities in various species. The *in vivo* administration of MDP derivatives to rodents typically produces a biphasic response, a relatively short inhibitory phase being followed by a more prolonged enhancement of some oxidase activities (5, 7, 8).

Inhibition of MFO activity by MDP compounds *in vivo* and *in vitro* is generally considered to result from the metabolic formation of an active MDP species, possibly a carbene (9, 10), that forms an inhibitory complex with cytochrome P-450. Cytochrome P-450 in hepatic microsomal fractions isolated from rats treated with MDP compounds is, in part, bound to the active MDP metabolite (8, 11, 12). The reduced MDP metabolite-cytochrome P-450 complex can be observed as the so-called Type III optical difference spectrum (11) with characteristic dual Soret region peaks at 427 and 455 nm. Recent studies with reconstituted systems incorporating purified cytochrome P-450 isozymes and appropriate antibodies have established that the Type III difference spectrum is associated primarily with the "phenobarbital-type" cytochrome P-450 (P-450$_b$) and not with the "3-methylcholanthrene type" (P-450$_c$) that exhibits a difference spectrum with a single absorbance maximum at 455 nm (8, 13, 14). The oxidized MDP metabolite-cytochrome P-450 complex yields a single absorbance maximum at 438 nm (1, 4, 11) and in this state the MDP metabolite can be displaced from the cytochrome by a variety of compounds such as 2-alkylbenzimidazoles, *n*-alkylcarbamates, alkanes, several MFO substrates, and other MDP compounds (12, 15–17); displacement cannot be effected by Type II ligands such as aniline or imidazole (15).

Interest in the inducing action of MDP compounds has been stimulated by the finding that repeated treatment of rats with isosafrole induces a novel cytochrome P-450 isozyme (P-450$_d$) (13, 18, 19). Cytochrome P-450$_d$ has been purified as its isosafrole metabolite complex and has been characterized in some detail (6, 13, 18, 19). The substrate specificity and/or spectrum of MFO activity of cytochrome P-450$_d$ remains unknown, since in reconsti-

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[1] The abbreviations used are: MDP, methylenedioxyphenyl; MFO, mixed-function oxidase; AMDB, 4-*n*-alkoxy-1,2-methylenedioxybenzene; ADM, aminopyrine *N*-demethylase; AHH, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase.

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tuted systems it exhibits poor catalytic activity toward almost all substrates tested, irrespective of whether it is complexed with, or displaced from, the isosafrole metabolite (13, 18). The only exceptions to this are the activity of displaced P-450$_d$ toward isosafrole (complex formation) and lanosterol (18). Despite the apparent lack of catalytic activity of cytochrome P-450$_d$, hepatic microsomal fractions from rats treated with isosafrole show enhanced levels of oxidase activity toward several substrates, including benzphetamine, *p*-nitroanisole, aniline, and benzo[*a*]pyrene (18); substantial increases in the latter have been shown to occur following *in vivo* treatment of rats with dihydrosafrole (8). It remains unclear whether the increased microsomal activity results from cytochrome P-450$_d$, cytochrome P-450$_b$, or cytochrome P-450$_c$ that are simultaneously induced by isosafrole (13), or from the presence of as yet unidentified isozymes.

In a study with six MDP compounds of varying structure, Bridges and Fennell (6) concluded that inducing capacity was related directly to the ability of the MDP compound to form a complex with cytochrome P-450. The present study, with a homologous series of 4-*n*-alkyl ethers of 1,2-methylenedioxybenzene, was undertaken to investigate further the possible relationships between the extent of MDP metabolite-cytochrome P-450 complex formation, the stability of the complexes, and the induction of cytochrome P-450 and MFO activity.

EXPERIMENTAL PROCEDURES

Chemicals. The methyl ether of sesamol (I) was prepared as previously described (20). The remaining AMDB derivatives (II–X, Table 1) were obtained in good yield by the reaction of sesamol (Aldrich Chemical Company, Milwaukee, Wisc.) with the appropriate alkyl halide (21). The preparation of 4-*n*-propoxy-1,2-methylenedioxybenzene (III) is typical. A mixture of sesamol (13.8 g, 0.10 mole), *n*-propylbromide (14.8 g, 0.12 mole), and anhydrous potassium carbonate (13.8 g, 0.10 mole) was heated under reflux in dry acetone (60 ml) for 48 hr. After removal of the acetone and addition of water (100 ml), the reaction mixture was extracted with three 50-ml portions of diethyl ether and the combined ether extracts were washed with three 25-ml portions of 10% sodium hydroxide solution. Alkali was removed by further washing with water, and the ether extract was dried over anhydrous sodium sulfate. The ether was removed and the resulting oil was distilled under vacuum to yield 12.7 g (70%) of 4-*n*-propyl-1,2-methylenedioxybenzene, b.p. 112–113° (3 mm Hg). Physical properties and elemental analyses (Schwarzkoff Microanalytical Laboratory, Woodside, N. Y.) of the AMDB derivatives are shown in Table 1.

Biochemicals were obtained from Boehringer Mannheim (Indianapolis, Ind). All other solvents and chemicals were of analytical reagent grade.

Animals. Male Sprague-Dawley-derived rats (200–250 g) were purchased from Blue Spruce Farms (Altamont, N. Y.). AMDB derivatives were administered i.p. in corn oil at a dose of 1 mmole/kg once daily for 3 consecutive days; control animals were given corn oil alone. One treatment group consisting of four animals was used for each AMDB derivative.

Microsomal preparations. Livers from each treatment group were pooled and hepatic microsomal fractions were prepared as described previously (23), except that 0.1 M potassium phosphate-buffered sucrose (pH 7.4) was used in place of Tris-HCl-buffered sucrose. Microsomes were stored as frozen pellets and used within 2 weeks of preparation. Protein was determined by the method of Lowry *et al.* (24), using bovine serum albumin as standard.

Enzyme assays. ADM activity was determined as previously described (25), utilizing the method of Nash (26) to measure formaldehyde generated.

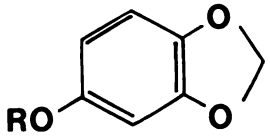
AHH activity was determined by the direct spectrofluorometric assay of Yang and Kicha (27), using an Aminco SPF-125 spectrofluorometer.

Optical difference spectroscopy. Cytochrome P-450 was measured by the method of Omura and Sato (28), using an Aminco-Chance DW-2 spectrophotometer and employing an extinction coefficient of 91 mM^{-1}cm^{-1} for the cytochrome P-450-ferrous carbonyl-spectral complex.

TABLE 1

Physical properties of AMDBs

Structure of the AMDB nucleus:



Compound no.	Substituent (*R*)	Boiling point (degrees/mm)	Elemental analysis			
			Calculated		Found	
			C	H	C	H
I	CH_3	85/6.0 (Lit. 108–110/15)[a]	—	—	—	—
II	C_2H_5	86–97/1.0	65.08	6.02	64.85	6.12
III	*n*-C_3H_7	112–113/3.0 (Lit. 67–69/0.08)[b]	66.69	6.67	66.65	6.74
IV	*n*-C_4H_9	127–128/3.0 (Lit. 87–88/0.1)[b]	68.06	7.21	68.18	7.30
V	*n*-C_5H_{11}	156–158/2.8 (Lit. 94–98/0.04)[b]	69.25	7.69	69.19	7.73
VI	*n*-C_6H_{13}	168–170/2.8	70.29	8.10	70.64	8.32
VII	*n*-C_7H_{15}	160–161/3.2	71.20	8.47	71.23	8.30
VIII	*n*-C_8H_{17}	155–157/2.0 (Lit. 110–113/0.07)[b]	72.01	8.79	71.85	9.38
IX	*n*-$C_{10}H_{21}$	185–187/3.8	73.40	9.35	73.35	9.41
X	*n*-$C_{12}H_{25}$	175–177/2.0	74.53	9.80	73.69	10.22

[a] Reported by Wilkinson (20).
[b] Reported by Beroza (22).

The AMDB metabolite-ferrocytochrome P-450 complex was measured at 37° with an Aminco-Chance DW-2 spectrophotometer using 1-cm cuvettes and 1-ml aliquots of microsomal suspensions (2 mg of protein per milliliter) in potassium phosphate buffer (0.1 M, pH 7.4). The absorbance difference between 455 nm and 490 nm was measured after the addition of 0.17 μmol of NADH to the sample cuvette, and the amount of cytochrome P-450 complexed with the AMDB metabolite was quantitated by applying an extinction coefficient of 75 $mM^{-1}cm^{-1}$ to the 455-nm/490-nm absorbance difference (12).

MDP-metabolite displacement. Microsomal pellets s from AMDB-treated rats were suspended in a small volume (3–5 ml) of potassium phosphate buffer (0.1 M, pH 7.4) to a protein concentration of approximately 60 mg/ml. Displacement of the AMDP-metabolite from the cytochrome P-450 complex was effected by two successive 30-min incubations (37°), each with a fresh aliquot (10 μmoles/ml of microsomal suspension) of 2-heptylbenzimidazole. Following the dual incubation, the microsomal suspension was loaded onto a Sephadex G-50 column (2.6 × 18 cm) and the microsomes were separated from excess displacer by elution with 0.1 M potassium phosphate buffer (pH 7.4) as described by Fisher *et al.* (18).

Data analysis. Data are expressed as the means ± standard error of the mean of estimations indicated for each data set. Statistical significance (criterion $p < 0.05$) of the difference between sample means was determined by Student's *t*-test for samples of unequal size (29). Regression equations for the quantitative structure-activity correlations were obtained using the SAS (Statistical Analysis System) option GLM (General Linear Models) procedure and the IBM 370 computer at Cornell University. For Compounds I–IV, *n*-alkoxy substituent π values used in the regression analyses were those reported by Hansch and Leo (30); values for the higher homologues of the series (V–X) were calculated on the principle of the additive character of π, employing an increment of 0.5 for each additional methylene unit.

RESULTS

Microsomal fractions from rats treated *in vivo* on 3 consecutive days with 1 mmole/kg (i.p.) of 1 of 10 AMDBs (I–X) contained levels of cytochrome P-450 (uncomplexed) that in general were not significantly different ($p > 0.05$) from those measured in microsomes from control animals (0.80 ± 0.01 nmole/mg of protein) (Table 2). However, the total cytochrome P-450 (uncomplexed cytochrome plus that complexed with the appropriate AMDB metabolite) content was clearly enhanced following *in vivo* treatment with AMDB derivatives, and increased with increasing *n*-alkoxy chain length to attain maximal levels 2- to 2.3-fold those in controls with compounds containing C_3–C_7 substituents (III–VII) before decreasing again with the higher homologues (Table 2).

AHH activities in the same microsomal fractions showed a trend similar to that of cytochrome P-450 levels, increasing progressively from Compounds I through VI and thereafter decreasing with increasing size of the *n*-alkoxy substituent. The maximal level of AHH activity (5.52 nmoles/mg of protein per minute) observed in microsomes from rats treated with the *n*-pentoxy derivative (VI) was 4.6-fold higher than that measured in controls (1.21 nmoles/mg of protein per minute).

In contrast to the pattern observed with respect to AHH activity, ADM activities in microsomal fractions from AMDB-treated rats were highest in animals receiving the three lowest homologues of the series (I–III) (Table 2) but were significantly ($p < 0.05$) lower than controls in animals receiving Compounds IV and V. ADM activities in microsomes from rats treated with Compounds VI–X were not significantly different ($p > 0.05$) from that measured in controls.

The AMDB metabolite-ferrocytochrome P-450 complex (455-nm spectral peak) in microsomal fractions from treated animals increased dramatically with increasing size of the *n*-alkoxy substituent from C_1 to C_4 (I–IV, Table 3). It remained at a high level in microsomes from rats treated with the C_4–C_7 derivatives but subsequently exhibited a marked decrease in intensity with Compounds VIII through X, the intensity of the latter being only 0.0033 absorbance units/mg of microsomal protein.

The relative stabilities of the several AMDB-ferricytochrome P-450 complexes were measured by their ease

TABLE 2

Monooxygenase activity and cytochrome P-450 content of hepatic microsomal fractions isolated from rats treated in vivo with AMDBs

Hepatic microsomal fractions were prepared from pooled livers of groups of four rats treated *in vivo* with each AMDB derivative (1 mmole/kg). The ferrocytochrome P-450-AMDB metabolite complex was determined from the ΔA (455–490 nm) after reduction of the microsomes with NADH; quantitation was effected using an extinction coefficient of 75 mM^{-1} cm^{-1}. Total cytochrome P-450 was determined as the sum of the complexed and uncomplexed forms of the cytochrome. All data are means ± standard error of the mean of three to six determinations.

Compound no. (alkoxy carbon chain length)	Uncomplexed cytochrome P-450 content[a]	AHH activity[b]	ADM activity[c]	Total cytochrome P-450 content[d]
I (1)	0.79 ± 0.01	1.56 ± 0.08[e]	3.32 ± 0.10[e]	0.91 ± 0.12
II (2)	0.95 ± 0.03[e]	2.87 ± 0.02[e]	3.54 ± 0.07[e]	1.44 ± 0.10[e]
III (3)	0.93 ± 0.04[e]	3.72 ± 0.14[e]	3.19 ± 0.10[e]	1.81 ± 0.12[e]
IV (4)	0.78 ± 0.01	3.89 ± 0.04[e]	1.94 ± 0.04[e]	1.81 ± 0.07[e]
V (5)	0.81 ± 0.04	4.08 ± 0.08[e]	2.07 ± 0.05[e]	1.82 ± 0.10[e]
VI (6)	0.79 ± 0.03	5.52 ± 0.17[e]	2.19 ± 0.11	1.80 ± 0.11[e]
VII (7)	0.77 ± 0.01	4.81 ± 0.12[e]	2.52 ± 0.13	1.65 ± 0.05[e]
VIII (8)	0.99 ± 0.01[e]	2.39 ± 0.06[e]	3.01 ± 0.21	1.28 ± 0.09[e]
IX (10)	0.94 ± 0.02[e]	1.23 ± 0.08	2.60 ± 0.10	1.03 ± 0.05[e]
X (12)	0.82 ± 0.01	1.17 ± 0.06	2.40 ± 0.05	0.86 ± 0.03

[a] Data are expressed as nanomoles per milligram of protein; levels in control microsomes were 0.80 ± 0.01 nmoles/mg of protein.

[b] Data are expressed as nanomoles of benzo[*a*]pyrene metabolized per milligram of protein per minute; levels in control microsomes were 1.21 ± 0.07 nmoles/mg of protein per minute.

[c] Data are expressed as nanomoles of formaldehyde produced per milligram of protein per minute; levels in control microsomes were 2.55 ± 0.13 nmoles/mg of protein per minute.

[d] Sum of the complexed and uncomplexed cytochrome P-450 (nanomoles per milligram of protein); variations are the sums of the variations of the two measurements.

[e] Significantly different from controls ($p \leq 0.05$, Student's *t*-test).

of displacement by 2-*n*-hepthylbenzimidazole as determined by two different methods. The data in Table 3 were obtained following incubation of microsomes with a high concentration of 2-*n*-heptylbenzimidazole (10 mM) and subsequent removal of the displacer prior to spectrophotometric measurement of the 438-nm peak. The data in Table 4 were obtained by direct spectrophotometric monitoring of the 438-nm peak in the cuvette in the presence of the displacer (12). Both data sets indicate that the metabolite-cytochrome P-450 complexes formed from the C_1–C_3 compounds (Fig. 1), and to a lesser extent those formed from the higher homologues (C_7–C_{12}), are relatively easily displaced by 2-*n*-heptylbenzimidazole. In contrast, the complexes formed from the C_4–C_6 derivatives are remarkably stable and appear unusually refractory to displacement under the conditions employed (Fig. 2).

Plots of total cytochrome P-450 levels, AHH activities, and AMDB metabolite-cytochrome P-450 complexes versus the π values for the *n*-alkoxy substituents (Fig. 3A, B, and C) suggested that the biological activity of the AMDB derivatives might be related to their hydrophobic character. Regression analysis was employed to investigate this relationship further. As anticipated from the shape of the plots shown in Fig. 3, neither total cytochrome P-450 levels, AHH activities, nor spectral complex formation were well correlated in linear relationships with π, and equations yielded r^2 values that accounted for only 14%, 8%, and 13% of the data variance, respectively (data not shown). However, equations in terms of π and π^2 (Eqs. 1–3, Table 5), describing parabolic relationships, indicated good correlations with each of these parameters; this was not the case with ADM activity (Eq. 4, Table 5). These results strongly suggest that levels of cytochrome P-450 (total), AHH activity, and spectral complex formation are interrelated and that ADM activity is not associated with these parameters.

Further regression analyses were performed to evaluate relationships between microsomal oxidase (AHH and ADM) activities, uncomplexed cytochrome P-450, total (complexed plus uncomplexed) cytochrome P-450, and 455-nm spectral complex formation (Table 6). High correlation coefficients were obtained for analyses relating AHH activity to total cytochrome P-450 levels and 455-nm spectral complex formation (Eqs. 5 and 6, Table 6) with about 83% and 89% of the data variance being accounted for, respectively. AHH activity was not significantly correlated with uncomplexed cytochrome P-450 levels, and, although a very tenuous link between ADM activities and levels of uncomplexed cytochrome P-450 might be inferred from Eq. 10 (Table 6) ($r = 0.164$; $r^2 = 38\%$), ADM activity was not well correlated with any of the three parameters tested.

TABLE 3

Effect of n-alkoxy chain length on the formation and stability (displaceability) of the AMDB complexes with cytochrome P-450

Displacement was effected by incubation with 2-*n*-heptylbenzimidazole of microsomal suspensions from rats treated *in vivo* with AMDB derivatives. Excess displacer was removed from the microsomes prior to assay as described under Experimental Procedures. All values are means ± standard error of the mean from at least two estimations.

Compound no. (alkoxy carbon chain length)	$\Delta A_{455-490\ nm} \times 10^3$/mg protein		% Complex displaced
	Spectral complex before displacement	Spectral complex after displacement	
I (1)	8.8 ± 1.1	0	100
II (2)	36.6 ± 1.4	0	100
II (3)	65.8 ± 1.5	0	100
IV (4)	76.9 ± 1.8	56.1 ± 0.5	27
V (5)	75.9 ± 1.0	42.0 ± 0.4	45
VI (6)	75.6 ± 1.5	35.3 ± 0.9	53
VII (7)	66.0 ± 0.7	7.3 ± 0.6	89
VIII (8)	28.4 ± 1.7	7.0 ± 1.6	75
IX (10)	6.5 ± 0.2	2.8 ± 0.4	56
X (12)	3.3 ± 0.1	1.0 ± 0	69

TABLE 4

Displacement of AMDB metabolite-ferricytochrome P-450 complexes with 2-n-heptylbenzimidazole

Microsomal suspensions (2 mg/ml) were divided equally between 1-cm cuvettes in an Aminco-Chance DW-2 spectrophotometer, and a baseline was recorded. Displacement was initiated by the addition of 2-*n*-heptylbenzimidazole to the sample cuvette in dimethyl sulfoxide (2 μl) to a final concentration of 400 μM. An equal volume of solvent was added to the reference cuvette. The optical difference spectrum was scanned between 380 and 500 nm until no additional changes in absorbance were observed; this was usually achieved in less than 1 hr with the readily displaced complexes but increased to 90 min with those that were more stable. The reciprocal of the absorbance difference between 438 and 490 nm was plotted against the reciprocal of the time at which the spectrum was recorded, as described by Elcombe *et al.* (12).

No. of carbon atoms in alkoxy substituent[a]	Apparent maximal spectral change[b]	Time to half-maximal displacement[c]
1	0.020	0.82
2	0.057	2.57
3	0.058	5.93
4	0.026	10.47
5	0.083	28.31
6	0.108	16.85

[a] For the heptoxy through dodecoxy metabolite complexes, determination of displacement parameters was not possible. Complex absorbance was too small and rates of displacement too low to permit accurate estimates of the parameters to be made.

[b] Obtained from the ordinate intercept of the double-reciprocal plot and expressed in $\Delta A_{438-490\ nm}$ per milligram of protein.

[c] Obtained from the abscissal intercept of double-reciprocal plot and expressed in minutes.

DISCUSSION

The results of this study have established that, as previously reported with isosafrole (13, 18, 19) and dihydrosafrole (8), a series of *n*-alkyl ethers of sesamol (4-hydroxy-1,2-methylenedioxybenzene) are effective inducers of cytochrome P-450 and AHH activity following *in vivo* administration to rats. Although no attempt has been made to identify or characterize the individual cytochrome P-450 isozymes induced by the several AMDB derivatives employed in this study, it seems probable that they are quite similar to those induced by isosafrole and dihydrosafrole. Since the AHH activity induced by both dihydrosafrole and the AMDBs is inhibited substantially (~80%) (data not shown) by an antibody to cytochrome P-450$_c$ purified from hepatic microsomes from β-naphthoflavone induced rats, and

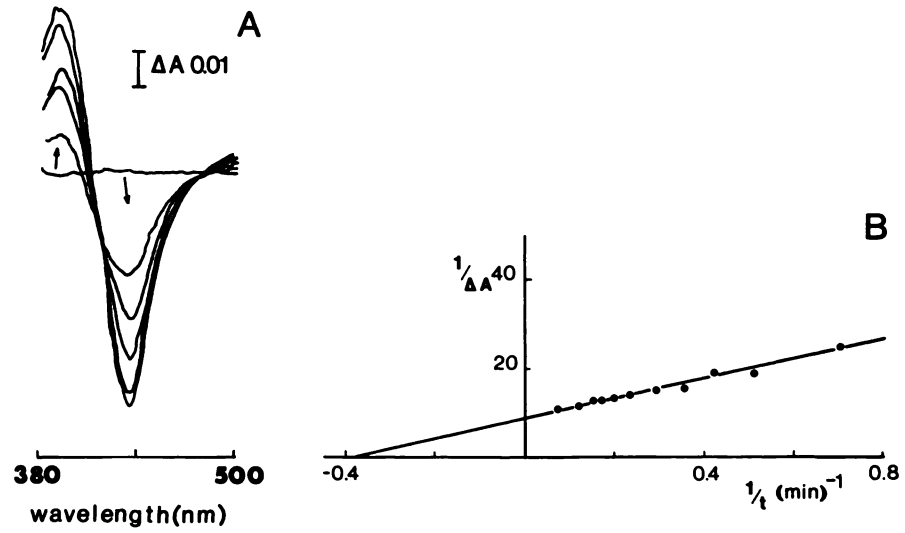


FIG. 1. *Displacement of the 4-ethoxy-1,2-methylenedioxybenzene (II) metabolite from its complex with ferricytochrome P-450 in the presence of 400 μM 2-n-heptylbenzimidazole*

The microsomal protein concentration was 2 mg/ml. A, *Arrows* indicate the direction in which the spectral change intensifies with time, and scans showing increasing absorbance intensity were at 2.26, 4.24, 4.80, 7.64, and 13.79 min, respectively. B, Data shown in Table 4 were derived from a double-reciprocal plot of the absorbance difference between 438 nm and 490 nm versus time of displacement.

since cytochrome P-450$_c$ is one of three major isozymes induced by isosafrole (13), it is possible that this isozyme is the one responsible for the observed increase in AHH activity following treatment of rats with MDP derivatives. This conclusion is supported in part by the recent finding that, in rabbits, isosafrole is an excellent inducer of cytochrome P-450$_{LM_4}$ (31), the same isozyme induced by 3-methylcholanthrene and β-naphthoflavone, although the rabbit P-450$_{LM_4}$ displays poor catalytic activity toward benzo[α]pyrene. Certainly it appears unlikely that enhanced AHH activity is due to induction of the isozyme P-450$_d$, the major hemoprotein in microsomes from isosafrole-induced rats (13), since although P-450$_d$ has some immunochemical relatedness to P-450$_c$ (32) and there is some homology between the mRNAs associated with the structural genes for cytochromes P-450$_c$ and P-

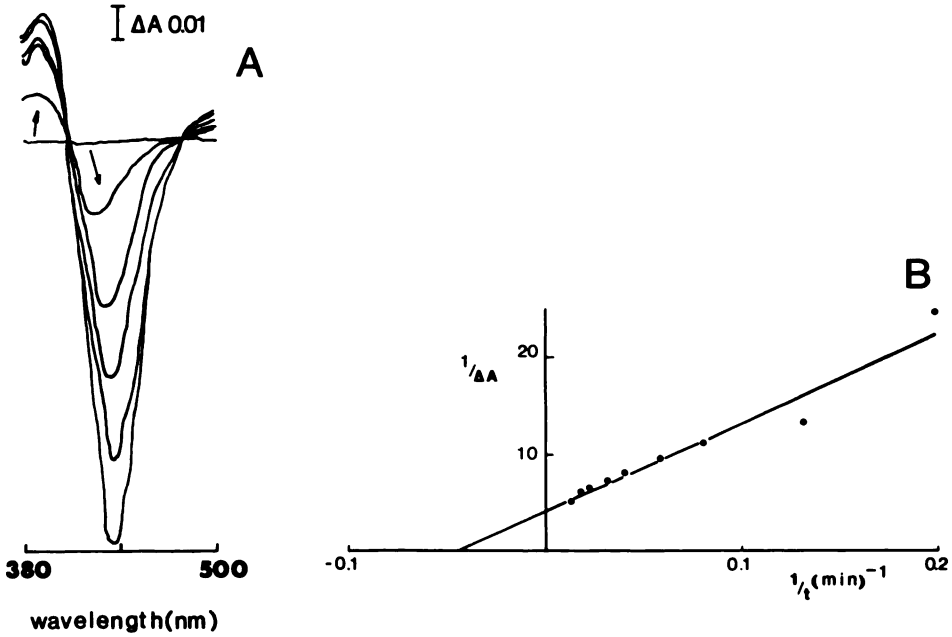


FIG. 2. *Displacement of the 4-n-hexoy-1,2-methylenedioxybenzene (VI) metabolite from its complex with ferricytochrome P-450 in the presence of 400 μM 2-n-heptylbenzimidazole*

The microsomal protein concentration was 1 mg/ml. A, *Arrows* indicate the direction in which the spectral change intensifies with time, and scans showing increasing absorbance intensity were at 5.06, 17.38, 32.39, 61.71, and 94.41 min, respectively. B, Data in Table 4 were derived from a double-reciprocal plot of the absorbance difference between 438 nm and 490 nm versus time of displacement.

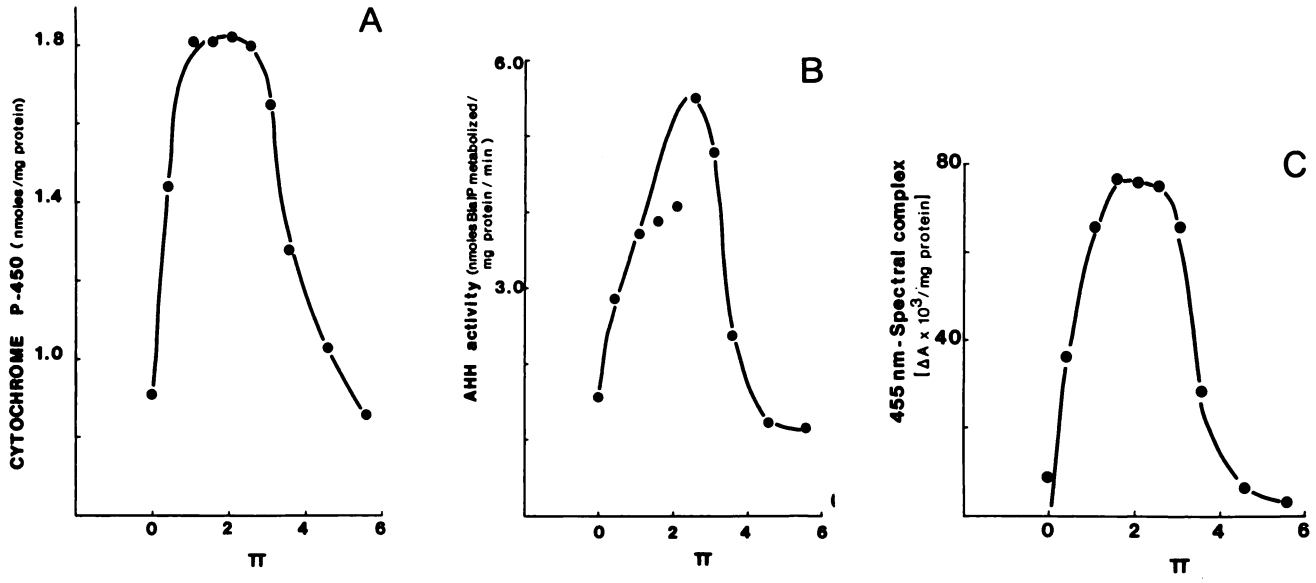


FIG. 3. *Relationship of total microsomal cytochrome P-450 levels (A), AHH activities (B), and MDP spectral complex formation (C) to the hydrophobic constant, π, of AMDB derivatives administered in vivo to rats*

TABLE 5

Correlation of various microsomal parameters with the hydrophobic constant π in microsomes from rats treated in vivo with a series of AMDB derivatives

Values of π for the *n*-alkoxy substituents in Compounds I–IV (−0.02, 0.38, 1.05, and 1.55, respectively) were from Hansch and Leo (30); π values for the *n*-alkoxy substituents in the remaining members of the series were calculated employing an increment of 0.5 for each additional methylene unit.

Eq. no.	Equation	r^a	S^b	F^c	r^2 (%)^d
1	P-450 total = $1.20 + 0.47\pi - 0.10\pi^2$ (0.14)[e] (0.02)	0.872	0.219	11.11	76
2	AHH activity = $2.04 + 1.86\pi - 0.39\pi^2$ (0.59) (0.10)	0.835	0.950	8.06	70
3	Spectral complex = $0.025 + 0.04\pi - 0.01\pi^2$ (0.01) (0.002)	0.858	0.18	9.78	74
4	ADM activity = $3.88 + 0.60\pi + 0.09\pi^2$ (0.30) (0.05)	0.646	0.482	2.50	42

[a] Multiple correlation coefficient.
[b] Standard deviation for the regression.
[c] Value of *F* ratio for regression.
[d] Percentage of data variance accounted for by regression equation.
[e] Figures in parentheses indicate standard error of parameter coefficients.

TABLE 6

Correlation of monooxygenase activity with cytochrome P-450 levels and spectral complex formation in microsomes from rats treated in vivo with a series of AMDB derivatives

Eq. no.	Monooxygenase activity	r^a	s	F	r^2 (%)
5	AHH = 3.52 [P-450$_{total}$] − 1.95 (0.556)[b]	0.913	0.660	40.0	83
6	AHH = 45.9 [spectral complex] + 1.08 (5.91)	0.940	0.553	60.2	88
7	AHH = −8.40 [P-450$_{free}$] + 10.25 (6.76)	0.402	1.48	1.55	16
8	ADM = −0.516 [P-450$_{total}$] + 3.42 (0.464)	0.366	0.549	1.24	13
9	ADM = −8.03 [spectral complex] + 3.04 (5.63)	0.450	0.527	2.03	20
10	ADM = 4.68 [P-450$_{free}$] − 1.29 (2.13)	0.614	0.466	4.84	38

[a] *r*, *s*, *F* and r^2 are defined in Table 5.
[b] Figures in parentheses indicate standard error of parameter coefficient.

450_d (33), little or no benzo[*a*]pyrene hydroxylase activity is observed in reconstituted systems incorporating P-450_d (13, 18).

The results presented here support the conclusion of Bridges and Fennell (6) that the inducing action of MDP compounds appears to be dependent on their ability to form metabolite complexes with cytochrome P-450. Although the data reported relate to an *in vivo* process and consequently may reflect the effects of several pharmacokinetic factors such as the distribution and transportation of AMDBs to the liver, as well as their interaction with cytochrome P-450, the equations in Table 6 demonstrate a good correlation between induction of cytochrome P-450 (complexed plus uncomplexed) and AHH activity and the ability of the AMDBs to form complexes with cytochrome P-450. Each of these parameters is related to the hydrophobic character of the *n*-alkoxy substituent of the AMDB (Table 5). The relationship with π is parabolic in nature (Fig. 3), optimal hydrophobicities (π_o) for AHH induction, spectral complex formation, and cytochrome P-450 (total) induction being 2.37, 2.30, and 2.30, respectively. These values correspond to an alkoxy chain length of five or six carbon atoms.

The results of previous studies in this laboratory on the inhibitory activity of a series of 1-alkylimidazoles implied the existence of a hydrophobic region accommodating nine or ten carbon atoms close to the active center of cytochrome P-450 (34). Considering the fact that the MDP ring is larger and more hydrophobic than the imidazole ring, it is possible that the two groups of compounds are interacting with the same hydrophobic area, although multiple substrate binding sites undoubtedly exist (35). It is also of interest that Dickins *et al.* (15) reported an apparent optimal log *p* value of 4.96, equivalent to a seven-carbon atom chain, with respect to the ability of a series of 2-*n*-alkylbenzimidazoles to displace the isosafrole metabolite-cytochrome P-450 complex.

A mechanistic explanation of the apparent relationship between the ability of MDP compounds to form complexes with cytochrome P-450 and to induce AHH activity is not immediately obvious. Since complex formation is dependent on the generation of an active metabolite species, possibly a carbene (9, 10), it is possible that formation of the same active species is a prerequisite for AHH induction. The recent report that isosafrole is capable of displacing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin from the mouse liver cytosolic Ah receptor (36) may be of relevance, although under the displacing conditions employed, metabolic activation of isosafrole would not be expected. AHH induction could also conceivably result from some kind of feedback mechanism resulting directly from the formation of the AMDB metabolite-cytochrome P-450 complex, but there is no precedence for this type of interaction. The fact that AHH activity correlates with total (rather than uncomplexed) cytochrome P-450 strongly suggests that AHH activity is catalyzed by an isozyme(s) that (a) is induced by AMDB derivatives and (b) is present primarily in a complexed state in the microsomes. Also, since AHH activity in microsomes from AMDB-induced rats is not affected by displacement of the metabolite-cytochrome P-450 complex, the presence of the 455-nm spectral complex per se does not appear to be inhibitory toward AHH activity. These characteristics further support the involvement of cytochrome P-450_c in the observed induction of AHH activity by AMDB derivatives, since, in reconstituted systems incorporating this isozyme, MDP compounds do not inhibit AHH activity despite their ability to form a 455-nm spectral complex (14). Furthermore, AHH activity is the same both before and after displacement of the 455-nm MDP spectral complex in microsomes from rats induced with β-naphthoflavone and treated *in vivo* with dihydrosafrole prior to sacrifice (8). The apparent insensitivity of AHH activity to the 455-nm spectral complex raises interesting questions regarding the nature of the complex.

In contrast to AHH activity, ADM activity was not induced by AMDB derivatives used in this study with the possible exception of the C_1–C_3 derivatives (Table 2). In fact, a slight inhibition of ADM activity was observed in microsomes from animals treated with Compounds IV and V (Table 2), and a weak correlation existed between ADM activity and uncomplexed cytochrome P-450 (Table 6).

Interpretation of the data with respect to ADM activity is difficult. Although the reaction is known to be catalyzed by cytochrome P-450_b, one of the three major isozymes induced by MDP compounds such as isosafrole (13), ADM activity is inhibited by MDP compounds (14) and it is probable that a substantial portion of the induced P-450_b is present as the inactive metabolite complex. Furthermore, ADM activity is probably not catalyzed exclusively by cytochrome P-450_b.

In agreement with the results of previous studies with isosafrole (12, 15, 17), the AMDB metabolite-cytochrome P-450 complexes are susceptible to displacement by 2-*n*-heptylbenzimidazole under oxidized conditions. However, the present study establishes that not all MDP complexes with cytochrome P-450 are of equal stability in the presence of 2-*n*-heptylbenzimidazole and that complexes generated from AMDB derivatives with *n*-butoxy to *n*-hexoxy substituents (Table 3 and 4) are not only the most intense but also the most stable with respect to displacement.

It has been suggested that MDP-complex formation involves bidentate binding within the active center of cytochrome P-450, a ligand interaction of the postulated MDP-carbene with the heme iron being combined with a hydrophobic interaction between MDP side chain and a lipophilic region adjacent to the heme (12, 37); disruption of the latter hydrophobic bonding by Type I and reverse Type I compounds has been suggested to be of primary importance in the displacement process (12). The data reported here support the contention that hydrophobic interactions are of importance in both the formation and stability of MDP-cytochrome P-450 complexes and suggest that the *n*-butoxy to *n*-hexoxy derivatives presumably define the approximate size of the 'lipophilic binding site.'

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