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ORGANIC SOLVENTS AS MODIFIERS OF ALDRIN EPOXIDASE IN RECONSTITUTED MONOOXYGENASE SYSTEMS AND IN MICROSOMES

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Abstract—To examine the response of individual cytochrome P-450 species catalysing the epoxidation of aldrin (Wolff T and Guengerich FP, *Biochem Pharmacol* **36**: 2581–2588, 1987), monooxygenase systems reconstituted from these species were assayed in the presence of 5% (v/v) = 0.87 M ethanol. The activity of cytochromes P-450$_{PB-B}$ and P-450$_{PB-D}$, two enzymes inducible by phenobarbital was increased seven-fold. The activity of two other P-450 enzymes purified from these animals was either inhibited by 50%, as observed for cytochrome P-450$_{PB-C}$ or remained unchanged, as noted with cytochrome P-450$_{PCN-E}$. Two P-450 enzymes purified from untreated rats, cytochromes P-450$_{UT-F}$ and P-450$_{UT-H}$, showed an inhibition by 50 and 20%, respectively, while the activity of cytochrome P-450$_{UT-A}$ was slightly increased by 50%. Indirect evidence that solvents enhance aldrin epoxidation by interacting with the hemoprotein was obtained by the finding that ethanol stimulated the activity of cytochrome P-450$_{PB-B}$ already, before addition of the lipid component, L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine. The K_m of cytochrome P-450$_{PB-B}$ for NADPH cytochrome P-450 reductase was not altered by ethanol indicating that the interaction between the two enzymes was not affected by the solvent. Other results indicate that the stimulatory solvent binds to a site, apart from the type I or type II binding site. The potency of various hydrophylic solvents to modify aldrin epoxidase activity was assayed in microsomes of rats pretreated with phenobarbital and of untreated male rats. Ethanol, *n*-propranol, *n*-butanol, acetone and tetrahydrofuran enhanced enzyme activity of phenobarbital pretreated rats to a maximal extent of two-fold and, at similar concentrations, inhibited the enzyme activity of untreated rats by 50%. The potency of these solvents correlated with their lipophilicity. Methanol and dimethylsulfoxide only slightly modified the activity of induced and noninduced animals. In the presence of 0.5 M *n*-propranol as the modifying agent, microsomal epoxidase activity of rats pretreated with pregnenolone-16α-carbonitrile, dexamethasone, 3-methylcholanthrene and of control rats was inhibited by 60–80%, whereas the activity of animals pretreated with phenobarbital, DDT, or the polychlorinated biphenyl mixture, Clophen A 50, was stimulated between two- and three-fold. The results reveal that organic solvents frequently used to dissolve monooxygenase substrates may considerably modify the activity of cytochrome P-450 dependent reactions, in particular when purified enzymes are assayed.

In vitro studies on the metabolism of xenobiotics by cytochrome P-450 dependent monooxygenases frequently require that the hydrophobic test compound is dissolved by an organic solvent to supply the enzyme with sufficient concentrations of the substrate. The solvent itself may interact with the metabolizing enzyme [1] and may inhibit the reaction studied [2]. Occasionally, monooxygenase dependent reactions were found to be stimulated *in vitro* [2–4]. To our knowledge, there is no systematic study on the question of how solvents affect the activity of individual cytochrome P-450 species. It is possible that a moderate degree of inhibition or stimulation of a microsomal monooxygenase activity is the result of much stronger effects of the solvent on particular cytochrome P-450 enzymes. Considering that these enzymes each exhibit different but overlapping substrate specificities or different preferences for particular reactions, a solvent may not only retard or increase the overall rate of a monooxygenase reaction *in vitro*, but may also alter the composition of pattern of microsomal metabolites.

To examine solvent effects on a particular reaction catalysed by various cytochrome P-450 enzymes, we have carried out an extensive study with a number of commonly applied organic solvents, using aldrin epoxidation as a model reaction. Aldrin epoxidation is catalysed by four rat liver cytochrome P-450 species inducible by phenobarbital (PB)§ and three other species predominantly occurring in the untreated male rat [5]. Cytochrome P-450 species inducible by polycyclic hydrocarbons and related compounds do not exhibit aldrin epoxidase activity [6, 7]. Because of its high sensitivity, aldrin epoxidation has been successfully used for the detection and quantification of cytochrome P-450 activity in various tissues, particularly in those of low enzymatic activity or minute sample size [8–16]. Earlier findings suggested that the activity of aldrin epoxidation may be modified by solvents: ethanol enhanced the activity of a major

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§ Abbreviations: PB, phenobarbital; MC, 3-methylcholanthrene; PCB, commercial mixture of polychlorinated biphenyls; DDT, 2,2′-bis-(4-chlorophenyl)-1,1,1-trichloroethane; PCN, pregnenolone-16α-carbonitrile; DEX, dexamethasone; DLGP, L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine. Enzymes: NADPH–cytochrome P-450 (cytochrome *c*) reductase (EC 1.6.2.4); glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

species purified from PB-treated rats [7] and was inhibitory for the microsomal activity of untreated rats [17].

In this paper, microsomal aldrin epoxidase activity of PB-treated rats was tested for modification by various aliphatic alcohols and other commonly used hydrophilic organic solvents. Furthermore, epoxidase activity of animals pretreated with various inducers was assayed for response to a modifying solvent. The results suggested that the response observed, i.e. whether stimulation or inhibition occurred, was probably due to the presence of particular cytochrome P-450 species. Therefore, the activity of P-450 enzymes purified from rat liver was assayed for modification in reconstituted monooxygenase systems. Two PB-inducible species showed a dramatic stimulation of the enzymatic activity. One of these enzymes, cytochrome P-450$_{PB-B}$, was further studied to explore by which kind of mechanism solvents enhance aldrin epoxidase activity.

MATERIALS AND METHODS

Chemicals. Enzymes and cofactors were purchased from Boehringer (Mannheim, F.R.G.). Aldrin and dieldrin, purity ≥99.9%, were obtained from Riedel de Haen (Seelze, F.R.G.). *n*-Hexane, ("zur Rückstandsananlyse") was from Merck (Darmstadt, F.R.G.) or was from Fisher Scientific (Fairlawn, NJ) ("HPLC grade"). Other organic solvents, analytical grade, were from Merck. PB (sodium salt) and MC were purchased from Fluka (Neu-Ulm, F.R.G.); the commercial PCB mixture, Clophen A-50, was from Bayer-Leverkusen (F.R.G.), PCN was a generous gift of Dr Schulte-Hermann (Institut für Krebsforschung, Wien, Austria), and DEX was obtained from Sigma Chemie (München, F.R.G.).

Pretreatment with inducers and preparation of microsomes. Wistar rats were obtained from the animal breeding station of the Gesellschaft für Strahlen- und Umweltforschung (Neuherberg, F.R.G.). For pretreatment with PB, males (180–220 g) received a single i.p. injection of 80 mg/kg in saline and subsequently 0.1% PB in their drinking water for one week. Lower doses were given via the drinking water containing 0.03 and 0.01% PB, respectively, and were administered for 10 days. Other inducers, dissolved in oil, were administered to male Wistar rats at about seven weeks of age as follows: MC was applied by gavage at doses of 40 mg/kg, 48 and 24 hr prior to killing; Clophen A-50 at a single oral dose of 500 mg/kg and the microsomes prepared 3 days later; DDT at a single dose of 200 mg/kg body wt 5 days prior to preparation of the microsomes. PCN and DEX were suspended in 1% Tween 80 and were administered by gavage to females in single doses of 20 mg/kg on 4 consecutive days and the animals killed day after the last dose.

Cytochrome P-450 enzymes were purified from liver microsomes of Sprague–Dawley rats obtained from Harlan Industries (Indianapolis, IN) and treated as described elsewhere [18]. Animals were killed 24 hr after the last dosage. Control animals were pretreated with the corresponding vehicles. Preparation of liver microsomes and determination of microsomal protein content and cytochrome P-450 level was performed as previously described [19].

Purification of cytochromes P-450. Enzymes were purified and antibodies were prepared according to procedures described elsewhere [20–22]. The nomenclature and its rationale for the cytochrome P-450 enzymes are presented elsewhere [20–22]. Other preparations in the literature that appear to correspond to the more significant ones dealt with in this report include the following: P-450$_{UT-A}$ [21]: male-specific P-450 [23], PB 2c [20], RLM 5 [24], P-450 h [24]; P-450$_{PCN-E}$ [21]: P-450$_{PCN}$ [25], PB 2a [20].

Incubation procedures. To assay the effect of solvents on the activity of purified P-450 enzymes, the standard incubation mixture prepared in small test tubes on ice contained, in 100 μl of 0.1 M phosphate buffer, pH 7.5: 10 pmol of purified cytochrome P-450, 20 pmol NADPH cytochrome P-450 reductase, 2 μg DLGP, 5 nmol aldrin (in 1 μl methanol), 5% (v/v) = 0.87 M ethanol and an NADPH-generating system consisting of 1 μmol glucose-6-phosphate, 0.05 μmol NADP and 0.1 I.U. glucose-6-phosphate dehydrogenase. The components were mixed using a vortex device after addition of each component. The reaction was started immediately after addition of the cofactors by placing the samples into a shaking water bath at 37° and was terminated after 5 min by replacing the samples on ice. Ten microlitres of a 0.1 μM methanolic solution of DDE, the internal standard, were added to every sample and the aqueous phase was vigorously extracted with 1 ml HPLC-grade *n*-hexane. After removing the aqueous phase the hexane extract was ready for analysis.

Aldrin epoxidase activity of microsomes was assayed as described elsewhere [6]. The aldrin concentration was 50 μM; microsomal protein concentration was 0.5 mg/ml. Organic solvents to be tested for their modifying effects were added to the incubation mixture during the preincubation period at 0°, prior to the addition of cofactors. Control activities in the absence of methanol were determined by using a microcrystalline suspension of aldrin prepared by ultrasonification according to the procedure given in Ref. 19. Dieldrin was quantified by electron-capture gas chromatography on capillary or packed columns, as described previously [5].

Partition coefficients. Octanol–water partition coefficients of dimethylsulfoxide and acetone were determined by UV spectroscopy. The aqueous phase consisted of 0.1 M phosphate buffer, pH 7.6. The maximal wavelength of absorbtion of dimethylsulfoxide was 238 nm in buffer and 243 nm in *n*-octanol. The corresponding data for acetone were 266 and 274 nm, respectively. P-values are means of three single determinations carried out with three different concentrations between 0.1 and 0.7 M of dimethylsulfoxide and of acetone.

RESULTS

Modifying effects of ethanol on aldrin epoxidase activity of various cytochrome P-450 enzymes purified from rat liver

Seven major purified rat liver cytochrome P-450 species known to exhibit aldrin epoxidase activity [5] were analysed for modification by 5% (v/v) =

Table 1. Effect of ethanol on aldrin epoxidase activity of various cytochrome P-450 species in reconstituted monooxygenase systems

P-450 enzyme	Modification of epoxidase activity due to ethanol (% of control)
$P\text{-}450_{UT\text{-}A}$	150 ± 24 (6)
$P\text{-}450_{UT\text{-}F}$	52 ± 5 (3)
$P\text{-}450_{UT\text{-}H}$	81 ± 20 (3)
$P\text{-}450_{PB\text{-}B}$	680 ± 23 (8)
$P\text{-}450_{PB\text{-}C}$	51 ± 16 (3)
$P\text{-}450_{PB\text{-}D}$	740 ± 160 (4)
$P\text{-}450_{PCN\text{-}E}$	106 ± 19 (4)

The composition of the reconstituted monooxygenase systems was the same as used for determination of aldrin turnover numbers [5]. Numbers of single determinations (±SD) performed at various molar ratios of cytochrome P-450 versus NADPH cytochrome P-450 reductase between 1:10 to 1:2 are given in parentheses. Ethanol concentrations was 0.87 M (5% v/v). For further experimental details see Materials and Methods. Turnover numbers in the absence of ethanol were: $P\text{-}450_{UT\text{-}A}$ 3.10, $P\text{-}450_{UT\text{-}F}$ 0.82, $P\text{-}450_{UT\text{-}H}$ 0.87, $P\text{-}450_{PB\text{-}B}$ 0.68, $P\text{-}450_{PB\text{-}C}$ 1.63, $P\text{-}450_{PB\text{-}D}$ 0.21, $P\text{-}450_{PB/PCN\text{-}E}$ 0.40 nmol dieldrin/min/nmol P-450, respectively.

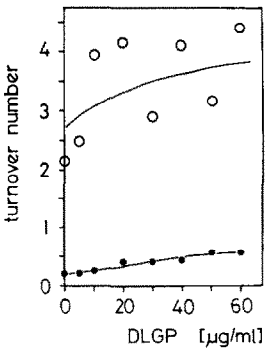

Fig. 1. Stimulation of cytochrome $P\text{-}450_{PB\text{-}B}$ dependent aldrin epoxidase by ethanol: requirement for DLGP. Enzyme activity was determined in a reconstituted monooxygenase system composed of 100 nM cytochrome $P\text{-}450_{PB\text{-}B}$ and 100 nM NADPH-cytochrome P-450 reductase in the presence (○—○) and in the absence (●—●) of ethanol. Concentration of aldrin was 20 µM and of ethanol 0.87 M.

0.87 M ethanol in reconstituted monooxygenase systems (Table 1). Cytochromes $P\text{-}450_{PB\text{-}B}$ and $P\text{-}450_{PB\text{-}D}$ purified from animals pretreated with PB showed a seven-fold increase in epoxidase activity. The activity of cytochromes $P\text{-}450_{UT\text{-}F}$ and $P\text{-}450_{UT\text{-}H}$, two enzymes purified from untreated animals, and of cytochrome $P\text{-}450_{PB\text{-}C}$, another form purified from animals pretreated with PB, was inhibited by ethanol. Epoxidase activity of $P\text{-}450_{UT\text{-}A}$, the major species of untreated males, was slightly enhanced by ethanol. No significant change was observed for the activity of $P\text{-}450_{PCN\text{-}E}$, a form inducible by PCN and by PB [21, 26].

Studies on the mechanism of the stimulation effect

The effect of ethanol on steady state kinetics of aldrin epoxidation by cytochrome $P\text{-}450_{PB\text{-}B}$ was determined in a reconstituted monooxygenase system consisting of 50 nM cytochrome P-450, 125 nM NADPH–cytochrome P-450 reductase and 20 µg/ml DLGP. Aldrin epoxidase activity was measured at substrate concentrations between 1 and 20 µM aldrin. The Lineweaver–Burk diagram revealed a K_m of 1 µM for aldrin. In the presence of 0.87 M ethanol the K_m value of this system was increased to 3.3 µM. In the same system, aldrin epoxidase activity was determined as a function of reductase concentration at a fixed concentration of cytochrome $P\text{-}450_{PB\text{-}B}$. The incubations were carried out at various reductase concentrations between 50 and 1000 nM and a fixed cytochrome P-450 concentration of 100 nM. The aldrin concentration was 50 µM. Ethanol did not significantly alter the K_m-value of cytochrome $P\text{-}450_{PB\text{-}B}$ for the reductase, which was 160 nM.

The role of the lipid factor, DLGP, was also examined (Fig. 1). Addition of the lipid was not particularly essential for the cytochrome to express enzyme activity under these conditions. Aldrin epoxidase activity in the absence of the lipid was only enhanced two-fold when increasing amounts of DLGP were added. Ethanol, at a concentration of 0.87 M, strongly stimulated enzyme activity in the absence of added lipid by a factor of about 10. Addition of lipid increased the enzyme activity by about 50% only.

To prove for the involvement of the type II binding site at the hemoprotein, the type-II binding compound, pyridine [27], was analysed for its modifying effect on aldrin epoxidase activity of microsomes from PB treated rats. No stimulation of enzyme activity was observed up to a concentration of 260 mM pyridine.

Modification of microsomal aldrin epoxidase activity by various organic solvents in untreated and PB-pretreated rats

A series of commonly used organic solvents inhibited aldrin epoxidase activity of untreated rats, except for methanol, which was ineffective up to a concentration of 3 M (Fig. 2, open circles). The alcohol concentration required to inhibit enzyme activity by 50% decreased with increasing length of the aliphatic hydrocarbon chain. The corresponding I_{50}-values were 1.3 M, 0.25 M and 0.05 M for ethanol, *n*-propanol and *n*-butanol, respectively. Of the non-alcoholic solvents, tetrahydrofuran was a strong inhibitor, similar to butanol. Acetone was moderately inhibitory and dimethylsulfoxide was slightly inhibitory.

By contrast, aldrin epoxidase activity in microsomes from PB treated animals was enhanced by most solvents. The maximum extent of enhancement was about two-fold of the control activity determined in the absence of the solvents (Fig. 2, closed circles). Dimethylsulfoxide had no effect on the enzyme activity up to a concentration of 0.8 M. Methanol moderately enhanced epoxidase activity. "S_{max}", the

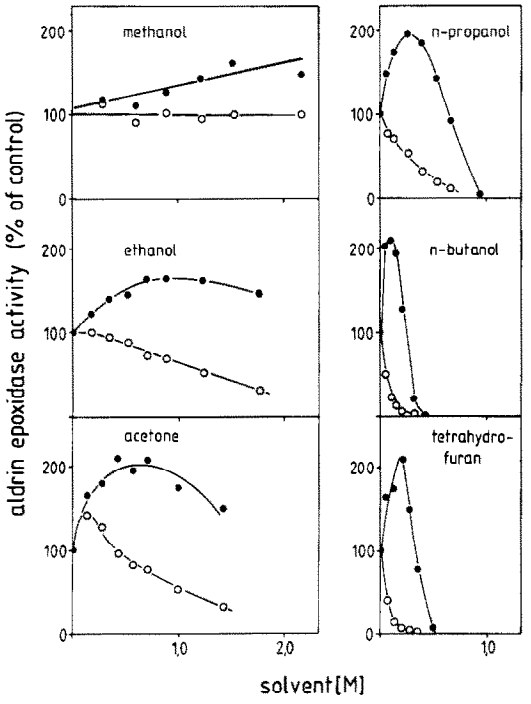

Fig. 2. Modification of microsomal aldrin epoxidase activity by organic solvents. Aldrin epoxidase was determined in the presence of the organic solvents indicated using liver microsomes of rats pretreated with PB (●—●) or of untreated rats (○—○). Control activities were: 9.0 and 3.8 nmol dieldrin/mg protein/min for PB- and untreated animals, respectively. Each point represents the mean of two independent determinations, the variation of which was 10%. The control activity in the absence of methanol was determined utilizing a microcrystalline, aqueous suspension of aldrin prepared by ultrasonication (see Materials and Methods).

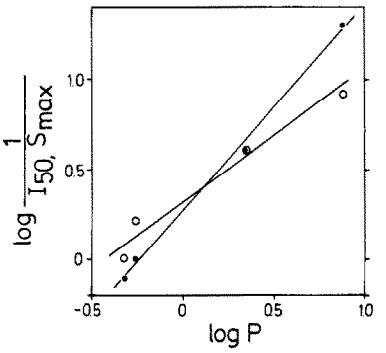

Fig. 3. Relation of I_{50} and S_{max} to the octanol water partition coefficient P. The data for I_{50} (●—●) and S_{max} (○—○) were taken from Fig. 2 and plotted in logarithmic scales.

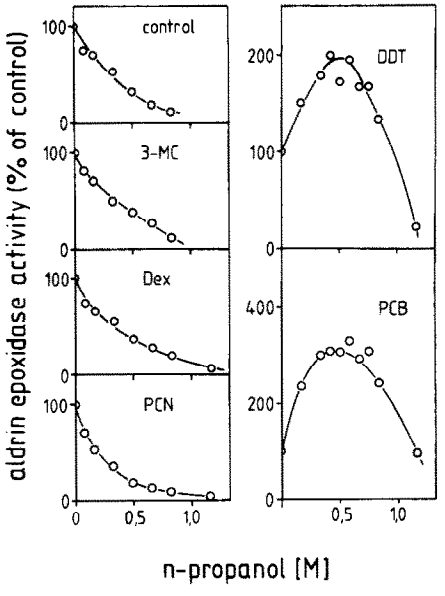

Fig. 4. Effect of *n*-propanol on microsomal aldrin epoxidase activity of variously pretreated rats. Enzyme activities were determined in the presence of increasing concentrations of *n*-propanol, as given in Materials and Methods. Aldrin epoxidase activity of animals pretreated with Clophen A-50, DDT and MC, was 97, 160 and 52%, respectively, of the activity of untreated males, which varied between 2.1 and 5.8 nmole dieldrin/mg protein/min. PCN and DEX increased the activity of untreated females of 0.62 ± 0.08 nmol dieldrin/mg protein/min by 407 and 422%, respectively. The curves represent the means of two individuals.

solvent concentration causing maximal stimulation, was: 0.12 M for *n*-butanol, 0.21 M for tetrahydrofuran, 0.25 M for *n*-propanol, 0.6 for acetone and 1.0 M for ethanol.

The stimulation effect of *n*-propranolol was analysed in microsomes prepared from animals pretreated with various doses of PB. A stimulation of aldrin epoxidase activity was still observable when the animals had received one third of the PB-dose generally applied. At one tenth of the dose, a bimodal response was observed indicating that propranolol was only effective as a stimulator of enzyme activity at high concentrations of about 0.5 M.

The potency of a solvent to inhibit or to enhance microsomal aldrin epoxidase activity was inversely correlated to its lipophilicity, expressed by the octanol/water partition coefficient P. A linear relationship between log P and $\log(1/I_{50})$ and $\log(1/S_{max})$ was observed (Fig. 3). This relationship corresponds to the equation log (1/concentration) = a log P + b, established by Hansch and Leo [28] for the relationship between the biologically effective concentration and the partition coefficient of a series of lipophilic compounds.

Modifying effect of n-*propanol on aldrin epoxidase activity of animals pretreated with various inducers*

n-Propanol inhibited microsomal aldrin epoxidase from animals pretreated with MC, PCN and DEX (Fig. 4). By contrast, the solvent stimulated the activity of PCB-pretreated animals by a factor of three, and the activity of DDT-pretreated animals two-fold.

DISCUSSION

Aliphatic alcohols, ketones and ethers are generally known as inhibitors of monooxygenase reactions. The present study revealed that, in addition to being inhibitors, these solvents were also capable of enhancing the rate of a monooxygenase reaction. The enhancement of aldrin epoxidase activity described here provides one of the rare examples that organic solvents stimulate a monooxygenase reaction. Another example is the enhancement of aniline *p*-hydroxylation by acetone observed with microsomes from control and PB-pretreated rats [2, 3]. Ethanol was shown to stimulate the activity of arylhydrocarbon hydroxylase in rat kidney microsomes [4]. The mechanism underlying these effects however, is not known.

The monooxygenase system reconstituted from cytochrome P-450$_{PB-B}$ provided a suitable system to further explore the enhancing effect of solvents. Ethanol in this system did not alter the K_m of cytochrome P-450$_{PB-B}$ for the reductase, indicating that ethanol did not increase the activity by improving the interaction between the reductase and the hemoprotein, a mechanism that is thought to underlie the stimulatory effect of phospholipids on ethoxycoumarin deethylation in reconstituted monooxygenase systems [29]. Furthermore, the phospholipid component was not essential, since ethanol had stimulated the activity of cytochrome P-450$_{PB-B}$ already, before the phospholipid factor was added to the system. Increasing amounts of the lipid stimulated the activity only to a moderate extent, irrespective of whether ethanol was present or not. It appears that solvents enhance PB-inducible aldrin epoxidase rather by directly interacting with the hemoprotein than by facilitating the interaction between the hemoprotein and the reductase or by directly affecting the lipid component.

Further evidence that organic solvents directly interact with the hemoprotein is derived from substrate binding spectra. Alcohols and ketones elicit a "modified type-II" spectral change. It was concluded that this type of spectral change was the result of an interaction at both the type I binding site, where most substrates bind and at another site, possibly the type II binding site [30] or a site apart from the type II binding site [31]. Our finding that the type II binding compound, pyridine [27], did not stimulate microsomal epoxidase activity from PB-treated animals indicates that the enhancing effect is not caused by interaction at the type II site. Likewise, binding at the type I binding site seems not to be involved in the enhancing effect, since solvents binding at this site would interfere with the metabolism of the substrate and would be inhibitory.

These findings lead us to postulate a solvent binding site or binding region at cytochromes P-450$_{PB-B}$ and P-450$_{PB-D}$, apart from the type I and type II substrate binding sites. This interpretation based on results on the catalytic activity of purified P-450 species, substantiates the previous assumption of a particular solvent binding site, which was deduced from microsomal binding spectra of alcohols and ketones [31] and of hydrocarbons dissolved in these solvents [32].

In microsomes the response of aldrin epoxidase activity to the modifying solvent varied with the type of induction: the activity of PB-, DDT- and PCB-induced animals was stimulated by the modifying solvent, while inhibition was the response observed for control, MC- and hormone-treated animals. These findings suggested that the response observed may be due to the predominating contribution of particular cytochrome P-450 enzymes. The two enzymes exhibiting the highest degree of stimulation in reconstituted monooxygenase systems, cytochromes P-450$_{PB-B}$ and P-450$_{PB-D}$, probably are responsible for the stimulation effect observed with microsomes from animals pretreated with PB and PCB. The relative portion of these P-450 enzymes is tremendously augmented from 2% to 50% and to 25% of total cytochrome P-450 by PB- and PCB-induction, respectively [20, 21]. Both inducers do not considerably alter the relative amount of other enzymes the activity of which is inhibited or slightly increased by solvents, such as cytochromes P-450$_{PB-C}$, P-450$_{UT-F}$, or is slightly affected, as observed for P-450$_{UT-A}$ and P-450$_{PCN-E}$ [20, 21]. The stimulation effect observed with microsomes from DDT-pretreated animals is in line with the finding that the pattern of monooxygenase activities induced by DDT resembles the pattern of activities after PB pretreatment [33]. The inhibition effect observed with untreated, MC- and hormone-pretreated animals, on the other hand, corresponds to the prevalence of inhibitable or insensitive enzymes, cytochromes P-450$_{PB-C}$, P-450$_{UT-F}$, P-450$_{UT-A}$ and P-450$_{PCN-E}$ and to the low abundance of cytochromes P-450$_{PB-B}$ and P-450$_{PB-D}$ in these microsomes [20, 21]. Major groups of aldrin epoxidizing cytochrome P-450 species thus may be roughly differentiated *in vitro* by employing the modifying effect of solvents.

Another aspect of this study to be discussed here is the possibility that the solvent effects observed for aldrin epoxidation may also pertain to the metabolism of other lipophilic monooxygenase substrates; in particular, when microsomes from animals pretreated with PB or PB-like inducers are applied. Regarding Michaelis–Menten kinetics, a modifying solvent present in every sample at the same concentration may alter the K_m and V_{max} value as compared to a procedure, where the substrate had been dissolved by a solvent exhibiting marginal modifying effects, such as methanol. If the test compound is added from one stock solution, the double reciprocal plots may deviate from linearity at high substrate concentrations, since at high substrate concentration, i.e. high solvent concentrations, the enhancing effect of the solvent becomes efficacious. A method to exclude solvent effects would be to employ solvent-free substrate suspensions prepared by ultrasonication. These suspensions have been successfully used to determine enzyme kinetic parameters for microsomal PCB-hydroxylation [19] and aldrin epoxidation, as shown in this paper. If not practicable, solvents of low lipophilicity, such as methanol or dimethylsulfoxide, are preferable.

In conclusion, our results show that organic solvents may considerably modify the activity of several major cytochrome P-450 species *in vitro*. Accordingly, the metabolite pattern of a lipophilic mono-

oxygenase substrate may be also varied. This conclusion may be significant for *in vitro* studies on the oxygenation of drugs, toxic compounds and carcinogens metabolized by distinct cytochrome P-450 species, particularly by PB-inducible enzymes [34–36].

REFERENCES

1. Imai Y and Sato R, Studies on substrate interactions with cytochrome P-450 in drug hydroxylation by liver microsomes. *J Biochem* **62**: 239–249, 1967.
2. Kawalek JC and Andrews AW, The effect of solvents on drug metabolism *in vitro*. *Drug Metab Disp* **8**: 380–384, 1980.
3. Anders MW, Acetone enhancement of microsomal aniline para-hydroxylase activity. *Arch Biochem Biophys* **126**: 269–275, 1968.
4. Wiebel FJ, Leutz JC, Diamond L and Gelboin HV, Aryl hydrocarbon (benzo[a]pyrene) hydroxylase in microsomes from rat tissues: differential inhibition and stimulation by benzoflavones and organic solvents. *Arch Biochem Biophys* **144**: 78–86, 1971.
5. Wolff T and Gungerich FP, Rat liver cytochrome P-450 isozymes as catalysts of aldrin epoxidation in reconstituted monooxygenase systems and microsomes. *Biochem Pharmacol* **36**: 2581–2588, 1987.
6. Wolff T, Wanders H and Deml E, Aldrin epoxidation, a highly sensitive indicator specific for cytochrome P-450-dependent monooxygenase activities. *Drug Metab Disp* **7**: 301–305, 1979.
7. Wolff T, Greim H, Huang MT, Miwa GT and Lu AYH, Aldrin epoxidation catalyzed by purified rat-liver cytochromes P-450 and P-448: high selectivity for cytochrome P-450. *Eur J Biochem* **111**: 545–551, 1980.
8. Van Cantfort J, Leonard-Poma M, Séle-Doyen MJ and Gielen JE, Differences in the biochemical properties of aldrin epoxidase, a cytochrome P-450-dependent monooxygenase, in various tissues. *Biochem Pharmacol* **32**: 2697–2702, 1983.
9. Siegers CP, Schümann S, Thies E, Böse-Younes H and Younes M, Aldrin epoxidase and dimethylhydrazine demethylase activities in tumorous and non-tumorous tissue of the human colon and rectum. *Cancer Lett* **23**: 39–43, 1984.
10. Wolff T, Aldrin epoxidation, a sensitive and specific assay of monooxygenase activity in small liver samples. In: *Fine Needle Aspiration Biopsy of the Liver* (Ed. Zbinden G), pp. 49–57. Pergamon Press, Oxford, 1980.
11. Williams FM, Woodhouse KW, Middleton D, Wright P, James O and Rawlins MD, Aldrin epoxidation kinetics in small samples of human liver. *Biochem Pharmacol* **31**: 3701–3703, 1982.
12. McManus ME, Boobis AR, Minchin RF, Schwartz DM, Murray S, Davies DS and Thorgeirsson SS, Relationship between oxidative metabolism of 2-acetylaminofluorene, debrisoquine, bufuralol, and aldrin in human liver microsomes. *Cancer Res* **44**: 5692–5697, 1985.
13. Wolff T and Strecker M, Lack of relationship between debrisoquine 4-hydroxylation and other cytochrome P-450 dependent reactions in rat and human liver. *Biochem Pharmacol* **34**: 2593–2598, 1985.
14. Schwartz LR, Götz R, Wolff T and Wiebel FJ, Monooxygenase and glucuronyltransferase activities in short term cultures of isolated rat hepatocytes. *FEBS Lett* **98**: 203–206, 1979.
15. Kremers P, Letawe-Goujon F, De Greave J, Duvivier J and Gielen JE, The expression of different monooxygenases supported by cytochrome P-450 in neonatal rats and in primary fetal hepatocytes in culture. *Eur J Biochem* **137**: 603–608, 1983.
16. Wiebel FJ, Wolff T and Lambiotte M, Presence of cytochrome P-450- and cytochrome P-450-dependent monooxygenase functions in hepatoma cell lines. *Biochem Biophys Res Commun* **94**: 466–472, 1980.
17. Wolff T, *In vitro* inhibition of monooxygenase dependent reactions by organic solvents. In: *Industrial and Environmental Xenobiotics* (Eds. Fouts JR and Gut I), pp. 196–199. Excerpta Medica, Elsevier, Amsterdam, 1978.
18. Kaminsky LS and Guengerich FP, Cytochrome P-450 isozyme/isozyme functional interactions and NADPH cytochrome P-450 reductase concentrations as factors in microsomal metabolism of warfarin. *Eur J Biochem* **149**: 479–489, 1985.
19. Wolff T and Hesse S, Species differences of mixed-function oxidase induction between rabbits and rats after pretreatment with polychlorinated biphenyls. *Biochem Pharmacol* **26**: 783–787, 1977.
20. Waxman DJ, Dannan GA and Guengerich FP, Regulation of rat hepatic cytochrome P-450: age dependent expression, hormonal imprinting, and xenobiotic inducibility of sex specific isozymes. *Biochemistry* **24**: 4409–4417, 1985.
21. Guengerich FP, Dannan GA, Wright ST, Martin MV and Kaminsky LS, Purification and characterisation of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic and immunological properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**: 6019–6030, 1982.
22. Kamataki T, Maeda K, Yamazoe Y, Nagai T and Kato R, Sex difference of cytochrome P-450 in the rat: purification, characterisation, and quantitation of constitutive forms of cytochrome P-450 from liver microsomes of male and female rats. *Arch Biochem Biophys* **225**: 758–770, 1983.
23. Cheng KC and Schenkman JB, Metabolism of progesterone and estradiol by microsomes and purified cytochrome P-450 RLM_3 and RLM_5. *Drug Metab Disp* **12**: 222–234, 1984.
24. Ryan DE, Iida S, Wood AW, Thomas PE, Lieber CS and Levin W, Characterization of three highly purified cytochromes P-450 from hepatic microsomes of adult male rats. *J Biol Chem* **259**: 1239–1250, 1984.
25. Newman SL and Guzelian PS, Identification of the cyanopregnenolone-inducible form of hepatic cytochrome P-450 as a catalyst of aldrin epoxidation. *Biochem Pharmacol* **32**: 1529–1531, 1983.
26. Elshourbagy NA and Guzelian PS, Separation, purification, and characterization of a novel form of hepatic cytochrome P-450 from rats treated with pregnenolone-16-α-carbonitrile. *J Biol Chem* **255**: 1279–1285, 1980.
27. Schenkman JB, Remmer H, Estabrook RW, Spectral studies of drug interaction with hepatic microsomal cytochrome. *Molec Pharmacol* **3**: 113–123, 1967.
28. Hansch C and Leo A, *Substituent Constants for Correlation Analysis in Chemistry and Biology*. Wiley and Sons, New York, 1979.
29. Müller-Enoch D, Churchill P, Fleischer S and Guengerich FP, Interaction of liver microsomal cytochrome P-450 and NADPH cytochrome P-450 reductase in the presence and absence of lipid. *J Biol Chem* **259**: 8174–8182, 1984.
30. Yoshida Y and Kumaoka H, Studies on the substrate-induced spectral changes of cytochrome P-450 in liver microsomes. *J Biochem (Tokyo)* **78**: 455–468, 1975.
31. Schenkman JB, Cinti DL, Orrhenius S, Moldéus P and Krashnitz R, The nature of the reverse type I (modified type II) spectral change in liver microsomes. *Biochemistry* **11**: 4243–4251, 1972.
32. Backes WL and Canady WJ, The interaction of hepatic cytochrome P-450 with organic solvents. *J Biol Chem* **256**: 7213–7227, 1981.
33. Hart LG and Fouts JR, Further studies on the stimulation of hepatic microsomal drug metabolizing

enzymes by DDT and its analogs. *Naunyn Schmiedeberg's Arch Exp Path Pharmakol* **249**: 486–500, 1965.
34. Greim HA, An overview of the phenomena of enzyme induction and inhibition: their relevance to drug action and drug interactions. In: *Concepts in Drug Metabolism* (Eds. Jenner P and Testa B), pp. 219–263. Marcel Dekker, New York, 1981.
35. Toftgard R, Haaparanta T, Eng L and Halpert J, Rat lung and liver microsomal cytochrome P-450 isozymes involved in the hydroxylation of n-hexane. *Biochem Pharmacol* **35**: 3733–3738, 1986.
36. Guengerich FP, Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res* **48**: 2946–2954, 1988.