Biochemical Pharmacology, Vol. 36, No. 16, pp. 2581–2588, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

RAT LIVER CYTOCHROME P-450 ISOZYMES AS CATALYSTS OF ALDRIN EPOXIDATION IN RECONSTITUTED MONOOXYGENASE SYSTEMS AND MICROSOMES

THOMAS WOLFF*† and F. PETER GUENGERICH‡

Institut für Toxikologie, Gesellschaft für Strahlen- und Umweltforschung, (München), D-8042 Neuherberg, Federal Republic of Germany, and ‡Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN, U.S.A.

(*Received* 13 *August* 1986; *accepted* 25 *January* 1987)

Abstract—To explore which rat liver cytochrome P-450 species are involved in aldrin epoxidation, we have studied the catalytic activities of a series of cytochrome P-450 isozymes purified from untreated and inducer-treated Sprague–Dawley rats. Of ten cytochrome P-450 forms analyzed, seven isozymes, listed in order of decreasing activity, catalyzed aldrin epoxidation: P-450$_{UT-A}$, P-450$_{PB-C}$, P-450$_{UT-H}$, P-450$_{PB-B}$, P-450$_{PCN-E}$, P-450$_{UT-F}$, and P-450$_{PB-D}$. P-450$_{UT-I}$, P-450$_{BNF-B}$, and P-450$_{ISF-G}$ were not very active at all. A novel aldrin metabolite, *endo*-dieldrin, was formed by cytochrome P-450$_{UT-F}$ in a 6-fold excess over dieldrin, which is the exo-isomer.

The activity of aldrin epoxidase furthermore was assayed in liver microsomes from Sprague–Dawley rats of diverse physiological status and after pretreatment with various inducers resulting in a peculiar pattern of cytochrome P-450 isozymes. Untreated animals, at an age of 3 weeks, showed similar enzyme activities in both genders. During maturation, the activity of males increased by 3-fold, while the activity in females did not significantly change during this period. Pretreatment with pregnenolone-16-α-carbonitrile or dexamethasone strongly increased the activity in females. Pretreatment with dexamethasone did not increase the activity of males. A 50% depression of epoxidase activity was noted for males pretreated with 5,6-benzoflavone. Phenobarbital pretreatment increased the activity of females by 12-fold and of males by 2-fold. Males responded to pretreatment with polychlorinated biphenyls in a strain dependent fashion: enzyme activity was increased 2-fold in Sprague–Dawley rats but was not altered in Wistar rats.

"Theoretical" values of microsomal epoxidase activity were calculated for weanling and adult Sprague–Dawley rats from turnover numbers and published data on the relative abundance of aldrin epoxidizing P-450 isozymes (Waxmann *et al.*, *Biochemistry* **24**, 4409, 1985). These values agreed with the activities determined. A similar statement can be made for male rats of both strains pretreated with inducers, when the ratio of enzyme activity of pretreated to control animals was used as a basis of comparison. The activity ratio of females pretreated with pregnenolone-16-α-carbonitrile, dexamethasone and phenobarbital, however, was much higher than the ratio calculated.

Our results reveal that aldrin epoxidation is a reaction indicative of male specific and of phenobarbital-inducible cytochrome P-450 isozymes in rat liver. It is further suggested that, when aldrin is the substrate, these isozymes in microsomes of male rats poorly interfere with each other or with "non-epoxidizing" isozymes. In microsomes of female rats, however, the activity of epoxidizing isozymes may be stimulated by female-specific isozymes.

Hepatic cytochrome P-450-dependent monooxygenases are key enzymes mediating the oxidative metabolism of a wide variety of foreign chemicals and endogenous compounds. Many cytochrome P-450 species composing this enzyme system differ in their preference for substrates and pathways of bioactivation and detoxication [1, 2]. The presence or absence of these species in the liver or in tissues where toxic lesions occur seems to be one of the critical factors involved in the expression of chemical toxicity.

However, it is sometimes difficult to assay enzymatic activity and more so the presence of particular isozymes comprising the entire cytochrome P-450 enzyme complex. Extrahepatic tissues or cells in culture generally exhibit low cytochrome P-450 activities. Tissue material, for example, from human liver biopsies, frequently is available only in minute amounts. The fact that many monooxygenase reactions are mediated by relatively unspecific enzymes poses another limitation for the analysis of individual cytochrome P-450 species in tissue preparations.

During the past years, the assay of aldrin epoxidase activity has become a widely used procedure to detect cytochrome P-450-dependent activities; in particular, to assay low tissue levels of cytochrome P-450. The sensitivity of this assay has permitted the detection of cytochrome(s) P-450 in various extrahepatic tissues [3, 4], in human and animal liver biopsies [5–8], in freshly isolated and in cultured fetal hepatocytes [9, 10], and in hepatoma cells in culture [11].

Two cytochrome P-450 species have been shown

* To whom all correspondence should be addressed.

previously as catalysts of aldrin epoxidation in rat liver: a major form inducible by PB* [12, 13] and a form inducible by pretreatment with PB or PCN [14]. A cytochrome P-448 species isolated from MC-treated rats exhibited only a marginal activity [13]. However, microsomal aldrin epoxidation cannot be attributed solely to the activity of those two isozymes. Male rats, for example, show an epoxidase activity much higher than expected from the relatively low abundance of PCN and PB inducible isozymes [12, 15] suggesting the existence of further aldrin epoxidizing P-450 isozymes. Therefore, we have undertaken a comprehensive study to find out which major cytochrome P-450 species purified from untreated and from rats pretreated with a number of commonly applied inducers [15] possess aldrin epoxidase activity.

Turnover numbers determined in reconstituted monooxygenase systems, however, may not necessarily represent the enzymatic activity of the corresponding cytochrome P-450 species in microsomes. As compared to reconstituted monooxygenase systems, microsomes usually exhibit a much lower ratio of NADPH-cytochrome P-450 reductase to cytochrome P-450. Furthermore, it is possible that a particular cytochrome P-450 isozyme in microsomes interferes with the enzymatic activity of another isozyme. Recent observations on warfarin hydroxylation at various positions of the molecule showed that the activity of a single isozyme could be depressed in presence of another isozyme [16]. As a consequence, rates of warfarin hydroxylation in microsomes were much lower than those calculated from turnover numbers and from the relative portion of individual isozymes.

To find out whether this was also true for aldrin epoxidation we have assayed specific epoxidase activities of various microsomal preparations of which the isozyme pattern had been determined immunochemically [15, 20]. Comparison of these activity values with activities calculated from turnover numbers was used to estimate whether a mutual interaction of P-450 isozymes had occurred.

MATERIALS AND METHODS

Chemicals. Enzymes and cofactors were purchased from Boehringer (Mannheim, F.R.G.). Aldrin and dieldrin, 99% purity, 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 (U.S.A.) ("HPLC grade"). Other organic solvents, analytical grade, were from Merck (Darmstadt, F.R.G.). PB (sodium salt) and MC were purchased from Fluka (Neu-Ulm, F.R.G.); the commercial PCB mixtures, Aroclor 1254 and Clophen A-50, were from Analabs, (New Haven, CT), and from Bayer-Leverkusen (F.R.G.), respectively; PCN was a generous gift of Dr. Schulte-Hermann (University of Marburg, F.R.G.), and DEX was from Sigma Chemie (München, F.R.G.).

Pretreatment with inducers and preparation of microsomes. Wistar and Sprague–Dawley rats were obtained from the animal breeding station of the Gesellschaft für Strahlen- und Umweltforschung (Neuherberg, F.R.G.). For pretreatment with PB, animals (180–220 g) were treated by 5 single i.p. injections of 80 mg/kg PB once a day (Sprague–Dawley) or received a single i.p. injection of 80 mg/kg in saline and subsequently 0.1% in their drinking water for one week (Wistar). MC in olive oil was administered by gavage at doses of 20 mg/kg, 48 and 24 hr prior to killing. BNF in olive oil was given i.p. once a day for 3 days at doses of 40 mg/kg and animals killed 24 hr after the last dose. Aroclor 1254 in olive oil was given to males in a single i.p. dose of 300 mg/kg 3 days prior to sacrifice. Clophen A-50 in olive oil was administered to male Wistar rats in a single oral dose of 500 mg/kg and the animals killed 4 days later. PCN and DEX were suspended in 1% Tween 80 and were administered by gavage to females in single doses of 50 mg/kg on 4 consecutive days. Males were treated with single doses of 50 mg DEX/kg on 3 consecutive days and killed 16 hr after the last dose.

Cytochrome P-450 isozymes were purified from liver microsomes of Sprague–Dawley rats obtained from Harlan Industries (Indianapolis, IN) and treated as described elsewhere [15]. 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 [18].

Purification of cytochromes P-450. Enzymes were purified and antibodies were prepared according to procedures described elsewhere [15, 19, 20]. The nomenclature and its rationale for the cytochrome P-450 isozymes are presented elsewhere [15, 19, 20]. Other preparations in the literature that appear to correspond to the more significant ones dealt with in this report include the following: P-450$_{UT-A}$ [15]: male-specific P-450 [21], PB 2c [20], RLM 5 [22], P-450 h [23]; P-450$_{PCN-E}$ [15]: P-450$_{PCN}$ [24], PB 2a [20].

Incubation procedures. The standard incubation mixture was prepared in small test tubes on ice and contained in 100 μl of 0.1 M phosphate buffer, pH 7.5: 2.5 to 25.0 pmol of purified cytochrome P-450, 10–100 pmol NADPH cytochrome P-450 reductase, 2 μg L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, 2 nmol aldrin (in 1 μl methanol), and an NADPH-generating system consisting of 1 μmol glucose-6-phosphate, 0.01 μmol NADP and 0.1 IU glucose-6-phosphate dehydrogenase/ml. 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

* Abbreviations: BNF: β-naphthoflavone = 5,6-benzoflavone; DDE: 2,2-bis-(4-chlorophenyl)-1,1-dichloroethylene; DEX: dexamethasone; ISF: isosafrole; MC: 3-methylcholanthrene; PB: phenobarbital; PCB: commercial mixture of polychlorinated biphenyls; PCN: pregnenolone-16α-carbonitrile.

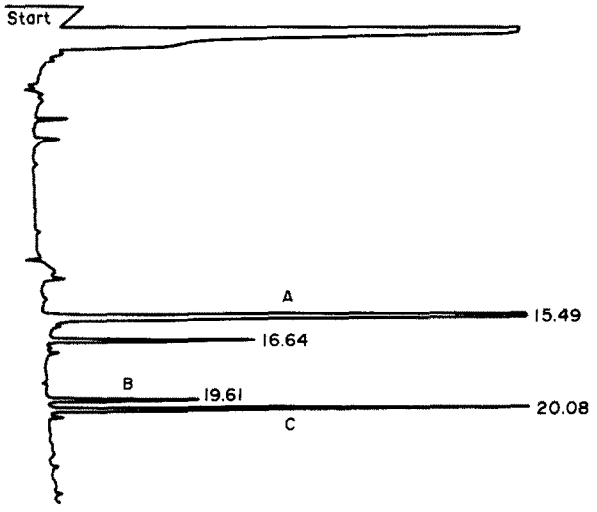

Fig. 1. Capillary gas chromatogram of a hexane extract from incubations containing P-450$_{UT-F}$. A = aldrin, B = dieldrin, C = novel metabolite (*endo*-dieldrin).

was vigorously extraced with 1 ml HPLC-grade n-hexane. After removing the aqueous phase the hexane extract was ready for analysis.

Incubations with microsomes were carried out as described elsewhere [12].

Immunoinhibition of microsomal aldrin epoxidation. Enzyme activity in liver microsomes prepared from untreated male Sprague–Dawley rats was assayed in the presence of polyclonal antibodies raised in rabbits to rat cytochrome P-450$_{UT-A}$ [15]. The incubation mixture contained in a final volume of 0.1 ml of 0.1 mM potassium phosphate buffer, pH 7.5: 0.1 μM microsomal cytochrome P-450, 50 μM aldrin (added from a 2 mM stock solution in methanol), various amounts of the antibody preparations, and cofactor concentrations as described before. Buffered suspensions of microsomes were preincubated with the antisera for 15 min at room temperature to facilitate formation of the antigen–antibody complex. The mixture was transferred to an ice bath. After addition of aldrin and the cofactor mixture the samples were incubated and further processed as described before.

Gas chromatography. Dieldrin was separated and quantified by electron-capture gas chromatography either on a capillary or on a packed column. Capillary gas chromatography was performed on a Model 3700 Varian gas chromatograph, equipped with a DB 210 column of 30 m length and 0.25 mm diameter. The oven temperature was 192°, and the injector and detector were heated to 270 and 400°, respectively. Helium was used as carrier gas at a pressure of 2 atm. DDE served as the internal standard. Under these conditions, the respective retention times of aldrin, DDE, and dieldrin were 2, 3 and 6 min. Hexane extracts were also analyzed on glass columns (6 ft length and 2 mm diameter) packed with 3% XE-60 on Chromosorb WAW DMCS (80–100 mesh). The column temperature was 200° and the injector and detector temperature was 250°. The flow rate of the carrier gas, argon/methane (90:10), was 30 ml/min.

Identification of endo-*dieldrin.* When hexane extracts from incubates containing cytochrome P-450$_{UT-F}$ were chromatographed on packed XE-60 columns, a double peak was observed consisting of dieldrin and a novel compound. Both compounds were separated on a DB-5 capillary column of 30 m length and 0.32 mm diameter. The oven temperature was programmed between 130 and 235° at a rate of 5°/min. Under these conditions, the retention times were 19.7 min and 20.1 min for dieldrin and the other compound, respectively (Fig. 1). The mass spectra of both compounds were very similar with respect to the peak pattern and the molecular ion which was m/e 362 (Fig. 2). The identification of the compound as *endo*-dieldrin was based on the GC-retention time and the mass spectrum which both showed identity to the corresponding data of an authentic sample prepared from aldrin [17]. Endrin, the other isomer of dieldrin (Fig. 3), showed a mass spectrum differing entirely from that of dieldrin and of *endo*-dieldrin.

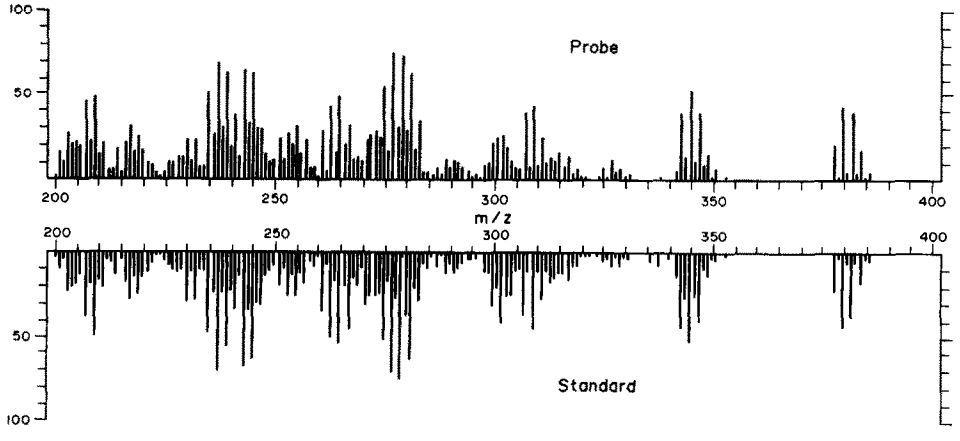

Fig. 2. Mass spectra of a novel metabolite formed by cytochrome P-450$_{UT-F}$ (upper part), and of an authentic sample of *endo*-dieldrin (lower part), prepared according to [17].

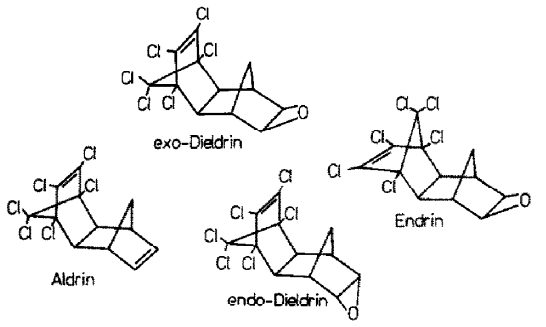

Fig. 3. Structures of some halogenated cyclodienes.

RESULTS

Aldrin epoxidase activity of various cytochrome P-450 isozymes in reconstituted monooxygenase systems

Aldrin epoxidase activity was determined in reconstituted monooxygenase systems containing each of ten different cytochrome P-450 isozymes purified from livers of untreated and pretreated Sprague–Dawley rats. The amount of dieldrin formed in these systems linearily depended on cytochrome P-450 concentration between 33 and 133 μM hemoprotein. The turnover numbers listed in Table 1 are calculated from determinations carried out within such a range of hemoprotein concentration.

Of the cytochrome P-450 isozymes purified from untreated rats, P-450$_{UT-A}$ had the highest activity of all isozymes assayed, whereas P-450$_{UT-I}$ was poorly active. Two other isozymes present in both genders, P-450$_{UT-F}$ and P-450$_{UT-H}$, exhibited moderate epoxidase activities.

Of the forms inducible by PB, P-450$_{PB-C}$ had the highest activity, followed by P-450$_{PB-B}$, P-450$_{PCN-E}$, and P-450$_{PB-D}$ in that order. Cytochrome P-450$_{BNF-B}$, the major form induced by BNF or MC, had only marginal activity and P-450$_{ISF-G}$, isolated from microsomes of rats pretreated with ISF, was inactive.

Table 1. Aldrin epoxidase activity of various purified cytochrome P-450 species in reconstituted monooxygenase systems

Cytochrome P-450 isozyme	Enzyme activity (nmol dieldrin formed/min/nmol cytochrome P-450)
UT-A	3.1 ± 1.0
PB-B	0.68 ± 0.19
PB-C	1.63 ± 0.29
PB-D	0.21 ± 0.02
PCN-E	0.40 ± 0.11
UT-F	0.35 ± 0.11*
BNF-B	0.005 ± 0.002
UT-H	0.94, 0.69†
UT-I	0.01, 0.01‡
ISF-G	n.d.§, 0.03‡

* Activity expressed as sum of *exo*- and *endo*-dieldrin.

† Determination performed at 33 and 67 nM cytochrome P-450.

‡ Determinations performed at 100 and 250 nM cytochrome P-450.

§ No dieldrin detectable.

Data represent means (±SD) of 4 single determinations performed at 33, 67, 100, and 133 nm cytochrome P-450, 0.25 μM NADPH-cytochrome P-450 reductase, and 30 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine.

Formation of a novel aldrin metabolite by cytochrome P-450$_{UT-F}$

A novel metabolite, slightly differing in retention time from dieldrin, was detected in the hexane extracts of monooxygenase systems reconstituted from P-450$_{UT-F}$, in addition to dieldrin. The compound was identified by mass spectrometry as the *endo*-isomer of dieldrin, which itself has the *exo*-configuration (Figs 1–3). *Endo*-dieldrin was formed in a 6-fold excess over (*exo*-)dieldrin. This ratio was observed under every experimental condition used, i.e. did not change under varying concentrations of cytochrome P-450 and NADPH-cytochrome *c* reductase.

Significance of turnover numbers determined in reconstituted monooxygenase systems for microsomal aldrin epoxidase activity

Experimental versus calculated activity in animals of different sex and age

Microsomal aldrin epoxidase activity in untreated Sprague–Dawley rats varied depending on animal sex (Table 2). The activity of males was more than 3-fold higher than of females. The sex dependence, however, was only expressed in adults; at the age of 3 weeks both genders had similar activities. Corresponding activities in Wistar rats were closely similar, with the exception that the enzyme of adult males was 50% more active compared to adult males of the Sprague–Dawley strain.

Aldrin epoxidase activities calculated from the data in Table 1 and published data on the relative abundance of P-450 isozymes [20] agreed well with microsomal activities determined in both male and female weanlings and adult male rats.

Experimental versus calculated values of enzyme activity in rats pretreated with inducers

Sprague–Dawley strain. PB was a strong inducer of aldrin epoxidase in females, but a moderate inducer in males. The enzyme was also induced in males pretreated with the PCB mixture, Aroclor 1254. Pretreatment with BNF, however, depressed the activity of males by 50%. Repeated administration of the glucocorticoid hormone, DEX, did not alter enzyme activity in males but strongly induced it in females (Table 3).

To compensate for the variation between control activities, the extent of alteration of enzyme activity was expressed as ratio of the activity of animals pretreated with inducers versus the activity of control animals. Similarly, a "theoretical" magnitude of alteration of enzyme activity was calculated from the "theoretical" activities of Sprague–Dawley rats pretreated with inducers and the corresponding values calculated for untreated controls, as listed in Table 2.

Table 3 shows that theoretical activity ratios in Sprague–Dawley males were consistent with the

Table 2. Age and sex dependence of microsomal aldrin epoxidase activity

Sex	Age	N	Cytochrome P-450 (nmol/mg protein)	Enzyme activity (nmol dieldrin/min/mg protein)	Calculated "Theoretical" enzyme activity
Male	Weanling	6	0.52 ± 0.10	0.6 ± 0.1	0.5 ± 0.1
Male	Adult	15	0.85 ± 0.08	2.5 ± 1.3	1.7 ± 0.4
Female	Weanling	3	0.37 ± 0.07	0.5 ± 0.0	0.4 ± 0.1
Female	Adult	12	0.81 ± 0.14	0.5 ± 0.2	0.5 ± 0.1

Liver microsomes were prepared from 3 weeks (weanling) and from 7 weeks (adult) old Sprague–Dawley rats. The data are statistical means (±SD) of N individual preparations. "Theoretical" enzyme activities for microsomal aldrin epoxidase were calculated from the turnover number of each cytochrome P-450 isozyme contributing to the entire microsomal activity listed in Table 1. Data on the abundance of these isozymes in microsomes were taken from the paper of Waxman *et al.* [20]. The corresponding figures were: $P\text{-}450_{UT\text{-}A}$: 0.01 and 0.34, $P\text{-}450_{UT\text{-}F}$: 0.16 and 0.15, $P\text{-}450_{PB\text{-}B}$ + $P\text{-}450_{PB\text{-}D}$: 0.07 and 0.02, $P\text{-}450_{PB\text{-}C}$: 0.18 and 0.19, $P\text{-}450_{PCN\text{-}E}$: 0.2 and 0.19 nmol cytochrome P-450/mg of microsomal protein for weanling and adult males, respectively. The corresponding figures for females were: $P\text{-}450_{UT\text{-}A}$: 0.02 and 0.02, $P\text{-}450_{UT\text{-}F}$: 0.21 and 0.15, $P\text{-}450_{PB\text{-}B}$ + $P\text{-}450_{PB\text{-}D}$: 0.03 and 0.03, $P\text{-}450_{PB\text{-}C}$ 0.15 and 0.25, $P\text{-}450_{PCN\text{-}E}$: 0.07 and 0.03.

ratios derived from experimentally-determined microsomal activities. Some variation was noted between theoretical and experimental data of animals pretreated with BNF and PCB. The alteration of enzyme activity following pretreatment with DEX is comparable to the activity change calculated for induction with PCN, since DEX induces the same major cytochrome P-450 isozyme as does PCN [24].

Wistar strain. In general, Wistar rats responded to pretreatment with inducers in a similar fashion as observed with Sprague–Dawley rats, except for pretreatment with PCB which, contrary to Sprague–Dawley rats, did not induce epoxidase activity, although the level of cytochrome P-450 was markedly augmented. MC, like BNF in Sprague–Dawley males, depressed epoxidase activity in Wistar males by 50%.

Theoretical activity ratios calculated for Sprague–

Table 3. Effect of various inducers on microsomal aldrin epoxidase activity of Sprague–Dawley rats

Inducing compound	Sex	Magnitude of alteration of: Cytochrome P-450 content	Enzyme activity (Controls = 1)	"Theoretical" enzyme activity
PB	Male	2.7 ± 0.2	1.8 ± 0.2	2.1 ± 0.5
	Female	1.9 ± 0.1	12.0 ± 0.4	3.8 ± 0.8
B-NF	Male	2.0 ± 0.1	0.4 ± 0.1	0.7 ± 0.2
PCB	Male	2.8 ± 0.3	2.0 ± 0.1	1.4 ± 0.4
DEX	Male	1.6 ± 0.1	0.9 ± 0.1	—
	Female	1.6 ± 0.3	5.5 ± 1.0	—
PCN	Male	—	—	0.9 ± 0.2
	Female	—	—	1.6 ± 0.3

The relative increase of "theoretical" enzyme activity is expressed as the ratio of enzyme activity calculated for inducer-pretreated animals to the enzyme activity calculated for untreated controls. Data on isozyme concentrations in microsomes of inducer-treated Sprague–Dawley rats were taken from references [20] and [15]. The corresponding data for males pretreated with PB, PCB, B-NF and PCN were: $P\text{-}450_{UT\text{-}A}$: 0.30, 0.27, 0.19 and 0.19, $P\text{-}450_{UT\text{-}F}$: 0.22, 0.15, 0.15 and 0.06, $P\text{-}450_{PB\text{-}B}$ + $P\text{-}450_{PB\text{-}D}$: 1.50, 1.29, 0.07 and 0.22, $P\text{-}450_{PB\text{-}C}$: 0.88, 0.36, 0.23 and 0.20, $P\text{-}450_{PCN\text{-}E}$: 0.66, 0.77, 0.11 and 1.07 nmol P-450/mg of microsomal protein, respectively. The corresponding values for PCN- and PB-treated females were: $P\text{-}450_{UT\text{-}A}$: 0.02 for both inducers, $P\text{-}450_{UT\text{-}F}$: 0.12 and 0.30, $P\text{-}450_{PB\text{-}B}$ + $P\text{-}450_{PB\text{-}D}$: 0.23 and 1.07, $P\text{-}450_{PB\text{-}C}$: 0.23 and 0.77, $P\text{-}450_{PCN\text{-}E}$: 0.70 and 0.19. Data on isozyme concentrations in microsomes from untreated Sprague–Dawley rats were taken from reference [20], as given in the legend of Table 2.

The data on the relative alteration of cytochrome content and enzyme activity are the means ± SD of microsomal preparations from 4 individuals. The mean variations listed under "theoretical" enzyme activity result from the variations of the individual isozyme activities as listed in Table 1.

Data on the abundance of isozyme $P\text{-}450_{UT\text{-}H}$ were not available. It is estimated that the contribution of this isozyme to the data listed in the column "theoretical" enzyme activity is below 0.1.

Table 4. Effect of various inducers on the activity of microsomal aldrin epoxidase in Wistar rats

Inducing compound	Sex	N	Magnitude of alteration of: Cytochrome P-450 content	Enzyme activity (Controls = 1)	"Theoretical" enzyme activity*
PB	Male	11	2.2 ± 0.2	2.3 ± 0.5	2.1 ± 0.5
MC	Male	11	1.5 ± 0.1	0.5 ± 0.1	—
Aroclor 1254	Male	4	2.6 ± 0.2	0.9 ± 0.1	—
Clophen A-50	Male	9	2.8 ± 0.5	1.1 ± 0.1	1.4 ± 0.4
DEX	Male	6	1.4 ± 0.2	0.6 ± 0.1	—
	Female	6	1.7 ± 0.1	4.2 ± 0.2	—
PCN	Male	—	—	—	0.9 ± 0.2
	Female	6	1.7 ± 0.2	4.1 ± 0.6	1.6 ± 0.3

* Values represent the means ±SD of "theoretical" enzyme activities calculated for Sprague–Dawley rats and were taken from the corresponding column in Table 3. Other data are means ±SD of microsomal preparations from N individuals.

Aldrin epoxidase activity in microsomes of untreated Wistar males and females was: 3.1 ± 1.1 and 0.62 ± 0.08 nmol dieldrin formed/mg protein/min, respectively; cytochrome P-450 content: 0.80 ± 0.15 and 0.51 ± 0.11 nmol/mg protein, respectively.

Dawley males also corresponded to ratios of microsomal activity determined with Wistar males. The results obtained with MC pretreated animals may be compared to the values calculated for BNF treatment, since MC closely resembles BNF as inducer [15]. Variations of experimental and theoretical values were also noted for animals pretreated with PCB and DEX.

Females of both strains pretreated with PB and glucocorticoids, however, exhibited a several-fold larger increase of epoxidase activity than was predicted (Tables 3 and 4).

Immunoinhibition of cytochrome P-450$_{UT-A}$ dependent aldrin epoxidase activity

Anti P-450$_{UT-A}$ strongly inhibited aldrin epoxidase activity in a monooxygenase system reconstituted from P-450$_{UT-A}$ (Fig. 4). The antibody inhibited microsomal aldrin epoxidase activity of untreated male Sprague–Dawley rats by 60% and of PB-treated males by 30%.

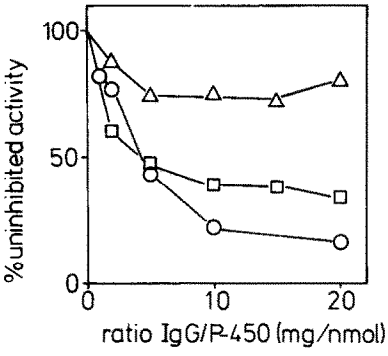

Fig. 4. Inhibitory effect of anti-P-450$_{UT-A}$ on aldrin epoxidase activity assayed in a reconstituted monooxygenase system containing P-450$_{UT-A}$ (○——○), in microsomes from untreated adult male rats (□——□), and in microsomes of PB-treated male rats (△——△). The curves represent the means of duplicate determinations in the reconstituted system and of 3 individual microsomal preparations. Details are given in the Experimental Section.

DISCUSSION

The results of the present study provide a quite comprehensive answer to our question of which major cytochrome P-450 isozymes in rat liver mediate aldrin epoxidation. In addition to the two forms previously shown as catalysts of this reaction [12–14], we detected epoxidase activity with five other forms purified from untreated and inducer-treated rats.

A novel and interesting finding is the high epoxidase activity determined with cytochrome P-450$_{UT-A}$, an isozyme characteristic for the male rat [20]. Since the female specific form, P-450$_{UT-I}$ [20], was almost inactive, and other isozymes present in both genders exhibited moderate activities, it is plausible that aldrin epoxidase activity of males is much higher than the activity of females. Further evidence, indicating a predominant role of isozyme P-450$_{UT-A}$ for epoxidase activity of untreated males, is provided by the immunotitration data.

Cytochrome P-450$_{UT-F}$ showed unique catalytic properties, since it converted aldrin into a novel metabolite, *endo*-dieldrin, in excess over (*exo*-)dieldrin, the metabolite generally observed as the result of aldrin epoxidation. This finding opens a new aspect concerning the molecular mechanism of aldrin epoxidation. Possibly, the geometry of the active site of cytochrome P-450$_{UT-A}$ is modified in the way that the substrate molecule (see Fig. 3) can be bound either with the methylene bridge of the cyclohexene ring adjacent or distant to the heme iron–oxo complex. These orientations may lead to formation of the two isomeric epoxides observed. Another explanation would be that the preparation consists of two isozymes closely similar in their immunochemical and physicochemical properties, but differing in their preference for one of the two orientations of the substrate molecule. At present it is difficult to discriminate precisely which of the two interpretations is the more likely one. In terms of

the criteria generally used to prove for homogeneity of purified proteins, the P-450$_{UT-F}$ preparation appears to be homogeneous.

Regarding isozymes susceptible to induction by commonly-used inducers, this work also confirms previous observations indicating that aldrin epoxidase is characteristic for PB-type inducers and is not related to activities expressed by cytochrome P-448. Of the four isozymes purified from PB-treated animals, cytochrome P-450$_{PB-C}$ and P-450$_{PB-D}$ were detected as novel isozymes catalyzing aldrin epoxidation. The two other isozymes assayed correspond to species already known as catalysts of this reaction. Isozyme P-450$_{PCN-E}$, is identical or closely similar to cytochrome "P-450$_{PCN}$" purified by Heuman *et al.* from the liver of PCN or DEX treated rats [26]. Indirect evidence that this form mediates aldrin epoxidation was based on the inducing effect of PCN and DEX and on the inhibitory effect of polyclonal antibodies raised toward the purified isozyme [14]. Isozyme P-450$_{PB-B}$ is closely similar to the main P-450 species purified from PB-pretreated rats by West *et al.* [25] exhibiting high aldrin epoxidase activity [13]. Regarding spectral and catalytic properties, isozyme P-450$_{BNF-B}$, the main form in BNF-treated animals is closely related or identical to cytochrome "P-448" purified previously from MC-treated rats [25]. Both preparations were almost inactive as catalysts of aldrin epoxidation. Cytochrome P-450$_{ISF-G}$, another isozyme related to cytochrome P-450$_{BNF-B}$, in terms of spectral and some enzymatic properties [15], also did not catalyze aldrin epoxidation.

The second aspect of this study is directed to the question of whether the turnover numbers determined for aldrin are a proper measure for the enzymatic activity of the corresponding isozymes of microsomes, i.e. whether or not isozymes mutually interfere, when aldrin is the substrate. In case of a mutual interaction, the "theoretical" microsomal activity calculated from the relative portions and turnover numbers of all epoxidizing isozymes should considerably deflect from the activity determined.

Our finding that theoretical epoxidase activities in untreated rats corresponded to activities determined suggests that no major reaction had occurred between single isozymes. This conclusion also pertains to male rats pretreated with inducers. With some minor variations, ratios of theoretical microsomal activity of pretreated versus control animals corresponded to the respective ratios of measured activity. The variations observed with PCB- and BNF-pretreated animals may be due to the circumstance that animals and experimental conditions used for immunochemical quantitation of isozymes slightly differed from those used for determination of microsomal epoxidase activities. In this context, it is interesting to note that the activity ratios determined in Wistar rats also corresponded to the ratios of theoretical activity calculated for Sprague–Dawley rats. This indicates that strain was not a major determinant for the accordance observed between predicted and experimental values.

However, the finding that in hormone- and PB-treated females the ratios of theoretical activity were considerably lower than the ratios of measured activity indeed suggests a mutual interaction of P-450 isozymes. Possibly, a relative abundant but inactive female-specific isozyme, such as P-450$_{UT-I}$, enhances the activity of other epoxidizing isozymes, such as P-450$_{PB-B}$, P-450$_{PB-C}$, P-450$_{PB-D}$, and P-450$_{PCN-E}$ by a yet unknown activation mechanism.

The present data, however, do not provide final evidence whether single isozymes do or do not interfere with each other in their microsomal environment when aldrin is the substrate. Further work using reconstituted monooxygenase systems is required to probe for mutual interactions of aldrin epoxidizing P-450 isozymes.

In conclusion, the aldrin epoxidase assay provides a comprehensive method of detecting the enzymatic activity of a variety of individual cytochrome P-450 species, characteristic for the male and the PB-treated rat. The induction of aldrin epoxidase in PCN-treated females, recently claimed to indicate a novel aldrin epoxidizing species [14], is not specific for PCN treatment, since induction of this form was also observed after pretreatment with PB [15, 26]. This is a need to further discriminate between individual isozymes. Methods to differentiate various aldrin epoxidizing species will be discussed elsewhere [28].

Acknowledgements—The expert technical help of Ms. Heide Wanders is gratefully acknolwedged. Ms. Linde Lattmann and Dr. Clausen are thanked for recording mass spectra and Dr. E. Lahaniatis for help in capillary gas chromatography of *endo*-dieldrin. This research was supported in part by USPHS grants ES 01590 and ES 00267. F.P.G. is a Burroughs Wellcome Scholar in Toxicology (1983–1988).

REFERENCES

1. A. Y. H. Lu and S. B. West, *Pharmac. Rev.* **31**, 277 (1980).
2. F. P. Guengerich and D. C. Liebler, *CRC Crit. Rev. Toxicol.* **14**, 259 (1985).
3. J. Van Cantfort, M. Leonard-Poma, J. Séle-Doyen and J. E. Gielen, *Biochem. Pharmac.* **32**, 2697 (1983).
4. C. P. Siegers, S. Schümann, E. Thies, H. Böse-Younes and M. Younes, *Cancer Lett.* **23**, 39 (1984).
5. T. Wolff, in *Fine Needle Aspiration Biopsy of the Liver* (Ed. G. Zbinden) p. 49. Pergamon Press, Oxford (1980).
6. F. M. Williams, K. W. Woodhouse, D. Middleton, P. Wright, O. James and M. D. Rawlins, *Biochem. Pharmac.* **31**, 3701 (1982).
7. M. McManus, A. R. Boobis, R. F. Minchin, D. M. Schwartz, S. Murray, D. S. Davies and S. S. Thorgeirsson, *Cancer Res.* **44**, 5692 (1985).
8. T. Wolff and M. Strecker, *Biochem. Pharmac.* **34**, 2593 (1985).
9. L. R. Schwarz, R. Götz, T. Wolff and F. J. Wiebel, *FEBS Lett.* **98**, 203 (1979).
10. P. Kremers, F. Letawe-Goujon, J. De Greave, J. Duvivier and J. E. Gielen, *Eur. J. Biochem.* **137**, 603 (1983).
11. F. J. Wiebel, T. Wolff and M. Lambiotte, *Biochem. biophys. Res. Commun.* **94**, 466 (1980).
12. T. Wolff, E. Deml and H. Wanders, *Drug Metab. Disp.* **7**, 301 (1979).
13. T. Wolff, H. Greim, M. T. Huang, G. T. Miwa and A. Y. H. Lu, *Eur. J. Biochem.* **111**, 545 (1980).
14. S. L. Newman and P. S. Guzelian, *Biochem. Pharmac.* **32**, 1529 (1983).
15. F. P. Guengerich, G. A. Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry* **21**, 6019 (1982).

16. L. S. Kaminsky and F. P. Guengerich, *Eur. J. Biochem.* **149**, 479 (1985).
17. S. Nitz, H. Parlar and F. Korte, *Chemosphere* **2**, 83 (1974).
18. T. Wolff and S. Hesse, *Biochem. Pharmac.* **26**, 783 (1977).
19. D. Larrey, L. M. Distlerath, G. A. Dannan, G. R. Wilkinson and F. P. Guengerich, *Biochemistry* **23**, 2787 (1984).
20. D. J. Waxman, G. A. Dannan and F. P. Guengerich, *Biochemistry* **24**, 4409 (1985).
21. T. Kamataki, K. Maeda, Y. Yamazoe, T. Nagai and R. Kato, *Archs Biochem. Biophys.* **225**, 758 (1983).
22. K. C. Cheng and J. B. Schenkman, *Drug Metab. Disp.* **12**, 222 (1984).
23. D. E. Ryan, S. Lida, A. W. Wood, P. E. Thomas, C. S Lieber and W. Levin *J. biol. Chem.* **259**, 1239 (1984).
24. N. A. Elshourbagy and P. S. Guzelian, *J. biol. Chem* **255**, 1279 (1980).
25. S. B. West, M. T. Huang, G. T. Miwa and A. Y. H. Lu *Archs Biochem. Biophys.* **193**, 42 (1979).
26. D. M. Heuman, E. J. Gallagher, J. L. Barwick, N. A Elshourbagy and P. S. Guzelian, *Molec. Pharmac.* **21** 753 (1982).
27. K. Maeda, T. Kamataki, T. Nagai and R. Kato *Biochem. Pharmac.* **33**, 509 (1984).
28. T. Wolff and F. P. Guengerich, in preparation.