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LACK OF RELATIONSHIP BETWEEN DEBRISOQUINE 4-HYDROXYLATION AND OTHER CYTOCHROME P-450 DEPENDENT REACTIONS IN RAT AND HUMAN LIVER

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Abstract—The effect of various inhibitors and inducers of cytochrome P-450 on the activity of microsomal debrisoquine 4-hydroxylation (DQH) was investigated in rat liver. DQH was strongly inhibited by SKF 525-A, 7,8-benzoflavone, and metyrapone. Pretreatment of animals with common inducers, such as phenobarbital, 3-methylcholanthrene, the commercial PCB mixture, Clophen A-50, dexamethasone and pregenenolone-16α-carbonitrile did not lead to induction of DQH, while most reference reactions, i.e. aldrin epoxidation, ethylmorphine demethylation, and benzo(a)-pyrene hydroxylation were induced under these conditions. DQH likewise was not induced by pretreatment of animals with the enzyme substrate DQ. The relationship between DQH and other cytochrome P-450 functions was also studied in untreated animals. Both genders of Wistar rats exhibited similar rates of DQH, but different activities of the reference reactions. DQH activity in Wistar females however, was 10 times higher than in females of the DA-strain, whereas the reference activities were similar in both strains. The studies were also extended to human liver. DQH activity in homogenates of various biopsy samples was neither correlated to the activity of aldrin epoxidation nor to AHH activity. The results indicate that DQH in the rat and in man reflects the activity of a cytochrome P-450 species not related to various other known cytochrome P-450 functions.

Various exogeneous factors and endogeneous determinants are known to influence the activity of cytochrome P-450-dependent monooxygenases with the consequence of large variations in the oxidation rate of drugs and foreign chemicals. Among endogeneous determinants, the polymorphism of drug hydroxylation has gained increasing interest, since it comprises the deficiency of a sizable portion of the population to perform particular oxidative detoxification reactions (for review see [1]).

The genetically controlled 4-hydroxylation of the anti-hypertensive agent, debrisoquine, has been subject of many investigations [2, 3]. This reaction was recently shown to be mediated by cytochrome P-450 [4, 5]. Numerous investigations have been carried out on genetic and ethnic factors regulating the expression of DQH† in man; however, there is little information whether this reaction is also susceptible to the effect of exogeneous factors, like inhibitors or inducers of cytochrome P-450. Furthermore, little is known on the relationship of cytochrome P-450 species mediating DQH to other well characterized species or families of cytochrome P-450. More information is needed of whether a defect in drug oxidation also concerns the oxidation of other potential toxicants and carcinogens, in order to estimate the risk of exposure to chemicals of the poor metabolizer phenotype.

Therefore, we have studied the relationship between DQH and other monooxygenase reactions in rat and human liver. The effect on DQH activity of monooxygenase inhibitors and inducers was examined in the rat. For induction studies, animals were pretreated with inducers of the major groups or forms of cytochrome P-450, namely PB, MC, PCBs and the hormone analogues, PCN and DEX, to assure the induction of a broad pattern of monooxygenase reactions. To probe for a correlation between the activity of DQH and other monooxygenase activities in untreated animals we took advantage of the known sex dependent variation of monooxygenase activities [6] and of the strain dependent differences of DQH activity [7]. In particular, females of the DA strain were included, which have been proposed as an animal model for the human phenotype of the poor metabolizer [7]. To evaluate the significance of these animal studies for man variations of DQH activity in relation to other monooxygenase activities were analyzed in homogenates of human liver biopsies.

MATERIALS AND METHODS

Chemicals. Debrisoquine, (3,4-dihydro-2-[1H]isoquinoline-carboxamidine sulfate) and 4-hydroxydebrisoquine were gifts of Hoffman–La Roche (Basle, Switzerland). Sodium phenobarbital and 3-

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† Abbreviations used: AHH, aryl hydrocarbon hydroxylase; 7,8-BF, 7,8-benzoflavone; DEX, dexamethasone; PCN, pregnenolone-16α-carbonitrile; DQ, debrisoquine; 4-OH-DQ, 4-hydroxydebrisoquine; DQH, debrisoquine 4-hydroxylation; MC, 3-methylcholanthrene; PB, phenobarbital; PCBs, commercial mixture of polychlorinated biphenyls.

methylcholanthrene were purchased from Fluka (Neu-Ulm, FRG), Clophen A-50, a commercial PCB mixture with an average chlorine content of 50%, from Bayer (Leverkusen, FRG), 16α-cyanopregnenolone was a gift of Dr. Schulte Hermann, University of Marburg, FRG, dexamethasone was bought from Sigma Chemie (München, FRG), metyrapone and 7,8-benzoflavone from Ega-Chemie (Steinheim, FRG), SKF 525-A from Smith, Kline and Dauelsberg (München, FRG), hexafluoroacetylacetone and toluene, analytical grade, were obtained from Baker Chemicals (Deventer, Netherlands) and were distilled before use. Enzymes and coenzymes were purchased from Boehringer (Mannheim, FRG). All other chemicals were obtained from Merck (Darmstadt, FRG) and were of analytical grade.

Pretreatment with inducers and preparation of microsomes. Wistar rats were obtained from our own breeding station and rats of the DA strain from the Zentralinstitut für Versuchstierzucht (Hannover, FRG). For pretreatment with PB, male Wistar rats (150–200 g) received a single i.p. injection of 80 mg/kg b.w. in saline, and subsequently 0.1% PB in their drinking water for 1 week. MC and Clophen A-50 dissolved in 0.4 ml olive oil, were administered by gavage to male Wister rats at doses of 20 mg/kg b.w. and 100 mg/kg b.w., respectively, 48 and 24 hr prior to killing. PCN and DEX were suspended in 1% Tween 80 and were administered by gavage to female Wistar rats in single doses of 50 mg/kg b.w. on 4 consecutive days. Animals were killed 24 hr after the last dosage. Along with each group of chemical pretreated animals, groups of control animals were pretreated with the corresponding vehicle.

For induction studies with DQ, relatively high doses were administered in order to achieve sufficiently high cellular levels of DQ required for induction [8]. Male Wister rats were either pretreated, similar to the regimen described for PB, by a single i.p. injection of 80 mg/kg b.w. and subsequently free access to 0.1% DQ in their drinking water for 1 week or received single oral doses of 100 mg/kg b.w. DQ on five consecutive days and were killed 24 hr after the last application.

Preparation of liver microsomes and determination of protein by the biuret method were performed as previously described [9]. Microsomes were used immediately upon preparation or a few days after storage at −80°. Enzyme activities did not significantly change during this period.

DQH-assay. The standard reaction mixtures were prepared in 1 ml "reactivials" (Wheaton) on ice and contained in 200 μl: 16 μmoles of potassium phosphate buffer (pH 7.4), 1.6 μmoles of glucose-6-phosphate, 0.3 units of glucose-6-phosphate dehydrogenase, 0.2 μmoles of $NADP^+$, 0.2 mg of microsomal protein and 200 nmoles of DQ. Blanks contained all components except $NADP^+$. Standards were obtained by adding known quantities of 4-OH-DQ to these incubation mixtures without subsequent incubation. The reaction was started by placing the vials into a waterbath shaker at 37° and terminated after 20 min of incubation by replacing the samples into an ice bath and immediate addition of 50 μl of saturated $NaHCO_3$. The amidino group of substrate and metabolite was derivatized according to Malcolm and Marten by addition of 500 μl of 5% hexafluoroacetylacetone solution in toluene and subsequent heating to 100° for 2 hr [10]. 50 μl of 3 N NaOH were added at room temperature and the samples vigorously shaken for 5 min. Following centrifugation at 4000 rpm, 5 μl aliquots of the toluene phase were used for gas-chromatographic analysis without further purification.

The amount of 4-OH-DQ formed by the standard incubation mixture containing 1 mg of microsomal protein/ml from untreated animals, the cofactors and 1 mM DQ linearly increased during an incubation period of 30 min. Assays were performed within 20 min. 4-OH-DQ was not detectable, when NADPH or microsomal protein were omitted from the incubation mixture. A linear relationship between the amount of 4-OH-DQ formed and the concentration of microsomal protein was observed between 0.1 and 2.0 mg of protein/ml incubate.

Gaschromatographic analysis. Instead of the GC-MS device, as described by Erdmansky and Goehl [11], we have used a Hewlett–Packard gas chromatograph, model 5710 A, equipped with a ^{63}Ni electron capture detector to quantify 4-OH-DQ. The instrument was fitted with a glass column of 1.8 m length and 2 mm diameter, packed with 3% OV-17 on Chromosorb W (AW-DMCS), 80–100 mesh (Applied Science). Argon-methane (90:10) was used as carrier gas at a flow rate of 40 ml/min. Injection port and detector were kept at 250° and the oven was heated to 190°. 4-OH-DQ was quantified on the base of peak height determination. External standard samples of 50, 100 and 200 ng 4-OH-DQ were run during each experiment.

Other monooxygenase assays. The activity of aldrin epoxidase was determined by electron capture gaschromatography according to Wolff *et al.* [12]. *N*-Demethylation of ethylmorphine was measured by the method of Nash [13] with modifications as described by Wolff [14]. AHH activity was assayed by the fluorimetric determination of hydroxybenzo(a)pyrene according to Wiebel *et al.* [15].

Assay of DQH and other monooxygenase reactions in human liver biopsies. Needle biopsies of human liver using the Menghini technique were obtained from patients with suspected liver diseases or Morbus Hodgkin. Enzyme activities were analyzed in tissue surplus to histological requirement that was made available to us. Samples of 5–10 mg wet wt were frozen immediately after withdrawal and stored at −80°. For enzyme analysis the samples were thawed in a 10-fold volume of 0.1 M potassium phosphate buffer, pH 7.4, and homogenized in a 1 ml Potter homogenizer (Pierce "Reactiware") at 1000 Upm. The samples were assayed within a storage period of several weeks. During this period, a minor decrease of aldrin epoxidase activity of 20–30% was noticed. The storage stability of AHH activity was not monitored. A decrease by 10–40% during a storage period of 6 months was reported for human liver AHH [16, 17]. The activity of DQH did not decrease under storage conditions [4]. The protein content of the homogenates was determined according to Lowry *et al.* [18] using crystalline, dried bovine serum albumin as standard.

RESULTS

Analytical method

Figure 1 shows a typical gaschromatogram of the toluene extract from a standard incubation mixture. The retention times of DQ and 4-OH-DQ were 1.6 and 3.6 min, respectively. The difference between the retention times of 2 min was sufficient for quantitative analysis of 4-OH-DQ, even in the presence of impurities originating from the derivatization procedure. The limit of detection was better than 5 ng of 4-OH-DQ-derivative/ml toluene phase extracted from the incubation mixture.

Inhibition of DQH

As shown in Fig. 2, the monooxygenase inhibitors, metyrapone, SKF 525-A, and 7,8-benzoflavone, strongly inhibited DQH. About 10 μM SKF 525-A and 25 μM 7,8-benzoflavone decreased the rate of the reaction by 50%. At a concentration of 50 μM both compounds almost completely inhibited the reaction. Metyrapone was less effective: 200 μM lowered the rate of the reaction by 80%. Strong inhibitory effects were also seen when the incubations were carried out under a 1:4 mixture of oxygen to carbon monoxide. Enzyme activity was 16 ± 6 pmoles 4-OH-DQ/mg protein/min, compared to 260 ± 88 pmoles in control incubations, where carbon monooxide was replaced by nitrogen.

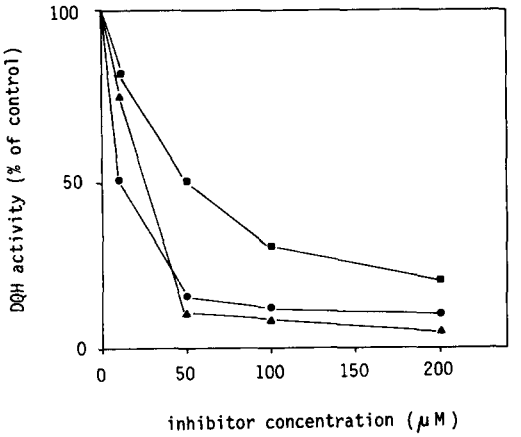

Fig. 2. Effect of monooxygenase inhibitors on DQH activity. DQH activity of control animals was determined in the presence of metyrapone ■---■, SKF 525-A ●---● and 7,8-benzoflavone ▲---▲. Substrate and protein concentration were given in the standard assay procedure. 100% of the specific activity of controls correspond to 220 pmoles 4-OH-DQ/mg protein × min. Each symbol represents the mean of three individual determinations.

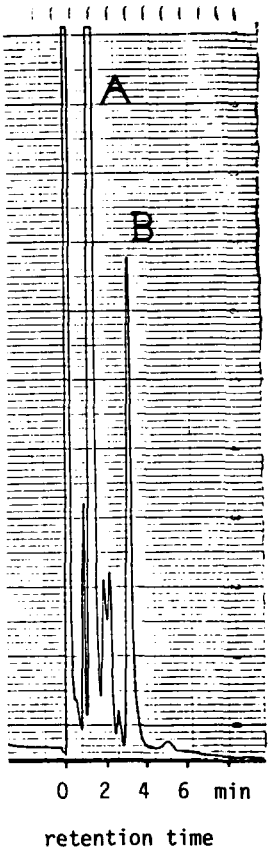

Fig. 1. Gaschromatographic trace of the toluene extract from an incubation mixture. The incubation was carried out under standard conditions, as described in Materials and Methods. Microsomes from male Wistar rats were used. A = DQ; B = 4-OH-DQ.

Effect of monooxygenase inducers

DQH activity was determined in liver microsomes of rats following pretreatment with various inducers, as shown in Table 1. None of all inducers tested increased the activity of DQH. Some inducers, such as PB and DEX, depressed the activity of this enzyme. The levels of cytochrome P-450 and the activities of aldrin epoxidase, ethylmorphine demethylase and AHH however, were substantially enhanced after every pretreatment, except for pretreatment with MC which only increased the level of cytochrome P-450 and the activity of AHH and for pretreatment with Clophen A-50 which did not induce aldrin epoxidase.

Since microsomal enzymes of differently pretreated animals may vary considerably in their substrate affinities we determined the hydroxylation rate of DQ as a function of the substrate concentration between 0.5 and 10 mM DQ. Figure 3 shows the double reciprocal plots of the data obtained from three different experimental groups of rats. The K_m values, expressed as the mean ± S.D. of three single microsomal preparations of three animals, were: 0.50 ± 0.06, 0.83 ± 0.22, and 0.62 ± 0.10 mM DQ for untreated, PB-pretreated and MC-pretreated animals, respectively. The corresponding V_{max} values were 0.55 ± 0.08, 0.22 ± 0.18, and 0.50 ± 0.07 nmoles 4-OH-DQ/mg protein × min, respectively.

Attempts were made to induce DQH by pretreatment with the enzyme substrate DQ, either given by gavage or via the drinking water (Table 1). However, both regimens led to a decrease by 50% of all enzymatic activities tested. The cytochrome P-450 level was decreased by 10–20%.

Table 1. Effect of pretreatment with inducers on the activity of DQH and other monooxygenase activities in rat liver

Inducer	Number of animals	Sex	Relative activities (% of controls)				
			Cytochrome P-450	Debrisoquine 4-hydroxylation	Aldrin epoxidation	Ethylmorphine demethylation	Benzo(a)pyrene hydroxylation
PB	(5)	♂	218 ± 16	62 ± 19	278 ± 26	226 ± 16	115 ± 8
MC	(5)	♂	164 ± 20	98 ± 18	48 ± 8	84 ± 10	510 ± 56
Clophen A-50	(2)	♂	290, 281	97, 98	200, 212	253, 273	566, 562
PCN	(6)	♀	174 ± 22	95, 111 (2)	407 ± 63	281 ± 63	274, 370 (2)
DEX	(2)	♀	137, 194	52, 55	410, 326	463, 640	320, 377
DQ (a)	(3)	♂	80 ± 11	51 ± 15	47 ± 14	52 ± 16	49 ± 8
(b)	(3)	♂	88 ± 12	56 ± 17	42 ± 9	46 ± 5	46 ± 17

Data are means ± S.D. of duplicate determinations performed with microsomes from male Wistar rats. The results are expressed as percent of control activities determined with microsomes from untreated animals. Control activities are given in Table 2. Maximal variation of duplicate determinations was ±5%. DQ was administered (a) by gavage, (b) in the drinking water, as described in Materials and Methods.

Strain and sex related differences of enzyme activities

DQH and other monooxygenase reactions were assayed in microsomes prepared from male and female Wistar rats and from females of the DA strain (Table 2). The activities of aldrin epoxidase, ethylmorphine demethylase, AHH and the total amount of cytochrome P-450 determined in microsomes of female Wistar rats were considerably lower as compared to males. By contrast, both genders shows the same rate of DQH. No relationship between DQH and the other monooxygenase activities was seen, when females of the Wistar strain were compared with females of the DA-strain. Levels of cytochrome P-450 and of the reference reactions were found similar in both strains. However, the rate of DQH in Wistar females was 10-fold higher than in DA females.

Monooxygenase activities in homogenates of human liver biopsies

To study the relationship between DQH and other monooxygenase reactions in man, human liver biopsies were used to determine the activity of DQH, aldrin epoxidase and AHH. Because of the limited amount of material available, the activities of DQH and aldrin epoxidase were determined in one group of randomly selected samples (Fig. 4a), and DQH and AHH in the other group of samples (Fig. 4b). The highest range of activity was observed for aldrin epoxidase. AHH activity was only 1% relative to the activity of DQH. Two samples showing marginal DQH activity had "normal" levels of aldrin epoxidase activity (Fig. 4a). The correlation coefficients of 0.45 and 0.27 indicate that DQH activity was neither correlated to the activity of aldrin epoxidase nor to AHH activity.

DISCUSSION

The results of the present study indicate that DQH is mediated by an enzyme that, in terms of cofactor need and effect of inhibitors, behaves like a 'classical'

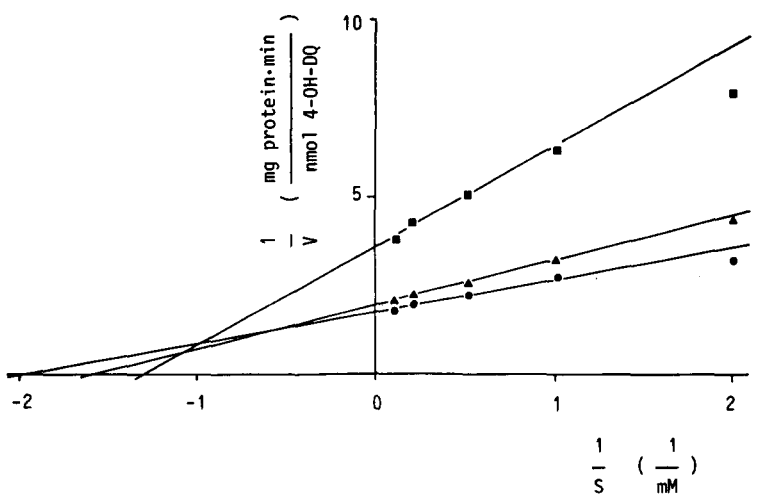

Fig. 3. Kinetics of DQH in control and inducer pretreated rats. The assays were performed with hepatic microsomes from rats pretreated with PB ■----■, MC ▲----▲, and from control rats ●----● using the standard incubation system. For further details see Materials and Methods. The substrate concentration was varied between 0.5 and 10 mM DQ. One experiment of three is shown for each experimental group.

Table 2. Sex and strain dependent variation of DQH and other monooxygenase activities in rat liver

Strain	Sex	(nmoles/mg protein) Cytochrome P-450	(nmoles product/mg protein × min) Debrisoquine 4-hydroxylation	Aldrin epoxidation	Ethylmorphine demethylation	Benzo(a)pyrene hydroxylation
Wistar	♂	0.80 ± 0.06	0.24 ± 0.03	2.47 ± 0.15	7.4 ± 1.5	0.16 ± 0.03
Wistar	♀	0.45 ± 0.08	0.22 ± 0.01	0.64 ± 0.12	3.4 ± 1.7	0.03 ± 0.01
DA	♀	0.49 ± 0.08	0.02 ± 0.01	0.58 ± 0.21	2.4 ± 0.9	0.02 ± 0.01

The results represent means ± S.D. of enzyme activities determined with microsomes from four animals in each experimental group. DQH activity was assayed at a substrate concentration of 0.2 mM and a protein concentration of 1 mg/ml.

cytochrome P-450 dependent monooxygenase. Our finding that typical inhibitors of monooxygenase reactions, such as SKF 525-A, 7.8-benzoflavone and metyrapone, also were inhibitors of DQH further substantiates previous results of others indicating the involvement of cytochrome P-450 [4, 5].

However, our findings also indicate striking differences between DQH and other known cytochrome P-450 functions. DQH was not inducible in rats pretreated with various inducers of major cytochrome P-450 species, such as PB, MC, PCB and the hormone analogues, PCN and DEX. PB is known as inducer of the reactions selected in this study to control the efficiency of the pretreatment with inducers, such as aldrin epoxidase, ethylmorphine demethylase, and AHH [12, 19]. Of these reactions only AHH is inducible by MC [12, 20]. PCBs are known to induce a broad pattern of monooxygenase activities including AHH and ethylmorphine demethylase [21]. Pretreatment of female rats with PCN causes a pattern of monooxygenase activites differing from that observed in animals pretreated with PB or MC [22, 23]. A similar type of induction is observed when DEX is given as inducer [24].

Recently, the existence of two enzymes of different substrate affinities, a high and a low K_m form, was deduced from the biphasic kinetics of DQH in untreated rats [25]. The DQH activities, listed in Table 1, were determined at a substrate concentration of 0.2 mM DQ. This concentration is supposed to be appropriate for the low K_m form of DQH. However, also the high K_m form of DQH was not inducible by PB and MC, as indicated by the V_{max} values obtained from the Lineweaver–Burk diagrams of Fig. 3.

The finding that commonly used inducers were ineffective as inducers of DQH was corroborated by the observation that pretreatment of animals with the substrate itself did not lead to induction of DQH. This result was unexpected, since many monooxygenase substrates are known to induce their own metabolism. The slight decrease of the cytochrome P-450 level and the general decrease of enzymatic

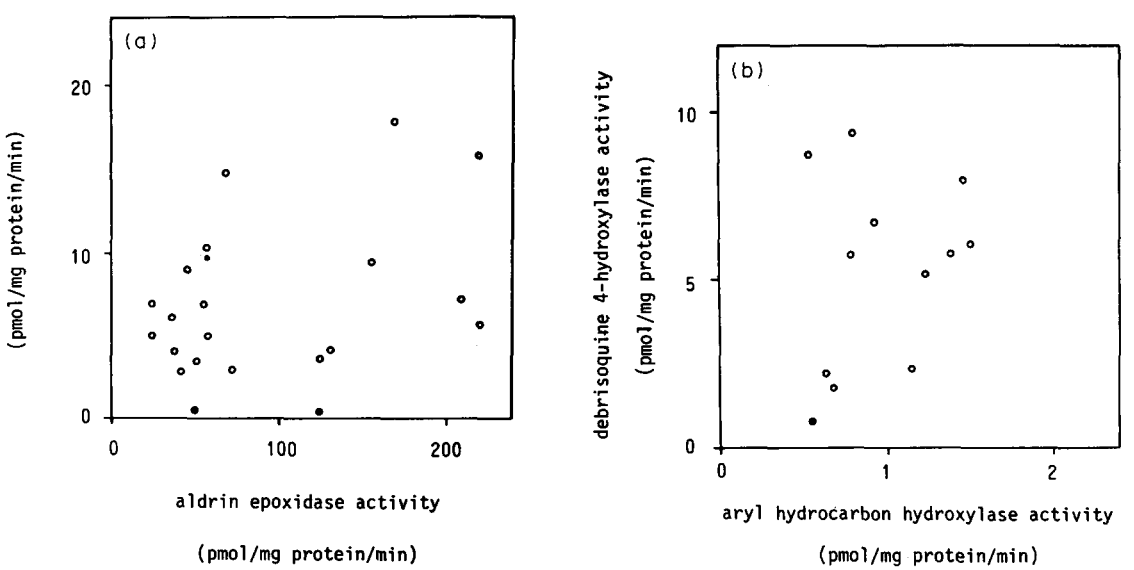

Fig. 4. (a) Correlation of DQH and aldrin epoxidation activity in human liver. Homogenates from 21 liver biopsies were analyzed. Concentration of DQ was 0.2 mM and of protein 1 mg/ml. Filled symbols represent the enzyme activities of poor metabolizers. The correlation coefficient r was 0.45. (b) Correlation of DQH and AHH activity in human liver. Homogenates from 12 liver biopsies were analyzed. Conditions of DQH assay were as described under Fig. 4. The correlation coefficient r was 0.27.

activities might be a consequence of the relatively high dose of DQ applied, possibly leading to a decreased blood flow in the liver. Administration of relatively high DQ doses however, was regarded necessary in order to achieve cellular levels of the drug sufficiently higher for induction.

The existence of noninducible cytochrome P-450 forms in rat liver was recently verified by the detection of two forms not inducible by PB, 5,6-benzoflavone, isosafrole, PCN and PCB [26]. However, these forms did not catalyze the hydroxylation of DQ*. Very recently, a minor cytochrome P-450 form termed "P-450$_{UT-H}$" with high activity towards DQ was detected in the liver of female SD rats [27]. Female DA rats had low amounts of this form. We assume that the DQH mediating, noninducible cytochrome P-450 species postulated in this study is similar to, if not identical with "P-450$_{UT-H}$".

The variation of DQH activity in relation to other monooxygenase activities in untreated animals of different sex and strain also indicates that DQH is mediated by a particular form of cytochrome P-450. DQH activity is known to vary largely between female Wistar and DA rats [7]. The activities of aldrin epoxidase, ethylmorphine demethylase and AHH, however, were similar in females of both strains. Moreover, in contrast to various other monooxygenase activities showing a sex dependency, DQH activity was similar in both genders of Wistar rats.

Similar to the enzyme from rat liver, DQH activity of human liver did not correlate to other monooxygenase activities, such as aldrin epoxidase or AHH, as indicated by the low correlation coefficients observed. Other authors, using human liver microsomes likewise reported that no correlation existed in between DQH and these and further monooxygenase activities, including aminopyrine *N*-demethylation, ethoxycoumarin deethylation and biphenyl-2-hydroxylation [28]. Whether these findings can be extended to the oxidation of chemical carcinogens and toxicants is subject of current investigations.

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