Table 2. Catalytic activities of purified human liver enzyme compared to the activity of the microsomes

| Substrate | Substrate concentration ($\mu$M) | Microsomes (nmol/min · nmol P-450) | Purified P-450 (nmol/min · nmol P-450) |
|---|---|---|---|
| Desmethylimipramine | 50 | 0.11 | 0.95 |
| Debrisoquine | 500 | 0.19 | 0.45 |
| Imipramine | 50 | — | 0.27 |

sequence analysis revealed that the preparation was not chemically homogeneous. The purified fraction could be assigned into one major and one minor component, with likely N-terminal amino acid sequences AlaLeuIle- and MetLysGlu-, respectively. In two different preparations, the major component was estimated to be present in an amount of about 50–70%. It is possible that one of the components in our preparation is identical with a non-heme protein contaminating some debrisoquine hydroxylase fractions prepared by Distlerath *et al.* [12].

The purified enzyme preparation hydroxylated DMI at a rate of 0.95 nmol/min · nmol cytochrome P-450, which is nine times faster than the rate found in the microsomes (on a cytochrome P-450 basis). It was also able to 4-hydroxylate debrisoquine and N-demethylate imipramine (Table 2). Five hundred $\mu$M debrisoquine inhibited the hydroxylation of DMI by 82%. These data indicate that our purified enzyme is identical to the major debrisoquine hydroxylating cytochrome P-450 in man. The rate of debrisoquine hydroxylation with our protein was similar to that of a recently purified human liver debrisoquine hydroxylase [12], further supporting this suggestion. Our protein might also be the one present in a human liver cytochrome P-450 preparation hydroxylating bufuralol [13].

Summary, we have purified a human liver cytochrome P-450 by chromatography on lauric acid AH Sepharose 4B and CM Sepharose CL-6B columns. The preparation exhibits a single major band on SDS-polyacrylamide gel electrophoresis ($M_r$ 51,500) and contains 8.5 nmol cytochrome P-450/mg protein. It oxidizes desmethylimipramine, debrisoquine and imipramine in a reconstituted system, and 500 $\mu$M debrisoquine inhibits the hydroxylation of DMI by 82%. The data indicate that we have purified a DMI hydroxylase which is identical to the major human liver debrisoquine hydroxylase. It also demethylates imipramine.

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## The influence of pretreatment on the urinary metabolite profile of pseudoracemic hexobarbital

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Hexobarbital (HB; Fig. 1) is a well-known model substrate for the assessment of changes in the activity of hepatic cytochrome P-450 (P-450 for short) in animals and man [1, 2]. Sleeping time following HB-administration [3], and pharmacokinetic parameters of HB, like half-life [4] and clearance [5–7] have been frequently used to characterize such changes. A complicating factor in the interpretation of these parameters is the fact that HB is a chiral compound. The two enantiomers, S(+)HB and R(−)HB, differ considerably in pharmacokinetics and in urinary metabolic pattern [8, 9]. Yet HB has been used as a model substrate nearly exclusively in the racemic form.

For an unambiguous interpretation of pharmacokinetic data of racemic HB as a reflection of changes in hepatic drug

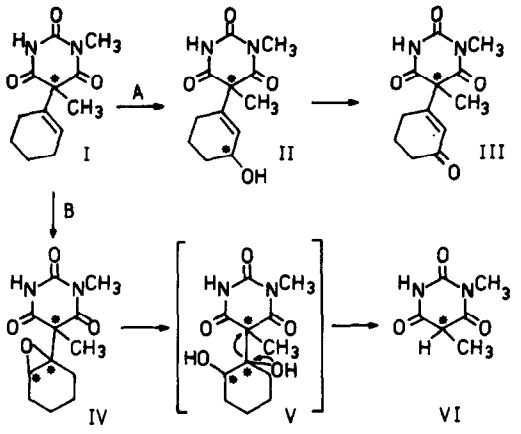


Fig. 1. Major metabolic pathways of racemic hexobarbital: (A) allylic oxidation of hexobarbital (I) leading to the formation of 3′-hydroxyhexobarbital (II) and 3′-ketohexobarbital (III); (B) epoxidation leading to 1′,2′-epoxyhexobarbital (IV) and 1,5-dimethylbarbituric acid (V).

metabolizing enzyme activity, at least two requirements should be fulfilled. Firstly, for each P-450 iso-enzyme involved in HB metabolism, the ratio of activities towards S(+)HB and R(−)HB should not be substantially altered. This was shown to be the case [2]. Secondly, inhibition or induction of P-450 activity should cause a quantitative and not a qualitative change in this activity. The possibility of qualitative changes in metabolism can be investigated (although not conclusively proven) by measuring the metabolites excreted in the urine.

Three urinary metabolites of HB can be quantitated: 3′-hydroxyhexobarbital (OH-HB), 3′-ketohexobarbital (K-HB) arising from the allylic oxidation pathway and 1,5-dimethylbarbituric acid (DMBA), originating from the epoxide-diol pathway (Fig. 1). Recently, a rapid and sensitive mass fragmentographic assay was developed for pseudoracemic HB consisting of equal molar fractions of S(+)HB and R(−)HB labeled with a trideuterated $N_1$-methyl group ($d_3$R(−)HB). With this procedure both the parent compounds in blood and the three major metabolites in urine could be stereoselectively quantitated [10]. Furthermore, effects of pretreatment on the metabolite pattern of each enantiomer of HB in the presence of its antipode may be studied.

In the present study, the metabolite profile of pseudoracemic HB was compared to the metabolite profile of separately administered enantiomers. Further, the effect of pretreating rats with carbon tetrachloride, phenobarbital and 3-methylcholanthrene on the urinary metabolite pattern was studied. This was done in an attempt to answer the question whether different P-450 iso-enzymes (showing negligible overlap in substrate specificity) participate in the metabolism of the enantiomers. This would show up in a difference in total recovery of the urinary metabolites, a shift in the relative contribution of the two metabolic pathways to total recovery, or difference in enantioselectivity, e.g. in the OH-HB over K-HB ratio for each enantiomer.

*Materials and methods*

S(+)-hexobarbital (S(+)HB) was a generous gift from Prof. J. Knabe, Universität des Saarlandes, Saarbrücken, F.R.G. The synthesis of $d_3$R(−)-hexobarbital ($d_3$R(−)HB) was described recently [10]. The metabolites 3′-hydroxyhexobarbital (OH-HB), 3′-ketohexobarbital (K-HB) and 1,5-dimethylbarbituric acid (DMBA) were synthesized as described previously [11]. Carbon tetrachloride was obtained from J. T. Baker (Deventer, The Netherlands), 3-methylcholanthrene (MC) from Sigma (St. Louis. MO) and the sodium salt of phenobarbital (PB; 5-ethyl-5-phenylbarbituric acid) from O.P.G. (Utrecht, The Netherlands).

Male Wistar rats from our own laboratory breed were used, weighing 180–220 g. With the exception of an untreated control group, they were subjected to the following pretreatment procedures via i.p. injection:
(a) Carbon tetrachloride ($CCl_4$), 0.8 ml·kg$^{-1}$, as a 1:3 v/v solution in sesame oil, 24 hr before the experiments.
(b) Sodium phenobarbital (PB), 75 mg·kg$^{-1}$ in water, administered daily for five consecutive days; the experiments were performed 24 hr after the last dose.
(c) 3-Methylcholanthrene (MC), 18 mg·kg$^{-1}$ sesame oil, daily for three consecutive days, the experiments were performed 24 hr after the last dose. Pseudoracemic HB was administered in a dose of 50 mg·kg$^{-1}$ via oral intubation.

The solution was prepared immediately before use by dissolving equimolar amounts of S(+)HB and $d_3$R(−)HB in an equivalent amountof 0.1 N NaOH and diluting with distilled water to a concentration of 10.0 mg·ml$^{-1}$. After administration of 0.50 ml per 100 g body weight, 24 hrs' urine was collected. Just prior to the experiments, 20 mg of sodium pyrosulphite was added to the urine receptacles to prevent possible oxidation of DMBA.

The extraction of unconjugated metabolites from urine was carried out according to a previously described procedure [9]. In the rat, conjugation of OH-HB's is quantitatively unimportant. Estimates range from zero [12] to a few percent [13]. The quantitative analysis of the metabolites of the respective enantiomers was carried out as described recently [10].

*Results and discussion*

The aim of this investigation was to study a possible reciprocal influence of S(+)HB and R(−)HB on each other's metabolite profile in the urine prior to and after pretreatment.

The results of all experiments are compiled in Table 1. Literature data on the urinary excretion of OH-HB, K-HB and DMBA following separate oral administration of 25 mg·kg$^{-1}$ of S(+)HB and R(−)HB [9] were also incorporated in Table 1 for comparison. Theoretically, it would be more elegant to study the rates of formation of the metabolites measured in the urine as well as the percentages of the dose excreted. This, however, is only a meaningful approach if the rate of urinary excretion of metabolites is considerably higher than the rate of their production, and if further metabolism does not occur. For studies with HB in the rat probably neither of these prerequisites apply [12]. The sum of OH-HB and K-HB

Table 1. The urinary metabolite profile of pseudoracemic hexobarbital compared with previous p.o. data obtained after administration of the enantiomers, and the influence of pretreatment

| % Dose | p.o.* | Control | PB | MC | $CCl_4$ |
|---|---|---|---|---|---|
| OH-HB | | | | | |
| S(+) | 7.8 ± 0.4 | 10.4 ± 1.6 | 9.0 ± 0.8 | 11.2 ± 1.5 | 11.1 ± 1.7 |
| $d_3$R(−) | 5.4 ± 0.6 | 4.4 ± 0.5 | 7.2 ± 0.4 | 5.4 ± 0.9 | 5.8 ± 0.8 |
| Sum | 13.2 ± 0.6 | 14.8 ± 1.4 | 16.2 ± 1.0 | 16.6 ± 1.2 | 16.9 ± 2.5 |
| K-HB | | | | | |
| S(+) | 9.6 ± 0.4 | 7.3 ± 0.8 | 6.2 ± 0.8 | 8.4 ± 1.1 | 12.0 ± 3.4 |
| $d_3$R(−) | 28.1 ± 0.9 | 16.9 ± 1.1 | 17.5 ± 1.5 | 21.3 ± 1.1 | 15.6 ± 3.6 |
| Sum | 37.7 ± 0.8 | 24.2 ± 1.6 | 23.7 ± 1.9 | 29.7 ± 1.7 | 27.6 ± 6.7 |
| DMBA | | | | | |
| S(+) | 4.6 ± 0.7 | 10.4 ± 1.2 | † | 5.4 ± 0.5 | 12.8 ± 2.5 |
| $d_3$R(−) | 5.9 ± 0.9 | 12.2 ± 3.5 | † | 5.6 ± 1.4 | 11.9 ± 6.4 |
| Sum | 10.5 ± 0.8 | 22.6 ± 4.1 | 4.9 ± 0.5 | 11.0 ± 1.4 | 24.8 ± 8.5 |
| Total | 61.4 ± 2.3 | 61.6 ± 4.7 | 44.8 ± 1.2 | 57.3 ± 3.0 | 70.3 ± 3.4 |
| Means ± SEM | N = 6 | N = 8 | N = 6 | N = 9 | N = 4 |

* The literature data on separately administered enantiomers were treated as if combined administration had been performed, and as if the effect of this on excretion patterns and recovery were non-existent.
† Not determined.

may not be a good reflection of allylic oxidation of HB, since on the one hand K-HB is probably further metabolized and on the other hand both OH-HB and K-HB may give rise to some DMBA, which is also the end-product of the epoxide-diol pathway.

In untreated rats, each enantiomer of pseudoracemic HB had a characteristic excretion pattern of OH-HB and K-HB. The percentage of OH-HB arising from $d_3$R(−)HB was up to a factor 2–3 lower than the percentage of OH-HB arising from S(+)HB. The percentage of K-HB arising from S(+)HB. The percentage of K-HB arising from S(+)HB was also a factor 2–3 lower than the percentage K-HB arising from $d_3$R(−)HB. These observations are in relatively good agreement with the corresponding data obtained upon separate oral administration of S(+)- and R(−)HB (Table 1). However, as compared to separate administration, the contribution of the allylic oxidation pathway metabolites to the total excretion was diminished, and this was balanced by a higher excretion of DMBA. The net result was, that both after separate and simultaneous administration the same percentage of the dose was excreted.

A comparison of the metabolite profile of pseudoracemic HB with and without pretreatments, showed that the percentage of the dose excreted as the sum of OH-HB, K-HB and DMBA increased in the order PB < MC < C < $CCl_4$. This is in agreement with results from a study in which racemic HB was administered simultaneously with antipyrine [7]. That PB-pretreatment results in a lower recovery of urinary metabolites of HB has also been demonstrated in the rabbit [12]. Pretreatment with PB and MC did not have large effects on the sum of OH-HB's and K-HB's nor on the stereoselectivity in their formation. The excretion of DMBA, however, was considerably lower than in the untreated rats.

A possible explanation might be the reaction of intermediary metabolites in the epoxide-diol pathway to undetectable products. Alternatively, DMBA might have been further metabolized to unidentifiable products. The possibility that the pathway leading to DMBA is neither preferentially mediated by PB-inducible nor by MC-inducible P-450 iso-enzymes is rather unlikely since compounds which are inefficiently metabolized by either of these iso-enzymes are rather uncommon [14, 15]. Finally, as stated previously, a certain fraction of DMBA may arise from OH-HB or K-HB [2]. Results obtained in man are not in disagreement with this hypothesis [16].

Following $CCl_4$ treatment, slightly higher amounts of all metabolites were excreted. A decreased P-450 content resulting in a higher urinary metabolite recovery has also been observed in rats with a portacaval shunt [17]. The origin of this effect is at present not know. Interestingly, $CCL_4$ pretreated rats converted OH-HB from $d_3$R(-)HB to K-HB less efficiently than other rats. Also for this phenomenon, the cause is unknown as yet.

In conclusion, the results of the present study indicate that there is some reciprocal effect of the enantiomers on each other's metabolism as far as the contribution of the different metabolic pathways is concerned. The total recovery, as well as stereoselective effects, e.g. the ratio OH-HB over K-HB, were essentially unchanged. Hence reciprocal influences do not attain a high degree of relevance.

The influence of pretreatment is mainly characterized by a considerable fluctuation in the recovery of DMBA. Since both the origin and the fate of DMBA are not completely clear, it is difficult to speculate on the relevance of this fluctuation. When considering all data available, there is no evidence to assume that pretreatment with $CCl_4$, PB, or MC results in other than quantitative changes in a number of P-450 iso-enzymes as reflected by HB metabolite profile. Of course, the absence of changes in relative substrate selectivity of P-450 iso-enzymes towards the HB enantiomers as a result of different pretreatments can only be conclusively established via *in vitro* experiments with purified iso-enzymes.

However, when focusing on the *in vivo* situation the present results can be interpreted as being in favour of the hypothesis that meaningful results may be obtained when racemic HB is used as a model substrate to reflect changes in (PB-inducible) P-450 activity. Measurement of urinary metabolites of HB is not likely to give relevant additional information in this regard. Furthermore, the measurement of urinary excretion of metabolites in induction or inhibition studies with HB does not provide information on the extent of the effects which is complementary to changes in (oral) clearance.

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# Monooxygenase activity of systems reconstituted with fractions from rats fed standard and low protein diets

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Weanling rats were fed *ad libitum* for 6 weeks either a standard diet containing 22% casein (St) or a low protein diet containing 6% casein (LP). The individual components of the microsomal cytochrome P-450-dependent monooxygenase system such as lipid, cytochrome P-450 and cytochrome P-450 reductase were isolated from the two dietary groups. Then, they were recombined in various combinations and the contributions of the individual components to the monooxygenase activity with respect to benzphetamine and ethylmorphine as substrates were studied. These metabolic activities were strongly decreased (86–95%) when incubations were carried out with fractions from the LP group compared to the St group. This decrease was predominantly (to about 60%) due to changes in cytochrome P-450. A smaller but considerable contribution (about 30%) to the decrease was due to changes in cytochrome P-450 reductase and very little (about 10%) of the decrease was due to changes in phospholipid composition. The observations indicate that low protein diet affects both the specific activities of the individual components and the interaction between cytochrome P-450, cytochrome P-450 reductase and phospholipids.

in the quantity of dietary protein depresses the activity of the cytochrome P-450 dependent microsomal monooxygenase (MO) system towards the substrates investigated [1–4]. These decreases may be accounted for either by decreased levels or by decreased catalytic activities of cytochrome P-450. Another mechanism by which the level of dietary protein intake may influence the specific activity of the MO system is through an effect on phospholipid composition [5, 6]. The work of I.U and COON in 1968 [7] has led to techniques allowing for the fractionation of the various components of the MO system, which when recombined allows for partial reconstitution of activity. With the availability of these procedures we report here the effect of low protein diet on the individual MO components. Moreover, in the present study a method was used which allowed the isolation of cytochrome P-450 at the low levels occuring in non-induced animals on a low protein diet.

## *Materials and methods*

Twenty weanling Sprague–Dawley male rats were fed for one week with a standard (St) diet containing 22% casein and then subdivided into two groups. Group 1 continued on the standard diet and group 2 received a low protein diet (LP) containing 6% casein given *ad libitum* for 6 weeks. Rats were starved overnight before killing and preparation of liver microsomes were carried out as previously described [8].

*Purification of cytochrome P-450.* Liver microsomal cytochrome P-450 was solubilized with sodium cholate and purified by affinity chromatography on ω-aminooctyl-sepharose 4B prepared as described [9] followed with a hydroxyl appatit column. The cytochrome P-450 was eluted with 50 mM phosphate buffer pH 7.25 from this column and then concentrated with ultra filtration (30 pm). The specific content of this fraction was 17 nmol of cytochrome P-450 per mg protein recovery of the first column was approximately 45% of total microsomal cytochrome P-450, and second column 27%, this fraction had not any contamination by other proteins juged by S.D.S. gel electrophores [10].