INFLUENCE OF INDUCERS AND INHIBITORS ON THE HYDROXYLATION PATTERN OF *N*-HEXANE IN RAT LIVER MICROSOMES

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1. Introduction

The cytochrome P450-linked monooxygenase system of liver microsomes has previously been considered as highly non-specific in view of the wide variety of chemically different endogenous and exogenous compounds known to act as substrates for this enzyme system [1]. More recently, however, increasing evidence has accumulated indicating the existence of several cytochrome P450-linked monooxygenases with different but overlapping substrate specificities. Such evidence includes different effects on hydroxylation in various positions of the same substrate molecule by inhibitors [2–4], storage of the microsomes [5] and by pretreatment of the animals with phenobarbital or polycyclic hydrocarbons [3, 4, 6, 7]. Most likely, these differences can be attributed to the existence of two different species of cytochrome P450.

Experiments described in the present paper support this assumptio and give evidence for a third form of cytochrome P450 participating in the ω-hydroxylation of *n*-hexane.

2. Materials and methods

Male rats (Wistar AF, Han) weighing 100–150 g were used. Sodium phenobarbital (80 mg/kg) or 3,4-benzpyrene (20 mg/kg, dissolved in olive oil) were injected intraperitoneally once daily for two days.

Liver microsomes were isolated as described previously [8] and protein was determined by the biuret method of Gornall et al. [9].

Incubation of liver microsomes with *n*-hexane and the isolation of the products were performed as previously described for the hydroxylation of *n*-heptane [4].

Metyrapone (SU 4885) (2-methyl-1,2-bis-(3-pyridyl)-1-propanone) was a gift from Ciba-Geigy, Basel, Switzerland, and 7,8-benzoflavone was obtained from Th. Schuchardt, Munich, Germany. All other chemicals were standard commercial products.

3. Results

When *n*-hexane was incubated with liver microsomes from untreated rats, hydroxylation occurred at all carbon atoms leading to a mixture of isomeric hexanols. Separation of the isomers by gas liquid chromatography and quantitation of each isomer gave the specific activities indicated in table 1. The standard deviation values were calculated for the eight different preparations and do not reflect variations in the ratios of the three isomers which showed much smaller deviations.

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Table 1
Hydroxylation of N-hexane in rat liver microsomes
$H_3C-CH_2-CH_2-\overset{3}{C}H_2-\overset{2}{C}H_2-\overset{1}{C}H_3$

Pretreatment	No. Prep.	Hexanol-1	Hexanol-2	Hexanol-3
		nmoles formed/min/mg protein		
None	8	0.4 ± 0.1	1.2 ± 0.6	0.2 ± 0.1
Phenobarbital	3	0.5 ± 0.1	6.6 ± 0.6	1.4 ± 0.1
3,4-Benzpyrene	7	0.2 ± 0.1	0.8 ± 0.3	0.7 ± 0.3

For the GLC-analysis of the *n*-hexanol isomers we used 4 m columns with DMCS-treated GLC-100 as solid phase coated with 0.15% Carbowax 20 M TPA as liquid phase. Heptanol-1 served as internal standard.
Each preparation consisted of 4 pooled livers. Three determinations were run from each preparation.

Calibration values were obtained by incubating the corresponding hexanols in equal amounts under the conditions of the hydroxylation assay. This proved to be necessary since a second hydroxylation of hexanol-2- and hexanol-3 seemed possible in view of the finding that they produce substrate binding spectra with cytochrome P450 when added to liver microsomes [10]. In the presence of excess *n*-hexane this was, however, shown not to occur. Therefore, we have substituted *n*-hexane by *n*-heptane in the calibration values. The heptanols formed did not interfere with the retention times of the hexanols.

When liver microsomes from phenobarbital-treated rats were used, a marked stimulation of the specific activities for hydroxylation in positions 2 and 3 was observed. In contrast hydroxylation in the ω-position was not affected. Pretreatment with 3,4-benzpyrene, on the other hand, did not change the overall hydroxylation rate (cf. [11]) but did markedly affect the ratio between the various isomers (cf. [4]). Thus, 3-hydroxylation increased 3-fold whereas the 1- and 2-hydroxylation activities decreased by 50% and 30%, respectively (table 1).

Metyrapone and 7,8-benzoflavone are inhibitors believed to interact preferentially with the phenobarbital- and 3,4-benzpyrene-induced forms of cytochrome P450, respectively [12, 13]. With liver microsomes from untreated and phenobarbital-treated rats, metyrapone in a 10^{-4} M concentration inhibited the hydroxylation of *n*-hexane in positions 2 and 3 by approx. 50% but had a stimulatory effect on the rate of ω-hydroxylation (table 2). With liver microsomes from 3,4-benzpyrene-pretreated rats, stimulation of the rate of hydroxylation in positions 2 and 3 occurred in the presence of metyrapone. 7,8-Benzoflavone, on the other hand, inhibited *n*-hexane hydroxy-

Table 2
Inhibition by metyrapone and 7,8-benzoflavone of *n*-hexane hydroxylation

Pretreatment	Metyrapone (10^{-4} M) % Activity in position			7,8-Benzoflavone (10^{-4} M) % Activity in position		
	1	2	3	1	2	3
None	97 ± 7	41 ± 5	35 ± 8	149 ± 7	40 ± 1	47 ± 6
Phenobarbital	71 ± 8	48 ± 5	36 ± 6	103 ± 4	59 ± 13	62 ± 12
3,4-Benzpyrene	175 ± 50	176 ± 50	113 ± 15	233 ± 60	45 ± 10	21 ± 10

7,8-Benzoflavone was added in acetone at a concentration which did not affect the pattern of isomers.
The ratios of isomers represent means of the ratios from each microsomal preparation consisting of four pooled livers. Three standard assays were run with and without inhibitors (= 100% activity).

lation in positions 2 and 3 independent of pretreatment but, in 3,4-benzpyrene-induced rat liver microsomes the inhibition is 100% higher in the 3-position. The ω-hydroxylation, again, is stimulated.

4. Discussion

In accordance with previous findings with other aliphatic hydrocarbons [4], liver microsomes were found to catalyze the hydroxylation of *n*-hexane in all positions. A preference for hydroxylation in position 2 (ω-1) observed can only be explained by steric factors involved, since the 2- and 3-positions are chemically equivalent.

The preferential stimulation of (ω-1)-hydroxylation by phenobarbital treatment and the change in isomer pattern after 3,4-benzpyrene administration to the rats support our previous assumption that more than one monooxygenase is involved in the hydroxylation of aliphatic hydrocarbons by liver microsomes [4]. Accordingly, the results of both the induction and inhibitor experiments suggest that the phenobarbital-induced cytochrome P450 form is equivalent to the (ω-1)-hydroxylase. In microsomes from 3,4-benzpyrene-induced rats the 2- and 3-position are equally attacked, however, the hydroxylation in the 3-position is much more sensitive towards 7,8-benzoflavone which may indicate that even after pretreatment with benzpyrene more than one monooxygenase is present. If this is true then the benzpyrene induced hemoprotein must have a preference for the attack in the 3-position.

The present work supports our earlier assumption of the existence of a separate ω-hydroxylase which is not inducible by either phenobarbital or 3,4-benzpyrene pretreatment of the animals [4]. Neither is it inhibited by metyrapone or 7,8-benzoflavone. Similar observations have been reported for the ω-hydroxylation of lauric acid in liver microsomes which in contrast to (ω-1)-hydroxylation is not inducible by phenobarbital treatment [3] and not inhibited by metyrapone or SKF 525-A [14]. In contrast, like in the present experiments a certain stimulation of the rate of *w*-hydroxylation was often observed in the presence of these inhibitors. The reason for this stimulatory effect is not yet clear. One obvious possibility is, however, that the various monooxygenases share a common reductase component and that more reducing equivalents are directed to the ω-hydroxylase in the presence of inhibitors binding to the other cytochrome P450 forms involved in the hydroxylation of the substrate studied.

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