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ω-AND (ω-1)-HYDROXYLATION OF 4-CHLOROPROPIONANILIDE IN LIVER MICROSOMES OF RABBITS TREATED WITH PHENOBARBITAL OR 3-METHYLCHOLANTHRENE

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Abstract—Hydroxylation of 4-chloropropionanilide to 4-chlorohydracrylanilide, ω-hydroxylation, and to 4-chlorolactanilide, (ω-1)-hydroxylation, by rabbit liver microsomes were found to be stimulated differently by treatment of rabbits with phenobarbital or 3-methylcholanthrene. ω-Hydroxylation was not stimulated by 3-methylcholanthrene, but increased to nineteen times the normal rate by treatment with phenobarbital. (ω-1)-Hydroxylation increased in rate by only 70 per cent after treatment with phenobarbital and by 200 per cent after treatment with 3-methylcholanthrene. On storage of microsomes for 18 days ω-hydroxylation decreased by 40 per cent and (ω-1)-hydroxylation remained unchanged. ω-Hydroxylation showed lower affinity for oxygen and higher sensitivity to the inhibiting effect of carbon monoxide than (ω-1)-hydroxylation. Hydroxylation of the acetic acid residue in 4-chloroacetanilide and acetophenone did not uniformly follow ω- or (ω-1)-hydroxylation of 4-chloropropionanilide. Treatment of rabbits with phenobarbital stimulated ω-hydroxylation of acetophenone and did not affect ω-hydroxylation of 4-chloroacetanilide.

IN INCUBATES of 10,000 *g* supernatant from rat liver homogenates with stearate Preiss and Bloch[1] found the (ω-1)-hydroxy derivative of the fatty acid in addition to ω-hydroxy stearate. Björkhem and Danielson[2] showed that isolated rat liver microsomes hydroxylate laurate, palmitate, and stearate in the ω- or (ω-1)-position. With laurate about equal amounts of the ω-hydroxy acid and the (ω-1)-hydroxy acid were found. With palmitate and stearate the proportion of ω-hydroxy acid was higher than that of (ω-1)-hydroxy acid. Some differences between microsomal ω- and (ω-1)-hydroxylation were observed. ω-Hydroxylation by rat liver microsomes was found to be more susceptible to inhibition by carbon monoxide than (ω-1)-hydroxylation. Treatment of rats with phenobarbital stimulated the (ω-1)-hydroxylation of laurate, palmitate and stearate, but not the ω-hydroxylation. Hamberg and Björkhem[3] found that only a small proportion, 5–10 per cent of the hydroxylation product of decanoate is the (ω-1)-isomer. Deuterium at the ω-C atom did not affect the rate of microsomal hydroxylation but deuteration at the (ω-1)-C-atom diminished the rate of its hydroxylation and reversed the ratio between L- and D-(ω-1)-hydroxydecanoate.

In the foregoing paper we have reported that the lowest fatty acid susceptible to (ω-1)-hydroxylation, propionic acid is also ω- or (ω-1)-hydroxylated by rabbit liver microsomes when offered as the 4-chloroanilide. This paper reports ω- and (ω-1)-hydroxylation of propionic acid in 4-chloropropionanilide by microsomes

from rabbits treated with phenobarbital or 3-methylcholanthrene. When ω- and (ω-1)-hydroxylation were found to be differently affected by the treatment with PB* or MC*, it appeared of interest to include into this study 4-chloroacetanilide and acetophenone which are known to be hydroxylated at the acetic acid residue.[4,5]

MATERIALS AND METHODS

Rabbit liver microsomes were prepared from male and female animals weighing 2·0–2·3 kg and fed on Altromin standard diet. Animals treated for 2 weeks with phenobarbital were given six daily subcutaneous injections of 25 mg/kg in the first week and 50 mg/kg in the second week. The animals were sacrificed the day after the last injection. 3-Methylcholanthrene, 10 mg/kg in olive oil, was injected intraperitoneally twice weekly. The animals were sacrificed the day after the fourth injection.

Liver homogenates were prepared by squeezing the livers through a sieve.[6] The pulp was suspended in 4 vol. of 0·25 M sucrose and centrifuged for 30 min at 9900 *g*. Microsomes were separated from the supernatant by centifugation for 120 min at 105,000 *g* and resuspended in 0·25 M sucrose. Protein content of microsomes was determined according to Szarkowska and Klingenberg.[7]

Microsome suspensions in 0·15 M phosphate pH 7·4, 3 mg protein/ml, fortified with 0·12 mM NADP, 10 mM glucose-6-phosphate, 350 IU glucose-6-phosphate dehydrogenase per litre, 6 mM magnesium chloride, and 12 mM nicotinamide were incubated with 10^{-3} M substrate at 37° under air for 15 min, if not otherwise stated.

Hydroxylation products of the chloroanilides were isolated and determined as described by Kiese and Lenk[8,9].

ω-Hydroxyacetophenone, a new metabolite described elsewhere, was extracted with ether from incubates of microsomes with acetophenone, isolated by TLC on silicagel PF_{254} Merck with cyclohexane: ethyl acetate (80:20), and measured by its absorbance at 242 nm.

RESULTS

The effect of phenobarbital or 3-methylcholanthrene treatment of rabbits on microsomal activity. In a series of experiments the increase in metabolite content of incubates with time was determined. All the metabolites measured increased in proportion to the incubation time for at least 20 min. Therefore, reaction rates were calculated from the amount of metabolite found after 15 min incubation.

The results of experiments on the hydroxylation of the acid residues in chloroanilides are summarized in Table 1. Microsomes from untreated rabbits hydroxylated chloropropionanilide in (ω-1)-position eight times more rapidly than in ω-position. Treatment of rabbits with PB stimulated both reactions. The rate of (ω-1) increased by about 70 per cent, but the rate of ω-hydroxylation was 19 times higher than in microsomes from untreated rabbits and exceeded the rate of (ω-1)-hydroxylation. Treatment of rabbits with MC had a different effect. The rate of ω-hydroxylation was not increased and (ω-1)-hydroxylation was three times more rapid than with microsomes of untreated animals.

The rate of microsomal hydroxylation of 4-chloroacetanilide to 4-chloroglycolanilide was increased by treatment of rabbits with MC but not by treatment with PB.

* PB = phenobarbital; MC = 3-methylcholanthrene.

TABLE 1. RATES OF HYDROXYLATION OF 4-CHLOROPROPIONANILIDE, 4-CHLOROACETANILIDE, AND ACETOPHENONE BY LIVER MICROSOMES FROM NORMAL RABBITS AND FROM RABBITS TREATED WITH PHENOBARBITAL OR 3-METHYLCHOLANTHRENE

Substrate:	Acetophenone		4-Chloroacetanilide		4-Chloropropionanilide				
Metabolite:	ω-Hydroxyacetophenone (μM/min)	P	4-Chloroglycolanilide (μM/min)	P	4-Chlorolactanilide (μM/min)	P	4-Chlorohydracrylanilide (μM/min)	P	$\frac{\omega - 1}{\omega}$
Normal rabbits	3·76 ± 2·27		0·31 ± 0·076		0·99 ± 0·087		0·13 ± 0·023		8·17 ± 1·63
Rabbits treated with phenobarbital	28·67 ± 1·09	<0·001	0·27 ± 0·058		1·67 ± 0·24	<0·02	2·51 ± 0·35	<0·001	0·68 ± 0·054
Rabbits treated with 3-methylcholanthrene	7·09 ± 2·92	>0·4	1·26 ± 0·12	<0·001	3·08 ± 0·093	<0·001	0·19 ± 0·042	0·3	19·4 ± 4·6

Protein content of microsome suspensions 3 mg/ml; incubation 15 min. The figures indicate the means + S.E. of 4–12 experiments. The last column shows the means of the ratios between (ω-1)- and ω-hydroxylation of 4-chloropropionanilide

The ω-hydroxylation of acetophenone, however, was increased eight-fold by treatment with PB and not, or very little, stimulated by treatment with MC.

The difference spectrum of microsomes + dithionite + carbon monoxide against microsomes + dithionite showed a maximum at 450 nm with microsomes from PB treated rabbits and a maximum at 448 nm with microsomes from MC treated rabbits.

Effect of storage on ω- and (ω-1)-hydroxylation by rabbit liver microsomes. Liver microsomes from rabbits treated with PB were incubated the day after their preparation with 4-chloropropionanilide and the rest of the same preparation was incubated after various days storage at 0°. The results are presented in Fig. 1. The rate of (ω-1)-hydroxylation was unchanged after 18 days storage of the microsomes. But the rate of ω-hydroxylation steadily decreased with the time of storage and had dropped in 18 days to 60 per cent of the first days rate.

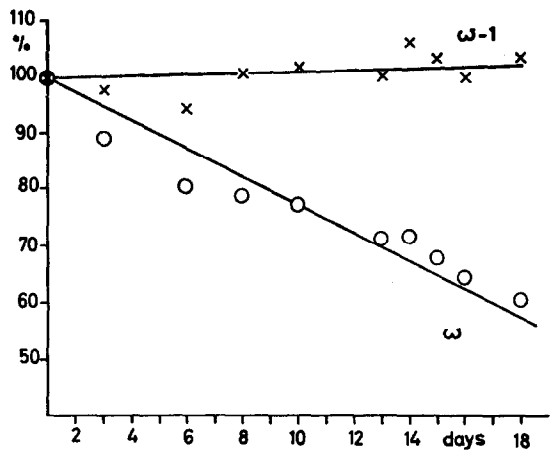

FIG. 1. Effect of storage on the rate of ω- and (ω-1)-hydroxylation of 4-chloropropionanilide by liver microsomes from rabbits treated with phenobarbital. The microsomes were stored at 0°. The symbols indicate the means of four experiments for days 1–13 and of three experiments for days 14–18. The rates observed on day 1 after the preparation of the microsomes were set to be 100. (×) Formation of 4-chlorolactanilide; (○) formation of 4-chlorohydracrylanilide.

Hydroxylation at low oxygen pressure and the effect of carbon monoxide. Since hydroxylation rates with liver microsomes from normal rabbits, in particular the ω-hydroxylation of 4-chloropropionanilide, are low, stimulated microsomes were used for studying hydroxylation at low oxygen pressure and in the presence of carbon monoxide. Microsomes from rabbits treated with PB were incubated with 4-chloropropionanilide and microsomes from rabbits treated with 3-methylcholanthrene were incubated with 4-chloroacetanilide.

Reaction rates were determined under air and under oxygen pressures of 87 mm and 33 mm Hg, in nitrogen and in carbon monoxide. The results, presented in Table 2, show that ω-hydroxylation of 4-chloropropionanilide has lower affinity for oxygen and is more susceptible to the inhibiting effect of carbon monoxide than (ω-1)-hydroxylation of the same substrate. At the oxygen pressure of 33 mm Hg the rate of ω-hydroxylation amounted to only 35 per cent of the rate under air (140 mm Hg of oxygen). Addition of the twenty-fold pressure of carbon monoxide diminished the rate

to less than one-third, i.e. to 10 per cent of the rate under air. At the same oxygen pressure, the (ω-1)-hydroxylation rate was 80 per cent of the rate under air, and the twenty-fold carbon monoxide pressure reduced it by half. The hydroxylation of 4-chloroacetanilide to 4-chloroglycolanilide showed the lowest susceptibility for carbon monoxide inhibition and oxygen affinity similar to (ω-1)-hydroxylation of 4-chloropropionanilide.

TABLE 2. HYDROXYLATION OF 4-CHLOROPROPIONANILIDE AND 4-CHLOROACETANILIDE BY RABBIT LIVER MICROSOMES AT LOW OXYGEN PRESSURES, AND THE EFFECT OF CARBON MONOXIDE

Substrate:			4-Chloropropionanilide		4-Chloroacetanilide
Metabolite:			4-Chlorolactanilide	4-Chlorohydracryl-anilide	4-Chloroglycol-anilide
O_2	N_2	CO			
88	587	—	96·1 ± 6·0	65·7 ± 3·9	82·2 ± 4·7
88	—	587	49·8 ± 7·1	19·5 ± 4·9	72·9 ± 3·9
34	641	—	80·8 ± 6·4	35·4 ± 6·0	65·4 ± 6·7
34	—	641	42·4 ± 5·5	8·8 ± 2·7	42·2 ± 7·5

Microsomes from rabbits treated with phenobarbital were used in experiments with 4-chloropropionanilide and microsomes from rabbits treated with methylcholanthrene in experiments with 4-chloroacetanilide. The first column shows the partial pressures of gases in mm Hg. The other figures indicate mean ±S.E. of hydroxylation rate in three to five, mostly four experiments, the rate under air being set to be 100.

DISCUSSION

The results of our experiments demonstrate that ω- and (ω-1)-hydroxylation of the propionic acid residue in 4-chloropropionanilide by rabbit liver microsomes are affected differently by treatment of rabbits with PB or MC. Treatment with PB stimulated both reactions, but to a very different extent. (ω-1)-Hydroxylation was less than twice as high as without treatment and ω-hydroxylation nineteen times higher than without treatment. MC stimulated only (ω-1)-hydroxylation. Stimulation by PB of ω-hydroxylation in rabbit liver microsomes has also been observed by Lu *et al.*[18] with dodecanoate, but the effect was much smaller than with 4-chloropropionanilide. In rats PB induces the production of the same microsomal cytochrome as found in microsomes of normal animals, i.e. cytochrome P-450. MC produces a different microsomal protein, cytochrome P-448.[10–13] Our experiments confirm results of Hildebrandt *et al.*[20] and Alvares *et al.*[21] which show that this is also true with rabbits. The results of the induction experiments, the differences in affinity for oxygen and in susceptibility to inhibition by carbon monoxide, and the different stabilities of activity on storage could be interpreted as evidence that ω-hydroxylation is catalysed, mainly or exclusively, by cytochrome P-450 and (ω-1)-hydroxylation by cytochrome P-448. Differences in substrate specificity between cytochrome P-450 and cytochrome P-448 have also been demonstrated by Lu *et al.*[14–16] with microsomal enzymes from rat liver. But the results with 4-chloroacetanilide and acetophenone question such conclusions. Hydroxylation of the acetic acid residue in 4-chloroacetanilide was not stimulated by PB, but the rate of ω-hydroxylation of acetophenone was eight times higher in microsomes of PB-treated rats than in microsomes of normal rats.

Studies of hydroxylation of medium-chain and long-chain fatty acids by rat liver

microsomes had results different from ours. Björkhem and Danielson[2] found no stimulation by PB of ω-hydroxylation of dodecanoate and even inhibition of ω-hydroxylation of hexadecanoate and octadecanoate. On the other hand, moderate stimulation of (ω-1)-hydroxylation by PB treatment and stronger inhibition by carbon monoxide of ω-hydroxylation than of (ω-1)-hydroxylation are in line with the results of 4-chloropropionanilide hydroxylation by rabbit liver microsomes.

Other investigators found that after PB treatment of rats there was a 2- to 3-fold increase in ω-hydroxylation of dodecanoate[17,18] and inhibition of ω-hydroxylation of octadecanoate.[19] The latter reaction is also inhibited by treatment of rats with MC.[19]

It is not known at present which structure is preferentially hydroxylated after changes in liver microsomes induced with PB or MC. When directly bound to the aromatic ring the acetic acid moiety is more rapidly hydroxylated after treatment with PB and when bound to the nitrogen of an arylamine is more rapidly hydroxylated after treatment with MC.

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