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MIXED FUNCTION OXYGENATION OF THE LOWER FATTY ACYL RESIDUES—I. HYDROXYLATION OF THE ACETIC, PROPIONIC, BUTYRIC, AND VALERIC RESIDUES BY RABBIT LIVER MICROSOMES*

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Abstract—Rates of hydroxylation of C-atoms in various positions [ω-, (ω-1)-, (ω-2)-] of an increasing chain length have been measured with the 4-chloroanilides of acetic, propionic, butyric, and valeric acid as substrates. The hydroxylation products were separated by t.l.c., quantified by u.v. spectroscopy and further characterized by n.m.r. and o.r.d. spectroscopy. The hydroxylation products in which an asymmetric centre had been introduced by oxygenation, were shown to be optically active, the sign of the optical rotation indicating an excess of the *S*- over the *R*-isomer. From the alteration of the hydroxylation pattern caused by previous treatment of rabbits with either phenobarbital or 3-methylcholanthrene it can be deduced that the ω, (ω-1), and (ω-2)-hydroxylation of the lower fatty acyl residues is not only catalysed by PB- and 3-MCh-inducible forms of cytochrome P-450 but also by the forms not inducible by either PB or 3-MCh.

Whereas ω- and (ω-1)-hydroxylation has been studied intensively *in vitro* with unbranched saturated fatty acids of long (C_{14}–C_{18}) and medium (C_8–C_{13}) chain length [1–5], and besides, ω-, (ω-1)-, and (ω-2)-hydroxylation was also found to occur in humans to whom labelled 3,6-dimethyloctanoic acid had been administered [6], reports on ω-, (ω-1)- or (ω-2)-hydroxylation of the lower fatty acids are scanty. Kuhn *et al.* [7], who detected the enhancing effect of amide or anilide formation on the ω-oxidation, found that *N*-phenyl-2,4-hexadienamide was ω-oxidized in the rabbit but that no ω-oxidation product was detected in the urine when the lower homolog *N*-phenyl-2-butenamide was administered. Instead, *N*-(4-hydroxyphenyl)-2-butenamide was found. The finding that even acetic acid, the lowest fatty acid susceptible to ω-oxidation, was ω-oxidized in rabbits when administered as an appropriately substituted anilide [8–13], offered an opportunity for studying ω-, (ω-1)-, and (ω-2)-hydroxylation of the lower fatty acids when offered as 4-chloranilides. In the first part of this study [14] the hydroxylation rates of the acetic and propionic acid residues have been determined and the effect of induction with either PB or 3-MCh on the hydroxylation pattern described. These experiments have been resumed because of methodological improvements and have been extended to the butyric and valeric residues. The effects of treatment with either PB or 3-MCh and of chain length on the specific ω-, (ω-1)-, and (ω-2)-hydroxylation activities have been investigated. The results of this study are meant as a contribution to differences in catalytic activity and substrate specificity of multiple forms of microsomal cytochrome P-450.

MATERIALS AND METHODS

Biologicals and chemicals. $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate-dehydrogenase were purchased from Fa. Boehringer & Söhne, Mannheim. NaF and $MgCl_2$ were purchased from Fa. Merck AG, Darmstadt. *N*-(4-chlorophenyl)-acetamide (AA) and *N*-(4-chlorophenyl)propanamide (PA), respectively, were prepared as previously described [9, 15]. *N*-(4-chlorophenyl)butanamide was prepared by the reaction of butanoylchloride with freshly distilled 4-chloroaniline and *N*,*N*-dimethylaniline.

After recrystallization from a 50% aqueous solution of ethanol the dried material melted at 102.5–103.5°, reported m.p. 104° [16]. *N*-(4-chlorophenyl)-pentanamide was prepared in the same way from pentanoylchloride. After recrystallization from a 50% aqueous ethanol solution the dried material melted at 102–103° (corr.), reported m.p. 104° [17].

Both 4-chloranilides were further characterized by n.m.r.-spectroscopy.

All melting points were determined with the Tottoli apparatus and corrected. u.v. Absorption spectra, n.m.r. spectra, and o.r.d.-curves were obtained by using a Cary 118 spectrophotometer (Varian), a

*Some of the results were briefly presented at the Joint Meeting of the German and British Pharmacological Society at Berlin [*Naunyn-Schmiedeberg's Arch. Pharmak.* **279**, R 31 (No. 49) (1973)].

Abbreviations: AA, 4-chloroacetanilide (C.A., *N*-(4-chlorophenyl)-acetamide); GA, 4-chloroglycolanilide (C.A., *N*-(4-chlorophenyl)-2-hydroxyacetamide); PA, 4-chloropropionanilide (C.A., *N*-(4-chlorophenyl)-propanamide); HA, 4-chlorohydracrylanilide (C.A., *N*-(4-chlorophenyl)-3-hydroxypropanamide); LA, 4-chlorolactanilide (C.A., *N*-(4-chlorophenyl)-2-hydroxypropanamide); BA, 4′-chlorobutyranilide (C.A., *N*-(4-chlorophenyl)-butanamide); VA, 4′-chlorovaleranilide (C.A., *N*-(4-chlorophenyl)-pentanamide); PNPE, propyl *p*-nitrophenyl ether (C.A., 1-nitro-4-propoxybenzene); PB, phenobarbital (C.A., 5-ethyl-5-phenyl-2,3,6-(1H,3H,5H)-pyrimidinetrione); B(a)P, (C.A., benzo(a)pyrene); 3-MCh, 3-methylcholanthrene (C.A., 1.2-dihydro-3-methyl-benz(j)aceanthrylene).

Varian HA-100 spectrometer, and a Cary 60 spectropolarimeter (Varian), respectively.

Methods. o.r.d. Curves of *N*-(4-chlorophenyl)-2-hydroxybutanamide, *N*-(4-chlorophenyl)-3-hydroxybutanamide, *N*-(4-chlorophenyl)-3-hydroxypentanamide, and *N*-(4-chlorophenyl)-4-hydroxypentanamide, isolated from microsomal incubates and dissolved in methanol, were recorded from 600 to 300 nm.

Animals and pretreatment. Male or female rabbits (Blausilber) of 2.0–2.5 kg weight were housed in stainless steel metabolic cages and fed a standard laboratory diet (Altromin GmbH, D-4937 Lage/Lippe, FRG) and water *ad lib.* For the treatment with PB an aqueous solution of sodium phenobarbital was adjusted to pH 7.4 and the suspension s.c.-injected once daily. The animals received a daily dose of 50 mg PB/kg and were killed on the day following the seventh injection.

For the treatment with 3-MCh a suspension of 3-MCh in olive oil was i.p.-injected on days 1, 4, 7 and 11 during two weeks. The animals received a daily dose of 10 mg 3-MCh/kg and were killed on the day following the fourth injection.

Microsome preparation. In the experiments with the control or the induced enzyme system, microsomes were prepared from the pooled livers of 4–10 male or female untreated or treated rabbits weighing 2.0–2.5 kg. Since sex differences in drug metabolism have not been found to exist in rabbits [18–20], no mention is made of the sex of the animals from which liver microsomes have been prepared.

Liver microsomes were prepared at 4° from pieces of liver by using a mincer as described earlier [21]. The pulp was suspended in 2 vol of 0.15 M KCl and homogenized for 30 min. The suspension was centrifuged for 30 min at 9900 *g* in an MSE centrifuge. The sediment was discarded and the supernatant centrifuged for 1 hr at 90,000 *g*. Half of the supernatant was replaced by 0.15 M KCl, the sediment homogenized, and the suspension centrifuged for another hr at 90,000 *g*. The supernatant was completely decanted and the sediment resuspended in 0.15 M KCl and centrifuged for 1 hr at 90,000 *g*. After complete removal of the supernatant the washing was repeated once more and the final pellet resuspended in 0.15 M potassium phosphate buffer, pH 7.4.

Assay of protein content. The protein content of microsomes was determined according to Robinson and Hogden [22] with the modification introduced by Szarkowska and Klingenberg [23].

Assay of ω-, (ω-1)-, and (ω-2)-hydroxylation activities. Microsomal incubates, 100 ml each in duplicate, contained 1.2 mM $NADP^+$, 10 mM glucose-6-phosphate, 350 i.u. of glucose-6-phosphate dehydrogenase per l, 6 mM $MgCl_2$, 0.1 mM NaF, and microsomal protein ranging from 1 to 3 mg/ml, depending on substrate; with AA 3 mg/ml and with PA, BA, and VA 1 mg/ml. The solvent was 0.15 M potassium phosphate buffer, pH 7.4. Substrates were added as methanol solutions (0.5 ml) at final concentrations of 10^{-3} M. The flasks were incubated for 15 min at 37° in a shaking bath. The frequency was 185 oscillations per min. Incubation was stopped by the addition of an equal volume of ethylacetate. After combination of the duplicates and removal of the organic layer the pH of the microsomal suspension was adjusted to 4.5 by the addition of 6 ml 2 M HCl. Another extraction with an equal volume of ethylacetate followed. The extracts were combined, dried with anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in a few ml of chloroform–methanol (80:20) and the solution streaked on to t.l.c. glass plates (20 × 40 cm) covered with a 0.6 mm layer of silica 60 PF_{254} supplied by Merck AG, Darmstadt.

After the reference compounds had been spotted on the plates, the chromatograms were developed twice with the solvent system chloroform–methanol (98:2) and then with 95:5 until the zones containing the hydroxy derivatives were resolved. Corresponding zones from parallel t.l.c. plates were removed and the metabolites quantitatively eluted with methanol (p.a.). The u.v. absorptions of the centrifuged eluates were scanned between 320 and 218 nm and the extinctions at 247 (GA) and 249 nm (all the other hydroxy derivatives) taken for the evaluation of the amounts of metabolite formed. This was achieved by means of extinction values of known amounts of metabolites, which had been either synthesized (GA) or isolated from microsomal incubates.

Substrate-induced binding spectra of PA, LA and HA. Six millilitres of a microsomal suspension with a protein concentration of 2 mg/ml (buffer: 0.15 M potassium phosphate, pH 7.4) was divided into *S* and *R*. A base line was recorded from 500 to 510 nm using an extension scale of 0.0–0.1. Ten microlitres each of a 0.175 M PA, 0.178 M LA, and 0.178 M HA in methanol were added to *S* to give final concentrations of 0.7 mM. The same amount of methanol was added to *R*. Absorbance was scanned between 500 and 370 nm, and the base line substracted from the experimental curves.

Statistics. In order to prove statistically significant differences among the means of three groups of observations, the results with the control enzyme system have been compared with those of the PB- or 3-MCh-stimulated enzyme system by means of the *t* test for independent observations. The hypothesis was tested that $\mu_1 \neq \mu_2$ and $\mu_1 \neq \mu_3$. Included in Table 2 are the computed values for *t* and d*f* (degrees of freedom) which have been compared with the corresponding *t* values from tables, and the probabilities (P) for accepting the hypothesis. Values of P = 0.95 indicate significance on the 5 per cent level.

RESULTS

Characteristics of ω- and (ω-1)-hydroxylation of PA

Omission of NADPH from the complete incubation system caused a complete block of ω- and (ω-1)-hydroxylation, and replacement of NADPH by 1.2 mM NADH, a pronounced drop in the specific ω- and (ω-1)-hydroxylation activity which was only 10–17 per cent of that seen with the complete system.

Addition of NADH to the complete system resulted in an increase in both activities. Reports in the literature suggested that there is a requirement for Mg^{2+} for mixed function oxygenation if buffers other than potassium phosphate buffer (pH 7.4) are used. The results indicate that omission of Mg^{2+} either from the complete system or from the complete

system to which NADH was added, caused no significant changes in (ω-1)-hydroxylation activity ($\bar{M}$ = 94.2 per cent of control), whereas ω-hydroxylation activity appeared slightly decreased ($\bar{M}$ = 82.1 per cent of control). Without any further attempt to prove statistical significance of this effect, Mg^{2+} was included in the following experiments. To test the effect of lipid peroxidation on ω- and (ω-1)-hydroxylation activity, EDTA, an inhibitor of lipid peroxidation, was added to the complete system. From the data of this experiment it can be deduced that addition of 100 μM EDTA did not enhance ω- and (ω-1)-hydroxylation activity which would have been the case if lipid peroxidation were prominent, but rather decreased it. No attempt was made to prove the statistical significance of this effect. The way in which addition of NADH affects the kinetics of ω- and (ω-1)-hydroxylation of PA is shown in a subsequent paper.

Stability on storage of the PB- and 3-MCh-inducible forms of cytochrome P-450 effecting ω- and (ω-1)-hydroxylation

As can be seen from Fig. 1 in [14], ω-hydroxylation activity with PA as substrate decreased on storage of PB-stimulated microsomes at 0° within 14 days by 40 per cent whereas (ω-1)-hydroxylation activity maintained its full activity during this period. The stability of the 3-MCh-induced enzyme system was tested with AA as substrate, and found to be stable over the period of observations, i.e. 18 days.

Prerequisites for determining formation rates

(1) *Time course of alteration of ω- and (ω-1)-hydroxylation activities during prolonged treatment of rabbits with PB.* After PB treatment, ω-hydroxylation, which is low in controls, increased up to the 7th day, then declined until the 21st day of injection and thereafter reached a level higher than that of (ω-1)-hydroxylation, which declined from a high level of activity until the 14th day of injection and thereafter also reached a steady state (Fig. 1). Based on the results of this experiment, PB-stimulated liver microsomes were routinely prepared from animals which had received seven daily injections of PB.

(2) *The dependence of specific ω-, (ω-1)-, and (ω-2)-hydroxylation activity on protein concentration.* Whereas only one hydroxy derivative was formed from AA, two hydroxy derivatives were formed from PA, and three from BA or VA. With hepatic microsomes from untreated animals a linear relationship was found between protein concentration and the rate of ω- and (ω-1)-hydroxylation of PA up to protein concentrations of 4 mg/ml. With BA as substrate, a linear relationship between the rate of ω-, (ω-1)-, and (ω-2)-hydroxylation and protein concentration was found to exist only up to 2 mg/ml. In order to test the effect of PB- and 3-MCh-induction on the relationship between the rate of ω- and (ω-1)-hydroxylation of PA and protein concentration, the specific activities of the induced enzyme systems were compared with that of the control system. As the results obtained with the PB-induced enzyme system indicate, the linear relationship between the rate of ω- and (ω-1)-hydroxylation and protein concentration was abolished at protein concentrations of 2 mg/ml, regardless of whether the period of treatment was 4 or 14 days. The same results were obtained with the 3-MCh-induced enzyme system. Therefore, protein concentrations used in the experiments with the substrates PA, BA, and VA were 1 mg/ml and 3 mg/ml for AA.

(3) *Progress curves for the ω- and (ω-1)-hydroxylation of PA.* In the optimal incubation conditions (see Methods) either ω- or (ω-1)-hydroxylation was linear with time for at least 20 min of incubation. Therefore the incubation period in the kinetic experiments was limited to 15 min.

(4) *Sensitivity of the assay method and interference with microsomal lipids.* When known amounts (0.05–1.8 mg) of synthetic GA, LA, HA or other metabolites were chromatographed and measured spectrophotometrically, the average recovery was 89 per cent. As a rule, amounts of metabolites from 0.1 to 2.65 mg could be assayed without interference. However, when the amounts of metabolites were small (< 0.1 mg), or on rare occasions with large amounts, the u.v. absorption spectrum of the eluted spot was distorted. Rechromatography of such samples changed the u.v. absorption curves to normal. Spectrophotometric determination of the re-chromatographed samples (< 0.1 mg) gave a recovery of 52–78 per cent of the original absorbance. Evidence was obtained

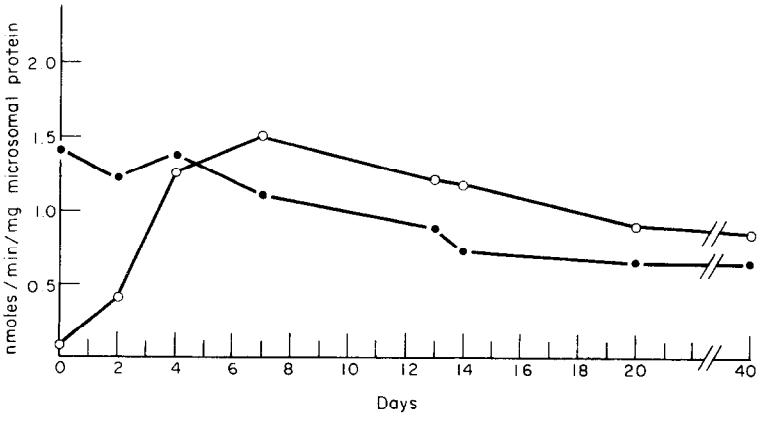


Fig. 1. Effect of daily s.c. injections of PB into rabbits on the specific ω- and (ω-1)-hydroxylation activity of liver microsomes with PA as substrate. Symbols represent the results of a single experiment: ω-hydroxylation (○) and (ω-1)-hydroxylation (●).

Table 1. Hydroxylation rates of C-atoms in different positions of lower fatty acyl residues, catalyzed by rabbit liver microsomes, and the effect of treatment of animals with either phenobarbital or 3-methylcholanthrene

Substrate	Metabolites	Controls	PB	3-MCh	Comparison: Controls → PB t (df)	Comparison: Controls → 3-MCh t (df)
N-(4-chlorophenyl)-acetamide (AA)	*N*-(4-chlorophenyl)-2-hydroxyacetamide (GA)	0.170 ± 0.021 (6)	0.080 ± 0.011 (5)	0.33 ± 0.022 (6)	3.518† (9)	5.136* (12)
	N-(4-chlorophenyl)-3-hydroxypropanamide (HA)	0.083 ± 0.008 (10)	1.237 ± 0.063 (17)	0.093 ± 0.011 (6)	13.81* (25)	0.71 (14)
N-(4-chlorophenyl)-propanamide (PA)	*N*-(4-chlorophenyl)-2-hydroxypropanamide (LA)	1.367 ± 0.121 (11)	1.015 ± 0.058 (17)	2.738 ± 0.525 (5)	2.905† (26)	3.562† (14)
	N-(4-chlorophenyl)-4-hydroxybutanamide (ω)	0.213 ± 0.017 (10)	0.296 ± 0.021 (11)	0.226 ± 0.019 (6)	2.988† (19)	0.481 (14)
N-(4-chlorophenyl)-butanamide (BA)	*N*-(4-chlorophenyl)-3-hydroxybutanamide (ω-1)	1.280 ± 0.107 (10)	1.969 ± 0.133 (11)	1.126 ± 0.086 (6)	3.975* (19)	0.996 (14)
	N-(4-chlorophenyl)-2-hydroxybutanamide (ω-2)	0.519 ± 0.072 (10)	0.322 ± 0.033 (11)	0.939 ± 0.182 (4)	2.57‡ (19)	2.652§ (12)
	N-(4-chlorophenyl)-5-hydroxypentanamide (ω)	0.196 ± 0.012 (10)	0.489 ± 0.034 (10)	0.155 ± 0.021 (6)	8.13* (18)	1.891‖ (14)
N-(4-chlorophenyl)-pentanamide (VA)	*N*-(4-chlorophenyl)-4-hydroxypentanamide (ω-1)	2.599 ± 0.158 (10)	3.140 ± 0.195 (9)	2.342 ± 0.209 (6)	2.169§ (17)	0.985 (14)
	N-(4-chlorophenyl)-3-hydroxypentanamide (ω-2)	0.383 ± 0.028 (10)	0.378 ± 0.026 (10)	0.344 ± 0.029 (6)	0.127 (18)	0.919 (14)

Microsomal protein concentrations varied between 1 and 3 mg per ml of incubate, substrate concentrations used 10^{-3} M. The figures indicate means of nM of hydroxylation product formed per min per mg of microsomal protein and standard error, with the number of experiments given in brackets. Columns 3–7 show the results of the statistical test for significance.* $P > 0.999$; † $P > 0.995$; ‡ $P > 0.99$; § $P > 0.975$; ‖ $P > 0.95$.

from chromatographic studies and n.m.r. spectroscopy that microsomal lipids were the agents which obscured the u.v. absorption curves of the metabolites.

Re-chromatography of amounts of metabolites from 0.1 to 2.65 mg which displayed normal u.v. absorption curves as a rule, also indicated the presence of microsomal lipids, although their proportion was too small to cause any distortion of the u.v. absorption curves. Spectrophotometric determination of the re-chromatographed samples gave recoveries of 60–98 per cent. The data of Table 2 constitute uncorrected originals or are corrected on the basis of the mean recovery of 89 per cent.

ω-Hydroxylation of AA

The identification of the oxygenation product of AA isolated from microsomal incubates as being GA was described earlier [9]. From the results shown in Table 2 it can be seen that the specific ω-hydroxylation activity of the control enzyme was low and that previous treatment with PB significantly decreased it, whereas 3-MCh caused a 2-fold increase.

ω- and (ω-1)-Hydroxylation of PA

The identification of the two hydroxy derivatives of PA isolated from microsomal incubates as being HA (ω-) and LA (ω-1) was described earlier, as was the assessment of the excess of the *S*-isomer of LA over the *R*-isomer [15]. As can be seen from Table 2, the specific ω-hydroxylation activity of the control enzyme system was low, but increased 15-fold after previous treatment with PB. In contrast, treatment with 3-MCh did not affect it. Specific (ω-1)-hydroxylation activity was high with the control enzyme system. PB-treatment was found to decrease this activity significantly, while previous treatment with 3-MCh increased it 2-fold. Thus, the hydroxylation pattern observed for the (ω-1)-C-atom of the propionic residue closely resembled that obtained for the ω-C-atom of the acetic moiety.

The results obtained with the acetyl and propionyl residues differ from those presented in Table 1 of [14]. The reason may be found in the effect of 12 mM nicotinamide which was included in the previous work. The effect of 12 mM nicotinamide on the kinetics of ω- and (ω-1)-hydroxylation is described in a forthcoming paper. In addition, the dependency of the discrete specific hydroxylation activities on microsomal protein concentration was neglected in the previous work.

Difference spectra

Addition of PA or its hydroxy derivatives LA and HA to suspensions of PB-stimulated rabbit liver microsomes caused the appearance of a type I binding spectrum with troughs between 422 and 430 nm and maxima between 405 and 390 nm. Attempts to estimate quantitative changes in the difference spectra by increasing the amounts of added substrates failed because addition of twice the amount of substrate increased turbidity of the suspension, but not the amplitude of the spectral changes.

ω-, (ω-1)-, and (ω-2)-Hydroxylation of BA

Ethylacetate extracts of microsomal incubates to which BA had been added prior to incubation contained three metabolites, the ω-hydroxy derivative being the most polar and the (ω-2)-hydroxy derivative the least polar compound. This was proved by the n.m.r. data shown in Table 2. The yield of several incubations enabled the identification of the metabolites by n.m.r. spectroscopy and o.r.d. measurements.

N-(4-*chlorophenyl*)-4-*hydroxybutanamide*. The crystallized ω-hydroxy derivative of BA melted at 105–106°. As can be seen from Table 1, the specific ω-hydroxylation activity was low with the control enzyme system and increased only to a small but significant extent after previous treatment with PB, whereas treatment with 3-MCh did not affect it.

N-(4-*chlorophenyl*)-3-*hydroxybutanamide*. The crystallized (ω-1)-hydroxy derivative of BA melted at 125–126°, and the u.v. spectrum in methanol solution had an absorption maximum at 247–248 nm (ε = 19,266). The n.m.r. data were indicative of a hydrogen bridge between the hydroxyl at C-3 and the carbonyl group by which a six-membered ring is formed. It was dextrarotatory and gave a specific rotation of

$$[\alpha]_{589}^{27} = +5.4°,\ [\alpha]_{420}^{27} = +12.9°,\ [\alpha]_{320}^{27} = +33.6°.$$

Whereas *S*-3-hydroxybutanoic acid itself is dextrarotatory [24], its sodium salt [24] and methylester [25] are also dextrarotatory. In contrast, *R*-3-hydroxybutanoic hydrazide is levorotatory [26], therefore *S*-3-hydroxybutanoic hydrazide must also be dextrarotatory. It can therefore be assumed that the amide and the anilide of *S*-3-hydroxybutanoic acid are also dextrarotatory. Thus the isolated metabolite constituted a mixture of the *S*(+) and *R*(−)-isomers with an excess of the *S*(+)-isomer. An assessment of the excess of the *S*(+)-isomer was not possible, since the optical properties of the pure synthetic compound are not known.

As can be seen from Table 1, the specific (ω-1)-hydroxylation activity was relatively high with the control enzyme system, and previous treatment with PB still increased it significantly, whereas 3-MCh-treatment did not affect it. The hydroxylation pattern of the ω- and (ω-1)-C-atom resembled closely that observed for the ω-C-atom of the propionic residue.

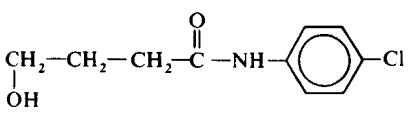
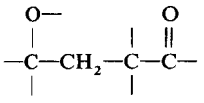
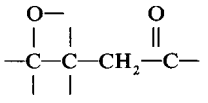
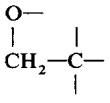
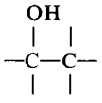
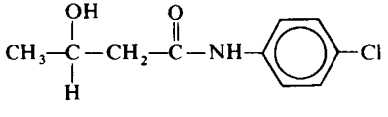
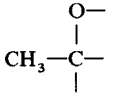
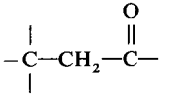
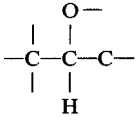
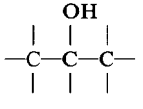
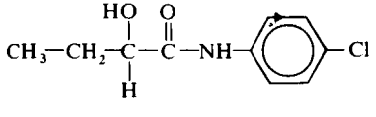
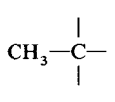
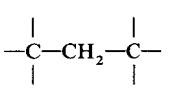
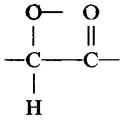
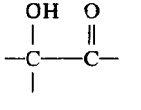
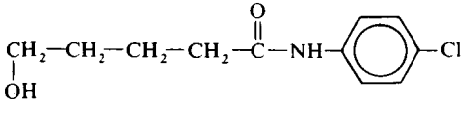
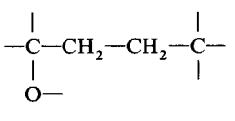
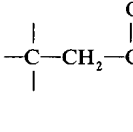
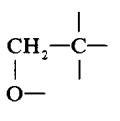
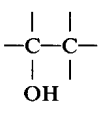
N-(4-*chlorophenyl*)-2-*hydroxybutanamide*. The crystallized (ω-2)-hydroxy derivative of BA melted at 131–132°; it was levorotatory, having a specific rotation of

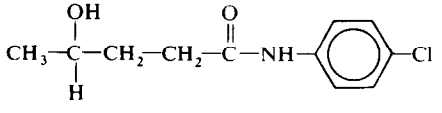
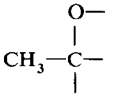
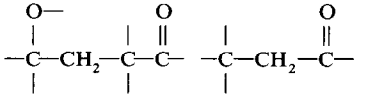
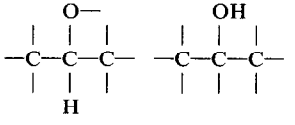
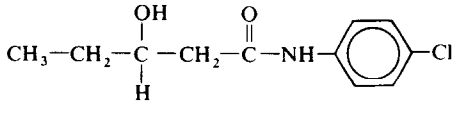
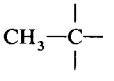
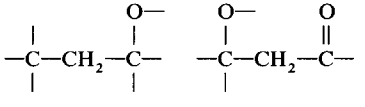
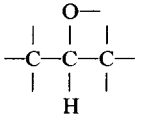
$$[\alpha]_{589}^{27} = -48.8°,\ [\alpha]_{420}^{27} = -107.3°,\ [\alpha]_{320}^{27} = -279.7°.$$

By analogy to *S*-lactic acid, *S*-2-hydroxybutanoic acid itself is dextrarotatory [27], whereas its Ba-salt [28] and its methylester are levorotatory [29]. Although the optical properties of the amide or the anilide are not known, it can be assumed (from the analogy with *S*-lactic acid) that both compounds should be levorotatory. The isolated metabolite thus constituted a mixture of the *S*(−) and *R*(+)-isomers with an excess of the *S*(−)isomer. The assessment of the excess of the *S*(−)isomer was not possible because of lack of optical data for the pure synthetic isomer.

As can be seen from Table 1, previous treatment

Table 2. Nuclear magnetic resonance data of oxygenation products of *N*-(4-chlorophenyl)-butanamide and *N*-(4-chlorophenyl)-pentanamide isolated from microsomal incubates and dissolved in d_6-DMSO (new compounds)

	Aliphatic H and —OH				Aromatic H	—NH—
						
N-(4-chlorophenyl)-4-hydroxybutanamide	1.72(2) m	2.36(2) t J = 8 c.p.s.	3.44(2) t + d After exch: t J = 5 and 8 c.p.s.	4.48(1) t Exch. ag. D	3/5-H 7.3 2/6-H 7.6 $A_2B_2(4)$	9.98(1) Exch. ag. D
						
N-(4-chlorophenyl)-3-hydroxybutanamide	1.11(3) d J = 7 c.p.s.	2.37(2) AB-system steric fixation by H-bridge	4.08(1) m	4.50(1) d Exch. ag. D J = 5 c.p.s.	3/5-H 7.1 2/6-H 7.6 $A_2B_2(4)$	9.95(1) Exch. ag. D
						
N-(4-chlorophenyl)-2-hydroxybutanamide	0.92(3) t J = 7 c.p.s.	1.66(2) m	3.97(1) Double doublet* J = 5 and 7 c.p.s.	5.8(1) broad	3/5-H 7.3 2/6-H 7.8 $A_2B_2(4)$	9.78(1) Exch. ag. D
						
N-(4-chlorophenyl)-5-hydroxypentanamide	ca. 1.5(4) m	2.30(2) t J = 7 c.p.s.	3.40(2) After exch. ag. D: t J = 6 c.p.s.	4.36(1) t Exch. ag. D	3/5-H 7.3 2/6-H 7.6 $A_2B_2(4)$	9.95(1) Exch. ag. D

							
N-(4-chlorophenyl)-4-hydroxypentanamide	1.06(3) d J = 6.5 c.p.s.	1.66(2) m	2.37(2) t J = 8 c.p.s.	3.62(1) m	4.45(1) d Exch. ag. D J = 5 c.p.s.	3/5-H 7.3 2/6-H 7.6 $A_2B_2(4)$	9.98(1) Exch. ag. D
							
N-(4-chlorophenyl)-3-hydroxypentanamide	0.87(3) t J = 7 c.p.s.	ca. 1.4(2) m	2.36(2) d J = 6 c.p.s.	3.86(1) t J = 6 c.p.s.		3/5-H 7.3 2/6-H 7.6 $A_2B_2(4)$	9.96(1) Exch. ag. D

* By inhibition of free rotation.

with PB caused a distinct and significant decrease of the specific (ω-2)-hydroxylation activity, wereas treatment with 3-MCh increased it nearly 2-fold. The hydroxylation pattern of the (ω-2)-C-atom resembled that seen with the ω-C-atom of the acetic and (ω-1)-C-atom of propionic residue. All three C-atoms have in common a position α to the carboxamide group.

ω-, (ω-1)-, and (ω-2)-Hydroxylation of VA

Ethyl acetate extracts of microsomal incubates to which VA had been added as substrate, were found by t.l.c. to contain three metabolites in substantial amounts and only traces of a fourth metabolite. No efforts were therefore made to isolate this last compound, which most like constituted the (ω-3)-hydroxy derivative of VA. The n.m.r. data of the other three metabolites (see Table 2) proved that they are (in decreasing order of polarity) the ω-, (ω-1)-, and (ω-2)-hydroxy derivatives of VA.

N-(4-*chlorophenyl*)-5-*hydroxypentanamide*. The crystallized ω-hydroxy derivative of VA melted at 117–118°. As can be seen from Table 1, the specific ω-hydroxylation activity with the control enzyme system was as low as with the butyric residue. PB-induction caused a more than 2-fold increase, whereas 3-MCh-induction decreased it.

N-(4-*chlorophenyl*)-4-*hydroxypentanamide*. The (ω-1)- hydroxy derivative of VA was recrystallized from water and melted at 118–119°. The u.v. spectrum had an absorption maximum at 247–248 nm (ε = 19,824), and it was dextrarotatory, the specific rotation being assessed as

$$[\alpha]_{589}^{27} = +2.5°, [\alpha]_{420}^{27} = +6.8°, [\alpha]_{320}^{27} = +13.1°.$$

S-4-hydroxypentanoic acid itself is dextrarotatory, and so are the corresponding Na-salt, amide, and hydrazide [30, 31]. It can be deduced that the anilide also will be dextrarotatory. The isolated (ω-1)-hydroxy derivative of VA therefore constituted a mixture of *S*(+) and *R*(−)-isomers with an excess of the *S*(+)-isomer. The assessment of the excess was not possible because the optical properties of the pure synthetic compound are not known.

As can be seen from Table 1, the specific (ω-1)-hydroxylation activity was higher with the control system as compared with the butyric residue; in fact, it was the highest of all the activities observed. Whereas PB induction increased it slightly but significantly, 3-MCh did not change the activity. The hydroxylation pattern of the ω- and (ω-1)-C-atoms resembled the pattern obtained for the corresponding C-atoms of the butyric residue and for the C-atom in the ω-position of the propionic residue.

N-(4-*chlorophenyl*)-3-*hydroxypentanamide*. The (ω-2)-hydroxy derivative of VA was dextrarotatory and showed a specific rotation of

$$[\alpha]_{589}^{27} = +10.5°, [\alpha]_{420}^{27} = +23.1°, [\alpha]_{320}^{27} = +56.8°.$$

S-3-hydroxypentanoic acid as well as the Ba-salt is dextrarotatory [32]. Since the ethylester of *R*-3-hydroxypentanoic acid is levorotatory [33], the corresponding *S*-isomer must be dextrarotatory. It can therefore be assumed that the amide or anilide of *S*-3-hydroxypentanoic acid will be dextrarotatory, too. The isolated metabolite thus constituted a mixture of the *S*(+)- and *R*(−)-isomer with an excess of the *S*(+)-isomer. The assessment of the excess was not possible since the pure synthetic isomer is not known.

As can be seen from Table 1, the specific (ω-2)-hydroxylation activity with the control enzyme system was as low as with the butyric residue; however, in contrast to the lower homolog, neither PB- nor 3-MCh affected it.

DISCUSSION

Three distinct hydroxylation patterns were observed as a result of exposure to either PB or 3-MCh. These patterns depended on the position of the C-atom in the carbon chain as follows:

1. With the C-atom in ω-position of the acetic, in (ω-1)-position of the propionic, and in (ω-2)-position of the butyric residues, all these positions being α to the carboxamide moiety, there was a decrease in the specific hydroxylation activity due to PB-induction and an increase due to 3-MCh-induction. This is also valid, if the aryl substituent at the N-atom of the acetamido group is replaced by another aromatic residue [34–36]. Differences in the velocity within the control or the induced enzyme systems probably reflect differences in substrate affinity. The decrease of the specific hydroxylation affinity due to PB-induction can be explained with an increase in the amount of PB-inducible form(s) of cytochrome P-450 (which do not participate in the oxygenation of these C-atoms)at the expense of those forms which do participate and are not inducible by PB. Such an assumption has been experimentally supported first by Welton and Aust [37]. The finding that the specific hydroxylation activity increased after 3-MCh-induction and that the increase was proportional to the specific activity of the control enzyme system (2-, 2-, and 1.8-fold), suggested that the C-atoms marked by a dashed line in Fig. 2 were oxygenated by 3-MCh-inducible forms of cytochrome P-450.

2. With the C-atom in the ω-position of the propionic and the C-atoms in ω- and (ω-1)-positions of the butyric and valeric residues, there was an increase in the specific hydroxylation activities due to PB-induction, and 3-MCh-pretreatment did not change it, except for the ω-hydroxylation activity of the valeric residue which was decreased. These C-atoms have been marked with a solid line (see Fig. 2). The observed decrease of the specific hydroxylation activity due to 3-MCh-induction points to an increase in the amount of the 3-MCh-inducible form(s) of cytochrome P-450 (which do not participate in the oxygenation of these C-atoms) at the expense of those forms which do participate and are inducible by PB. Experimental support for this assumption has been given by Haugen *et al.* [38] and Atlas *et al.* [39]. Since the increase of the specific hydroxylation activity due to PB-induction was not proportional to the activity of the control enzyme system (15.0-, 1.4-, 1.5-, 2.5-, and 1.2-fold), participation of other forms of cytochrome P-450 can be assumed which are neither inducible by PB or 3-MCh. A participation of such forms of cytochrome P-450 and of PB-inducible forms of the hemoprotein could, however,

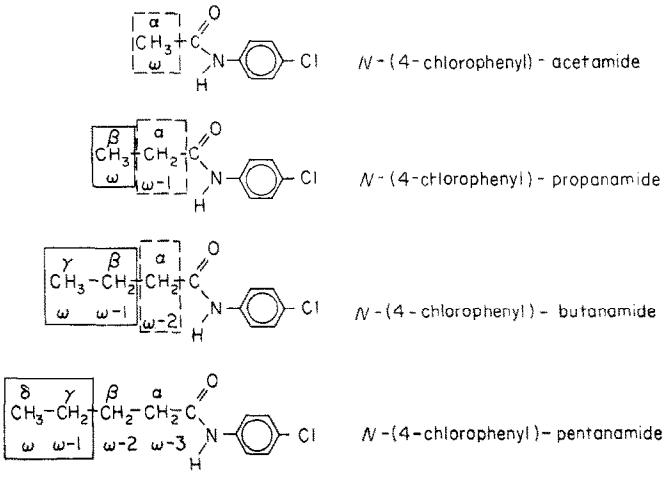


Fig. 2. Substrate specificity of the control, PB-, and 3-MCh-stimulated mixed function oxygenase system in hepatic microsomes from rabbits. C-atoms in various positions of carbon chains which are oxygenated by 3-MCh-inducible form(s) of cytochrome P-450 hemoprotein and share a distinct hydroxylation pattern, are marked by a dashed line, whereas those which are oxygenated by PB-inducible form(s) and have a different hydroxylation pattern in common, are marked by a solid line.

explain the differential increase of the specific hydroxylation activity. Since there is no evidence for the participation of the PB-inducible form of cytochrome P-450 in the ω-hydroxylation of the acetic moiety, it may be assumed that it is the size of the acetyl moiety that does not meet the steric requirements for these forms of cytochrome P-450.

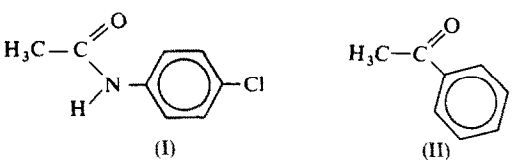
3. With the C-atom in the (ω-2)-position of the valeric residue there was no change in the specific hydroxylation activity, either by PB- or by 3-MCh-induction.

There is no evidence for the participation of either PB- or 3-MCh-inducible forms of cytochrome P-450 in the hydroxylation of this C-atom.

Whereas the velocities of ω-, (ω-1)-, and (ω-2)-hydroxylations of the valeric residue differ by an order of 10, a pronounced drop in the hydroxylation rate of the (ω-3)-C-atom was observed. Due to this low velocity insufficient amounts of the hydroxy derivative were accumulated to enable its identification.

Other examples of substrate specificity and differences in catalytic activity of the multiple forms of cytochrome P-450 have recently been published by Kremers *et al.* [40] and Stohs *et al.* [41]. Evidence for the participation of cytochrome P-450 and the other components of the electron transport chain, respectively, functioning in hepatic microsomes of mammals, in the ω- [1, 2, 3, 14] and (ω-1)-oxygenation [3, 14] of long-chain fatty acids, of medium [2, 3], and of short chain length [14], have been presented by several investigators. The effect of inducing the hepatic microsomal monooxygenases in rats or rabbits with either PB or 3-MCh- on the specific ω- or (ω-1)-hydroxylation activity with respect to dodecanoic, hexadecanoic, and octadecanoic acid have been investigated by Wada *et al.* [1], Lu *et al.* [2], and Björkhem and Danielson [3]. Whereas Lu *et al.* reported on an enhancing effect of the PB-induction in rats or rabbits on the ω-hydroxylation of dodecanoic acid, Björkhem and Danielson, using rat liver preparations, observed no effect of PB-induction on ω-hydroxylation. In addition, the same authors found that the specific ω-hydroxylation activity of hexadecanoic and octadecanoic acid decreased, whereas the specific (ω-1)-hydroxylation activity of dodecanoic, hexadecanoic, and octadecanoic acid increased. Wada *et al.* [1], investigating the effect of PB- or 3-MCh- induction in rats on the rate of ω-hydroxylation of octadecanoic acid, observed a decrease in the velocity with either enzyme preparation.

Different results were obtained from experiments on the effect of PB-induction or induction with either 3-MCh or B(a)P in mice, rats or guinea pigs on the specific ω-, (ω-1)-, (ω-2)-, and (ω-3)-hydroxylation activities with PNPE [42], n-pentane [43], n-hexane [44, 45], and n-heptane [46], respectively. This is not surprising, since these substrates are not acids or derivatives of acids and hence differences in substrate affinity for the multiple forms of cytochrome P-450 are expected. As was demonstrated earlier, ω-hydroxylation of the acetic moiety of AA (I) was enhanced 4-fold by previous treatment of rabbits with 3-MCh, whereas ω-hydroxylation of the acetic moiety of acetophenone (II) was enhanced 7-fold by PB-induction [14]. It can be expected that due to even small changes in the chemical structure of substrates,



(I) (II)

differences in the partition coefficients and in substrate affinity for the multiple forms of cytochrome P-450 arise which cause alterations in the hydroxylation pattern.

In all cases in which an asymmetric centre had been introduced by oxygenation, the *S*(L)-isomer had been

formed in excess of the *R*(D)-isomer; the optical purity of the isolated metabolites, however, could be determined only in one case: 90–92 per cent of the (ω-1)-derivative of PA, LA, consisted of the *S*(−)-isomer [14]. In all the other cases a determination of the optical yield was not possible because of lack of data for the pure optical isomers. This finding is in accordance with the results of experiments reported by Hamberg and Björkhem on the mixed function oxygenation of deuterated decanoic acids [4] in which the *S*(L)-isomer had been formed in large excess (75 per cent) of the *R*(D)-isomer (25 per cent).

In similar experiments with deuterated dodecanoic acids however, Björkhem and Hamberg [5] found that the (ω-1)-derivative consisted of an excess (56–60 per cent) of the *R*(D)-isomer over the *S*(L)-isomer (40–44 per cent). The results presented here indicate that with PA, BA, and VA as substrates predominantly the *S*(L)-H of the (ω-1)-C-atom had been replaced by OH and that this feature of the mixed function oxygenases can be extended to the (ω-2)-C-atom.

The three types of spectral changes observed on the addition of various (drug) substrates to microsomal suspensions have been interpreted as a manifestation of substrate binding to microsomal cytochrome P-450 species which precedes enzymic oxygenation. The similarity between the spectral dissociation constant K_s and the Michaelis constant K_m for the oxidative metabolism of some type I substrates was taken as support for this hypothesis. The finding, however, that not only PA, but also its ω- and (ω-1)-hydroxy derivative displayed a type I difference spectrum does not support such a hypothesis, unless it is assumed that either hydroxy derivative serves as substrate for secondary oxygenation effected by the same oxygenase, an assumption which cannot be excluded, but lacks experimental support at present time. Ellin *et al.* [47] observed a type I difference spectrum on adding dodecanoic acid to rat kidney cortex microsomes, whereas its ω-hydroxy derivative gave a reverse type I spectrum, and its (ω-1)-hydroxy derivative did not cause any spectral change at all. The apparent capacity of cytochrome P-450 to combine with a great number of chemically different compounds points to a lack of structural specificity of the binding sites for substrates [48]. Interpretation of the spectral changes, therefore, seems difficult as long as our knowledge of the nature of the multiple forms of cytochrome P-450 is deficient [49], and binding cannot be correlated with specific metabolic routes.

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