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ω- AND (ω-1)-HYDROXYLATION OF 4-CHLOROPROPIONANILIDE BY RABBITS AND RABBIT LIVER MICROSOMES*

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Abstract—4-Chlorolactanilide, 4-chlorohydracrylanilide, 4-chloroglyceranilide, and 4-chloroglycolanilide were isolated from urine of rabbits injected with 4-chloropropionanilide. The product of (ω-1)-hydroxylation amounted to nearly 30 per cent of the dose and the ω-OH derivative to about 4 per cent. An average 91 per cent of the 4-chlorolactanilide was the L-(−)-isomer. After injection of DL-4-chlorolactanilide only a small shift was found in the ratio between L- and D-isomer of the 4-chlorolactanilide isolated from urine. Rabbit liver microsomes hydroxylated 4-chloropropionanilide more rapidly in the (ω-1)-position than in the ω-position. Ninety-three per cent of the 4-chlorolactanilide isolated was the L-(−)-isomer. 4′-chloromandelanilide isolated from urine after injection of 4′-chlorophenylacetanilide was 94% L-(+)-isomer and methylphenylcarbinol isolated after injection of ethylbenzene was 75% D-(+)-isomer. As all optically active metabolites show the same relative configuration at the asymmetric center, only the configuration at the (ω-1)-C-atom determines the hydrogen which is replaced by a hydroxyl group, the rest of the molecule affecting only binding to the enzyme and rate of hydroxylation.

THE BIOCHEMICAL oxidation of medium-chain and long-chain fatty acids in the ω- and (ω-1)-position has been studied *in vivo* and *in vitro*; a review of the results is being published.[1] Experimental data on the ω- and (ω-1)-oxidation of the lower fatty acids are scanty. Whereas the amide or anilide of unsaturated C_6 fatty acid was found to be ω-oxidized by rabbits, no ω-oxidation product was detected in the urine, when the anilide of unsaturated C_4 acid was administered to rabbits.[2] The discovery that certain *N*-phenylacetamides are transformed into glycolamides and oxanilic acids in rabbits[3–7] established that acetic acid, the lowest fatty acid susceptible to ω-oxidation, is also ω-oxidized *in vivo*, if administered in an appropriate form, e.g., as 4-chloroanilide. It would be interesting to see whether the 4-chloroanilide of propionic acid, the lowest fatty acid susceptible to (ω-1)-oxidation, is oxidized in ω-, (ω-1)- or either position. Since it has recently been shown that hydroxylation at the penultimate carbon atom of an phenylalkane,[8,9] medium-chain,[10] or long-chain fatty acid[11,12] is stereoselective with retention of the absolute configuration, the product of (ω-1)-hydroxylation of 4-chloropropionanilide, 4-chlorolactanilide, was expected to be optically active. In order to assess the importance of certain structures for the stereoselective hydroxylation, structurally related compounds, such as 4′-chlorophenyl-

* Some results were briefly presented at a meeting of the Deutsche Pharmakologische Gesellschaft at Mainz 1970. [*Naunyn-Schmiedeberg's Arch. Pharmakol.* **266**, 390 (1970)].

acetanilide and ethylbenzene, were included in this study. This communication deals with the ω- and (ω-1)-oxygenation of 4-chloropropionanilide and the stereoselective hydroxylation of 4'-chlorophenylacetanilide and ethylbenzene in rabbits and by rabbit liver microsomes *in vitro*.

MATERIALS AND METHODS

Materials. NMR data of the new compounds synthesized are listed in Table 1. NMR spectra of other compounds synthesized and of all metabolites isolated also were recorded in order to confirm their structure. TMS was used as standard.

All melting points, determined with the Tottoli-apparatus, are corrected.

In ORD measurements c is g/100 ml of solution.

The preparation and properties of 4-chloroglycolanilide have been described earlier.[4]

4-Chloropropionanilide was prepared from 4-chloroaniline (3·8 g), *N,N*-dimethylaniline (3·6 g) and propionylchloride (2·8 g) in ether. After recrystallization from 50% aqueous ethanol the m.p. was 140°. Chattaway[13] gave 141°. 4'-Chlorophenylacetanilide was prepared from phenylacetylchloride and 4-chloroaniline in ether. After recrystallization from benzene the m.p. was 167°. Walther and Grossmann[14] gave 163–164°.

All 4-chloroacylanilides described below were synthesized by heating 4-chloroaniline and the respective acid according to Elbs and Sinner[15]. L-(-)-4-Chlorolactanilide from L(+)-lactic acid and 4-chloroaniline, after recrystallization from cyclohexane, m.p. 102·5–104°, specific rotation $[\alpha]_{390}^{22°} = -69{\cdot}1°$ ($c = 0{\cdot}192$ in methanol) $[\alpha]_{589}^{22°} = -22{\cdot}3°$ ($c = 3{\cdot}52$ in methanol), $\lambda_{max} = 248$ nm, $\epsilon_{248} = 18{\cdot}1 \times 10^3$. Brand and Priesner[16] gave m.p. 106° and $[\alpha]_{589} = -22°$ ($c = 10{\cdot}0$ in 90% aqueous ethanol). D,L-4-Chlorolactanilide was obtained by mixing L(-)- and D(+)-4-chlorolactanilide; it contained a slight excess (0·5%) of the D(+)-isomer.

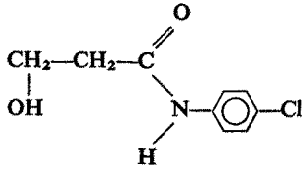
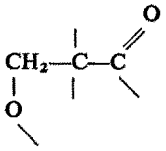
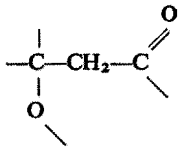
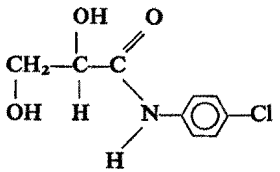
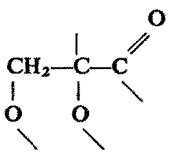
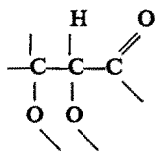
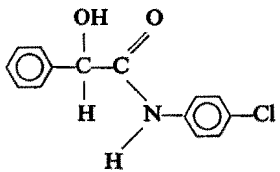
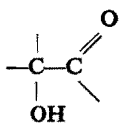
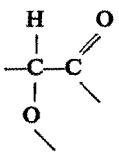
4-Chlorohydracrylanilide (new compound), poor yield. After purification by TLC and recrystallization from benzene the m.p. was 101–102°; in methanol $\lambda_{max} = 249$ nm, $\epsilon_{249} = 19{\cdot}0 \times 10^3$. D,L-4-Chloroglyceranilide (new compound), poor yield. After purification by TLC the oily product crystallized on treatment with chloroform; m.p. 111–112°; in methanol $\lambda_{max} = 247$ nm, $\epsilon_{247} = 21{\cdot}1 \times 10^3$.

L(+)-4'-Chloromandelanilide (new compound). After recrystallization from 50% aqueous ethanol m.p. 107–108°, specific rotation $[\alpha]_{390}^{12°} = +34{\cdot}5°$, $[\alpha]_{589}^{12°} = +20{\cdot}9°$ ($c = 0{\cdot}365$ in methanol); in methanol $\lambda_{max} = 250$ nm, $\epsilon_{250} = 21{\cdot}1 \times 10^3$.

Ethylbenzene and optically inactive methylphenylcarbinol were commercial products. In methanol methylphenylcarbinol showed absorbance maxima at 267 (ϵ = 81·17), 264 ($\epsilon = 137{\cdot}96$), 257 ($\epsilon = 185{\cdot}33$), 251 ($\epsilon = 153{\cdot}75$) and 241 nm ($\epsilon = 84{\cdot}22$).

Methods. For the isolation of 4-chloropropionanilide metabolites the urine collected in 24 hr was adjusted to pH 6·8, centrifuged, and incubated with 300–400 mg of β-glucuronidase (Sigma bacterial type I) and 2 ml of chloroform at 37° for 6 hr. The urine was extracted three times with equal volumes of ether. The combined extracts were dried, reduced to a volume of about 350 ml, and extracted one or two times with 15 ml of 1 N NaOH to remove colored and acid impurities. After being dried over sodium sulfate, the ether was evaporated. The residue, dissolved in a mixture of chloroform and methanol, was applied to several silica gel thin-layer plates PF_{254}

TABLE 1. NUCLEAR MAGNETIC RESONANCE SPECTRA OF METABOLITES OF 4-CHLOROPROPIONANILIDE AND 4′-CHLOROPHENYLACETANILIDE WHICH ARE NOT YET DESCRIBED. NMR-SIGNALS OF METABOLITES ISOLATED FROM URINE OF RABBITS DOSED WITH 4-CHLOROPROPIONANILIDE OR 4′-CHLOROPHENYLACETANILIDE RECORDED WITH A VARIAN HA-100

			Aromatic H	—NH—
4-Chlorohydracrylanilide in d_5-pyridine	4·36 ppm (2) *t* *J* = 6 cycles/sec	2·98 ppm (2) *t* *J* = 6 cycles/sec	7·38 ppm 8·01 ppm } A_2B_2 (4)	10·96 ppm (1) broad
				
4-Chloroglyceranilide in d_6-DMSO	3·62 ppm (1) d 3·63 ppm (1) d *J* = 5 cycles/sec; *J* = 4 cycles/sec	4·04 ppm (1) double d *J* = 4 cycles/sec; *J* = 5 cycles/sec	7·33 ppm 7·75 ppm } A_2B_2 (4)	9·75 ppm (1) broad
				
4′-Chloromandelanilide in d_6-DMSO	6·42 ppm (1) d *J* = 4 cycles/sec exch. against D	5·11 ppm (1) d *J* = 4 cycles/sec after exch. against D:s	between 7·2 ppm and 7·8 ppm (9)	10·02 ppm (1) exch. against D

(Merk). Authentic compounds were cochromatographed. Incubation of the urine with glucuronidase and extraction with ether were repeated until chromatography of the extracts failed to detect metabolites. Chromatograms were developed several times with chloroform: methanol (95:5 or 97:3) until the three major zones had separated.

Corresponding zones of all chromatograms were eluted with analytical grade methanol. After the absorbance of the eluates at 247 nm had been read, the metabolites were crystallized and identified by NMR- and i.r.-spectroscopy. Crystals of 4-chlorolactanilide for ORD measurements to estimate the excess of the L(−)-isomer were obtained in a yield of 70–80 per cent of the amount optically determined in the eluate.

Metabolites of 4-chloropropionanilide in microsome suspensions were isolated by the same method except that washing of the ether extracts with 1 N NaOH was omitted. TLC of ether extracts of the suspensions had to be repeated in order to obtain pure products.

4′-Chloromandelanilide was isolated from urine of rabbits dosed with 4′-chlorophenylacetanilide by the same procedure as used for the isolation of 4-chlorolactanilide. Other metabolites observed in TLC of urine extracts were not isolated.

The same method was used to isolate methylphenylcarbinol from urine of rabbits dosed with ethylbenzene. Cyclohexane: ethyl acetate (70:30) was used as developing fluid in TLC. After elution with analytical grade methanol, reading the absorbance of 257 nm, and evaporation of the solvent under reduced pressure the yellowish oil was dissolved in tetrachlorocarbon for NMR spectroscopy.

All ORD measurements were carried out with a Cary-60 spectropolarimeter. ORD curves of synthetic L(−)-4-chlorolactanilide and synthetic L(+)-4′-chloromandelanilide were recorded in methanol solution from 600 to 320 nm (see Fig. 1). The dependence of the rotation angle on the concentration was measured with synthetic L(−)-4-chlorolactanilide and synthetic L(+)-4′-chloromandelanilide in methanol at 390 nm, 5 cm light path, and room temperature; it was found to be linear. The excess of L-isomer in isolated 4-chlorolactanilide and 4′-chloromandelanilide was estimated under the same experimental conditions.

Data reported in Pickard and Kenyon[17] were used for calculating the proportions of stereoisomers in methylphenylcarbinol isolated from urine.

NMR spectra were recorded with a Varian HA 100.

Rabbit liver microsomes were prepared according to Jagow *et al.*[18] One series of rabbits was treated with 5 daily subcutaneous injections of sodium phenobarbital 25 mg/kg, followed by 5 daily injections of 50 mg/kg. Another series received 4 intraperitoneal injections, 3 days apart, of 3-methylcholanthrene, 20 mg/kg, in olive oil.

Microsome suspensions (3 mg of protein/ml) in 0·15 M phosphate buffer pH 7·4, 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 4-chloropropionanilide at 37° in air for 1 hr *in batches of 20 ml* and extracted three times with equal volumes of ether. Protein contents were determined by the method of Gornall *et al.* as modified by Szarkowska and Klingenberg.[19]

For intraperitoneal injection *N*-acylanilides were suspended in a solution of 0·25% agar in 0·9% sodium chloride.

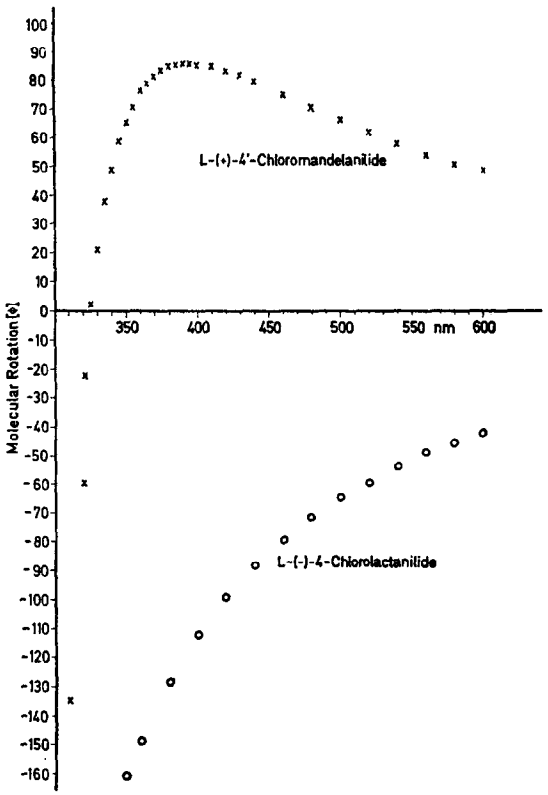


FIG. 1. ORD curves of synthetic L(+) 4'-chloromandelanilide in methanol at 12°; optical path 1 cm, $c = 3{\cdot}5$ and synthetic L(−) 4-chlorolactanilide in methanol at 22°; optical path 1 cm, $c = 3{\cdot}52$.

RESULTS

Metabolites of 4-chloropropionanilide in rabbit's urine. Five rabbits were injected intraperitoneally with 860 mg of 4-chloropropionanilide (50 mg/kg). From urine collected during the first and second day after the injection nearly 40 per cent of the dose was recovered as metabolites which were identified as 4-chlorolactanilide, 4-chlorohydracrylanilide, 4-chloroglyceranilide, and 4-chloroglycolanilide.

Infra-red and NMR spectra of the metabolites were found to be identical with those of synthetic compounds. 4-Chloropropionanilide and 4-chloroacetanilide were not detected in the urine. Results presented in Table 2 show 4-chlorolactanilide to be a major metabolite.

For ORD measurements 10·28 mg of 4-chlorolactanilide isolated from urine was dissolved in 3 ml of methanol. The rotation of −0·101° showed that 92 per cent of the metabolite was the L(−)-isomer. In three more series of experiments with five rabbits only 4-chlorolactanilide was isolated from urine. The portion of L(−)-isomer was found to amount to 91·4, 90·4 and 91·4 per cent.

Urinary excretion of 4-chlorolactanilide by rabbits after intraperitoneal injection of D,L-*4-chlorolactanilide.* The appearance of optically active 4-chlorolactanilide in urine of rabbits may be caused by stereoselective hydroxylation of 4-chloropropion-

TABLE 2. EXCRETION OF METABOLITES OF 4-CHLOROPROPIONANILIDE BY RABBITS

	Urine (ml)	Incubations	4-Chlorolactanilide			4-Chlorohydracryl-anilide		4-Chloro-glyceranilide	4-Chloroglycolanilide	
			mg	% of Dose of 4-chloropro-pionanilide*	Portion of L(−)-isomer (%)	mg	% of Dose of 4-chloropro-pionanilide*	% of Dose of 4-chloropro-pionanilide*	mg	% of Dose of 4-chloropro-pionanilide*
0–24 hr	825	14	210·0	25·6	92	26·1	3·9	<0·5	41·5	5·4
24–48 hr	690	5	21·4	2·6		2·3	0·4		1·9	0·3

* Corrected on the basis of recoveries of known amounts added to urine (88% with L(−)-4-chlorolactanilide or 4-chloroglycolanilide and 71% with 4-chlorohydracrylanilide).

Metabolites isolated from the urine of 5 rabbits after an intraperitoneal dose of 860 mg of 4-chloropropionanilide (50 mg/kg). The figures in column three indicate the numbers of incubations of one urine sample, each with freshly added glucuronidase.

anilide or by stereoselective metabolism of the optically inactive hydroxylation product. In order to test the latter possibility, 6 rabbits were injected i.p. 5 times, at 2 h intervals with a mixture of D(+)- and L(−)-4-chlorolactanilide, 4 mg/kg, 50·5 per cent of the mixture being the D(+)-isomer.

From a total of 120 mg of DL-4-chlorolactanilide 65·5 mg were recovered from first day's urine and 1·4 mg from second day's urine. Corrected on the basis of recovery of known amounts, the sum amounted to 63 per cent of the dose. For ORD measurements 13·63 mg of 4-chlorolactanilide isolated from urine was dissolved in 3 ml of methanol. The rotation of the sample was found to be −0·040°. Therefore, 62·6 per cent of the metabolite was L(−)-isomer. The D(+)-isomer amounting to 50·5 per cent of the dose applied was diminished by 13·1 per cent in its proportion during the passage through the organism. In addition to 4-chlorolactanilide, 4-chloroglycolanilide, amounting to 2·3 per cent of the dose of 4-chlorolactanilide, was isolated from urine.

In a similar experiment with three doses of the same mixture of D(+)- and L(−)-4-chlorolactanilide, 10 mg/kg, injected at 3 hr intervals, 70 per cent of the dose was recovered from urine. From the rotation of −0·0185° observed with 10·31 mg of metabolite in 3 ml of methanol the proportion of L(−)-isomer was calculated to be 57·7 per cent.

Hydroxylation of 4-chloropropionanilide by rabbit liver microsomes. Microsomes prepared from rabbits not previously treated with phenobarbital or 3-methylcholanthrene did not produce 4-chlorolactanilide in amounts sufficient for correct determination of the optical rotation.

Microsomes prepared from rabbits treated with phenobarbital produced more 4-chlorolactanilide and 4-chlorohydracrylanilide from 4-chloropropionanilide, an average of 11·5 and 4·2 μg/ml of suspension being determined after 1 hr incubation.

From ether extracts from 560 ml of microsome suspension 7·17 mg of rechromatographed 4-chlorolactanilide was isolated. Dissolved in 3 ml of methanol it showed a rotation of −0·0735° indicating 93·9% L(−)-isomer in the metabolite.

Rabbits treated with 3-methylcholanthrene yielded even more active microsomes. After 1 hr incubation with 4-chloropropionanilide 45 μg of 4-chlorolactanilide and 0·9 μg of 4-chlorohydracrylanilide/ml of suspension were found. From ether extracts of 340 ml of suspension 12·34 mg of rechromatographed 4-chlorolactanilide was isolated. Dissolved in 3 ml of methanol it showed a rotation of −0·122° which proves that 92 per cent of the product was L(−)-isomer.

4′-Chloromandelanilide in urine of rabbits dosed with 4′-chlorophenylacetanilide. Seven rabbits were injected intraperitoneally with 1315 mg of 4′-chlorophenylacetanilide (50 mg/kg). Only 4′-chloromandelanilide was isolated from the urine. It was identified by its IR and NMR spectrum. 4-Chloroglycolanilide and other metabolites were detected in the urine.

Results summarized in Table 3 show that the production of 4′-chloromandelanilide from 4′-chlorophenylacetanilide is much slower than the hydroxylation of 4-chloropropionanilide, only 4·4 per cent of the dose of 4′-chlorophenylacetanilide being recovered as 4′-chloromandelanilide in urine collected in 2 days.

The solution of 10·39 mg of the metabolite in 3 ml of methanol showed a rotation of +0·054° indicating that 94·4 per cent of the metabolite was L(+)-isomer of 4′-chloromandelanilide.

TABLE 3. EXCRETION OF 4′-CHLOROMANDELANILIDE BY RABBITS AFTER ADMINISTRATION OF 4′-CHLOROPHENYLACETANILIDE

	Urine (ml)	Incubations with glucuronidase	4′-Chloromandelanilide mg	% of Dose of 4′-chlorophenylacetanilide*
0–24 hr	865	6	33·8	3·1
24–48 hr	1100	4	13·5	1·3

4′-Chloromandelanilide isolated from urine of severn rabbits collected after intraperitoneal injection of 1315 mg of 4′-chlorophenylacetanilide (50 mg/kg).
* Corrected on the basis of recoveries of known amounts added to urine (76·8 per cent).

Methylphenylcarbinol in urine of rabbits dosed with ethylbenzene. Six rabbits were injected intraperitoneally with a total of 5·16 g of ethylbenzene. From 425 ml of urine collected in 24 hr 268 mg of methylphenylcarbinol was isolated. Its u.v. and NMR spectrum was identical with that of authentic methylphenylcarbinol. For ORD measurements 215·45 mg of methylphenylcarbinol was dissolved in 5 ml of methanol. With $\lambda = 589$ nm and 5 cm optical path the rotation was found to be $+0{\cdot}445°$; $[\alpha]_D^{27} = +20{\cdot}65°$. Using Pickard and Kenyon's[17] data on dextrorotatory methylphenylcarbinol, $[\alpha]_D = +41{\cdot}94°$, the portion of dextrorotatory isomer was calculated as amounting to 75 per cent.

DISCUSSION

The results of our experiments show that the propionic acid residue in 4-chloropropionanilide is ω- or (ω-1)-hydroxylated *in vivo* and *in vitro*, 4-chlorolactanilide being the major and 4-chlorohydracrylanilide the minor metabolite in either case. 4-Chlorolactanilide and 4-chlorohydracrylanilide found in the urine of rabbits account for nearly 33 per cent of the dose of 4-chloropropionanilide injected. The metabolic fate of the major portion of 4-chloropropionanilide is not yet known. Since 4-chloropropionanilide is deacylated by rabbit liver microsomes *in vitro*,[6] part of it is likely to be deacylated also *in vivo*. The presence of 4-chloroglycolanilide in urine of rabbits injected with 4-chloropropionanilide indicates that part of the 4-chloroaniline formed *in vivo* is acetylated, then hydroxylated, and excreted as 4-chloroglycolanilide.

The ratio between the amounts of (ω-1)- and ω-hydroxylation product determined in incubates of liver microsomes with 4-chloropropionanilide was found to be affected by previous administration of phenobarbital or 3-methylcholanthrene to the rabbits and will be discussed in more detail in the next paper. In the present study microsomes from rabbits treated with phenobarbital or 3-methylcholanthrene were only used to increase the yield of 4-chlorolactanilide for accurate results of ORD measurements.

The largest portion, namely more than 90 per cent, of 4-chlorolactanilide, isolated from urine of rabbits injected with 4-chloropropionanilide was found to be the L(−)-isomer. Since crystalline 4-chlorolactanilide was obtained from the methanol eluates in yields of 70–80 per cent of the amount determined by reading the u.v. absorbance at 247 nm, there is no doubt about the excretion of optically active 4-chlorolactanilide in urine of rabbits dosed with 4-chloropropionanilide.

Urinary excretion of an optically active hydroxylation product does not prove stereoselective hydroxylation. It does so, however, if stereoselective disposal of a racemic metabolite cannot account for the proportion of stereoisomers found in urine as the results of experiments with D,L-4-chlorolactanilide show.

The results of experiments with liver microsomes *in vitro* support the conclusions drawn from results of experiments with rabbits. Microsomes from livers of rabbits treated with phenobarbital or 3-methylcholanthrene transformed 92 per cent of the 4-chloropropionanilide hydroxylated to 4-chlorolactanilide into the L(−)-isomer.

As shown in Fig. 2, the optically active metabolites of ethylbenzene and 4′-chlorophenylacetanilide, namely dextrorotatory methylphenylcarbinol and dextrorotatory 4′-chloromandelanilide, have the same relative configuration at the asymmetric center as levorotatory 4-chlorolactanilide. This proves that with the three substrates the L-hydrogen at the (ω-1)-C-atom is exposed to the attack by the oxygenase. The rest of the molecule seems to have an unspecific effect favoring the binding of the substrate to the enzyme. L-configuration at the asymmetric center is also found with the (ω-1)-hydroxylation products of octadecanoic acid,[11,12] and decanoic acid.[10]

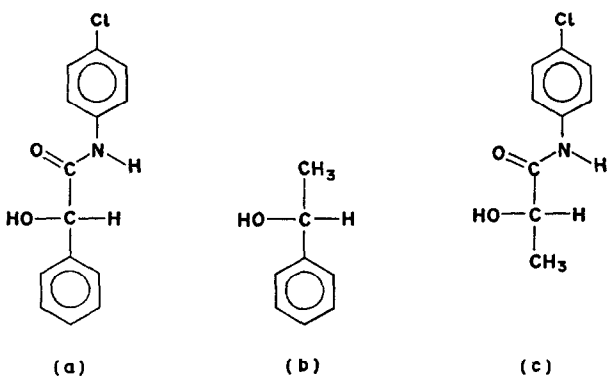


FIG. 2. Relative configurations, according to Mislow,[27] of the optically active metabolites of 4′-chlorophenylacetanilide, ethylbenzene, and 4-chloropropionanilide. (a) (+)-4′-Chloromandelanilide, (b) (+)-methylphenylcarbinol, (c) (−)-4-chlorolactanilide.

The (ω−1)-hydroxylation of 4-chlorolactanilide is also an α-hydroxylation of the propionic acid residue. α-Hydroxylation of medium chain and long-chain fatty acid has been observed in rat brain,[20] with the mitochondrial fraction prepared from livers of mice,[21] rats,[22] and guinea-pigs,[23] and in higher plants.[24] But the products of this hydroxylation mechanism show D-configuration at the asymmetric center.[25,26] Thus, the α-C in 4-chloropropionanilide and 4′-chlorophenylacetanilide is hydroxylated by the (ω−1)-hydroxylation mechanism which Heinz *et al.*,[12] Jones,[11] and Hamberg and Björkhem[10] elucidated with tritium or deuterium labelled octadecanoic and decanoic acids.

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