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# REGIOSELECTIVITY AND REACTIVITY IN MICROSOMAL HYDROXYLATION OF A SERIES OF N-ACYL- AND N-SULFONYLAMINES IN RATS

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## ABSTRACT:

**Regioselectivity and overall reactivity in the hydroxylation of a series of substituted N-benzoyl- and benzenesulfonyl aliphatic and alicyclic amines with rat liver microsomes were investigated. The hydroxylation occurred predominantly at the position γ to the nitrogen atom. *para*-Alkyl-substituted benzoylamines were hydroxylated at both the benzylic positions as well as the γ-position, whereas *para*-substituted benzenesulfonylamines were hydroxylated mainly at the benzylic position. The relative overall reactivity of primary, secondary, and tertiary carbon atoms was about 1:3:8, and the contribution of substituents adjoining to the reaction site was negligible. Benzylic hydroxylation proceeded stereoselectively, whereas γ-hydroxylation gave optically inactive metabolites; the inter- and intramolecular isotope effects in the hydroxylation were 1.6 and 7.2, respectively.**

Many drugs possess the N-acylamine or N-sulfonylamine moiety in their chemical structure. One of the important processes in metabolism of such drugs is oxidation at saturated aliphatic carbon atoms catalyzed by the cytochrome P-450 mono-oxygenase system. When the mono-oxygenation reaction occurred at the α-carbon atom of aliphatic groups substituted on a nitrogen atom, formation of dealkylated amines and aldehydes is observed (1, 2). On the other hand, mono-oxygenation at the other aliphatic carbon atoms produces hydroxylated metabolites (3, 4).

We have been interested in the regioselectivity and reactivity in this hydroxylation of N-acylamines and N-sulfonylamines, because such information may contribute to drug design from the viewpoint of the control of biotransformation and pharmacokinetics *in vivo*. The aim of the present study was to define the effect of structure on the overall regioselectivity and reactivity in the hydroxylation of a series of substituted N-benzoyl- and benzenesulfonyl aliphatic and alicyclic amines by enzymes of rat liver microsomes.

## Materials and Methods

**Spectrometric Analyses.** Absorption spectra were measured in methanol with a Shimadzu spectrophotometer (model UV-210A). IR spectra were recorded in Nujol with a Hitachi spectrophotometer (model 260-30). Proton-NMR spectra were obtained in $CDCl_3$ containing tetramethylsilane as an internal standard with a Varian spectrometer (model EM-360). Mass spectra were obtained with a double focusing mass spectrometer (JEOL, model JMS-01SG) with use of a direct-insertion probe technique. Specific rotations were measured in methanol with a digital polarimeter (JASCO, model DIP-140).

**Chromatography.** Thin-layer chromatography was carried out on silica gel plates ($60F_{254}$, E. Merck). The solvent system used for development was acetone/benzene/ether/25% ammonia, 30:20:5:1.5 (v/v). Compounds were visualized under UV illumination (254 nm). Quantitative evaluation of TLC was carried out with a Zeiss UV Chromatoscanner at the wavelength of 240 nm.

High-pressure liquid chromatography was carried out with a Shimadzu liquid chromatograph (model LC-3A), monitored at 240 nm in a 25-cm × 4.6-mm i.d. column prepacked with Zorbax ODS (E. I. DuPont). Chromatography was performed in reverse-phase mode with a solvent system of methanol/water, 4:1 (v/v) at a flow rate of 1 ml/min.

**Synthesis of Substrates.** The structures of N-acylamines and N-sulfonylamines used as substrates are shown in tables 1 and 2. The substrates were prepared from corresponding amines by a general method (5). Benzoyl-, *p*-toluoyl-, *p*-ethylbenzoyl-, benzenesulfonyl-, *p*-toluenesulfonyl, and *p*-ethylbenzenesulfonyl chloride were purchased commercially.

Heptamethyleneimine was prepared by Beckmann rearrangement (6) of cycloheptanone oxime (7) followed by reduction with $LiAlH_4$ (8). 4-Hexenylamine and phenylpropylamine were prepared by reduction of 4-hexenonitrile (9) and β-phenylpropionitrile, respectively, with $LiAlH_4$. Synthesis of piperidine-4-$d_1$ was carried out by reduction (10) of pyridine-4-$d_1$ (11) with sodium in ethanol. Reduction of pyridine-$d_5$ with sodium in ethanol-$d_1$ by the same method gave piperidine-$d_{10}$. All other amines were available commercially. The purity of all substrates was shown to be >98% by TLC and HPLC.

Spectroscopic data for some substrates were as follows:

**II.** IR:2930, 1620 (amide), 1440, 1270 $cm^{-1}$; NMR:δ 7.30 (s, 5, phenyl), 3.50 (broad s, 4, $CH_2NCH_2$), 1.62 [broad s, 6, $(CH_2)_3$]; MS:*m/Z* 189 ($M^+$), 188, 105.

**IV.** IR:2930, 1620 (amide), 1420, 1300 $cm^{-1}$; NMR:δ 7.35 (s, 5, phenyl), 3.50 (broad s, 4, $CH_2NCH_2$), 1.65 [broad s, 10, $(CH_2)_5$].

**V.** IR:2930, 1630 (amide), 1440, 1270 $cm^{-1}$; NMR:δ 7.35 (s, 5, phenyl), 4.40-3.95 (m, 2, N—$CH_{eq}$), 3.15-2.60 (m, 2, N—$CH_{ax}$), 1.85-1.10 (m, 5, $CH_2CHCH_2$), 0.98 (d, 3, $CH_3$).

**VII.** NMR:δ 7.80-7.05 (m, 5, phenyl), 6.20 (broad s, 1, NH), 4.25-3.55 (m, 1, —NCH), 2.30-0.80 [m, 10, $(CH_2)_5$].

**VIII.** NMR:δ 7.35 (s, 5, phenyl), 3.40 (t, 2, $NCH_2$), 3.00 (s, 3, $NCH_3$), 1.85-1.00 [m, 4, $(CH_2)_2$], 0.88 (t, 3, $CH_3$).

**XII.** NMR:δ 7.85-7.05 (m, 5, phenyl), 5.50-5.15 (m, 2, CH=CH), 3.60-3.10 (m, 2, $NCH_2$), 2.25-1.30 [m, 7, $(CH_2)_2$ and $CH_3$].

**XIII.** NMR:δ 7.85-7.00 (m, 10, phenyl), 6.35 (broad s, 1, NH), 3.50 (q, 2, N—$CH_2$), 2.73 (t, 2, $CH_2$—phenyl), 2.20-1.65 (m, 2, C—$CH_2$—C).

**XIV.** NMR:δ 7.40-7.05 (m, 4, phenyl), 3.55 (broad s, 4, $CH_2NCH_2$), 2.38 (s, 3, $CH_3$), 1.63 [broad s, 6, $(CH_2)_3$].

**XVIII.** NMR:δ 7.80-7.10 (m, 4, phenyl), 3.40-2.85 (m, 4, $CH_2NCH_2$), 2.40 (s, 3, $CH_3$), 1.95-1.10 [m, 6, $(CH_2)_3$].

**XIX.** NMR:δ 8.20-7.25 (m, 4, phenyl), 4.50-4.00 (m, 1, N—CH—$CH_3$), 3.95-3.40 (m, 1, N—$CH_{eq}$—C), 3.30-2.90 (m, 1, N—$CH_{ax}$—C), 2.78 (q, 2, $CH_2$—phenyl), 1.55 [broad s, 6, $(CH_2)_3$], 1.30 (t, 3, $CH_3$—C—phenyl), 1.10 (d, 3, N—C—$CH_3$).

**XX.** NMR:δ 7.30 (s, 5, phenyl), 3.75-3.25 (m, 4, $CH_2NCH_2$), 2.25-1.05 (m, 5, $CH_2CHCH_2$); MS:*m/z* 190 ($M^+$), 189, 188, 105.

**XXI.** NMR:δ 7.30 (s, 5, phenyl); MS:*m/z* 199 ($M^+$), 198, 197, 105.

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**Preparation of Reference Compounds.** The reference compounds of the metabolites of each of N-acyl- and N-sulfonylamines were obtained in large scale by perfusion of isolated rat livers. The perfusion system and procedure were almost the same as those reported in a previous paper (12) except that the bile duct was ligated. Each sample of the perfusates was extracted three times with chloroform. The combined chloroform layer was dried over $K_2CO_3$. After removing $K_2CO_3$ by filtration, chloroform was evaporated and the residue was subjected to preparative TLC on a silica gel plate as described above. The metabolites developed on the chromatogram were detected under UV light (254 nm) and analyzed with a UV chromatoscanner. The corresponding zone was scraped off the plate and the metabolite was eluted with methanol.

The isolated metabolites, hydroxyl compounds, were identified by elemental and spectroscopic analyses and/or they were analyzed after they were converted to the corresponding keto compounds by oxidation with chromic oxide (13). The purity of the reference compounds was >94%, as shown by chromatographic analyses. The structures of the metabolites are shown in tables 1 and 2; their spectral properties and specific rotations were as follows:

**Ia.** IR:3350 (alcohol), 1600 (amide), 1450, 1100 $cm^{-1}$; NMR:$\delta$ 7.55-7.30 (m, 5, phenyl), 4.60-4.40 (m, 1, CH—O), 3.80-3.45 (m, 4, $CH_2NCH_2$), 2.20-1.95 (m, 2, $CH_2$); MS:*m/z* 191 ($M^+$), 190 ($M^+-1$), 105 ($C_6H_5CO$).

**IIa.** IR:3370 (alcohol), 1610 (amide), 1440, 1270, 1070 $cm^{-1}$; NMR:$\delta$ 7.30 (s, 5, phenyl), 4.20-3.50 (m, 3, N—$CH_{eq}$ and CH—O), 3.50-3.00 (m, 2, N—$CH_{ax}$), 2.20-1.15 (m, 4, $CH_2$—C—$CH_2$); MS:*m/z* 205 ($M^+$), 204, 105. ***Keto derivative of IIa.*** NMR:$\delta$ 7.40 (s, 5, phenyl), 3.85 (t, 4, $CH_2NCH_2$), 2.50 (t, 4, $CH_2COCH_2$); IR:1710 (ketone), 1620 $cm^{-1}$.

**IIIa.** IR:3350 (alcohol), 1630 (amide), 1420, 1280, 1060 $cm^{-1}$; NMR:$\delta$ 7.35 (s, 5, phenyl), 4.35-3.10 (m, 5, $CH_2NCH_2$ and CH—O), 2.15-1.30 [m, 6, $(CH_2)_3$]; $[\alpha]^{20}_D = 0°$ (c = 0.037, methanol). ***Keto derivative of IIIa.*** NMR:$\delta$ 7.35 (s, 5, phenyl), 4.35-3.90 (m, 2, $NCH_2CO$), 3.85-3.35 (m, 2, $NCH_2$), 2.80-2.40 (m, 2, $CH_2CO$), 2.15-1.50 [m, 4, $(CH_2)_2$].

**IIIb.** NMR:$\delta$ 7.35 (s, 5, phenyl), 4.05-3.20 (m, 5, $CH_2NCH_2$ and CH—O), 2.20-1.30 [m, 6, $CH_2$—C—$(CH_2)_2$]; $[\alpha]^{20}_D = 0°$ (c = 0.029, methanol). ***Keto derivative of IIIb.*** NMR:$\delta$ 7.35 (s, 5, phenyl), 4.05-3.40 (m, 4, $CH_2NCH_2$), 2.70 (t, 4, $CH_2COCH_2$), 2.10-1.55 (m, 2, $CH_2$).

**IVa.** NMR:$\delta$ 7.35 (s, 5, phenyl), 3.95-3.20 (m, 5, $CH_2NCH_2$ and CH—O), 2.25-1.45 [m, 8, $CH_2$—C—$(CH_2)_3$]. ***Keto derivative of IVa.*** NMR:$\delta$ 7.35 (s, 5, phenyl), 3.85-3.15 (m, 4, $CH_2NCH_2$), 2.90-2.30 (m, 4, $CH_2COCH_2$), 2.25-1.40 [m, 4, $(CH_2)_2$].

**IVb.** NMR:$\delta$ 7.30 (s, 5, phenyl), 3.70-3.10 (m, 4, $CH_2NCH_2$ and CH—O), 2.65-1.55 [m, 8, $(CH_2)_2$—C—$(CH_2)_2$]. ***Keto derivative of IVb.*** NMR:$\delta$ 7.25 (s, 5, phenyl), 3.85-3.05 (m, 4, $CH_2NCH_2$), 2.70-1.30 [m, 8, $(CH_2)_2CO(CH_2)_2$].

**Va.** IR:3380 (alcohol), 1600 (amide), 1440, 1280, 1120, 970 $cm^{-1}$; NMR:$\delta$ 7.35 (s, 5, phenyl), 4.15-3.10 (m, 4, $CH_2NCH_2$), 1.80-1.40 (m, 4, $CH_2$—C—$CH_2$), 1.25 (s, 3, $CH_3$). NMR spectrum after oxidation of **Va** with $CrO_3$ was not changed from that of **Va**.

**VIa.** NMR:$\delta$ 7.35 (s, 10, $C_6H_5CO$ and C—$C_6H_5$), 3.70-3.10 (m, 4, $CH_2NCH_2$), 3.00 (s, 2, $CH_2$), 1.85-0.90 (m, 4, $CH_2$—C—$CH_2$); MS:*m/z* 295 ($M^+$), 294, 190 ($M^+-C_6H_5CO$), 156, 148, 105. The oxidation product of **VIa** with $CrO_3$ showed an NMR spectrum identical to that of **VIa**.

**VIIa.** NMR:$\delta$ 7.80-7.05 (m, 5, phenyl), 4.30-3.50 (m, 2, —NCH and CH—O), 2.30-1.10 [m, 8, $CH_2$—C—$(CH_2)_3$]; MS:*m/z* 219 ($M^+$), 218, 105. ***Keto derivative of VIIa.*** NMR:$\delta$ 7.80-7.10 (m, 5, phenyl), 4.50-4.00 (m, 1, —NCH), 2.80-2.20 (m, 4, $CH_2COCH_2$), 2.10-1.20 [m, 4, $(CH_2)_2$].

**VIIIa.** NMR:$\delta$ 7.60-7.20 (m, 5, phenyl), 3.95-3.15 (m, 3, $NCH_2$ and CH—O), 3.03 (s, 3, $NCH_3$), 2.25-1.55 (m, 2, $CH_2$), 1.35 and 1.20 (d, 3, $CH_3$); MS:*m/z* 207 ($M^+$), 189 ($M^+-H_2O$), 162, 149, 136, 105. ***Keto derivative of VIIIa.*** NMR:$\delta$ 7.55-7.20 (m, 5, phenyl), 3.70 (t, 2 $NCH_2$), 3.02 (s, 3, $NCH_3$), 2.80 (t, 2, $CH_2CO$), 2.18 —s, 3, $CH_3$); MS:*m/z* 205 ($M^+$), 162, 148, 135, 105.

**IXa.** NMR:$\delta$ 7.95-7.20 (m, 5, phenyl), 3.65-3.25 (m, 2, $NCH_2$), 3.40 (t, 2, $CH_2O$), 2.20-1.40 (m, 2, $CH_2$).

**Xa.** NMR:$\delta$ 7.95-7.25 (m, 5, phenyl), 3.90-3.20 (m, 3, $NCH_2$ and CH—O), 2.20-1.50 (m, 2, $CH_2$), 1.25 (d, 3, $CH_3$).

**XIa.** NMR:$\delta$ 7.90-7.30 (m, 5, phenyl), 3.80-3.40 (m, 2, $NCH_2$), 2.05-1.50 (m, 2, $CH_2$), 1.25 [s, 6, $(CH_3)_2$].

**XIIa.** NMR:$\delta$ 7.90-7.10 (m, 5, phenyl), 5.70-5.35 (m, 2, CH=CH), 4.20-3.25 (m, 3, $NCH_2$ and CH—O), 2.45-1.30 (m, 5, $CH_2$ and $CH_3$); MS:*m/z* 219 ($M^+$), 201 ($M^+-H_2O$), 156, 149, 141, 105.

**XIIIa.** NMR:$\delta$ 7.80-7.10 (m, 10, phenyl), 4.90 (t, 1, CH—O), 3.95-3.30 (m, 2, $NCH_2$), 2.40-1.70 (m, 2, $CH_2$).

**XIVa.** NMR:$\delta$ 7.50-7.15 (m, 4, phenyl), 4.25-3.00 (m, 5, $CH_2NCH_2$ and CH—O), 2.40 (s, 3, $CH_3$), 2.25-1.25 (m, 4, $CH_2$—C—$CH_2$).

**XIVb.** NMR:$\delta$ 7.50-7.15 (m, 4, phenyl), 4.64 (s, 2, $CH_2$—O), 3.80-3.20 (m, 4, $CH_2NCH_2$), 1.65 [broad s, 6, $(CH_2)_3$].

**XVa.** NMR:$\delta$ 8.10-7.15 (m, 4, phenyl), 4.00-3.20 (m, 3, $NCH_2$ and CH—O), 3.05 (s, 3, $NCH_3$), 2.80 (q, 2, $CH_2$—phenyl), 2.30-1.60 (m, 2, $CH_2$), 1.40 and 1.25 (d, 3, O—C—$CH_3$), 1.30 (t, 3, $CH_3$—C—phenyl); $[\alpha]^{20}_D = 0°$ (c = 0.020, methanol).

**XVb.** NMR:$\delta$ 8.00-7.20 (m, 4, phenyl), 4.95 (q, 1, CH—O), 3.45 (t, 2, $NCH_2$), 3.05 (s, 3, $NCH_3$), 1.90-1.00 [m, 4, $(CH_2)_2$], 1.50 (d, 3, $CH_3$—C—phenyl), 0.90 (t, 3, $CH_3$); $[\alpha]^{20}_D = -15.8°$ (c = 0.085, methanol).

**XVIa.** NMR:$\delta$ 7.95-7.30 (m, 5, phenyl), 4.00-2.80 (m, 5, $CH_2NCH_2$ and CH—O), 2.10-1.30 (m, 4, $CH_2$—C—$CH_2$); MS:*m/z* 241 ($M^+$), 240, 141.

**XVIIIa.** NMR:$\delta$ 7.80-7.15 (m, 4, phenyl), 3.95-2.80 (m, 5, $CH_2NCH_2$ and CH—O), 2.43 (s, 3, $CH_3$), 2.05-1.35 (m, 4, $CH_2$—C—$CH_2$). ***Keto derivative of XVIIIa.*** NMR:$\delta$ 7.80-7.15 (m, 4, phenyl), 3.40 (t, 4, $CH_2NCH_2$), 2.50 (t, 4, $CH_2COCH_2$), 2.43 (s, 3, $CH_3$).

**XVIIIb.** NMR:$\delta$ 7.80-7.30 (m, 4, phenyl), 4.73 (s, 2, $CH_2$—O), 3.15-2.80 (m, 4, $CH_2NCH_2$), 2.00-1.00 [m, 6, $(CH_2)_3$].

**XIXa.** NMR:$\delta$ 8.20-7.20 (m, 4, phenyl), 4.55-4.00 (m, 1, N—$CHCH_3$), 3.95-3.40 (m, 2, N—$CH_{eq}$ and CH—O), 3.35-2.90 (m, 1, N—$CH_{ax}$), 2.80 (q, 2, $CH_2$—phenyl), 1.80-1.20 (m, 4, $CH_2$—C—$CH_2$), 1.35 (t, 3, $CH_3$—C—phenyl), 1.05 (d, 3, N—C—$CH_3$); MS:*m/z* 283 ($M^+$), 282, 169; $[\alpha]^{20}_D = 0°$ (c = 0.033, methanol).

**XIXb.** NMR:$\delta$ 8.00-7.20 (m, 4, phenyl), 4.95 (q, 1, CH—O), 4.50-4.00 (m, 1, N—CH—$CH_3$), 3.95-3.30 (m, 1, N—$CH_{eq}$), 3.15-2.75 (m, 1, N—$CH_{ax}$), 1.85-1.10 [m, 6, $(CH_2)_3$], 1.55 (d, 3, $CH_3$—C—phenyl), 1.05 (d, 3, N—C—$CH_3$); $[\alpha]^{20}_D = -8.8°$ (c = 0.023, methanol).

Elemental analyses (C, H, N) for compounds **IIa**, **IIIa**, **IIIb**, **IVa**, **IVb**, **Va**, **VIIa**, **XIVa**, **XIVb**, **XIXa**, and **XIXb** were within 0.3% of the theoretical values.

Synthesis of N-benzoyl-4-hydroxy-4-$d_1$-piperidine (**XXa**) was carried out as follows: The oxidation (13) of 4-hydroxypiperidine with $CrO_3$ gave 4-piperidinone, which was converted (5) to **XXa** by N-benzoylation followed by reduction (14) with sodium borodeuteride. NMR:$\delta$ 7.30 (s, 5, phenyl), 4.20-3.55 (m, 2, N—$CH_{eq}$), 3.55-2.95 (m, 2, N—$CH_{ax}$), 2.20-1.00 (m, 4, $CH_2$—C—$CH_2$); MS:*m/z* 206 ($M^+$), 205, 204, 105. Compound **XXIa** was prepared by hydroxylation of **XXI** in the isolated rat liver system described above.

The reference compounds of N-demethylated metabolites of the substrates **VIII** and **XV** were prepared by the reaction of *n*-butylamine with the corresponding acyl chlorides (5).

**Microsomal Hydroxylation.** Male Sprague-Dawley rats, weighing 200–250 g, were used in all experiments. Rats were fed *ad lib.* on laboratory chow and tap water before use.

Liver homogenates were prepared in 4 volumes of cold 0.25 M sucrose solution in a Potter-Elvehjem glass homogenizer with a Teflon pestle. Liver microsomal fractions were prepared from the homogenates by the conventional differential ultracentrifugation technique (15). Microsomal protein was determined by the method of Lowry *et al.* (16). The incubation mixture contained 0.4 mM substrate, 4 mM glucose 6-phosphate, 0.5 mM NADPH, 5 mM $MgCl_2$, 0.5 units of glucose 6-phosphate dehydrogenase, 0.1 M Tris-HCl buffer (pH 7.4), and an appropriate amount of microsomal suspension (containing 3.0 mg of protein) in a total volume of 1 ml. Incubation was conducted at 37°C with shaking. The mixtures were incubated for 0, 5, 10, 15, 20, and 30 min to determine the relation among the amounts of the substrate, metabolites, and reaction time. The reaction was terminated by the addition of 3 volumes of methanol. After centrifugation at 2000 *g* for 10 min, 2-ml aliquots of the supernatant fluid were evaporated to dryness *in vacuo*. The residue obtained was dissolved into 500 $\mu$l of methanol, and 5-$\mu$l aliquots were subjected to HPLC for analysis of the unchanged substrate and metabolites. The metabolites were characterized by comparative TLC and HPLC with the reference compounds

TABLE 1

*Metabolites and rates of hydroxylation of N-acyl- and N-sulfonylamines*

The incubation of substrates with liver microsomes of a rat was carried out three times for each substrate. The overall rates (nmol/mg protein/min) were corrected for the number of hydrogen atoms at the reaction site (means ± SD).

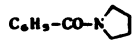
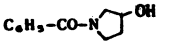
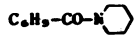
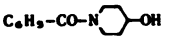
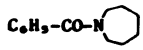
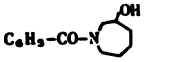
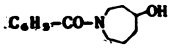
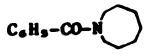
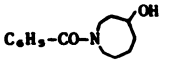
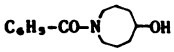
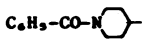
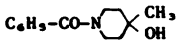
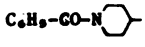
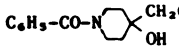
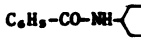
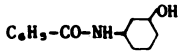
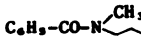
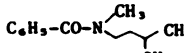
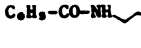
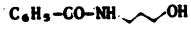
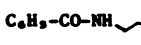
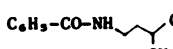
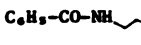
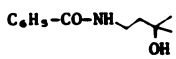
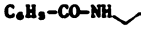
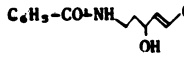
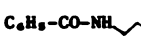
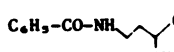
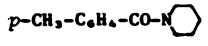
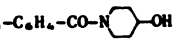
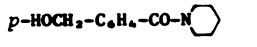
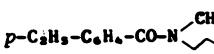
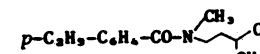
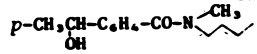
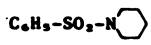
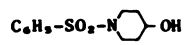
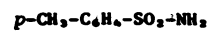
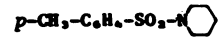
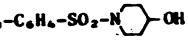
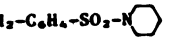
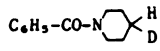
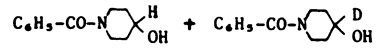
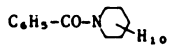
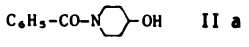
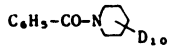
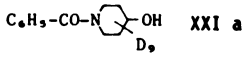
| Substrates | Metabolites | Rates |
|---|---|---|
| | | *nmol/mg protein/min/H atom* |
|  I |  I a | 0.12 ± 0.03 |
|  II |  II a | 1.56 ± 0.30 |
|  III |  III a | 0.24 ± 0.08 |
| |  III b | 0.71 ± 0.13 |
|  IV |  IV a | 0.68 ± 0.15 |
| |  IV b | 0.18 ± 0.05 |
|  V |  V a | 2.50 ± 0.35 |
|  VI |  VI a | 1.21 ± 0.22 |
|  VII |  VII a | 0.90 ± 0.15 |
|  VIII |  VIII a | 0.88 ± 0.20 |
|  IX |  IX a | 0.21 ± 0.06 |
|  X |  X a | 0.62 ± 0.15 |
|  XI |  XI a | 1.60 ± 0.25 |
|  XII |  XII a | 0.65 ± 0.18 |
|  XIII |  XIII a | 0.88 ± 0.21 |
|  XIV |  XIV a | 1.25 ± 0.19 |
| |  XIV b | 1.33 ± 0.26 |
|  XV |  XV a | 0.90 ± 0.11 |
| |  XV b | 1.24 ± 0.16 |
|  XVI |  XVI a | 0.40 ± 0.10 |
|  XVII | No metabolites | – |
|  XVIII |  XVIII a | 0.31 ± 0.05 |
| |  XVIII b | 2.55 ± 0.32 |
|  XIX |  XIX a | 0.30 ± 0.06 |
| |  XIX b | 2.07 ± 0.36 |

TABLE 2

*Inter- and Intramolecular isotope effects*

The values, determined by the mass-spectral method, are given as means ± SD ($N = 5$).

| Substrates | Metabolites | Isotope effects |
|---|---|---|
|  XX |  II a XX a | Intramolecular 7.2 ± 0.8 |
|  II |  II a | Intermolecular 1.6 ± 0.2 |
|  XXI |  XXI a | |

prepared above. The formation of all metabolites showed a linear correlation with the incubation time for at least 15 min. In order to examine the formation of glucuronide of the hydroxylated metabolite, the residue obtained above was dissolved in 0.2 M acetate buffer (pH 4.8) and incubated with β-glucuronidase (5000 units/ml) at 37°C for 24 hr. The mixture was subjected to HPLC for analysis of hydrolyzed products.

**Measurements of Isotope Effects.** The intra- and intermolecular isotope effects were determined in the microsomal hydroxylation of N-benzoylpiperidine-$d_1$ (XX), and in the microsomal competitive reaction of unlabeled (II) and $d_{10}$-labeled (XXI) N-benzoylpiperidine, respectively. A total volume of 500 ml of incubation mixture contained 0.4 mM XX, or 0.2 mM II and 0.2 mM XXI. After incubation for 10 min at 37°C, the reaction mixture was extracted three times with chloroform and dried over $Na_2SO_4$. Solvent was evaporated and residue was subjected to TLC. Unlabeled and/or labeled 4-hydroxy-N-benzoylpiperidine was isolated from the corresponding zone on a silica gel plate. The deuterium content of the products was determined by the mass-spectral method; ratios of the deuterated metabolites, **XXa/IIa** (intramolecular isotope effect) or **IIa/XXIa** (intermolecular isotope effect), were calculated from the relative intensities of $m/z$ 206/205 or 205/214 with use of a standard curve prepared in advance.

## Results

**Metabolites and Rates of Hydroxylation.** The metabolites produced by incubation of N-acylamines and N-sulfonylamines with rat liver microsomes, and the overall rates of hydroxylation are shown in table 1.

The hydroxylation of N-benzoylamines (**I-XIII**) predominantly took place at the γ-position. N-Benzoylpiperidine (**II**) was hydroxylated about 10 times faster than the pyrrolidine derivative (**I**). The rate of hydroxylation at the γ-position of N-benzoylhexamethyleneimine (**III**) was about three times larger than that at the β-position, and N-benozylheptamethyleneimine (**IV**) was hydroxylated at the γ-position about four times faster than at the δ-position. Also, in the cases of N-benzoylpiperidine derivatives having substituents at the γ-positions (**V** and **VI**), the γ-position was still the only reaction site. N-Benzoylcyclohexylamine (**VII**) was hydroxylated exclusively at the γ-position and a metabolite hydroxylated at the δ-position was not detected.

The hydroxylation of N-benzoyl derivatives of aliphatic amines was observed to be slower than that of cyclic amines. The relative overall reactivity of primary, secondary, and tertiary carbon atoms in a series of N-monoalkylbenzamides (**IX, X, and XI**) was about 1:3:8. An olefinic double bond (**XII**) and a phenyl ring (**XIII**) adjoining the reaction site did not contribute to the increment of the reactivity of the reaction site, suggesting that the rate-determining step in this hydroxylation did not involve the formation of a free carbonium ion. N-Benzoylpiperidine derivatives having alkyl substituents at the *para*-position (**XIV** and **XV**) were hydroxylated at both the γ-position and benzylic positions.

The hydroxylation of the γ-position of N-benzenesulfonylpiperidine (**XVI**) was much slower than that of **II**. Therefore, N-*p*-tosylpiperidine (**XVIII**) and N-(*p*-ethylbenzenesulfonyl)-2-methylpiperidine (**XIX**) were hydroxylated predominantly at the benzylic position. However, *p*-toluenesulfonamide (**XVII**) was not metabolized at the benzylic position.

The formation of the N-demethylated metabolites and of the glucuronide was not detected in the microsomal incubation mixture.

**Optical Activity and Isotope Effects.** Specific rotations ($[\alpha]_D^{20}$) of some hydroxylated metabolites were: **IIIa**, 0°; **IIIb**, 0°; **XVa**, 0°; **XVb**, −15.8°; **XIXa**, 0°; **XIXb**, −8.8°. The metabolites hydroxylated at benzylic positions of racemic substrates showed some optical activity, whereas the hydroxylation at β- or γ-positions of N-acyl- and N-sulfonylamines gave optically inactive products.

The inter- and intramolecular isotope effects in the hydroxylation of N-benzoylpiperidine were determined by measuring the isotope ratios by mass spectrometry. As shown in table 2, the inter- and intramolecular isotope effects were 1.6 and 7.2, respectively, demonstrating a relatively large difference.

## Discussion

Based on the metabolites obtained, the hydroxylation of the N-substituted benzoylamines **I-XIII** occurred predominantly at the γ-position, and neither aromatic hydroxylation nor N-dealkylation was detected. The regioselectivity at the γ-position observed in this study was higher than that in the similar microbiological hydroxylation (17, 18). In addition, the metabolites formed by rat liver enzymes were only hydroxylated products, and keto derivatives were not detected, whereas the oxygenation with microorganisms gave a mixture of hydroxyl and ketonic products. Chlorpropamide, 3-(*p*-chlorobenzenesulfonyl)-1-*n*-propylurea, a hypoglycemic agent, has been reported to be hydroxylated at the β- and γ-positions in rats *in vivo* (19) showing the similar observations obtained in this study.

N-Benzoylcyclohexylamine (**VII**) was hydroxylated exclusively at the γ-position with rat liver microsomes, whereas the δ-position was also hydroxylated in the microbiological transformation (14). The cyclohexylamine moiety in amantadine (20), an antiparkinsonism drug, and glibenclamide (21), an antidiabetic agent, was hydroxylated only at the γ-position in rats.

It was observed in this study that **XIV** and **XV** were hydroxylated at both the γ-position and benzylic positions to yield **XIVa, XVa** and **XIVb, XVb**, respectively, whereas **XVIII** and **XIX** were hydroxylated predominantly at the benzylic position to give **XVIIIb** and **XIXb**. Tolazamide, 1-(hexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea, a hypoglycemic agent, was hydroxylated exclusively at the benzylic position in rats (22). The biotransformation of the other *para*-substituted phenylsulfonylurea derivatives such as tolbutamide, having tosyl and *n*-butylamino moieties, occurred at the benzylic position, but the hydroxylation at the γ-position was not observed in rats or in humans (23).

It is worth mentioning that there is no significant difference of regioselectivity and stereoselectivity between the metabolic hydroxylation of drugs and of the substrates used in this study. Therefore, it can be noted that the metabolic studies *in vitro* on substrates having simple structures are useful tools to discuss the biotransformation of drugs having complicated structures.

In the next place, in order to get some informations on the mechanism of the hydroxylation with liver microsomes, stereoselectivity and isotope effect in this hydroxylation were studied. The metabolites hydroxylated at $\beta$- or $\gamma$-positions, *i.e.* **IIIa**, **IIIb**, **XVa**, and **XIXa**, showed no optical activity, whereas the benzylic hydroxylation (**XVb** and **XIXb**) was found to give optically active products, suggesting a difference of pattern of reaction between $\beta$- or $\gamma$-hydroxylation and benzylic hydroxylation. The $\gamma$-hydroxylated metabolite of tolazamide isolated from human urine also showed no optical activity (22). The inter- and intramolecular isotope effects in the hydroxylation at the $\gamma$-position were 1.6 and 7.2, respectively.

As for the mechanism of the rate-determining step, homolytic hydrogen abstraction has been proposed for aliphatic hydroxylation (24–25), though carbene-like oxygen insertion has been accepted as an alternative mechanism (3). According to a general concept of enzymatic reaction, however, the enzymatic reaction is usually multistage and the character of the rate-determining step often masked by nature of the overall reaction steps. On the other hand, the observed large intramolecular isotope effect and the optical nonactivity of $\gamma$-hydroxylated metabolites may indicate that homolytic hydrogen abstraction by a relatively less reactive radical species is one of the possible mechanisms of the rate determining step of the hydroxylation at a saturated carbon atom.

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