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Studies on Drug Metabolism. XIII.[1] Possible Role of Liver Microsomal Cytochrome P-450 in the Oxidative N-Demethylation of *p*-Substituted Dimethylaniline, N-Methyl-N-alkylaniline and N-Methyl-N-acylaniline Derivatives

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N-Demethylation of *p*-substituted dimethylaniline derivatives, N-methyl-N-alkylaniline derivatives and N-methyl-N-acylaniline derivatives was investigated. The rates of N-demethylation of several series of *p*-substituted dimethylaniline depended upon their lipid solubilities. The more lipid soluble the amine was, the more readily it was demethylated. The *p*-substituted dimethylanilines of high lipid solubilities revealed small Km and Ks values, whereas those of low lipid solubilities exhibited large Km and Ks values. N-Demethylation activity of the high lipid soluble *p*-substituted dimethylaniline was intensely stimulated by phenobarbital administration in comparison with that of the low lipid soluble *p*-substituted dimethylaniline. N-Demethylation of *p*-substituted dimethylaniline of low lipid solubility was inhibited more strongly than that of high lipid solubility by SKF 525-A, but this relation between lipid solubility and inhibitory rate was not observed in carbon monoxide inhibition. The studies on N-demethylation of N-methyl-N-alkylaniline derivatives revealed that the rate of N-demethylation did not always depend on the lipid solubilities of amines. It was suggested that steric hindrance about the nitrogen atom interfered with N-demethylation of N-methyl-N-alkylaniline derivatives. In the studies on N-demethylation of N-methyl-N-acylaniline derivatives, the difference of N-demethylation rate between N-methyl-N-propyonylaniline and N-methyl-N-benzoylaniline seemed to depend on the spectral change per P-450 rather than lipid solubility.

Many drugs which contain a N-methyl group are metabolized in the body by oxidative N-demethylation mechanism. The enzyme system which is present in mammalian liver tissue and catalyzes the oxidative N-demethylation of lipid soluble N-methyl compounds, was first described by Mueller and Miller.[3] La Du, *et al.*[4] subsequently have demonstrated that this enzyme system is localized in the microsomal fraction of liver homogenates. It is known that the reaction, the N-demethylation, requires oxygen and NADPH, and then yields as primary products formaldehyde and the corresponding demethylated compound. Gaudette and Brodie[5] have demonstrated a relationship between the lipid solubility of the substrates and their metabolic rates. Demethylation studies made by MacMahon[6] demonstrated that lipid solubility was rate limiting factor for demethylation and that the reaction might proceed by direct oxidative attack the less hindered methyl group carbon as suggested by Brodie, *et al.*[7] rather than the highly hindered nitrogen as proposed by Horning's group.[8,9]

On the contrary, Brodie[10] has suggested that in the first part of the reaction the N-methyl compound is oxidized to the alkyl N-oxide and this intermediate N-oxide is then degraded.

1) Part XII: H. Kitagawa and T. Kamataki, *Chem. Pharm. Bull.* (Tokyo), **19**, 827 (1971).
2) Location: *1-33 Yayoicho, Chiba-shi, Chiba.*
3) G.C. Mueller and J.A. Miller, *J. Biol. Chem.*, **202**, 579 (1953).
4) B.N. La Du, L. Gaudette, N. Trousof and B.B. Brodie, *J. Biol. Chem.*, **214**, 741 (1955).
5) L.E. Gaudette and B.B. Brodie, *Biochem. Pharm.*, **2**, 89 (1959).
6) R.E. MacMahon, *J. Med. Pharm. Chem.*, **4**, 67 (1961).
7) B.B. Brodie, J.R. Gillette and B.N. La Du, *Annu. Rev. Biochem.*, **27**, 427 (1958).
8) M.S. Fish, C.C. Sweeley, N.M. Johnson, E.P. Lawrence and E.C. Horning, *Biochim. Biophys. Acta*, **21**, 190 (1956).
9) M.S. Fish, N.M. Johnson, E.P. Laurence and E.C. Horning, *Biochim, Biophys. Acta*, **18**, 564 (1955).
10) B.B. Brodie, *Ann. Rev. Biochem.*, **57**, 427 (1958).

This communication presents a study of the effects of structure as well as chemical and physical properties on the rate of N-demethylation of dimethylaniline derivatives, N-methyl-N-alkylaniline derivatives and N-methyl-N-acylaniline derivatives.

Material and Method

1. **Animals**——Male rats of Wistar strain weighing about 90—150 g were used.

2. **Chemicals**——Substrates were mainly dissolved as hydrochloride salts.

3. **Preparation of Liver Microsomal Fraction**——Rats were killed by decapitation and the liver was immediately removed and homogenized with 2 volumes of ice cold 1.15% KCl solution in a Teflonglass homogenizer. The homogenate was centrifuged at $9000\times g$ for 20 minutes and the supernatant was recentrifuged at $105000\times g$ for 60 minutes in a HITACHI ultracentrifuge model 55-P. The $9000\times g$ supernatant from pooled liver of 5—10 rats was used as the enzyme source. All further procedures for the preparation of the enzyme were carried out at 0—4°.

4. **Preparation of Incubation Mixture**——The incubation mixture consisted of 1 ml of $9000\times g$ supernatant, 0.1 ml NADP (0.4 μmoles), 0.1 ml glucose-6-phosphate (20 μmoles), 0.1 ml magnesium chloride (37.5 μmoles), 0.1 ml nicotinamide (50 μmoles), 0.1 ml semicarbazide (50 μmoles), 2.3 ml of 0.2M phosphate buffer, pH 7.4, 0.2 ml of various substrates (usually 8 μmoles) in a final volume of 4 ml. The mixture was incubated at 37° for 30 minutes. Formaldehyde formation was determined by the method of Nash.[11] The data were supported by at least another repeated experiments.

5. **Induction by Phenobarbital**——Seventy mg/kg of phenobarbital sodium (in 0.9% saline) was injected subcutaneously once a day for 3 consecutive days and the last injection was done 24 hr before sacrifice.

6. **Measurement of the Lipid Solubility**——Eight μmoles of amines in 20 ml heptane were shaken with an equal volume of 0.2M phosphate buffer, pH 7.4, for 30 minutes. The concentrations of amines in heptane layer both before and after shaking were determined by ultraviolet measurement.

7. **Measurement of Microsomal Cytochrome P-450**——Contents of microsomal cytochrome P-450 were determined according to Omura and Sato,[12] using a HITACHI recording spectrophotometer model EPS-3T with an integrating spare accessory. The microsomal proteins were measured according to Lowry, *et al.*[13]

8. **Measurement of Spectral Change**——The difference spectra were recorded using a HITACHI recording spectrophotometer, Model EPS-3T with an integrating sphare accessory, as described by Schenkman *et al.*[14] For recording of substrate-induced difference spectra, 0.2 ml of substrate HCl was added to the sample cuvette and 0.2 ml of water was added to the reference cuvette containing 3 ml of the same microsomal suspension. The baseline was substracted from the change in absorbance caused by addition of different substrates to sample cuvette. Spectra were obtained at room temperature.

Result and Discussion

The rates of N-demethylation of *p*-substituted dimethylaniline derivatives are presented in Fig. 1. *p*-Dimethylaminochlorobenzene, *p*-dimethylaminomethylbenzene, *p*-dimethylaminobenzaldehyde and dimethylaniline were more rapidly N-demethylated than *p*-dimethylaminophenol and *p*-dimethylaminobenzoic acid. Difference of N-demethylation rates between the former and the latter was inclined to increase as incubation time goes on. Moreover, in N-demethylation rates, there was significant difference between *p*-dimethylaminobenzoic acid and *p*-dimethylaminophenol but not among rapidly demethylated compounds.

Data in Table I show partition coefficients, Michaelis constants (Km) and spectral dissociation constants (Ks) of *p*-substituted dimethylanilines. *p*-Dimethylaminochlorobenzene, *p*-dimethylaminomethylbenzene, *p*-dimethylaminobenzaldehyde and dimethylaniline belonged to the high lipid solubility group, and *p*-dimethylaminophenol and *p*-dimethylaminobenzoic acid belonged to the low lipid solubility one. These results suggest that the highly lipid soluble compounds are demethylated easily and that the solubility may be the main limiting factor

11) T. Nash, *Biochem. J.*, **55**, 416 (1953).
12) T. Omura and R. Sato., *J. Biol. Chem.*, **239**, 2370 (1964).
13) O.H. Lowry, H.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
14) J.B. Schenkman, H. Remmer and R.W. Estabook, *Mol. Pharmacol.* **3**, 113 (1967).

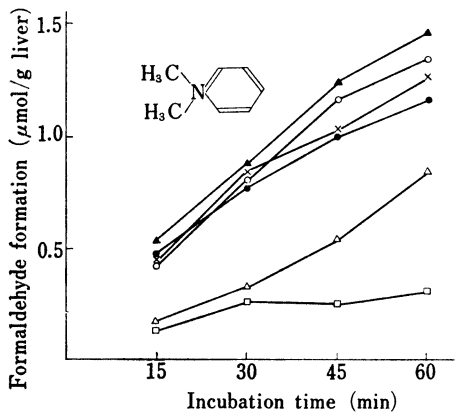


Fig. 1. Liver Microsomal Enzymatic N-Demethylation Rates of *p*-Substituted Dimethylaniline Derivatives

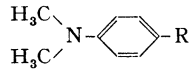
—▲—: R=Cl —●—: R=CH_3
—○—: R=H —△—: R=OH
—×—: R=CHO —□—: R=COOH

for N-demethylation. But it is not always considered that the difference of N-demethylation rates between *p*-dimethylaminophenol and *p*-dimethylaminobenzoic acid is caused by the difference of their lipid solubility.

Table II shows substrate induced spectral change per P-450 and basicity of nitrogen. These data show that the substrate induced spectral change per P-450 are correlated with the lipid solubility in the *p*-substituted dimethylaniline derivatives. These suggest that the difference of N-demethylation rates between *p*-dimethylaminophenol and *p*-dimethylaminobenzoic acid is not caused by lipid solubility and spectral change per P-450 but basicity of nitrogen.

The effects of phenobarbital administration on N-demethylation of *p*-substituted dimethylaniline derivatives are shown in Fig. 2. N-Demethylation activity of high lipid soluble

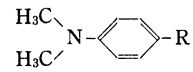
Table I. Physico-biochemical Properties of *p*-Substituted Dimethylaniline Derivatives



R	Partition coefficient[a)]	Km (mM)	Ks (mM)
-CH_3	0.99 (255 mμ)	0.31	0.13
-H	0.99 (252 mμ)	0.38	0.59
-Cl	0.99 (260 mμ)	0.34	0.32
-CHO	0.96 (316 mμ)	0.43	6.79
-COOH	0.02 (290 mμ)	1.04	1.59
-OH	0.07 (238 mμ)	0.78	55.6

a) $\frac{\text{heptane-pH 7.4 buffer}}{\text{heptane (known)}}$

Table II. Absorption Maxima and Minima and Relative Change in Absorbance of Rat Liver Microsomes Induced by *p*-Substituted Dimethylaniline Derivatives



R	ΔA/mg prot.[a)]	ΔA/ΔA P-450[a)]	Absorption (mμ) Minimum	Absorption (mμ) Maximum	Basicity[b)] (Hammett constant)
-Cl	0.017	0.154	421	380	+0.23
-H	0.017	0.148	421	383	0.00
-CH_3	0.012	0.104	420	382	−0.17
-CHO	0.024	0.213	414	—	+0.22
-COOH	0.008	0.075	418	—	+0.45
-OH	0.011	0.094	418	—	−0.37

a) The change in optical density (ΔA) observed on addition of 0.18 mM *p*-substituted dimethylaniline derivatives. (ΔA; $OD_{420-380}$ mμ, ΔA P-450; $OD_{450-490}$ mμ)
b) J. Clar and D.D. Perrm, *Quart. Res.*, (London) **18**, 295 (1964)

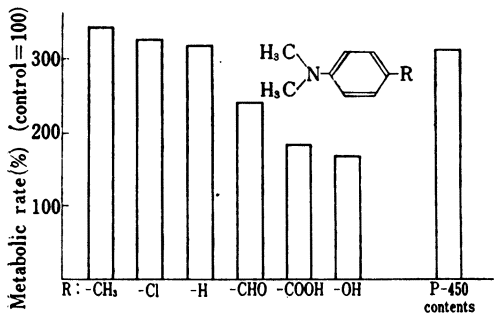


Fig. 2. Differences of N-Demethylation Rates in Phenobarbital Induced Liver amoung *p*-Substituted Dimethylaniline Derivatitives

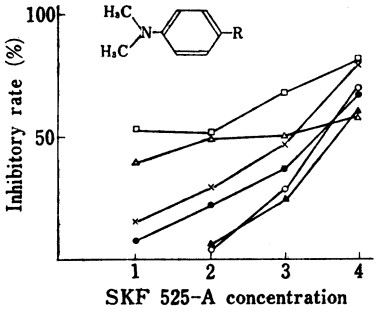


Fig. 3. N-Demethylation Rates between *p*-Substituted Dimethylaniline Derivatives in Various Concentration of SKF 525-A *in vitro*.

SKF 525-A concentration
1: 5×10^{-5}M 2: 1×10^{-4}M
3: 5×10^{-4}M 4: 1×10^{-3}M
—▲—: R=-Cl —×—: R=-CHO
—○—: R=-H —△—: R=-OH
—●—: R=-CH_3 —□—: R=-COOH

compounds was stimulated in parallel with increased P-450 contents. N-Demethylation activity of high lipid soluble compounds was increased about 3-fold by phenobarbital administration, but N-demethylation activity of low lipid soluble compounds was increased about 1.6-fold to 1.8-fold. These results suggest that in the compounds of high lipid solubility, lipid solubility will no longer be the only rat limiting factor of N-demethylation. On the contrary, in the compounds of low lipid solubility, the penetration of substrate into lipid membrane will be the rate limiting step for N-demethylation, therefore, lipid solubility will be able to be rate limiting factor of N-demethylation. For this reason, it is considered that the N-demethylation activity of compounds of low lipid solubility is not stimulated in parallel with increased P-450 contents.

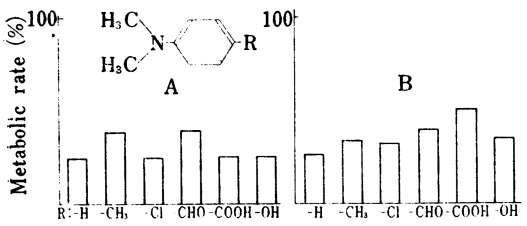


Fig. 4. Effects of Carbon Monoxide on Enzymatic N-Demethylation Rates of *p*-Substituted Dimethylaniline Derivatives

Metabolic rate is represented by following formula in the experiment A and B.

A $\frac{80\%\ CO:20\%O_2}{80\%\ N_2:20\%O_2}\times100$

B $\frac{95\%\ CO:5\%O_2}{95\%\ N_2:5\%O_2}\times100$

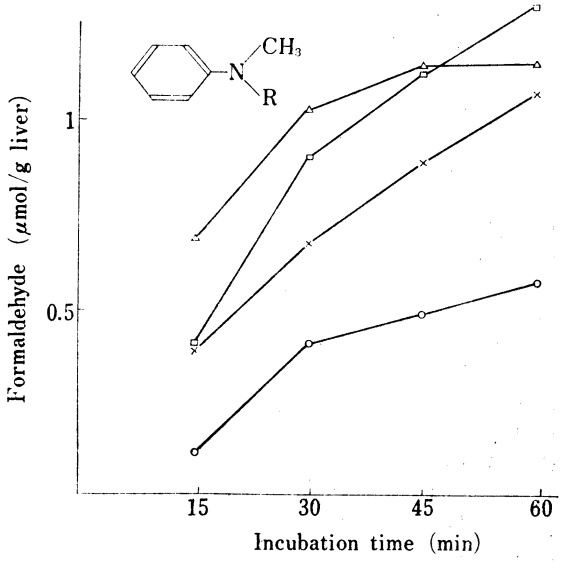


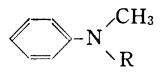
Fig. 5. Enzymatic N-Demethylation Rates of N-Methyl-N-Alkylaniline Derivatives

—□—: R=-CH_3 —×—: R=-$CH(CH_3)_2$
—△—: R=-C_2H_5 —○—: R=-$CH_2C_6H_5$

Fig. 3 and 4 show the inhibitory effects of SKF 525-A and carbon monoxide, in various concentrations, on N-demethylation of *p*-substituted dimethylanilines. The *p*-substituted dimethylanilines of low lipid solubility presented a tendency to be inhibited more strongly than those of high lipid solubility in low concentration of SKF 525-A, but this inclination disappeared in high concentration of SKF 525-A. These results suggest that the differences of inhibitory rate among dimethylaniline derivatives in low concentration of SKF 525-A are caused by the differences in the affinity of substrates for the enzyme. In the inhibitory rates of carbon monoxide on N-demethylation, no correlation were observed with the lipid solubilities of substrates. These data suggest that the inhibition mechanism by carbon monoxide may be different from that of SKF 525-A.

The demethylation rate curves for N-methyl-N-alkylaniline derivatives are presented in Fig. 5. Activity of N-demethylation was inclined to decrease as N-alkyl group was becoming larger.

TABLE III. Partition Coefficients of N-Methyl-N-Alkylaniline Derivatives



R	Partition coefficient[a]	$\Delta A/\Delta A$ P-450[b]
$-CH_3$	0.98 (252 mμ)	0.283
$-C_2H_5$	0.96 (255 mμ)	0.304
$-CH<^{CH_3}_{CH_3}$	0.94 (260 mμ)	0.285
$-CH_2-C_6H_5$	0.91 (295 mμ)	—[c]

a) $\frac{\text{heptane-pH 7.4 phosphate buffer}}{\text{heptane (known)}}$

b) ΔA is the optical density values observed on addition of 0.18 mM N-methyl-N-alkylaniline derivatives (ΔA; $OD_{420-380}$ mμ, ΔA P-450; $OD_{450-490}$ mμ)

c) We could not observe N-methyl-N-benzylaniline induced spectral change.

The partition coefficients and spectral change per P-450 of N-methyl-N-alkylaniline derivatives are presented in Table III. In all of these N-methyl-N-alkylaniline derivatives, their lipid solubilities and spectral change per P-450 were of approximately the same. These results suggest that the N-demethylation rate is influenced by the size of N-alkyl group. It appears that steric hindrance about the nitrogen atom interferes with N-demethylation. It is considered that the steric hindrance about the nitrogen atom is more effective in the reaction proceeding by direct oxidative attack on nitrogen atom than in that by direct hydroxylation on N-methyl carbon. It is shown that steric hindrance about the nitrogen is effective in preventing N-demethylation of N-methyl-N-alkylaniline derivatives. It suggests that N-methyl-N-alkylaniline N-oxides are formed by the N-demethylation of N-methyl-N-alkylaniline derivatives.

Inhibitory effects of SKF 525-A in various concentration on N-demethylation of N-methyl-N-alkylaniline derivatives are shown in Fig. 6. Inhibitory activity was inclined to decrease as the size of alkyl group became larger. It is well known that carbon monoxide and SKF 525-A do not inhibit N-oxidation of dimethylaniline.[15,16] These data suggest that N-methyl-N-alkylaniline N-oxide formation increases as alkyl group is becoming larger. It is considered that N-demethylation of dimethylaniline is inhibited more intensely than that of N-methyl-N-benzylaniline since the production of N-methyl-N-benzylaniline N-oxide may be greater than that of dimethylaniline N-oxide.

15) J.M. Machinist and D.M. Ziegler, *Fed. Proc.*, **25**, 735 (1966).
16) D.M. Ziegler and F.H. Pettit, *Biochemistry*, **5**, 2932 (1966).

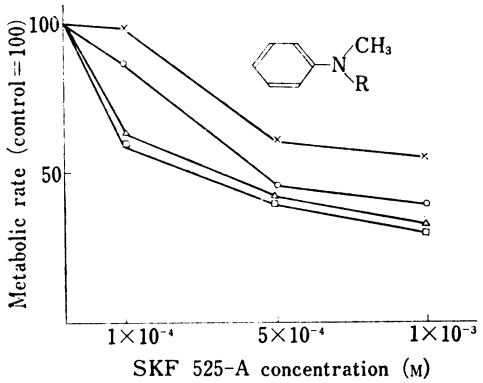


Fig. 6. Effects of SKF 525-A in Various Concentrations on N-Demethylation of N-Methyl-N-Alkylaniline Derivatives

—□—: R=-CH_3 —○—: R=-$CH(CH_3)_2$
—△—: R=-C_2H_5 —×—: R=-CH_2-C_6H_5

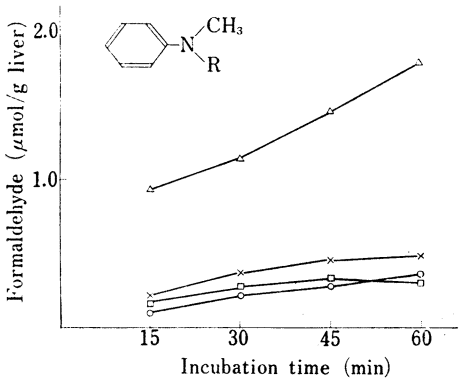
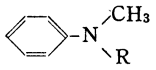


Fig. 7. Enzymatic N-Demethylation Rate of N-Acyl-N-Methylaniline Derivatives

—△—: R=-CO-C_6H_5 —○—: R=-$COCH_3$
—×—: R=-CHO —□—: R=-COC_2H_5

TABLE IV. Partition Coefficients of N-Acyl-N-Methylaniline Derivatives



R	Partition Coefficient[a)]	$\Delta A/\Delta A$ P-450[b)]
-CHO	0.49 (240 mμ)	—[c)]
-$COCH_3$	0.37 (238 mμ)	—[c)]
-COC_2H_5	0.75 (238 mμ)	0.026
-COC_6H_5	0.98 (250 mμ)	0.085

a) $\frac{\text{heptane-pH 7.4 phosphate buffer}}{\text{heptane (known)}}$

b) ΔA is the optical density values observed on addition of 0.18 mM N-methyl-N-acylaniline derivatives ($\Delta A_{420-380}$ mμ, ΔA P-450: $OD_{450-490}$ mμ)

c) We could not observe N-methyl-N-formylaniline induced spectral change and N-methyl-N-acetylaniline induced spectral change.

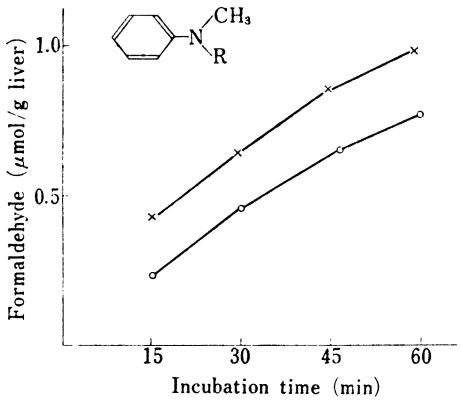


Fig. 8. Difference of N-Demethylation Rates between N-Methyl-N-benzylaniline and N-Methyl-N-benzoylaniline

R=CH_2-C_6H_5 : —○—
R=CO-C_6H_5 : —×—

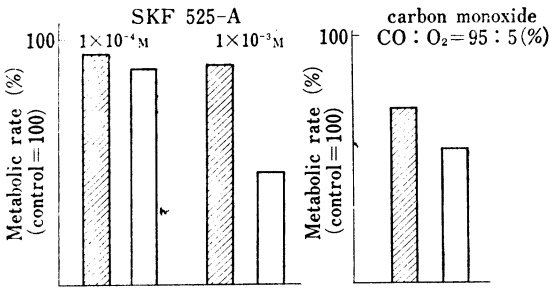


Fig. 9. Effect of SKF 525-A and Carbon Monoxide on N-Demethylation Rates of N-Methyl-N-benzyl-aniline and N-Methyl-N-Benzoylaniline

▨: N-methyl-N-benzylaniline
□: N-methyl-N-benzoylaniline

Demethylation rate curves of N-methyl-N-acylaniline derivatives are shown in Fig. 7.

Partition coefficients and substrate induced spectral change per P-450 of N-methyl-N-acylaniline derivatives are presented in Table IV. N-Methyl-N-benzoylaniline was rapidly demethylated in comparison with N-methyl-N-formylaniline and N-methyl-N-acetylaniline. It is considered that the differences in N-demethylation rates of these compounds result from differences in lipid solubilities. But it is considered that the fact that N-methyl-N-benzoylaniline was demethylated more rapidly than N-methyl-N-propionylaniline, may be due mainly to the difference in spectral change per P-450. These results suggest that in demethylation of N-methyl-N-acylaniline derivatives, steric hindrance about the nitrogen atom does not interfere with N-demethylation.

The rate of N-demethylation and the effects of SKF 525-A or carbon monoxide on demethylation of N-methyl-N-benzylaniline and N-methyl-N-benzoylaniline are presented in Fig. 8. and 9. It is of interest that demethylation of N-methyl-N-benzoylaniline, was inhibited more strongly than that of N-methyl-N-benzylaniline, not only in low concentration but also in high concentration of SKF 525-A. It is considered that N-methyl-N-benzoylaniline does not form its N-oxide, probably, because steric hindrance about nitrogen atom does not, in itself, interfere with N-demethylation. On the contrary, it is suggested that N-methyl-N-benzylaniline formed its N-oxide. And it is well known that carbon monoxide or SKF 525-A do not inhibit N-oxide formation of dimethylaniline. It seems possible that these differences in inhibitory rates are caused by the difference in metabolic pathway.