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Demethylation Studies—III. The *in vitro* Demethylation of Dialkylmethylamines

ROBERT E. MCMAHON and NELSON R. EASTON, *The Lilly Research Laboratories, Indianapolis, Indiana*

Many drugs which contain an *N*-methyl group are metabolized in the body by oxidative *N*-demethylation. In recent years it has been established that the enzyme system (or systems) which is responsible for this reaction is localized in the submicroscopic particles of mammalian liver cells. The reaction, an oxidation, requires oxygen and TPNH and yields as primary products formaldehyde and the corresponding demethylated compound.[1,2,3] This enzyme system is little understood and has not as yet been obtained in a soluble form, but it is now becoming clear that lipid solubility is a major factor in determining whether or not a substrate will be *N*-demethylated.[4,5] The more fat-soluble the amine is the more readily it will be demethylated. This appears to be true in both the *in vitro* and *in vivo* system.[5]

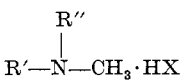
Although a great deal of work has been done with substrates that contain aromatic rings in the molecule, little is known concerning the demethylation of purely aliphatic substituted methylamines. It has now been found[6] that aliphatic methylamines are indeed readily demethylated if they are sufficiently lipid-soluble. This communication presents a study of the effect of structure and physical properties upon the rate of *N*-demethylation of several series of aliphatic methylamines.

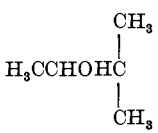
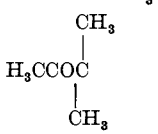
Experimental

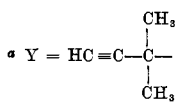
Substrates. The preparation of several of the substrates needed for this work has been described earlier by Ainsworth and Easton.[7] Those amines used in this work, but not described in reference 7, were prepared by procedures to be published[7,8] and are tabulated

in Table I. The pK_a's were determined by D. O. Woolf by potentiometric titration of very dilute solutions of the hydrochloride salts in water. A paper discussing the influence of chemical

Table I. Dialkylmethylamine hydrohalides



R′	R″	HX	m.p., °C	Formula	Nitrogen	
					Calcd.	Found
n-propyl	*n*-propyl	HCl	172–175	$C_7H_{18}ClN$	9·24	9·04
n-butyl	*n*-butyl	HCl	122–124	$C_9H_{22}ClN$	7·79	7·51
s-butyl	*s*-butyl	HBr	115–117	$C_9H_{22}BrN$	6·25	5·98
i-butyl	*i*-butyl	HCl	126–128	$C_9H_{22}ClN$	7·79	7·98
n-amyl	*n*-amyl	HCl	123–124	$C_{11}H_{26}ClN$	6·74	6·51
n-propyl	*n*-amyl	HCl	122–124	$C_9H_{22}ClN$	7·79	7·55
n-butyl	*n*-amyl	HCl	118–119	$C_{10}H_{24}ClN$	7·23	7·43
Y[a]	ethyl	HCl	185–186	$C_8H_{16}ClN$	8·67	8·56
Y	*n*-propyl	HCl	145–147	$C_9H_{18}ClN$	7·97	8·08
Y	*n*-butyl	HCl	150–152	$C_{10}H_{20}ClN$	7·38	7·56
Y	*s*-butyl	HCl	135–137	$C_{10}H_{20}ClN$	7·38	7·47
Y	benzyl	HCl	186–188	$C_{13}H_{18}ClN$	6·26	6·24
	*i*-propyl	HCl	136–138	$C_9H_{22}ClNO$	7·16	7·20
	*i*-propyl	HCl	167–168	$C_9H_{20}ClNO$	7·23	7·32

[a] Y = 

structure upon base strength will be published separately. Stock solutions of the water-soluble salts of the substrate amines were made up in distilled water for the demethylation studies.

Demethylation studies. Demethylation studies were carried out using the microsomes plus soluble fraction prepared from the livers of adult male rats by the procedure described by Axelrod.[2] The conditions under which the various substrates were incubated with enzyme preparation are given in the footnotes of the Tables and Figures found in the text. The formaldehyde formed in the reaction (trapped as the semicarbazone) was measured spectrophotometrically after reaction with 2,4-diketopentane in ammonium chloride solution. Details of the methods used in these studies have been published.[5, 9] Although many different batches of enzyme were used for this work, each set of data presented in the Tables was obtained with a single batch of microsomes.

Results and Discussion

A reasonable explanation of the discrimination among substrates shown by the *N*-demethylating enzyme system is that the enzyme is protected by a lipid barrier and that only those compounds which are lipid-soluble enough to penetrate the barrier are acted upon by the enzyme.[4, 5] If lipid solubility is the limiting factor, then it seemed reasonable to assume that purely aliphatic methylamines would serve as substrates provided they were sufficiently fat-soluble. Such was the case.

For example, preliminary experiments showed that di-*n*-amylmethylamine (Compound V, Fig. 1) was as readily demethylated as is propoxyphene,[5] a good substrate for the enzyme system. Fig. 1 presents a series of curves showing the rate of demethylation of five selected substrates. The curves are typical enzyme rate curves showing a drop in rate with time due to loss of enzyme activity, inhibition by product, depletion of substrate, etc. It should be observed, however, that the rates are sufficiently linear for the first 15 min to allow the use of 15-min rates as an approximation of the initial rate.

In Fig. 2 the effect of concentration upon rate is presented in the form of a Lineweaver–Burke plot.[10] It is interesting to note that the maximum rate at enzyme saturation is very nearly the same for the three substrates. The 'half-saturation values' ($-K_m$), however, show a wide variation. The meanings of these observations are as yet obscure. In the work discussed below, substrate

concentrations were high enough so that the rate measured did not differ greatly from maximum rates.

The distribution of an amine between a buffered aqueous phase and a lipid phase is governed by two factors: the relative solubility of the free base in each phase and the base strength which regulates the amount of free base available for distribution between the phases. The effect of both of these factors upon the

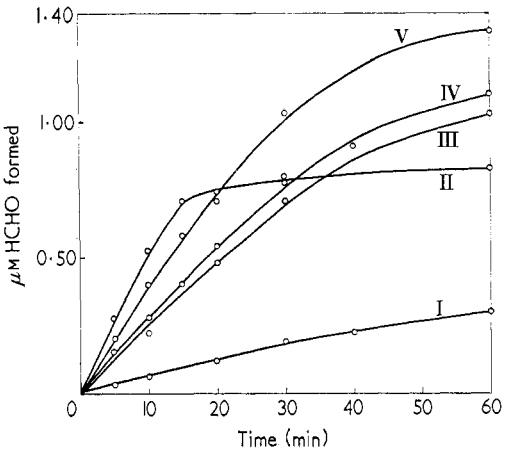


Fig. 1. Conditions: Each incubation flask contained 25 μM nicotinamide, 25 μM $MgCl_2$, 0·5 μM TPN+, 10 μM glucose-6-phosphate (sodium salt) 45 μM semicarbazide (neutralized), 0·5 ml of 0·5M phosphate buffer, pH 7·4, supernatant fraction containing microsomes from 200 mg of rat liver, substrate and sufficient water to bring the volume to 3 ml. Substrates were present at an initial concentration of 5×10^{-3}M. The flasks were incubated with shaking at 37° in air for the indicated length of time.

The compounds shown are *N*-*t*-amyl-*N*-*t*-butyl methylamine (I), 3-ethyl-3-(*N*-*i*-propyl-*N*-methylamino)-pentyne-1 (II), 3-methyl-3-(*N*-*t*-butyl-*N*-methylamino)butyne-1 (III), 3-methyl-3-(*N*-*i*-propyl-*N*-methylamino) butanone-2 (IV), and *N*,*N*-di-*n*-amylmethylamine (V).

N-demethylation rate has been investigated. In Table II the rate of demethylation of a series of di-(*n*-alkyl)-methylamines is presented. As all of these amines are of approximately the same base strength, the only important variant is lipid solubility which should, in this well-defined case, parallel molecular weight. As one would predict, the 'initial' (15-min) rate of demethylation increases regularly with increasing molecular weight. In the case

of di-(*n*-butyl)methylamine (3) and *n*-amyl-*n*-propylmethylamine (4), which are of equal molecular weight, the symmetrically substituted one is the more active substrate.

In Table III amines of equivalent molecular weight but of differing base strength are considered. Although the initial demethylation rates correlate well with decreasing base strength as predicted, one cannot overlook a new variable which has been introduced, i.e. steric hindrance caused by an increase in branching

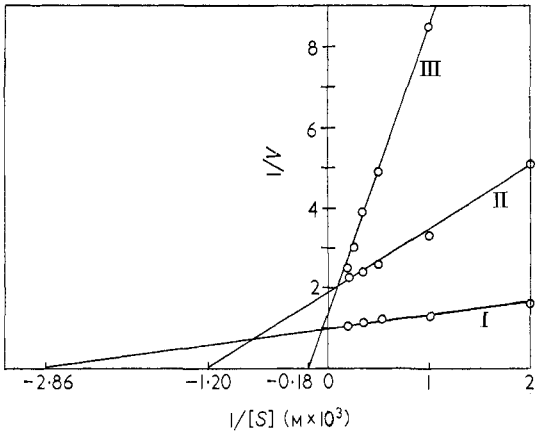


Fig. 2. Conditions: As shown in Fig. 1. The rate V is expressed as μM of HCHO formed per 200 mg of liver in the 15-min period of incubation. $[S]$ is the concentration of substrate. The substrates used were 3-methyl-3-(*N*-benzylmethylamino)butyne-1 (I), *N*,*N*-di-*n*-amylmethylamine (II), and 3-methyl-3-(*N*-isopropylmethylamino)butanone-2 (III).

on the carbon atoms attached to nitrogen. It seemed possible that the decreased activity of the highly branched substrates might be due in part to the effect of this hindrance about the nitrogen as well as to the increased base strength of such highly hindered amines. In order to gain some insight into this possibility, the series of amines shown in Table IV was studied. In this series substitution on the carbons adjacent to the nitrogen has been held constant and near maximum. Groups that effect base strength have been introduced into the side chain. It is seen that compounds 1 and 2, in which the pK_a is appreciably

Table II. Demethylation of some dialkylmethylamines $R'R''NCH_3$

No.	R′	R″	μM HCHO formed per gram liver in: 15 min	60 min	pK_a
1	*n*-amyl	*n*-amyl	1·77	5·15	10·4
2	*n*-amyl	*n*-butyl	1·44	3·40	10·4
3	*n*-butyl	*n*-butyl	0·46	1·47	10·5
4	*n*-amyl	*n*-propyl	0·39	1·23	10·4
5	*n*-propyl	*n*-propyl	0·06	0·17	10·4

Conditions: As shown in Fig. 1. Substrate concentrations: 3×10^{-3}M.

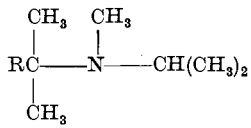
lowered, are good substrates for the *N*-demethylating enzyme, whereas the other three compounds which are strong bases are very poor substrates. It appears then that steric hindrance about the nitrogen is not, in itself, effective in preventing demethylation. Either the lowered pK_a or the actual presence of the —C≡CH or $-COCH_3$ group must be responsible. That this second possibility is unlikely is seen by examination of the final series of compounds (Table V). In this series the acetylene group is present in all cases; substitutions have been made on the nitrogen in such

Table III. Demethylation of some dialkylmethylamines $R'R''NCH_3$

No.	R′	R″	μM HCHO formed per gram of liver in: 15 min	60 min	pK_a
1	*i*-butyl	*i*-butyl	0·95	1·55	10·1
2	*n*-butyl	*n*-butyl	0·65	2·06	10·4
3	*n*-amyl	*n*-propyl	0·55	1·65	10·4
4	*s*-butyl	*s*-butyl	0·25	0·70	11·1
5	*t*-amyl	*i*-propyl	0·25	0·75	11·2
6	*t*-amyl	*t*-butyl	0·15	0·50	11·9

Conditions: As shown in Fig. 1. Substrate concentration: 5×10^{-3}M. Di-(*t*-butyl)methylamine was not available and *t*-amyl-*t*-butylmethylamine was substituted.

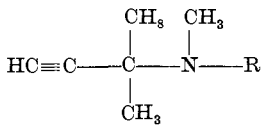
Table IV. Demethylation of some dialkylmethylamines



No.	R	μM HCHO formed per gram of liver in: 15 min	60 min	pK_a
1	—C≡CH	1·95	3·20	8·7
2	—$COCH_3$	1·80	6·05	9·3
3	—$CH{=}CH_2$[a]	0·20	0·50	11·3
4	—CH_2CH_3	0·10	0·40	11·2
5	—$CHOHCH_3$	0·20	0·20	11·4

Conditions: As shown in Fig. 1. Substrate concentration: 3×10^{-3}M. [a] The *N*-isopropyl derivative of this amine was not available and the *N*-*t*-butyl analogue was substituted.

Table V. Demethylation of some dialkylmethylamines



No.	R	μM HCHO formed per gram liver in: 15 min	60 min	pK_a
1	methyl	0·60	0·95	7·9
2	ethyl	0·65	1·10	8·2
3	*n*-propyl	1·65	2·25	8·2
4	*i*-propyl	1·20	2·30	8·7
5	*n*-butyl	3·20	3·85	8·2
6	*s*-butyl	2·30	2·55	8·8
7	*t*-butyl	1·75	4·05	9·3
8	benzyl	3·70	6·50	7·1

Conditions: As shown in Fig. 1.

a way as to influence both pK_a and molecular weight. In the series where R is methyl (1), ethyl (2), *n*-propyl (3), or *n*-butyl (5), the change in pK_a is small and rates increase as molecular weight increases, demonstrating the effect of lipid solubility. The effect of increasing base strength at equal molecular weight is also seen. The isopropyl (4) analogue has a higher pK_a than the unbranched analogue (5) and a lower rate of demethylation. In the series R equals *n*-butyl (5), *s*-butyl (6), *t*-butyl (7), pK_a increases and rate decreases in the expected manner. In compound 8 the introduction of the benzyl group serves to both increase lipid solubility and lower pK_a to produce one of the most active substrates encountered in these studies.

The importance of lipid solubility is best explained in terms of a model in which the *N*-demethylating enzyme system located in the microsomes is protected from substrates in the surrounding aqueous phase by a lipid barrier. Only those substrates which are sufficiently lipid-soluble to allow penetration of the barrier could be demethylated by these enzymes. It seems apparent that a more sophisticated understanding of these matters awaits the preparation of this enzyme in a soluble form. It would then be possible to study enzyme–substrate interaction without the interfering presence of the 'lipid barrier'. It is encouraging to note that recently two groups of workers[11, 12] have succeeded in solubilizing the aromatic hydroxylase activity of microsomes. Significantly, solubilization was achieved by treatment of purified microsomes with lipase preparations.

The finding that steric hindrance about the nitrogen atom does not in itself interfere with *N*-demethylation is of interest. It suggests that the reaction may proceed by direct oxidative attack upon the less hindered methyl carbon as suggested by Brodie *et al.*[3] rather than upon the highly hindered nitrogen as proposed by Horning's group.[13, 14]

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Summary. It has been demonstrated that dialkylmethylamines are suitable substrates for the *N*-demethylating enzyme found in the microsomal fraction from liver. There is an empirical relationship between

the two properties, molecular weight and base strength, and the rate at which demethylation occurs, i.e. the rate of *N*-demethylation increases not only with increasing molecular weight but also with decreasing base strength. It is proposed that these two factors exert their influence by their effect upon lipid solubility and that the rate of demethylation correlates with fat solubility in the same manner as observed with the aryl-alkylamines studied earlier.[5]

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