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Demethylation Studies—I. The Effect of Chemical Structure and Lipid Solubility

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One of the recent landmarks in the developing field of drug metabolism was the discovery by Brodie *et al.*[1] of the oxidative enzyme systems which are localized in the microsomal fraction of mammalian liver. These enzymes are distinguished by an unusual lack of specificity[2] and appear to act upon lipid-soluble foreign materials which enter the body. Gaudette and Brodie[3] have indeed demonstrated a relationship between the lipid solubility of the substrates and their tendency to be metabolized. The net result of the action of the microsomal enzymes is the conversion of a lipid-soluble compound into a more polar form which can be excreted through the kidney.

Although the microsomal enzyme systems are by no means the only enzymes involved in the biotransformation of drugs, their importance is great enough to warrant extensive study. For example, a knowledge of the relationship of chemical structure to enzyme action would be valuable for medicinal chemists in the designing of new drugs.

Among the reactions for which the microsomal enzymes are responsible is the oxidative demethylation of substituted methylamines. Early studies of this reaction were carried out by Axelrod[4] and by La Du *et al.*[5] As part of a study of the effects which changes in chemical structure (and the attendant changes in physical properties) have upon drug metabolism, we have examined the demethylation of a group of arylalkyldimethylamines. Both *in vitro* and *in vivo* work is reported and attention is given to the problem of species differences. The data secured in these experiments yield a good correlation with lipid solubility.

Experimental

Labelled Amines

γ-Phenylpropyldimethyl-^{14}C-amine and phenethyldimethyl-^{14}C-amine were prepared in good yield by reductive alkylation of the corresponding secondary amine with radioformaldehyde and Pd–C/H_2. The procedure which was developed earlier[6] for the preparation of erythromycin-^{14}C was employed. γ-(*p*-Chlorophenyl)propyldimethyl-^{14}C-amine and benzyldimethyl-^{14}C-amine were prepared from the secondary amines by alkylation with radioformaldehyde and formic acid (*cf.* Tarpey *et al.*[7]). The radiochemical yield was better than 75 per cent in each case. The preparation of (±)-demethyl propoxyphene carbinol and its conversion to (±)-propoxyphene carbinol-*N*-methyl-^{14}C and (±)-propoxyphene-*N*-methyl-^{14}C has been reported by Pohland *et al.*[8] All of the labelled amines were isolated and purified as their hydrochloride salts.

Each of the labelled amines proved to be identical with an authentic sample of known material with respect to melting point, X-ray diffraction pattern, paper chromatographic behaviour, and lipid–buffer distribution. Some of the properties of these labelled compounds are presented in Table I.

Table I. Physical properties of *N*-methyl labelled amines

	Name	m.p. °C of HCl salt[a]	pK_a (H_2O)	$K_{heptane}$[b]	R_f
I	±-Propoxyphene	170–173	8·9	141	0·79
II	±-Propoxyphene carbinol	229–230	9·4	43·5	0·50
III	γ-(*p*-Chlorophenyl)-propyl dimethylamine	155–156	9·6	3·1	0·43
IV	γ-Phenylpropyldimethyl-amine	145–146	9·5	0·85	0·26
V	Phenethyldimethylamine	165–167	9·3	0·54	0·13
VI	Benzyldimethylamine	178–180	$8·9_5$	1·18	0·08

[a] Corrected.
[b] Partition coefficient expressed as the ratio of total base concentration in the heptane layer to total base concentration in 0·1 M phosphate buffer (pH 7·4) layer.

Paper Chromatography

Paper chromatographic separations were carried out in the buffered system described previously.[9, 10] Tertiary amines, (±)-demethyl propoxyphene and (±)-demethyl propoxyphene carbinol were made visible on the chromatograms with Dragendorff reagent. Secondary amines were detected with the nitroprusside spray developed by Sweeley and Horning.[11] (±)-Demethyl propoxyphene and (±)-demethyl propoxyphene carbinol do not react well with this reagent. Phenols were located by spraying with a solution of *p*-nitrophenyldiazonium fluoborate in alcohol followed by 5 per cent sodium carbonate. Radioactive areas were detected with an automatic scanning device.

Lipid–Water Distribution

Solutions (5×10^{-3}M) of the amines to be studied were prepared in pH 7·4 0·1M phosphate buffer. The solutions were then equilibrated with an equal volume of heptane (Phillips pure grade, 99 mole per cent minimum). The concentration of amine in each layer was measured both by ultraviolet measurement and by radioactivity assay in the case of the labelled amines. The partition coefficients calculated from these data are shown in Table I.

In Vitro *Demethylation Studies*

The microsome plus soluble fraction was prepared from the livers of adult male mice, rats, and guinea pigs as described by Axelrod.[4] These preparations showed little loss of activity when stored at −15° for several weeks.

For the purpose of determining demethylation rates, varying amounts of substrate were incubated in 20-ml beakers for 1 h at 37° in air with the following mixture: microsome plus soluble fraction from 200 mg of liver, 25 μM of nicotinamide, 25 μM of $MgCl_2$, 45 μM of semicarbazide, 0·25 of μM TPN^+, 11 μM of glucose 6-phosphate, 0·5 ml of 0·25M phosphate buffer (pH 7·4), and sufficient water to bring the final volume to 3 ml. The resulting incubate was assayed for formaldehyde by the method of Cochin and Axelrod[12] as follows. The incubation mixture was added to 4 ml of a 10 per cent solution of $ZnCl_2$ and, after stirring, 2 ml of a saturated solution of $Ba(OH)_2$ was added. After centrifugation,

a 5 ml sample of supernatant was removed and treated with 2 ml of double strength Nash reagent.[13] The colour was developed by heating at 60° for 30 min, and the tubes were read at 415 mμ. Known amounts of formaldehyde carried through the entire procedure served as standards.

A typical set of data is presented in Table II. Runs made at other concentrations of substrate show the same relative rates.

Table II. *In vitro* demethylation rates

Compound	μM HCHO per g liver per h		
	Guinea pig	Rat	Mouse
I	5·25	5·32	7·45
II	6·31	7·87	7·90
III	4·44	4·47	5·80
IV	4·35	4·22	3·85
V	3·89	2·87	3·40
VI	1·75	2·89	3·60

Conditions: Incubation flasks contained 25 μM of nicotinamide, 25 μM of $MgCl_2$ soluble plus microsome fraction from 200 mg liver, 0·25 μM of TPN^+, 11 μM of glucose 6-phosphate, 45 μM of semicarbazide, 0·5 ml of 0·25M phosphate buffer, pH 7·4, substrate and sufficient water to bring the volume to 3 ml. Five micromoles of substrate was used for the rat and mouse experiments and 2 μM in the case of the guinea pigs. The flasks were incubated with shaking for 1 h at 37° in air.

An exception was found in the case of the microsomal system from guinea pigs, where large substrate concentrations (>10 μM) led to substrate inhibition of the enzyme, and a confusing picture developed.

In Vivo *Demethylation Studies*

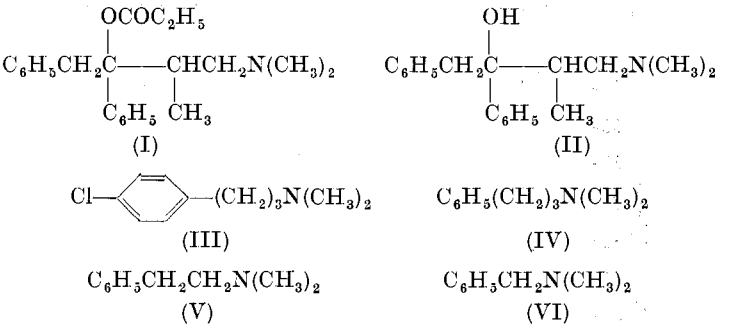
All of the *in vivo* work was carried out in male animals which had been fasted overnight with access to water. Rats averaged 150 g in weight, mice 25 g and guinea pigs 200 g. Solutions containing 5 μM/ml of radioactive substrates were prepared in 0·9 per cent saline. All injections were made by the intraperitoneal route at a dose of 50 μM/kg body weight. The rate and extent of radiocarbon dioxide elimination was followed by the procedure described in detail elsewhere.[14] The data presented

for rats and for guinea pigs represent the average from three animals. The mouse data were obtained by pooling the radiocarbon dioxide from three animals before analysis.

Urinary Metabolites

Twenty-four-hour urine collections were made from groups of three animals. The urine was collected in the presence of the antibacterial agent, thimerosol. An aliquot was dissolved in a toluene–2-propanol–DPO mixture for counting.[15] The urine samples were adjusted to pH 10, extracted three times with an equal volume of diethyl ether, and the radioactivity content of the extract determined. The extracts were examined by paper chromatography in order to identify the radioactive metabolites.

The six arylalkyldimethyl amines chosen for this study were (±)-propoxyphene (I), (±)-propoxyphene carbinol (II), γ-(*p*-chlorophenyl)propyldimethylamine (III), γ-phenylpropyldimethylamine (IV), phenethyldimethylamine (V), and benzyldimethylamine (VI).



(+)-Propoxyphene is an analgesic of considerable interest, since its addiction liability has been found to be substantially less than that of codeine.[16] The compound was first prepared by Pohland and Sullivan[17] and was later resolved by these same workers.[18] The (+)-form is responsible for the analgesic activity. The metabolism of (±)-propoxyphene has been investigated by Lee, Scott, and Pohland,[19] who found it to be metabolized through demethylation. (±)-Propoxyphene carbinol, the alcohol from which propoxyphene is derived, is without analgesic properties.

Oxidative demethylation of substituted methylamines by the microsomal enzyme system yields the dealkylated amine and formaldehyde as primary products.[4, 5] In *in vitro* experiments, the

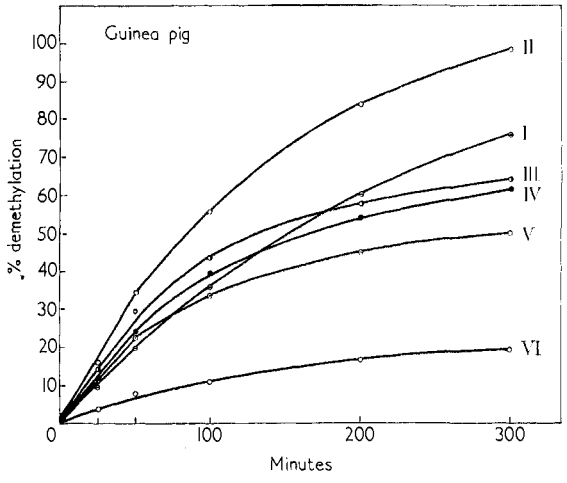


Fig. 1. *In vivo* demethylation rates in the guinea pig.

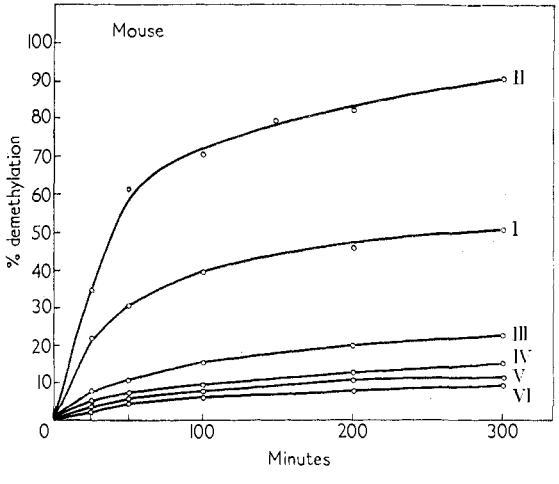


Fig. 2. *In vivo* demethylation rates in the mouse.

rate of demethylation is easily followed by determining the rate of formaldehyde formation. In Table II, the rate of enzymatic

demethylation of each of the six amines in each of the three species is tabulated. In all three species, the demethylation rates fall in the descending order II > I > III > IV > V and VI. Phenethyldimethylamine (V) and benzyldimethylamine (VI) were demethylated at about the same rate by rat or mouse microsomes, but in the case of the guinea pig system, V was the better substrate of the two.

In vivo demethylation studies were carried out with the same six amines labelled in the *N*-methyl group. The details of the labelling procedures are considered in the experimental section.

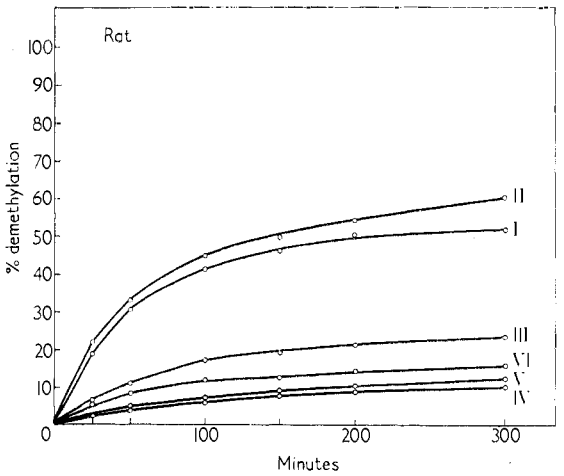


Fig. 3. *In vivo* demethylation rates in the rat.

When demethylation occurs in the whole animal, the formaldehyde formed initially undergoes further oxidation and is finally expired as carbon dioxide. Thus, by following the rate and extent of elimination of respiratory radiocarbon dioxide, one can study *in vivo* demethylation of drugs which have been labelled in an *N*-methyl group. It should be pointed out that, since each amine possesses two methyl groups on the nitrogen, the rate of demethylation is actually twice the rate at which radiocarbon dioxide is respired.

In Figs. 1, 2 and 3, the demethylation rate curves for each labelled amine in each species under study are presented. The

dose in each case was 50 μM/kg, administered by the intraperitoneal route. In both the guinea pig (Fig. 1) and the mouse (Fig. 2), the relative rates of demethylation for the six compounds under study were identical to those found in the microsomal enzyme studies reviewed above. In terms of extent of demethylation, the guinea pig was the more efficient of the two species, particularly in the case of compounds III, IV, V, and VI. In the rat, the order was again the same, with the exception of γ-phenylpropyldimethylamine (IV) which was the poorest substrate in this species.

In order to assess the meaning of the above results, it was felt that some information concerning other routes of metabolism should also be collected. Table III summarizes our current

Table III. Urinary metabolites in the rat

	Amine	% Radioactivity found in urine in 24 h	Metabolites identified
I	(±)-Propoxyphene	17	(±)-Demethylpropoxyphene
II	(±)-Propoxyphene carbinol	33	(±)-Demethylpropoxyphene carbinol
III	γ-(*p*-Chlorophenyl)propyl dimethylamine	63	γ-(*p*-Chlorophenyl)propyl-methylamine, unchanged amine
IV	γ-Phenylpropyldimethyl amine	82	γ-(*p*-Hydroxyphenyl)propyl-dimethylamine
V	Phenethyldimethylamine	88	Unchanged amine
VI	Benzyldimethylamine	92	Unchanged amine

knowledge concerning urinary excretion of radioactive metabolites by the rat. The proportion of the radioactivity administered appearing as urinary metabolites was lowest for (±)-propoxyphene and (±)-propoxyphene carbinol. In the case of the other four amines, excretion of radioactivity in the urine was much greater. In addition to the large proportion of radioactivity expired as carbon dioxide in the case of (±)-propoxyphene, Lee *et al.*[19] have shown that an appreciable amount is excreted in faeces. In the present work it was found that excretion of radioactivity in faeces was negligible except for (±)-propoxyphene-^{14}C.

The radioactive metabolites present in the non-polar solvent

extracts from the urines were identified by paper chromatography and suitable colour tests. The only metabolite found in the propoxyphene urine was (±)-demethylpropoxyphene, the primary product of the demethylation reaction. This is in agreement with the earlier studies of Lee *et al.*[19] The demethylated product was also the major metabolite in the case of (±)-propoxyphene carbinol. The urinary metabolites of γ-(*p*-chlorophenyl)propyldimethylamine (III) also included the demethyl compound and, in addition, some unchanged drug appeared. The only metabolite of compound IV identified was γ-(*p*-hydroxyphenyl)propyldimethylamine, the product of ring hydroxylation. Urine from rats receiving either compound V or VI contained the unchanged amine as the only radioactive metabolite.

The radioactive metabolites which have been identified so far do not by any means account for all of the radioactivity present in urine. After extraction of the metabolites referred to above, there still remain in the urine considerable amounts of radioactivity in the form of more polar metabolites which have not as yet been identified.

Discussion

Gaudette and Brodie[3] have proposed, as a working hypothesis, that the microsomal enzymes are protected in some manner by a lipoidal barrier which is penetrated only by fat-soluble compounds. The *in vitro* data obtained in the present work correlate well with such a model. The relative *in vitro* demethylation rates (Table II) for the six amines were found to correlate well among the three species studied, indicating the generality of the system under study. These rates show a very good positive correlation with lipid solubilities (Table I) expressed as a distribution ratio between heptane and pH 7·4 buffer. Such a result is predicted by the Gaudette and Brodie proposal. Heptane was chosen as a model for the lipid phase, since it gave a wide variation in distribution constants among the amines under study. The distributions of drugs between heptane and buffer have also served as useful models in relating lipid solubility to intestinal absorption[20] and to the passage of drugs into cerebrospinal fluid.[21]*

* There are two factors which influence the distribution of an amine between a buffer solution and a lipid phase. One influence is the inherent lipid solubility of the amine which determines the distribution of the unionized amine. The

Extension of these studies to the whole animal also proved of interest. The overall correlation between *in vitro* and *in vivo* results is good, as is the correlation with lipid solubility. The rather good correlation of *in vitro* data with *in vivo* results is particularly interesting considering that in the whole animal such factors as absorption, distribution, plasma binding, kidney excretion, etc. can influence the result.

It is also of interest to note that, although the absolute rates of demethylation vary between species, the relative order of importance of the amines as demethylation substrates does not vary much from species to species. It would seem that certain generalities concerning species differences might be developed if enough data were available.

The data here summarized thus show a definitive correlation between demethylation rate and lipid solubility both in the isolated enzyme system and in the whole animal, i.e., the rate-limiting step in demethylation appears to be controlled by the lipid-solubility of the substrate. At present the best representation of the rate-limiting step is the penetration of a lipoidal barrier which surrounds the enzyme.

Certain exceptions to the basic generalization appeared, however, and warrant further discussion. The most obvious failure of the generalization is with (±)-propoxyphene and (±)-propoxyphene carbinol. Although the carbinol is less lipid-soluble than (±)-propoxyphene, it is the better substrate of the two in all three species and in both *in vivo* and *in vitro* experiments. Both of these compounds are extremely lipid-soluble and should have no difficulty passing a lipoidal barrier. It may be that the extent of microsomal enzyme action may depend not only on lipid solubility

other factor is the base strength of the amine. This determines the concentration of unionized amine available in the aqueous phase for distribution. The lipid solubility may be expressed either as the ratio of the concentrations of unionized amine in the two phases or as the ratio of the total (ionized plus unionized) concentrations of amine in the two layers. In this discussion the latter, or 'measured', distribution coefficient is used. With the single exception of benzyldimethylamine (VI), the *relative* lipid solubilities are the same whichever method is used. When the 'measured' partition coefficient is used, as is done in Table I, VI appears to be more fat-soluble than IV or V. Based on the partition of unionized amine only, VI is found to be the least fat-soluble of the six amines, which is more in line with the demethylation data presented in this paper. We are grateful to a referee for bringing out these points in his report.

but upon chemical structure as well. The effect of chemical structure may show up only among highly lipid-soluble compounds where penetration of the lipid barrier is not rate-limiting. It would be of interest to study substrate specificity among a group of substrates all possessing the same fat solubility (preferably high fat solubility).

The results with *p*-chloro substituted amine (III) were also of interest. Although the chloro substitution increased the lipid solubility considerably over the unsubstituted analogue (IV), the increase in demethylation was not great. This is reminiscent of the findings in the study of the metabolism of ethoxybutamoxane[22] and chlorethoxybutamoxane.[14] Although the introduction of a chloro substituent into ethoxybutamoxane increased drug potency, it had no effect whatsoever upon the metabolism of the compound.

A third interesting observation is that in the whole rat (Fig. 3), compound IV (γ-phenylpropyldimethylamine) is out of line and is actually the poorest substrate of the six amines. Information obtained in the urine studies (Table III) perhaps supplies the answer. This compound in the rat undergoes a competing reaction, i.e. ring hydroxylation to yield *p*-hydroxyphenylpropyldimethylamine. There was no evidence that ring hydroxylation occurred in the case of the other five amines.

Among the least lipid-soluble amines, particularly V and VI, excretion of unchanged drug through the kidney was appreciable. Thus, it would seem that, as a generalization, fat-soluble dimethylamines are demethylated in the body to the more polar secondary amines, whereas less fat-soluble (more polar) ones can be excreted unchanged. Furthermore, certain very water-soluble methylamines[3] are dealkylated by enzymes located in the mitochondria.

We have extended this work more recently to wholly aliphatic amines and find a similar influence of lipid solubility upon demethylation.[23]

Summary. The enzymatic demethylation of a series of six arylalkyldimethylamines has been studied in three species: mouse, rat, and guinea pig. Both *in vitro* studies employing microsomal preparations from liver and studies in whole animals were carried out. From the data obtained, it is obvious that there is a positive correlation between fat solubility and rate of demethylation. The discrimination among substrates shown by

the demethylating enzyme(s) of the liver microsomal fraction seems to be based upon the lipid solubility of the substrate. In addition, it was found that there was reasonably good correlation between the demethylation rates found in the three species and between the *in vitro* and the *in vivo* results.

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