Demethylation Studies. V. The *in Vivo* and *in Vitro* N-Demethylation of N,N-Dimethyl-3,5,7-trimethyladamantane-1-carboxamide[1]

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The fate of N,N-dimethyl-^{14}C-3,5,7-trimethyladamantane-1-carboxamide has been studied in the rat. The main route of metabolism was found to be oxidative demethylation. The rate of demethylation was enhanced by phenobarbital treatment and was inhibited by DPEA. *In vitro* and *in vivo* studies show this amide to be a much more effective substrate for the demethylase enzyme than was the previously studied amide, N,N-dimethyldiphenylacetamide. The results suggest that the highly symmetrical trimethyladamantyl moiety possesses better transport and binding properties than does the folded aromatic diphenylmethyl system.

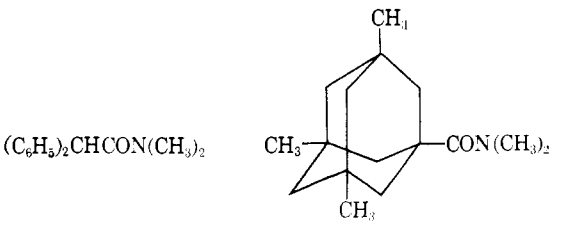
Oxidative N-demethylation is one of the most commonly encountered pathways by which drugs are metabolized.[2] The reaction is catalyzed by enzymes localized in the endoplasmic reticulum of the hepatic cell. Both oxygen and TPNH are required as cofactors. Mechanistically the reaction can be viewed as a special case of microsomal hydroxylation.[2]

$$>NCH_3 \longrightarrow [>NCH_2OH] \longrightarrow >NH + HCHO$$

The intermediate methylolamine formed by the hydroxylation reaction dissociates into the dealkylated amine and formaldehyde. Since formaldehyde is easily determined by colorimetric methods, numerous *in vitro* studies of N-demethylation have been reported. Furthermore, *in vivo* N-demethylation can be quantitated by utilizing N-methyl-^{14}C substrates. In this case appearance of $^{14}CO_2$ in expired air serves to measure the rate of demethylation.

In vitro and *in vivo* studies have established certain basic facts concerning the nature of the microsomal dealkylation process. For example, it is clear that in a series of related tertiary amines there is a positive correlation between lipid solubility and demethylation rate.[3,4] Also it has been found that steric hindrance about the nitrogen atom has no effect on rate.[4,5] A free base rather than the ionized amine serves as substrate. The basicity of the amine does not appear to have an effect on rate. Indeed neutral compounds such as N-methylamides have been found to be suitable substrates.[2] Although the *in vivo* demethylation of amides was reported as early as 1939,[6] it was not until 1961 that the work of Hodgson and Casida[7] demonstrated this to be a microsomal reaction. More recently reports from this laboratory have described both *in vitro* demethylation of a variety of amides[8] and the *in vivo* demethylation of the herbicide, diphenamid (N,N-dimethyldiphenylacetamide, I).[9]

Studies on the demethylation of amides have now been extended to compounds derived from the studies of Gerzon and associates.[10] These workers have reported the preparation and properties of some carboxamides containing the symmetrically methylated blocking group, 3,5,7-trimethyladamantyl.[11] This particular group appears to possess unique lipid properties and for that reason we were interested in investigating its possible effect on the demethylation reaction. In this paper N-demethylation studies on the amide, N,N-dimethyl-3,5,7-trimethyladamantanecarboxamide (II), are described and compared with the previous studies on diphenamid. Radiocarbon labeling was used to facilitate this investigation.



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Materials and Methods

Labeled Compounds.—N,N-Dimethyl-^{14}C-3,5,7-trimethyladamantanecarboxamide (II) was synthesized by treating 167 mg (0.75 mmol) of 3,5,7-trimethyladamantanecarboxylic acid with 0.20 ml (1.16 mmol) of oxalyl chloride in 1 ml of dry benzene containing 1 drop of a very dilute solution of pyridine in benzene. The reaction mixture was allowed to stand at room temperature for 2 hr. The solvent was removed *in vacuo* and the residual oil, 3,5,7-trimethyladamantanecarbonyl chloride, was added to 48.6 mg (0.60 mmol, 0.1 mCi) of ^{14}C-dimethylamine hydrochloride. A solution of 2.0 ml of 2.5 *N* NaOH was added immediately and the flask was stoppered and shaken vigorously. Colorless crystals separated within 10 min and after cooling in an ice bath for 1 hr were collected by filtration. The product, after washing with 10 ml of cold H_2O and drying *in vacuo*, amounted to 142 mg (95%), mp 99–100°; specific activity, 0.71 μCi/mg.

N-Methyl-^{14}C-3,5,7-trimethyladamantanecarboxamide (III) was synthesized by the general procedure described above, employing 417 mg (1.88 mmol) of 3,5,7-trimethyladamantanecarboxylic acid, 0.50 ml (5.80 mmol) of oxalyl chloride, and 108.5 mg (1.77 mmol, 0.5 mCi) of ^{14}C-methylamine hydrochloride. The product amounted to 390 mg (94%), mp 159–160°; specific activity, 1.20 μCi/mg.

Chromatographic Methods.—Paper chromatography was carried out on Whatman No. 1 paper. A 5 *N* NH_4OH saturated *n*-BuOH solution was employed for development of crude urine samples. An *n*-BuOH–AcOH–H_2O (4:1:5) system was employed for the separation of ^{14}C-urea from urine hydrolysates. Urea was identified by spraying the developed paper strip with a 5% solution of phenol in 95% EtOH, drying the tape at 90° for 10 min, and spraying the tape with 5% aqueous NaOCl. The urea

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spot appeared light green in color. Thin layer chromatography was carried out on silica GF (Merck A. G.) plates employing one of five solvent systems for development: (1) C_6H_6–Et_2NH (19:1 v/v), (2) EtOAc, (3) EtOH–EtOAc (1:1 v/v), (4) MeOH–EtOAc (7:3 v/v), and (5) C_6H_6–AcOH (9:1 v/v).

Demethylation *in Vitro*.—Livers were removed from 300-g male rats that had been sacrificed by decapitation. The livers were immediately homogenized in 4 vol of 0.1 *M* phosphate buffer (pH 7.4) in a Potter–Elvehjem homogenizer at 0° using a Teflon pestle. About 1.5 min of grinding time was required to produce a preparation of maximum activity. The homogenate was then centrifuged at 15,000*g* for 30 min and the supernatant fraction containing the soluble plus microsomal fraction was removed by decantation.

Livers were removed from 300-g male rats pretreated with phenobarbital (40 mg/kg) daily for the 4 days immediately prior to the day of sacrificing and processed to the 15,000*g* supernatant fraction as above.

For the *in vitro* demethylation experiments a mixture of 1 ml of supernatant fraction (200 mg of liver) together with 300 μmol of phosphate buffer (pH 7.4), 0.5 μmol of TPN^+, 11 μmol of glucose 6-phosphate, 45 μmol of semicarbazide, 50 μmol of $MgCl_2$, and 50 μmol of nicotinamide in H_2O (2 ml) was added to 5 μmol of substrate. The cofactor concentrations were chosen as those that gave maximum formaldehyde production from butynamine.[12] The reaction mixtures were then incubated in air with shaking at 37° for 1 hr. The reactions were terminated by the addition of 4 ml of 10% $ZnCl_2$ and the formaldehyde formed was determined by the method of Cochin and Axelrod.[13]

Inhibition experiments were carried out in a manner similar to that described above except that various quantities of DPEA ranging from 5×10^{-6} *M* were added to the reaction mixture. The substrate employed in these experiments was N,N-dimethyl-3,5,7-trimethyladamantanecarboxamide (II).

Demethylation *in Vivo*.—The rates of *in vivo* demethylation were determined by following the rates of $^{14}CO_2$ expiration after intraperitoneal administration of the ^{14}C-labeled dimethyl- and monomethylamides of 3,5,7-trimethyladamantanecarboxylic acid. For these experiments, 150-g male rats were used at a dose of 40 mg/kg in polyethylene glycol solution. In order to determine the rate of expiration of $^{14}CO_2$, a radiorespirometer similar to that developed and described by Tolbert[14,15] was employed. In our instrument the rat cage and the ionization chamber had a volume of 500 ml. A flow rate of 500 ml/min of air was employed.

In the inhibition studies DPEA (2,4-dichloro-6-phenylphenoxyethylamine hydrochloride) was given intraperitoneally (50 μmol/kg) 10 min before administration of the amide. In the induction studies, male rats (150 g) were given a single dose of phenobarbital (40 mg/kg) daily for 4 days immediately prior to the administration of the amide.

Urinary Excretion Studies.—The *in vivo* fate of II and its N-methyl-^{14}C analog (III) was studied in 200-g male Purdue–Wistar rats. After dosing with 40 mg of radiolabeled amide/kg ip, the animals were kept in stainless steel metabolism cages. Urine samples were collected for 24 hr and the radioactive content was determined by liquid scintillation counting.

In order to investigate the nature of the urinary metabolites, the 24-hr urine samples were first extracted with CH_2Cl_2, acidified to pH 5.5 with AcOH, and incubated for 18 hr with 1.0 ml of Glusulase solution (mixture of β-glucuronidase and sulfatase, Endo Products, Inc.)/100 ml of urine. The liberated metabolites present were then extracted with CH_2Cl_2 and studied by chromatographic methods.

Results

As an initial step in the investigation, a comparative study of the demethylation of I and II and their monomethyl analogs was carried out in the *in vivo* rat liver microsomal system. The demethylation of a typical lipid-soluble tertiary amide, *d*-propoxyphene, which is readily demethylated *in vitro*[16] was also used for comparison. The results of this study which are summarized in Table I show II to be a much more active substrate than is the previously studied I. As would be expected, microsomes from phenobarbital-pretreated rats showed substantially increased activity. Surprisingly, however, while the diphenamid and propoxyphene demethylation rates increased about threefold, the rate of demethylation of II increased almost eight times. Thus with phenobarbital-induced microsomes, II was an even more active substrate than the tertiary amine, *d*-propoxyphene. The corresponding secondary amides were inactive as substrates using either the control or induced microsomes.

TABLE I

In Vitro DEMETHYLATION RATES

Substrates	Demethylation rate —HCHO formed, mμmol/hr[a]— Control	Phenobarbital induced
$TMACON(CH_3)_2$ (II)	420	3210
Diphenamid (I)	110	360
$TMACONHCH_3$ (III)	0	0
Nordiphenamid	0	0
d-Propoxyphene	730	2040

[a] Each incubation flask contained 1 ml of the supernatant fraction (15,000*g*) from 200 mg of liver (either from control rats or phenobarbital-induced rats), 300 μmol of phosphate buffer (pH 7.4), 50 μmol of nicotinamide, 50 μmol of $MgCl_2$, 45 μmol of semicarbazide, 0.5 μmol of TPN^+, 11 μmol of glucose 6-phosphate, 5 μmol of substrate, and enough H_2O to make 3 ml. After incubation in air at 37° for 1 hr the formaldehyde formed was determined by the method of Cochin and Axelrod.[13]

TABLE II

THE EFFECT OF DPEA ON THE RATE OF DEMETHYLATION OF N,N-DIMETHYL-3,5,7-TRIMETHYLADAMANTANECARBOXAMIDE[a]

Concn of DPEA, *M*	mμmol of HCHO formed/hr	% inhib
5×10^{-4}	0	100
1×10^{-4}	60	81
5×10^{-5}	90	72
1×10^{-5}	190	41
5×10^{-6}	300	6
0	360	...

[a] Incubation conditions: substrate (5 μmol) and inhibitor in H_2O (1 ml) were placed in the incubation flask and to this was added 2 ml of a mixture containing 1 ml of the supernatant fraction (15,000*g*) from 200 mg of rat liver, 300 μmol of phosphate buffer (pH 7.4), 50 μmol of nicotinamide, 50 μmol of $MgCl_2$, 45 μmol of semicarbazide, 0.5 μmol of TPN^+, and 11 μmol of glucose 6-phosphate. The flasks were incubated at 37° for 1 hr in air. The formaldehyde formed was determined by the method of Cochin and Axelrod.[13]

2,4-Dichloro-6-phenylphenoxyethylamine (DPEA) is a potent inhibitor of N- and O-dealkylation.[17,18] The data in Table II demonstrate that DPEA is indeed an effective *in vitro* inhibitor of the demethylation of II. For example, the substrate present at 1.7×10^{-3} *M* and a concentration of DPEA of 1×10^{-5} *M* leads to 41% inhibition.

The *in vivo* demethylation of II and its monomethyl analog, III, was investigated next. By following the rate of $^{14}CO_2$ expiration after intraperitoneal administration of the N-methyl-^{14}C-labeled amides it was

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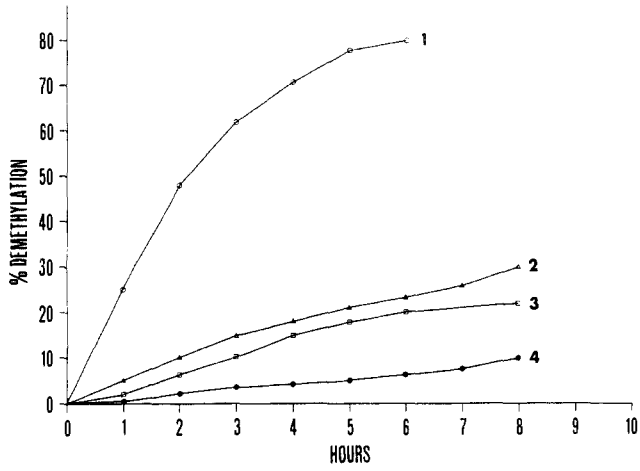


Figure 1.—Relative extent of demethylation of various N-methyl-labeled amides: (1) compound II, (2) compound I, (3) compound III, and (4) nordiphenamid. The per cent demethylation shown for the tertiary amides is twice the per cent $^{14}CO_2$ recovery. The data shown represent average values from three rats.

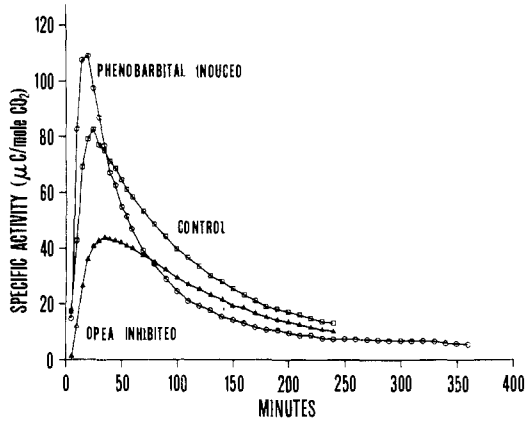


Figure 2.—Specific activity of respired $^{14}CO_2$ following administration of N,N-dimethyl-^{14}C-3,5,7-trimethyladamantane-1-carboxamide (II) to control, phenobarbital-induced, and DPEA-treated rats. Each curve represents average values for six rats.

possible to determine the rate and amount of N-demethylation occurring during a period of 6 hr. The rates of demethylation of these amides compared to the rates obtained for diphenamid and nordiphenamid are shown in Figure 1. As might be expected from the *in vitro* results cited above, the adamantane amide II is considerably more readily demethylated than is diphenamid I. Demethylation is clearly the main route by which II is metabolized in the intact rat. Unlike the *in vitro* experience, the secondary amides do undergo *in vivo* demethylation although the observed rates are rather slow.

Pretreatment of rats with DPEA (16 mg/kg ip) immediately prior to administration of labeled II caused a decrease in the rate of demethylation as measured by the specific activity of the respired $^{14}CO_2$ (Figure 2). This effect of DPEA is also reflected in decreased total amount of demethylation occurring during the first 6 hr following administration of the drug (Table III). Pretreatment of rats for 4 days with 40 mg/kg of phenobarbital, prior to the administration of the ^{14}C-labeled dimethylamide substantially increased the initial rate of demethylation (Figure 2).

TABLE III

EFFECT OF DPEA AND PHENOBARBITAL ON RATES OF DEMETHYLATION OF II AND III IN THE INTACT RAT

	% demethylation in 300 min with substrate[a]	
Treatment	II	III
None	71.2[b]	20.0
DPEA	48	9.3
PB	79.6	20.8

[a] Data for II were collected from seven rats, for III from four rats. [b] This figure is twice the per cent of the radioactivity found as $^{14}CO_2$, since only one of the N-methyl groups is removed.

However, the total amount of demethylation occurring in the first 6 hr was not greatly effected (Table III).

Pretreatment of rats with DPEA prior to administration of the secondary amide, N-methyl-^{14}C-3,5,7-trimethyladamantanecarboxamide (III), also lowered both the initial rate of the N-demethylation (Figure 3) and the extent of demethylation over the 6-hr test period. Phenobarbital pretreatment caused a significant increase in the initial rate of demethylation of III but did not appreciably affect the over-all extent of demethylation (Table III).

Since the amides II and III possess rather unique structures, these studies were extended to include an investigation of the nature of the urinary metabolites. The main route of metabolism of II is of course N-demethylation, but this removes only one methyl group. Thus, most of the metabolites should still retain the second methyl group and are still radioactive. Urine from rats receiving II contained an average of 38% of the administered dose. One-fourth of this (*i.e.*, 9.5% of the administered dose) could be recovered directly by solvent extraction (CH_2Cl_2). This extract was found to contain no unchanged II and only a trace of the demethylated amide III. Rather, the radioactivity was present as an unknown amide more polar than II or III. An additional amount of this unknown amide, equivalent to 4% of the dose, was obtained by treatment of the extracted urine with β-glucuronidase and then extracted again.

This unknown amide was then purified by preparative thin layer chromatography (solvent systems 2 and 3). Its ir spectrum was consistent with that of a

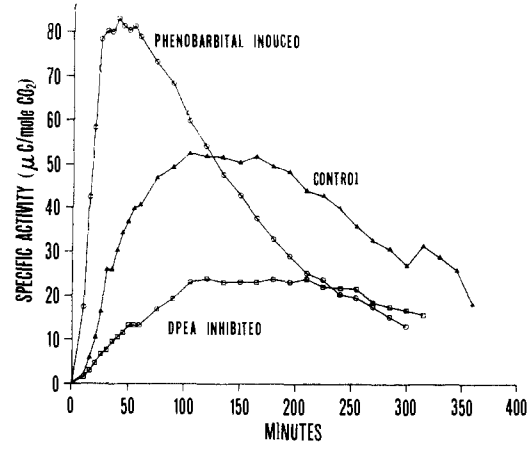
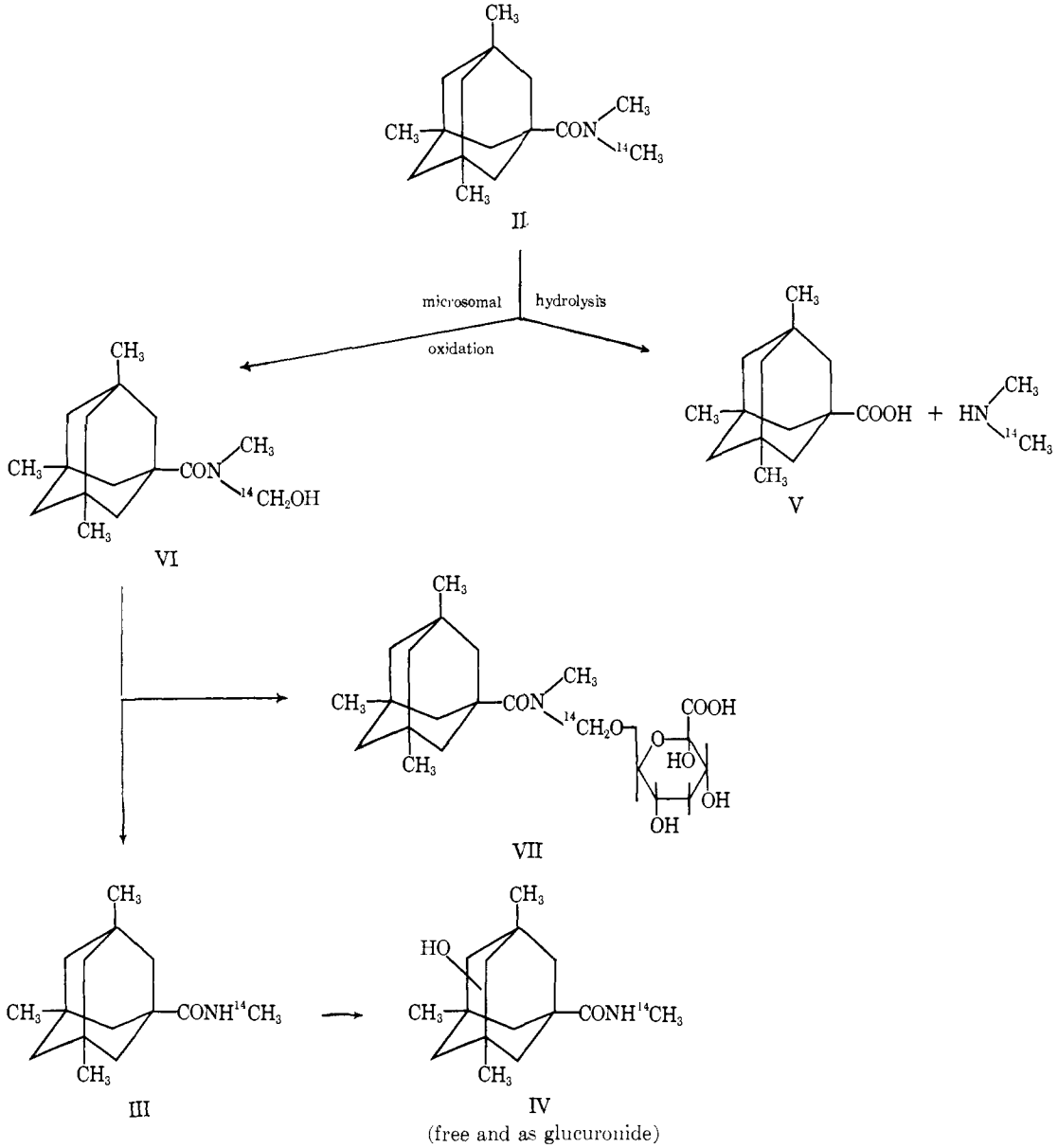


Figure 3.—Specific activity of respired $^{14}CO_2$ following administration of N-methyl-^{14}C-3,5,7-trimethyladamantane-1-carboxamide (III) to control, phenobarbital-induced, and DPEA-treated rats. Each curve represents average values for six rats.

SCHEME I

SUMMARY OF PATHWAYS BY WHICH N,N-DIMETHYL-^{14}C-3,5,7-TRIMETHYLADAMANTANECARBOXAMIDE IS METABOLIZED IN THE RAT



monomethyl amide of an aliphatic hydroxycarboxylic acid. These data and those obtained from nmr studies suggested that this unknown metabolite was indeed hydroxylated N-methyl-3,5,7-trimethyladamantanecarboxamide (IV, Scheme I). Further work with model compounds will be necessary to establish whether hydroxylation has occurred on the CH_3 or CH_2 carbon.

Three additional radioactive metabolites were identified in urine after removal of IV. These were dimethylamine (7.6%), formic acid (1.9%), and urea (1.1%). The dimethylamine was recovered from urine and identified as its N-benzoyl derivative formed directly in urine by the Schotten–Bauman procedure. Formic acid was identified by chromatography and by conversion to ^{14}CO by treatment of the sodium salt with concentrated H_2SO_4. Urea-^{14}C was identified and quantitated by paper chromatography (see Experimental Section).

The appearance of dimethylamine among the metabolites implies that one route of metabolism of II is hydrolysis and that 3,5,7-trimethyladamantanecarboxylic acid (V) should also be formed. This implication was confirmed by the isolation and identification of this acid from urine. Since it was nonradioactive no attempt was made to quantitate it.

Finally, evidence for the presence of one further minor (1%) metabolite VII was obtained. VII is the glucuronide of the supposed hydroxymethyl demethylation intermediate VI. It was identified using the procedure described earlier.[9] The results of the metabolite studies on II are summarized in Figure 4.

The *in vivo* metabolism of N-methyl-^{14}C-3,5,7-trimethyladamantanecarboxamide (III) in the rat was also investigated. Twenty-four hours after intraperitoneal administration of this labeled amide, 55% of the injected radioactivity was found in the urine. Using the methods described above for the tertiary amide metabolism study, the major urinary metabolite was hydroxylated amide (IV), accounting for 27.5% of the administered dose and present again in the urine both in the free and conjugated forms. The remainder of the radioactivity in the urine consisted of a mixture of formic acid, urea, and an unidentified metabolite.

Discussion

The nature of the blocking groups present in the two amides, N,N-dimethyldiphenylacetamide (diphenamid, I) and N,N-dimethyl-3,5,7-trimethyladamantane-1-carboxamide (II), contrast sharply with one another. While both the diphenylmethyl group and the 3,5,7-trimethyladamantyl groups are considered "lipid-soluble" blocking groups, they differ markedly from each other both sterically and electronically. Sterically the diphenylmethyl group presents two planar benzene rings, presumably inclined toward one another at the tetrahedral angle in the fashion of butterfly wings (*cf.* Figure 4). Electronically the rings represent two aromatic systems capable of binding through π-electron interaction, with a variety of structural components in an enzyme or receptor site surface. In contrast, the 3,5,7-trimethyladamantyl group is a bulky, symmetrical, rigid, cyclic group representing a unit of a diamond lattice. Electronically this group presents only methyl and methylene groups to the receptor surface.

With these considerations in mind, the results of the present study took on added interest. In order to be acted upon by the endoplasmic reticulum (ER) bound enzymes (the so-called microsomal oxygenases), a substrate must itself be taken up and bound by the ER. The finding that the adamantane amide (II) is a much better substrate than diphenamid (I) probably means that II produces a better fit to the catalytic site than does I.

Although nothing is known about the nature of this substrate binding pocket, these results allow certain speculations. It seems apparent that a geometric fit is of greater importance than is the capability of electronic binding through the π electrons of the planar aromatic rings. This conclusion is in harmony with our earlier work on the dealkylation of aliphatic amines,[4]

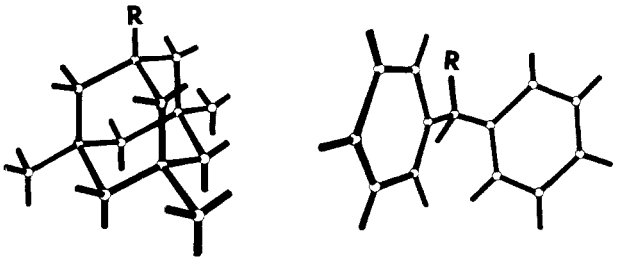


Figure 4.—Three-dimensional representation of structures of the diphenylmethyl group and the 3,5,7-trimethyladamantyl group.

which demonstrated that totally aliphatic structures could be excellent substrates and that steric hindrance *per se* did not interfere. In considering the nature of the binding pocket it may well be profitable to adopt a view similar to the diamond-lattice concept which Prelog[19] has used so successfully to define substrate requirements for the keto reductase from *Curvularia falcata*. The present studies also suggest that the receptor site in the CNS at which the adamantane amides act[11] may share certain of the characteristics of the microsomal enzyme binding site. Indeed the CNS receptor site may well be similarly associated with a membrane structure.

These suggestions are, of course, only speculative but are recorded in the hope that they may stimulate further investigations both into the nature of substrate binding to the microsomal oxygenases as well as into the special properties of the rigid adamantane nucleus.

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The Preparation of Vinblastine-4-acetyl-*t* and Its Distribution in the Blood of Rats

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High specific activity vinblastine-*t* has been prepared by acetylating deacetylvinblastine with acetic anhydride-*t*. It was isolated and purified by absorption and ion-exchange chromatography. The blood radioactivity reached a peak 1–2 hr after giving rats small intraperitoneal doses of the labeled alkaloid. When the blood collected in this period was centrifuged, about three-quarters of the tritium was found in the "buffy coat" region. The radioactivity was almost entirely present as unchanged vinblastine.

The dimeric *Vinca* alkaloids vinblastine and vincristine are used in the treatment of malignancy, particularly Hodgkin's disease and acute lymphocytic leukemia.[1] Only small doses of these drugs are tolerated, *e.g.*, bone marrow depression and leucopenia may develop if the weekly dose of vinblastine exceeds 0.3 mg/kg.[2] Therefore, information on the metabolism and fate of the alkaloids has been limited and consists mainly of a study of the excretion and tissue distribution of tritiated vinblastine prepared by the Wilzbach method.[3] Vinblastine tritiated by this method is difficult to purify[3,4] and, in view of the small doses which are tolerated, of inconveniently low specific activity.

This article describes an alternative method for preparing tritiated vinblastine with a specific activity 150 times greater than we have obtained by the Wilzbach procedure. It is being used for metabolic studies in rats. The method is based on a reaction sequence de-

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