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SHORT COMMUNICATIONS

The demethylation *in vitro* of N-methyl barbiturates and related compounds by mammalian liver microsomes

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LARGELY through the extensive researches of T. C. Butler and his associates, it is now well established that many N-methylated barbiturates and related heterocyclic compounds undergo N-demethylation *in vivo* (see Fishman[1] for a review and bibliography of the Butler studies). Their investigations implicate the liver as the site of demethylation, and it seems a reasonable assumption that the demethylations are catalyzed by the oxidative enzyme systems present in the liver microsomal fractions. These enzymes, which require NADPH and oxygen as cofactors, are responsible for many reactions involved in the metabolism of drugs, including the N-dealkylation of amines.[2] The data presented below show that the N-demethylation of N-methyl barbiturates is indeed another example of the microsomal N-demethylation reaction.

For our initial studies N,N′-dimethylbarbital was chosen as the model substrate. This compound, first reported by Bush and Butler,[3] has been reported[4] to be rapidly demethylated to N-methylbarbital in the dog. The removal of the second methyl group proceeds at a much slower rate. For this experiment a soluble fraction containing microsomes was prepared by centrifugation of a rat* liver homogenate at 15,000 × *g*. A microsomal pellet was then prepared by recentrifugation of this soluble fraction at 80,000 × *g* for 1 hr.[5] Experiments using microsomes prepared in this manner are summarized in Table 1.

TABLE 1. THE DEMETHYLATION OF N,N-DIMETHYLBARBITAL BY RAT LIVER MICROSOMES

Substrate (5 μmoles)	Additions	HCHO formed/hr (mμmoles)
N,N-dimethylbarbital	Microsomes	0
	Microsomes + 4·5 μmoles NADPH	67
	Microsomes + 9 μmoles NADPH	180
	Microsomes + soluble fraction + 1 μmole NADP + 12 μmoles glucose-6-phosphate	125
	Soluble fraction + 4·5 μmoles NADPH	0
Butynamine	Microsomes + 4·5 μmoles NADPH	580

Incubation conditions: Each flask contained 300 μmoles phosphate buffer (pH 7·4), 50 μmoles nicotinamide, 50 μmoles magnesium chloride, 45 μmoles semicarbazide plus 5 μmoles substrate, and further additions as noted in the table. Microsomes equivalent to 0·4 g liver were added in 1 ml 0·1 M phosphate buffer. The soluble fraction was equivalent to 0·2 g liver and was the supernatant (80,000 × *g*) from the microsome preparation. The incubation flasks were incubated in air at 37° for 1 hr. Formaldehyde was determined by the method of Cochin and Axelrod.[16]

It is clear from the data in Table 1 that the enzymes responsible for the N-demethylation of dimethylbarbital do indeed reside in the microsomal fraction from liver. Furthermore the requirement for NADPH is shown, although it can be replaced by a NADPH-generating system composed of NADP, glucose-6-phosphate, and soluble fraction as a source of glucose-6-phosphate dehydrogenase. (Because of its convenience, this latter system is used in the remaining experiments described below.)

* In all cases livers from male Purdue–Wistar rats (200 g) were used.

Although N,N′-dimethylbarbital is demethylated by this system the rate, though appreciable, is much less than that shown by a typical amine substrate such as butynamine.[5] The following experiments bear on this point.

A preliminary study was made in which it was found that the rate of demethylation of N,N′-dimethylbarbital remained constant for a period of 1 hr. This is in contrast to results with amines in which the rate begins to fall off with time much earlier.[5, 6] It was also found that incubation in oxygen instead of air resulted in the same rate of demethylation. A study was now made in which the effect of substrate concentration upon rate was determined.

Since the rates are constant over a 1-hr period, they were considered to represent the "initial rate." The results at various substrate concentrations were then plotted according to the method of Lineweaver and Burk[7] and the maximum rate (an infinite substrate concentration) found to be 21 mμmoles/min/g liver. By the same procedure V_{max} for N-methylbarbital was only 4·5 mμmoles/min/g liver. In the same batch of microsomes the V_{max} for butynamine was found to be 316 mμmoles/min/g liver.

Thus it is seen that, although N,N-dimethylbarbital is a satisfactory substrate for the demethylase system *in vitro*, the rate is much less than that commonly found for amines. The presence of the basic nitrogen in the amines must facilitate the reaction in some way. This is probably not an effect upon binding, however, since the $-K_m$ value found for dimethylbarbital ($1{\cdot}4 - 10^{-4}$ M) is comparable to that reported[8] for meperidine ($3{\cdot}8 \times 10^{-4}$ M) and much smaller than that found for butynamine (6 to 7×10^{-3} M). The much lower rate found for N-methylbarbital compared to that of dimethylbarbital correlates well with the *in vivo* work reported by Butler;[4] as he pointed out that the difference is too great to be explained only on the basis of the frequency factor because of the presence of two N—CH_3 groups in one molecule and one in the other.

Table 2 summarizes the effect of various inhibitors upon the rate of demethylation of dimethylbarbital. As is true of several other[9, 10] reactions catalyzed by liver microsomes cyanide, bipyridyl,

TABLE 2. THE EFFECT OF VARIOUS INHIBITORS UPON THE RATE OF DEMETHYLATION N,N-DIMETHYLBARBITAL

Inhibitor	Concentration of inhibitor (M)	Inhibitions (%)
$CuSO_4$	$1{\cdot}7 \times 10^{-3}$	100
	$1{\cdot}7 \times 10^{-4}$	49
$Hg(OAc)_2$	$1{\cdot}7 \times 10^{-3}$	100
	$1{\cdot}7 \times 10^{-4}$	24
KCN	$1{\cdot}7 \times 10^{-3}$	0
	$3{\cdot}3 \times 10^{-4}$	0
2,α-Bipyridal	$1{\cdot}7 \times 10^{-3}$	0
	$3{\cdot}3 \times 10^{-4}$	0
Iodoacetamide	$1{\cdot}7 \times 10^{-3}$	5
	$3{\cdot}3 \times 10^{-4}$	0
DPEA	1×10^{-4}	100
	1×10^{-5}	79

Incubation conditions: Substrate and inhibitor in 1 ml of water were placed in the incubation flask and to this was added 2 ml of a solution containing the supernatant fraction (15,000 × *g*) from 200 mg of rat liver, 300 μmoles phosphate buffer (pH 7·4), 50 μmoles nicotinamide, 50 μmoles magnesium chloride, 45 μmoles semicarbazide, 0·5 μmole $NADP^+$, and 11 μmoles glucose-6-phosphate. The flasks were incubated at 37° in air for 30 min.

and iodoacetamide are not effective inhibitors. On the other hand, certain metal ions such as Cu^{2+} and Hg^{2+}, which are known to inhibit microsomal oxidations, were found to be inhibitors in the present case. DPEA, (2,4-dichloro-6-phenylphenoxyethylamine), a powerful inhibitor of the N-demethylation of many tertiary amines,[8] inhibits the demethylation of dimethylbarbital very effectively. Thus the effect of inhibitors upon the demethylation of dimethylbarbital is very similar to that observed in other microsomal oxidations.

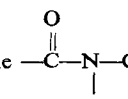
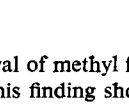
Selected other compounds containing the  group were also examined as possible substrates for microsomal N-demethylase. The results are reported in Table 3. Although microsomes from rabbit liver are more active than those from rat liver, the relative rates of demethylation correlate well. 5-Ethyl-3-methyl-5-phenylhydantoin, N-methylbarbital, and hexobarbital (all of which have been reported to undergo demethylation *in vivo*) were found to be substrates although none was so active as dimethylbarbital. The finding that N-(*p*-chlorophenylsulfonyl)-N′-methylurea undergoes

TABLE 3. THE RATE OF N-DEMETHYLATION OF VARIOUS AMIDES AND RELATED COMPOUNDS BY RAT LIVER MICROSOMES

Substrate	Demethylation rate (mμmoles HCHO/hr) Rat	Rabbit
N,N-dimethylbarbital	155	314
5-Ethyl-3-methyl-5-phenylhydantoin	82	78
N-(*p*-chlorophenylsulfonyl)-N′-methylurea	67	0
N,N-dimethyldiphenylacetamide	62	128
N-methylbarbital	28	63
Hexobarbital	12	23
Methohexital	0	48
N-methyldiphenylacetamide	0	23

Incubation conditions: Each incubation flask contained the supernatant fraction (15,000 × *g*) from 200 mg liver, 300 μmoles phosphate buffer (pH 7·4), 50 μmoles nicotinamide, 50 μmoles magnesium chloride, 45 μmoles semicarbazide, 0·5 μmole NADP, 11 μmoles glucose-6-phosphate, 5 μmoles substrate, and sufficient water to yield final volume of 3 ml. After incubation in air at 37° for 1 hr the formaldehyde formed was determined by the method of Cochin and Axelrod.[16]

dealkylation *in vitro* corroborates the finding by Welles *et al.*[11] that the corresponding N′-propyl analog (chlorpropamide) is dealkylated *in vivo*. The observation that methohexital is not demethylated by rat liver microsomes correlates well with the recently published *in vivo* findings.[12] It is interesting that simple amides such as the herbicide N,N,dimethyldiphenylacetamide are active substrates, although the related secondary amide is demethylated only by rabbit microsomes. The metabolism *in vivo* of these two amides is at present unknown.

The results of this study, taken together with the studies of Hodgson and Casida[13] on the demethylation of carbamates, shows that the removal of methyl from the grouping  is a general reaction catalyzed by liver microsomes. This finding shows that a basic nitrogen is not a requisite structural feature for N-demethylation. Thus the substrate is probably not bound to the enzyme by an ionic bond, which argues against any dehydrogenation mechanism such as that proposed for monamine oxidation.[14] Furthermore a mechanism that involves initial N-oxide formation[15] would be quite unlikely on chemical grounds in the case of amides. The most acceptable mechanism proposed is that of Brodie *et al.*[8] which involves direct hydroxylation of the methyl group as the initial reaction step.

This is a general mechanism which is equally satisfactory for the dealkylation of amines, arylethers, amides, carbamates, and barbiturates.

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The Lilly Research Laboratories,
Eli Lilly and Company,
Indianapolis, Ind., U.S.A.

R. E. McMAHON

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Gamma aminobutyric acid and 5-hydroxytryptamine concentrations in neurons and glial cells in the medial geniculate body of the cat

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GAMMA aminobutyric acid (GABA) results from the decarboxylation of glutamic acid and is transaminated to succinic semialdehyde. Both GABA and the enzymes accounting for its turnover have been measured in different brain structures and in different fractions of ultracentrifuged brain cells.[1, 2] However, which type of cells in the brain contains GABA has been in question.

5-Hydroxytryptamine (5-HT), similarly, has been assigned its place in certain brain structures and in certain fractions of ultracentrifuged brain cells;[3, 4] some of the enzymes accounting for its formation and degradation have a characteristic distribution.[5, 6] Which type of cells in the brain contains 5-HT has also been in question.

In the cat each medial geniculate body weighs about 28 mg and protrudes from the brain stem so that it is easily dissected free. Removal of cortical areas known as AI, AII, Ep, SII, temporal, and insular results in retrograde degeneration and gliosis in all portions of the principal division of the medial geniculate body.[7, 8] The degenerative process requires 6 weeks and leaves about 17 mg of tissue consisting of glia, presynaptic axon tips from the neurons of the inferior colliculus, and an insignificant number of neurons. This technique of obtaining a large sample of glial cells without intact neurons seemed useful in answering the above questions.

Cats were anesthetized and under aseptic conditions the proper cortical areas were removed by suction. The hole in the skull was covered by a thin sheet of dime silver so that compression by the