ether (3 × 100 ml.). The ether solutions from the second extraction were combined, dried over magnesium sulfate, and filtered, and the ether was removed by distillation *in vacuo*, yielding a purple residual liquid. The crude product was purified by distillation *in vacuo*, employing a microdistillation apparatus (24-cm. Vigreux column), yielding 10.9 g. (17.0%) of pure V, b.p. 60 65° (0.01–0.02 mm.); n_D^{25} 1.4465; IR (chloroform): 1580 cm.$^{-1}$ (C=N); UV (95% ethanol): 208 nm. (ϵ 15,000).

Anal.—Calc. for $C_{13}H_{14}F_6N_2$: C, 50.00; H, 4.52; F, 36.51; N, 8.97. Found: C, 50.07; H, 4.57; F, 36.23; N, 8.76.

Evaluation of Repellency—To minimize contact with the skin, Compounds II–V were evaluated as repellents against *Aedes aegypti* (L.) mosquitoes employing a standard cloth method (6); *N,N*-diethyl-*m*-toluamide was included in the tests as a standard for comparison. One-third square foot of a cotton stocking was treated with 1 g. of the compound in solution (10% solution in a volatile solvent, usually acetone). Two hours after treatment, the stocking, on the arm of a human subject, was exposed for 1 min. in a cage of mosquitoes. If less than five mosquitoes bit the subject through the stocking, the test was repeated at 24 hr. and then weekly thereafter until five bites were received in 1 min.

RESULTS AND DISCUSSION

As tested, Compounds II–V were found to be effective on cloth against *Aedes aegypti* (L.) mosquitoes for 7 days as compared to *N,N*-diethyl-*m*-toluamide and *N,N*-diethylbenzamide, which are effective under the same conditions for more than 21 days. To our knowledge, Compounds I–V are the only amidines that have been evaluated as insect repellents. The effectiveness of these specific agents and the structural similarities between amidines and amides and imides, which were found (7) to be the largest single class of compounds effective for 5 hr. or more upon dermal application, indicate that amidines as a class possess considerable potential as insectifugal agents.

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ACKNOWLEDGMENTS AND ADDRESSES

Received March 15, 1973, from the *Department of Medicina Chemistry, College of Pharmacy, University of Tennessee Medical Units, Memphis, TN 38103*

Accepted for publication July 19, 1973.

Abstracted in part from a dissertation to be submitted by D. D. Garner to the University of Tennessee in partial fulfillment of the Doctor of Philosophy degree requirements.

Supported by Research Contract DA-49-193-MD-2636 from the U. S. Army Medical Research and Development Command, Washington, D. C.

The authors gratefully acknowledge the assistance of Dr. D. E. Weidhaas, Mr. I. H. Gilbert, and their colleagues of the Entomology Research Division, Agricultural Research Service, U. S. Department of Agriculture, Gainesville, Fla., in the evaluation of mosquito repellency.

• American Foundation for Pharmaceutical Education Fellow, 1972–1973.

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Metabolism and Anticonvulsant Activity of Deuterated *N*-Demethyldiazepam

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Abstract □ The substitution of the hydrogen at the C_3-position of *N*-demethyldiazepam with deuterium shortens the anticonvulsant activity in mice from 20 to 5 hr. This pharmacological effect may be partly explained by a decrease in the ability of deuterated *N*-demethyldiazepam to protect mice from pentylenetetrazol-induced convulsions in contrast to the effect exerted by the C_3-unlabeled analog. Furthermore, the weaker pharmacological action of deuterated *N*-demethyldiazepam may in part be due to the lesser accumulation of the hydroxylated metabolite, oxazepam, in the brain of mice treated with deuterated *N*-demethyldiazepam than with the unlabeled analog. The lesser accumulation of oxazepam from deuterated *N*-demethyldiazepam than from the unlabeled analog is due to a reduced C_3-hydroxylation of this compound by liver microsomal enzymes, as shown by experiments *in vitro*.

Keyphrases □ Diazepam, *N*-demethyl, deuterated—metabolism and anticonvulsant activity, mice □ *N*-Demethyldiazepam, deuterated—metabolism and anticonvulsant activity, mice □ Anticonvulsant activity—deuterated *N*-demethyldiazepam, metabolism, mice □ Oxazepam—accumulation after administration of deuterated *N*-demethyldiazepam, mice

N-Demethyldiazepam, a major metabolite of diazepam, possesses pharmacological properties similar to the parent compound (1, 2). In the liver microsomal system, it undergoes a process of hydroxylation in position C_3 with the formation of oxazepam (3). The administration of *N*-demethyldiazepam in mice results in an accumulation of oxazepam in brain (4) where it is retained for several hours. Because oxazepam exerts

Table I—Relative Rates of Hydroxylation of Labeled and Unlabeled *N*-Demethyldiazepam

Substrate Added, *N*-Demethyldiazepam, mcg.	Substrate Recovered Unchanged[a], mcg.	Oxazepam Formed[a], mcg.
Unlabeled		
30	28 ± 2	0.5 ± 0.02
50	41 ± 3	2.7 ± 0.08
100	87 ± 3	6.4 ± 0.19
300	270 ± 5	24.2 ± 0.05
Labeled		
30	25 ± 2	—
50	44 ± 2	—
100	88 ± 3	—
300	247 ± 6	3.2 ± 0.09

[a] Results are expressed as the mean ± *SD* (n = 4).

an anticonvulsant activity comparable to that of *N*-demethyldiazepam, the consequence of C_3-hydroxylation in mice is a prolonged action of the administered drug due to accumulation of oxazepam in the brain.

Several drugs are oxidized by liver microsomal enzymes at a specific carbon–hydrogen bond. If breaking of the carbon–hydrogen bond is rate limiting in the oxidative process, substitution of hydrogen by deuterium at the suspected site of oxidation may result in a decreased rate of oxidation.

The purpose of this study was to evaluate the consequence of such C_3-substitution in *N*-demethyldiazepam on the metabolism and pharmacological activity in mice.

EXPERIMENTAL

The deuterated *N*-demethyldiazepam[1] (7-chloro-1*H*-3,3-dideutero-5-phenyl-1,4-benzodiazepin-2-one) had 84% of dideuterated molecules. Male, albino Swiss mice, weighing 20–25 g., were used in all experiments.

Drug Administration—*N*-Demethyldiazepam and deuterated *N*-demethyldiazepam were administered by the tail at 5 mg./kg. i.v. Drugs were dissolved in a solvent containing propylene glycol–glycofurol–benzyl alcohol–water (30:30:2:48).

The ED_{50} was determined using male, albino Swiss mice and is the dose (in micrograms per kilogram) protecting 50% of the mice from the mortality induced by phenylenetetrazol at 120 mg./kg. i.p. The method of Litchfield and Wilcoxon (5) was used for calculating the confidence limits. The ED_{50} for *N*-demethyldiazepam was 198 mcg./kg. (confidence limits of 177–221 mcg./kg.), and for deuterated *N*-demethyldiazepam it was 288 mcg./kg. (confidence limits of 252–329 mcg./kg.). The ED_{50} was calculated from data obtained 5 min. between drug and pentylenetetrazol administration, when the injected drug, according to previous observations (4), is practically unmetabolized.

Chemical Determinations—The preparation of brain extracts was made according to the method previously described (6). GLC analyses were carried out by using a gas chromatograph[2] equipped with a ^{63}Ni electron-capture detector (voltage of 42 v.). The stationary phase was 3% OV-17 on Gas Chrom Q (100–120 mesh) packed into a 1-m. glass column (2 mm. i.d., 4 mm. o.d.). The flow rate of the carrier gas (nitrogen) was 33 ml./min., and the column temperature was 245°.

Preparations of Liver Microsomes—Albino Swiss mice were given 40 mg./kg. i.p. of phenobarbital in 0.9% NaCl twice a day for 4 days. This treatment is known to increase the hydroxylation of *N*-demethyldiazepam by liver microsomal enzymes (7). After the last injection, the animals were deprived of food for 24 hr. and then sacrificed by decapitation. The livers were removed, chilled in ice, and homogenized in ice-cold 1.15% potassium chloride solution (1:4 w/v). The homogenate was centrifuged[3] at 105,000×*g* for 1 hr. The precipitated liver microsomes were suspended in 1.15% potassium chloride solution. Protein determinations were performed according to Lowry *et al.* (8).

Table II—Brain Levels of *N*-Demethyldiazepam and Its Metabolite, Oxazepam, after Administration (5 mg./kg. i.v.) of Unlabeled and Labeled *N*-Demethyldiazepam to Mice

N-Demethyldiazepam	Hours after Treatment	Benzodiazepine Brain Level, mcg./g. ± *SE*		Mice Protected against Pentylenetetrazol (120 mg./kg. i.p.), %
		N-Demethyldiazepam	Oxazepam	
Unlabeled	1	4.52 ± 0.18	2.37 ± 0.02	100
Labeled	1	5.66 ± 0.25	0.21 ± 0.01	100
Unlabeled	3	0.62 ± 0.01	3.93 ± 0.07	100
Labeled	3	3.50 ± 0.20	0.38 ± 0.01	66
Unlabeled	10	<0.002	0.75 ± 0.02	100
Labeled	10	0.74 ± 0.04	0.09 ± 0.007	16
Unlabeled	20	<0.002	0.09 ± 0.01	33
Labeled	20	0.09 ± 0.01	0.04 ± 0.02	0

Incubation *In Vitro*—The incubation mixture, as previously reported (3), consisted of 2.5 ml. of microsomal suspension equivalent to 1 g. of liver, NADP (1.5 μmoles), glucose 6-phosphate (50 μmoles), glucose 6-phosphate dehydrogenase (0.5 unit), magnesium chloride (35 μmoles), nicotinamide (50 μmoles), 1.4 ml. of 0.2 *M* phosphate buffer (pH 7.4), 300 mcg. of deuterated or nondeuterated *N*-demethyldiazepam, 0.45 ml. of 1.15% potassium chloride, and water to obtain a final volume of 5 ml. The mixtures were incubated in a metabolic shaker[4] at 37° under air for 30 min. Recovery studies of *N*-demethyldiazepam and oxazepam, both deuterated and nondeuterated, were performed with microsomal boiled mixtures containing ingredients identical to those in the experimental samples.

Determination of Substrates and Metabolites—At the end of the incubation time, 5 ml. of the mixture was extracted twice with 10 ml. of fresh peroxide-free ether. The combined ether extracts were dried, redissolved in a suitable volume of acetonitrile, and then submitted to the GLC procedure previously described (6). This procedure allows the simultaneous determination of the formed metabolite and the disappearance of the added substrate.

RESULTS

Table I shows the relative rate of hydroxylation *in vitro* of *N*-demethyldiazepam bearing hydrogen or deuterium in position 3. It is evident that more oxazepam is formed from *N*-demethyldiazepam than from the deuterated analog. However, the recovery of the added substrate is incomplete with the deuterated *N*-demethyldiazepam, suggesting alternative metabolic pathways or a tighter binding to liver microsomes.

Table II summarizes the findings obtained *in vivo*. Mice injected with deuterated *N*-demethyldiazepam showed higher levels of this compound in the brain compared to animals receiving the hydrogenated compound. Conversely, the brain levels of oxazepam, the hydroxylated metabolite, were much lower with deuterated than with hydrogenated *N*-demethyldiazepam. Because of the lower levels of brain oxazepam, the duration of the antipentylenetetrazol activity exerted by the deuterated compound is much shorter than for the C_3-unlabeled drug.

DISCUSSION

The present study indicates that the substitution of the hydrogen at the C_3-position of *N*-demethyldiazepam with deuterium resulted in a shortening of the anticonvulsant activity in mice. This finding

[1] Received as a gift from Prof. G. C. Jommi, Laboratory of Organic Chemistry, Department of Sciences, Milan University, Milan, Italy.
[2] Model GI, Carlo Erba, Milan, Italy.
[3] Rotor 40 ft., Beckman model L ultracentrifuge.
[4] Dubnoff.

may be partly explained by the fact that deuterated *N*-demethyldiazepam is less effective than the hydrogenated analog as an inhibitor of the mortality induced by pentylenetetrazol in mice. The ED_{50} was 198 mcg./kg. for *N*-demethyldiazepam and 288 mcg./kg. for the deuterated analog.

However, there is also a decreased accumulation of the hydroxylated metabolite, oxazepam, in the brain of mice treated with the deuterated analog compared to the C_3-unlabeled *N*-demethyldiazepam. This finding is in agreement with previous studies indicating that the long duration of action of diazepam and *N*-demethyldiazepam in mice is related to the formation and persistence of brain oxazepam (9). The lack of accumulation of oxazepam from C_3-deuterated *N*-demethyldiazepam is due to a reduced C_3-hydroxylation of this benzodiazepine *in vitro* compared to the C_3-unlabeled compound, as shown by the experiments utilizing liver microsomal enzymes.

The deuterated *N*-demethyldiazepam may be less hydroxylated than the C_3-unlabeled analog for one of the following reasons: (*a*) slower rate of cleavage of the carbon–deuterium bond, (*b*) lower affinity for the active site of the enzymes involved in the hydroxylation, and (*c*) more stable complex with the enzyme. While these possibilities are open to experimental investigations, it remains established that the presence of oxazepam in the brain is the main factor accounting for the prolonged antipentylenetetrazol effect exerted by diazepam or *N*-demethyldiazepam in mice.

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ACKNOWLEDGMENTS AND ADDRESSES

Received February 22, 1973, from *Istituto di Ricerche Farmacologiche "Mario Negri," Via Eritrea, 62-20157, Milano, Italy.*

Accepted for publication July 12, 1973.

Supported by Grant 1 PO1 GMI 8376-O2 PTR from the National Institutes of Health, Bethesda, MD 20014

The technical assistance of M. C. Reschiotto in greatly appreciated.

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COMMUNICATIONS

Effect of Hydroxy Group on Coacervate Formation by Sodium Hydroxybenzoates with Benzalkonium Chloride

Keyphrases ☐ Coacervate formation, sodium hydroxybenzoates and benzalkonium chloride—effect of position of hydroxy group ☐ Hydroxybenzoates—coacervate formation with benzalkonium chloride, phase transition diagrams, effect of hydroxy position

Sir:

Shah *et al.* (1) reported coacervate formation by sodium salicylate (sodium *o*-hydroxybenzoate) with benzalkonium chloride.

The phase transition diagram of this system (Fig. 1) illustrates two main regions: a biphasic coacervate system (I) and a monophasic equilibrium solution (II). The biphasic coacervate system shows two distinct regions. In one region (B) the coacervate phase has a higher density than the equilibrium liquid and it settles to the bottom of the container, and in the other region (T), the coacervate phase is lighter than the equilibrium liquid and floats on the top of the liquid.

This communication reports the effect of the presence and position of the hydroxy group on coacervate formation between hydroxybenzoates and benzalkonium chloride.

The coacervate systems are obtained by mixing various concentrations of sodium *m*-hydroxybenzoate, sodium *p*-hydroxybenzoate, and sodium benzoate with benzalkonium chloride in water. Figure 1 is the superimposed phase transition diagram of sodium *o*-, *m*-, and *p*-hydroxybenzoates. The phase regions for the hydroxybenzoates are equivalent, differing only in the positions of the boundary lines, and are as identified previously (1). Figure 2 shows a phase transition diagram of the sodium benzoate and benzalkonium chloride coacervate system.

The sodium benzoate system (Fig. 2) shows a lighter coacervate phase (T) throughout the biphasic coacervate region. The systems near the upper and lower transition lines showed the property of birefringence during flow. The coacervate phase in all three systems was observed to be isotropic under examination with a polarizing microscope.

From our previous (1) and present data, it is our belief that the density of the coacervate phase is a function of the molecular weight of the anionic electrolyte, the amount of this electrolyte associated with the micellar aggregation, and the size of the micelles.