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PHYSICAL EVIDENCE FOR THE OXIDATIVE DEMETHYLATION *IN VITRO* OF 1-NAPHTHYL *N*-METHYLCARBAMATE BY THE UDENFRIEND CHEMICAL HYDROXYLATION SYSTEM

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Abstract—1-Naphthyl *N*-methylcarbamate (carbaryl) was oxygenated in the Udenfriend chemical hydroxylation system as modified by Malling (H. V. Malling, *Mutation Res.* **3**, 537, 1966). The *N*-methyl oxidation product, 1-naphthyl *N*-(hydroxymethyl)carbamate, and the *N*-demethylation product, 1-naphthyl carbamate, were isolated and identified by mass and/or infrared spectrometry. These results very strongly suggest that the system is, in fact, capable of *N*-methyl oxidation and demethylation. This capability may apply to *N*-alkyl groups in general.

IN 1952 UDENFRIEND *et al.*[1] first reported the development of a chemical hydroxylation system, consisting of ascorbic acid, ferrous salt, EDTA, phosphate buffer and molecular oxygen, which was capable of introducing hydroxyl groups into the aromatic ring structures of tyramine, quinoline and acetanilide. These observations were then extended by Udenfriend *et al.*[2] and Brodie *et l.*[3] to show that the products produced by the chemical system from several compounds were identical to those produced *in vivo*. Boyland *et al.*[4] later compared the hydroxylation properties of the Udenfriend system with those of rat liver microsomes, and found that similar products were produced from compounds with simple ring structures. On the other hand, these investigators found both qualitative and quantitative differences between the chemical and metabolic hydroxylation products produced from compounds possessing complex, highly condensed ring structures. These authors also demonstrated the ability of the Udenfriend system to produce dihydrodiol products as well as phenols from aromatic compounds. In addition, the system has been shown capable of degrading aliphatic compounds by a series of sequential oxidative decarboxylations.[5,6]

More recently, the product mixtures produced by the Udenfriend chemical hydroxylation system from both dimethyl- and diethylnitrosamine have been shown to be mutagenic in biological test systems in which the parent compounds were not.[7,8] These nitrosamines are believed to be converted *in vivo* to the required active metabolites, the actual proximal mutagens and carcinogens, by pathways requiring an initial oxidative dealkylation step.[9–12] Thus, the genetic evidence suggested that the Udenfriend system was capable of the *N*-alkyl oxidative dealkylation of nitrosamines. To our knowledge, no conclusive demonstration of the *N*-alkyl oxidative

dealkylation capability of the Udenfriend system exists, with respect to any compound investigated in the system. However, the studies of Hanaki and Ishidate[13] on the effects of the system on 4-*N*-dimethylaminoazobenzene and the studies of Preussmann[12] on the effects of the system on various nitrosamines suggested such a capability.

The purpose of the present study was to demonstrate by physical methods that the Udenfriend chemical hydroxylation system was, in fact, capable of *N*-alkyl oxidative dealkylation. 1-Naphthyl *N*-methylcarbamate* was chosen as the model compound, since its *N*-methyl oxidation and demethylation products are stable in their pure forms, and authentic standards are available.

MATERIALS AND METHODS

1-Naphthyl *N*-methylcarbamate (carbaryl)† was recrystallized from ethanol, and a total of 100 mg was added to three 1-liter Erlenmeyer flasks as a methylene chloride solution (33·3 mg/flask), employing sterile techniques. After the solvent was evaporated at room temperature, a 500-ml portion of the Udenfriend hydroxylation medium,[2] as modified by Malling[7] but at pH 7·2, was aseptically added to each flask. The mixtures were then acrated with oxygen for 6 hr at room temperature. Since preliminary studies‡ with carbaryl, labeled with ^{14}C in the C_1 position of the naphthyl ring, had indicated little alteration of parent compound in the Udenfriend system with nitrogen aeration, such a control was not repeated. The mixtures were pooled after the aeration and extracted with an equal volume of methylene chloride.§ The solvent was evaporated under vacuum and the desiccated solid was weighed and redissolved in acetone. The solution was streaked on ten 100-μm thin-layer plates prepared from Silica gel G-F_{254} obtained from E. Merck. To remove organoextractable impurities from the Silica gel, thin-layer plates were routinely prewashed in methylene chloride. After sample application and development for 15 cm, compounds were visualized with short- and long-wavelength u.v. light, following procedures previously reported for commercial 250 μm thin-layer plates.[14]

The initial separation of desmethylcarbaryl (R_f = 0·29) and *N*-CH_2OH-carbaryl (R_f = 0·20) from carbaryl (R_f = 0·59) and other alteration products was accomplished in 19:1 (v/v) benzene–ethanol. For each compound, the Silica gel bands from each of ten thin-layer plates were pooled, and the compounds were eluted with acetone. At this and all subsequent concentration steps, the organic extracts were evaporated to dryness with a stream of ultra high purity nitrogen, in order to avoid possible air oxidation and contaminants from rotary evaporation apparatus that might interfere with mass and infrared spectrometry. To maximize the recoveries of polar carbaryl alteration products from the Silica gel, acetone was used for all but the final elutions for each compound. Methylene chloride, although less efficient than acetone for extracting hydroxylated alteration products, was utilized to obtain all final

* Like physostigmine and certain other carbamic acid esters, 1-naphthyl *N*-methylcarbamate is a potent cholinesterase inhibitor and is, therefore, employed as an insecticide.

† Abbreviations: carbaryl, 1-naphthyl *N*-methylcarbamate; desmethylcarbaryl, 1-naphthyl carbamate; *N*-CH_2OH-carbaryl, 1-naphthyl *N*-(hydroxymethyl)carbamate. These compounds were obtained from Union Carbide Corp.

‡ R. K. Locke and V. W. Mayer, unpublished work.

§ Organic solvents used in all procedures were "distilled-in-glass" reagents (Burdick & Jackson Laboratories). Reagent grade absolute ethanol and methanol were used.

extracts to be analyzed spectrometrically because of its relative freedom from impurities.

The isolated carbaryl in methylene chloride was quantitated by measuring absorbance at 280 nm, and was analyzed spectrometrically without further purification. The desmethylcarbaryl was further purified on a single thin-layer plate in 3:1 (v/v) chloroform–acetonitrile (R_f = 0·70), in which it co-chromatographed with an unknown product which fluoresced under long-wavelength u.v. light. Subsequent chromatography in 49 : 1 (v/v) chloroform–methanol (R_f = 0·46) yielded a pure material which was extracted into methylene chloride and analyzed by i.r. spectrophotometry. The *N*-CH_2OH-carbaryl was similarly purified in 3:1 (v/v) chloroform–acetonitrile (R_f = 0·48), and the organic extract was analyzed by mass and i.r. spectrometry.

Infrared spectra were obtained with micro KBr discs[15,16] by using a Perkin–Elmer i.r. grating spectrophotometer, model 621, equipped with a 6X dual condensing accessory. Mass spectra were obtained with an Atlas CH-4B mass spectrometer, using a direct probe inlet system. Spectra were compared with those of authentic standards.

RESULTS

The solubility of carbaryl in the Udenfriend hydroxylation system was less than its reported water solubility of 99 μg/ml;[17] therefore, after the oxygen aeration period, the undissolved excess was extracted into methylene chloride together with the alteration products. Of the 100 mg of carbaryl treated, 54·6 mg of material was obtained in the organic extract. Of this amount, 44·4 mg represented unaltered carbaryl, as evidenced by absorbance measurements and mass and infrared spectra. The remaining 10·2 mg represented organoextractable alteration products. Apparently 45·4 mg of the starting material had been converted to water-soluble products. Thin-layer chromatography of the organoextractable products with known standards revealed many ring-hydroxylated products, whose nature and relationship to metabolites produced from carbaryl by mammals, plants, insects and man will be discussed elsewhere.

The production of the *N*-demethylation product of carbaryl, desmethylcarbaryl, by the Udenfriend hydroxylation system was demonstrated by comparison of the i.r. spectrum of the total isolated product with that of an authentic standard (Fig. 1). Because of the instability of the isolated desmethylcarbaryl, as shown by a change in i.r. spectrum after brief storage of the original KBr disc, a mass spectrum could not subsequently be obtained nor could the compound be quantitated by absorbance measurements. However, a comparison of the short-wavelength u.v. absorbance of the banded compound prior to the final isolation with that of known standards suggested the production of a total of approximately 45 μg desmethylcarbaryl. In contrast to the instability of the isolated product, a standard of desmethylcarbaryl was stable in KBr discs for at least 6 months, as evidenced by an identical i.r. spectrum

The production of the *N*-methyl oxidation product of carbaryl, *N*-CH_2OH-carbaryl, by the Udenfriend system was proven by a comparison of the mass spectrum of an aliquot of the isolated product with that of a known standard (Fig. 2). Because the isolated *N*-CH_2OH-carbaryl was unstable, the subsequent i.r. spectrum of the remainder of the isolated product was not identical to that of a standard, but indicated that the compound was present in a mixture. Although exact quantitation

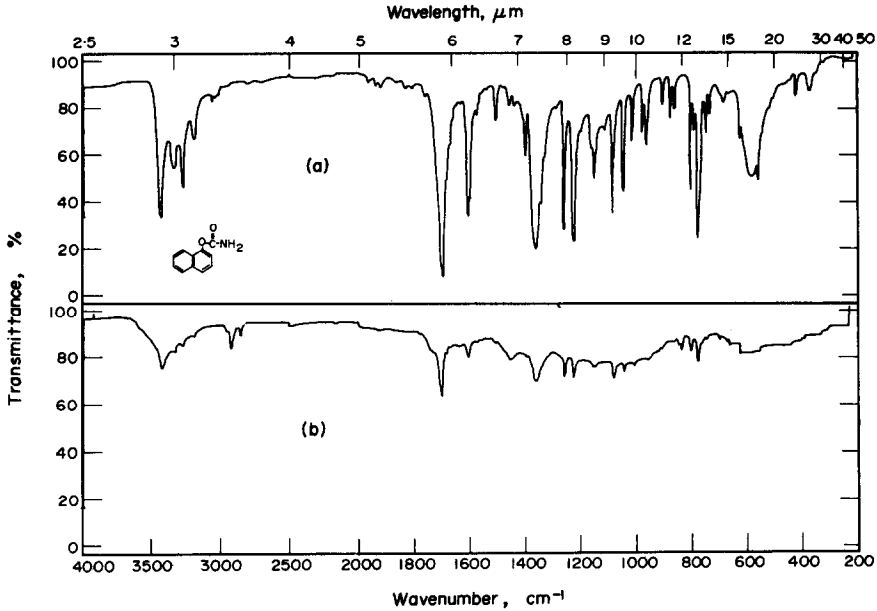

FIG. 1. Infrared spectrum of authentic desmethylcarbaryl (a) and of desmethylcarbaryl isolated, following oxygenation, from the Udenfriend chemical hydroxylation system containing carbaryl (b). Spectra were obtained as described in Materials and Methods.

could not be obtained, the short-wavelength u.v. absorbance of the banded compound prior to final elution suggested that a total of approximately 330 μg *N*-CH_2OH-carbaryl had been produced. As opposed to the instability of the isolated *N*-CH_2OH-carbaryl, a standard was stable for at least 6 months (as a powder or in KBr discs), as shown by an identical i.r. spectrum.

DISCUSSION

The present study strongly suggests that the Udenfriend chemical hydroxylation system is capable of effecting the *N*-methyl oxidation and dealkylation of 1-naphthyl *N*-methylcarbamate. It is likely that the first step of this reaction sequence is the formation of the primary alcohol, *N*-CH_2OH-carbaryl. This compound might then lose

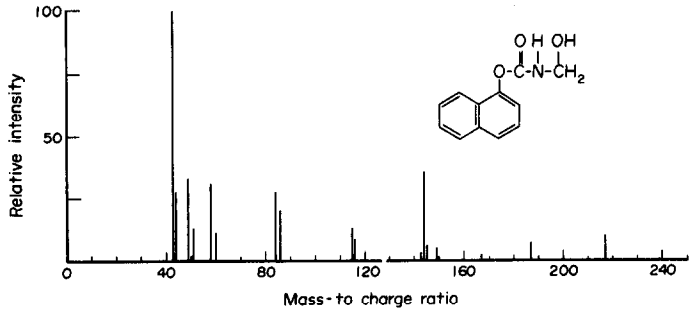

FIG. 2. Mass spectrum of *N*-CH_2OH-carbaryl isolated, following oxygenation, from the Udenfriend chemical hydroxylation system containing carbaryl. The spectrum was obtained as described in Materials and Methods.

formaldehyde to form the demethylation product, desmethylcarbaryl. The general tendency of hydroxymethyl groups to dissociate from the parent compound with the formation of formaldehyde is well known.[18] On the other hand, the primary alcohol group of *N*-CH_2OH-carbaryl might be further oxidized by the Udenfriend system to the aldehyde group and subsequently to the carboxylic acid function. The demethylation product, desmethylcarbaryl, could then be formed by decarboxylation with the liberation of CO_2. The postulation that *N*-CH_2OH-carbaryl is the initial product in either of these oxidative demethylation sequences is supported by the fact that with a great excess of carbaryl, the amount of *N*-CH_2OH-carbaryl produced (*ca.* 330 μg) by the Udenfriend system was approximately 7-fold greater than the amount of desmethylcarbaryl produced (*ca.* 45 μg).

The instability of the isolated *N*-CH_2OH-carbaryl and desmethylcarbaryl in the dry state or in KBr discs, as opposed to the stability of standards under the same conditions, can be best explained by the production of extraneous products during the oxygenation of carbaryl in the Udenfriend system, which were extractable into methylene chloride, co-chromatographed with the isolated products in the thin-layer systems used, and subsequently catalyzed their decomposition. This hypothesis is strengthened by the fact that we have isolated radiolabeled *N*-CH_2OH-carbaryl from biologically produced conjugates by using the same thin-layer chromatographic systems described in the present study, and the compound has remained stable in the dry state for several months, as evidenced by two dimensional thin-layer chromatography with an authentic standard. This fact would tend to eliminate solvent and Silica gel impurities as causative agents for the decomposition of the *N*-CH_2OH-carbaryl and desmethylcarbaryl isolated from the Udenfriend chemical hydroxylation system. The pH of the Udenfriend system remained at 7·2 following the oxygenation period, and it is, therefore, unlikely that the decomposition of these carbamates could be ascribed to the pH of the methylene chloride extract.

The evidence presented here that the Udenfriend chemical hydroxylation system is, in fact, capable of *N*-methyl oxidative demethylation might presumably indicate a general capability for *N*-alkyl oxidative dealkylation. It has already been shown[5] that the system is capable of the effective oxidative degradation of long-chain alkyl groups, although short-chain alkyl groups were less effectively degraded. Our data are also consistent with reports[7,8] that mutagenic product mixtures are produced by the Udenfriend chemical hydroxylation system from both dimethyl- and diethylnitrosamine, which are believed to require conversion to active metabolites by pathways requiring an initial oxidative dealkylation step.[9–12]

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REFERENCES

1. S. Udenfriend, C. T. Clark, J. Axelrod and B. B. Brodie, *Fedn Proc.* **11**, 300 (1952).
2. S. Udenfriend, C. T. Clark, J. Axelrod and B. B. Brodie, *J. biol. Chem.* **208**, 731 (1954).
3. B. B. Brodie, J. Axelrod, P. A. Shore and S. Udenfriend, *J. biol. Chem.* **208**, 741 (1954).
4. E. Boyland, M. Kimura and P. Sims, *Biochem. J.* **92**, 631 (1964).
5. R. P. Geyer, S. Kydd and M. Ryan, *Archs Biochem. Biophys.* **70**, 129 (1957).
6. M. Mazelis, *Nature, Lond.* **189**, 305 (1961).
7. H. V. Malling, *Mutation Res.* **3**, 537 (1966).

8. V. W. MAYER, *Molec. gen. Genet.* **112,** 289 (1971).
9. P. EMMELOT, I. J. MIZRAHI and E. KRIEK, *Nature, Lond.* **193,** 1158 (1962).
10. H. DRUCKREY, R. PREUSSMANN, D. SCHMÄHL and M. MULLER, *Naturwissenschaften* **48,** 134 (1961).
11. K. Y. LEE, W. LIJINSKY and P. N. MAGEE, *J. natn. Cancer Inst.* **32,** 65 (1964).
12. R. PREUSSMANN, *Arzneimittel-Forsch.* **14,** 769 (1964).
13. A. HANAKI and M. ISHIDATE, *Biochim. biophys. Acta* **57,** 180 (1962).
14. R. K. LOCKE, *J. agric. Fd Chem.* **20,** 1078 (1972).
15. J.-Y. T. CHEN, *J. Ass. off. analyt. Chem.* **48,** 380 (1965).
16. J.-Y. T. CHEN and R. W. DORITY, *J. Ass. off. analyt. Chem.* **53,** 978 (1970).
17. D. E. H. Frear (Ed.), *Pesticide Index*, 4th Edn. p. 88. College Science Publishers, State College, Pennsylvania (1969).
18. J. F. WALKER, *Formaldehyde*, 2nd Edn., p. 172. ACS Monograph-Reinhold, New York (1953).