COMMUNICATIONS

Thin-Layer Chromatography of 1-Naphthyl *N*-Hydroxy,*N*-methylcarbamate and Its Application in Two *in vitro* Studies Involving Carbaryl

1-Naphthyl *N*-hydroxy,*N*-methylcarbamate (*N*-hydroxycarbaryl) cochromatographs with carbaryl (1-naphthyl *N*-methylcarbamate) or 5-hydroxycarbaryl (5-hydroxy-1-naphthyl *N*-methylcarbamate) in several tlc systems commonly used for the separation of carbaryl metabolite aglycones. Two tlc systems are described which separate *N*-hydroxycarbaryl from carbaryl, 5-hydroxycarbaryl, 4-hydroxycarbaryl (4-hydroxy-1-naphthyl *N*-methylcarbamate), α-naphthol, *N*-hydroxymethylcarbaryl (1-naphthyl *N*-hydroxymethylcarbamate), 5,6-dihydro-5,6-dihydroxycarbaryl (5,6-dihydro-5,6-dihydroxy-1-naphthyl *N*-methylcarbamate), and 5,6-dihydro-5,6-dihydroxynaphthol. One of these systems has been utilized in preliminary studies which suggest that *N*-hydroxycarbaryl, among other hydroxylation products, is produced from carbaryl by an *in vitro* hydroxylation system previously thought incapable of N oxidation. The same tlc system was used to investigate the nature of the water-soluble metabolites produced from $^{14}C_1$-naphthyl-labeled carbaryl by rat liver cubes maintained in culture medium. Preliminary studies indicate that approximately 3% of these metabolites may represent N–O conjugates of *N*-hydroxycarbaryl. These thin-layer chromatographic indications of *N*-hydroxycarbaryl must still be confirmed by independent means such as infared and mass spectrometry.

N-hydroxy compounds are of great interest because of their potential mutagenic and carcinogenic effects. The carcinogenicity of 2-acetylaminofluorene (Cramer *et al.*, 1960; Miller *et al.*, 1960) and 2-naphthylamine (Radomski and Brill, 1970) has been related to the production of *N*-hydroxy derivatives from these compounds. 1-Naphthylamine and 2-naphthylamine are inactive, but the *N*-hydroxy derivatives are mutagenic for *E. coli* with respect to auxotroph reversion studies (Belman *et al.*, 1968; Mukai and Troll, 1969; Perez and Radomski, 1965). Recently it has been shown that hydroxylated derivatives of 1-naphthylamine and 2-naphthylamine produced by an *in vitro* hydroxylation system (but not the parent compounds) are mutagenic for *Saccharomyces cerevisiae*, suggesting the *in vitro* production of the *N*-hydroxy derivatives (Mayer, 1971). Possible *N*-hydroxy metabolites of the carbamate pesticides present in the food supply may pose a serious toxicological hazard to man. For this reason, analytical techniques are needed to investigate carbamate metabolites for the presence of *N*-hydroxycarbamate aglycones. The thin-layer chromatographic (tlc) behavior of the *N*-hydroxy derivative of carbaryl, a commonly used carbamate insecticide, was therefore investigated to develop a method for distinguishing 1-naphthyl *N*-hydroxy,*N*-methylcarbamate from other carbaryl metabolite aglycones.

MATERIALS AND METHODS

Chromatographic Standards. Commercial carbaryl (1-naphthyl *N*-methylcarbamate, Union Carbide Corp., New York, N.Y.) was recrystallized from ethanol (mp 138.0–138.5°C). α-Naphthol was obtained from Allied Chemical Corp., New York, N.Y. Union Carbide Chemicals Co.; New York, N.Y., was the source of the following compounds: 4-hydroxy-1-naphthyl *N*-methylcarbamate; 5-hydroxy-1-naphthyl *N*-methylcarbamate; 1-naphthyl *N*-hydroxy,*N*-methylcarbamate; 1-naphthyl *N*-hydroxymethylcarbamate; 5,6-dihydro-5,6-dihydroxy-1-naphthyl *N*-methylcarbamate; and 5,6-dihydro-5,6-dihydroxynaphthol.

Thin-Layer Chromatography. For tlc, 10 μl of methylene chloride solutions containing 1 mg of the chromatographic standards per milliliter were applied to commercial 250-μ Silica Gel F-254 tlc plates (EM Reagents Division, Brinkmann Instruments, Inc., Westbury, N.Y.). The solvent systems utilized are shown in Table I.

The absolute methanol and toluene used were "Baker Analyzed" reagents (J. T. Baker Chemical Co., Phillipsburg, N.J.), and "Pharmco" absolute ethanol was used (Publicker Industries, Inc., Philadelphia, Pa., 200-proof). All other solvents were "distilled-in-glass reagents" (Burdick and Jackson Laboratories, Inc., Muskegon, Mich.). The solvent front was 15 cm above the origin in all cases. Standards were detected by viewing with short-wave uv light (Chromato-Vue, Model C-3, Ultraviolet Products Inc., San Gabriel, Calif.).

RESULTS AND DISCUSSION

The R_f values shown in Table I were obtained for authentic standards of *N*-hydroxycarbaryl and various common carbaryl metabolite aglycones by tlc in the various systems. *N*-Hydroxycarbaryl cochromatographed with carbaryl in Systems I through VIII and with 5-hydroxycarbaryl in Systems IX through XII. In Systems IV and V, *N*-hydroxycarbaryl cochromatographed with both carbaryl and 5-hydroxycarbaryl.

Ether–hexane solvent systems (Systems I through III) have been widely used in the separation and tentative identification of aglycones of metabolites produced from carbaryl by mammals and by a liver microsome system (Leeling and Casida, 1966), by human embryonic lung cells in culture (Baron and Locke, 1970), by bean plants (Kuhr and Casida, 1967), and by tobacco cells in suspension culture (Locke and Baron,

Table I. R_f Values of Authentic Standards of *N*-Hydroxycarbaryl, Carbaryl, and Various Carbaryl Metabolite Aglycones

Compound	Solvent system[a]													
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Carbaryl	0.17	0.34	0.39	0.85	0.47	0.19	0.76	0.69	0.42	0.86	0.83	0.50	0.77	0.63
N-Hydroxycarbaryl	0.16	0.35	0.39	0.83	0.48	0.13	0.74	0.71	0.31	0.79	0.79	0.45	0.46	0.45
5-Hydroxycarbaryl	0.09	0.27	0.32	0.81	0.46	0.06	0.59	0.70	0.27	0.77	0.75	0.43	0.31	0.30
4-Hydroxycarbaryl	0.05	0.23	0.27	0.77	0.44	0.05	0.58	0.69	0.22	0.66	0.67	0.41	0.22	0.25
N-Hydroxymethyl-carbaryl	0.05	0.20	0.22	0.67	0.41	0.06	0.52	0.65	0.17	0.50	0.58	0.37	0.26	0.23
α-Naphthol	0.37	0.48	0.49	0.89	0.52	0.27	0.82	0.73	0.43	0.90	0.89	0.51	0.67	0.72
5,6-Dihydro-5,6-dihydroxycarbaryl	0.00	0.05	0.06	0.26	0.27	0.00	0.18	0.47	0.04	0.09	0.22	0.17	0.04	0.07
5,6-Dihydro-5,6-dihydroxynaphthol	0.03	0.14	0.16	0.36	0.37	0.03	0.29	0.52	0.06	0.18	0.45	0.23	0.04	0.10

[a] Solvent systems (v/v): I. ether–hexane, 1:1; II. ether–hexane, 4:1; III. ether–hexane, 9:1; IV. chloroform–acetonitrile, 2:1; V. ethyl acetate–ethanol, 98:2; VI. petroleum ether (bp 30–60°C)–acetone, 4:1; VII. petroleum ether (bp 30–60°C)–acetone, 7:3; VIII. acetonitrile–hexane, 9:1; IX. chloroform–acetonitrile, 4:1; X. methylene chloride–acetonitrile, 4:1; XI. ethyl acetate–toluene, 2:1; XII. acetonitrile–toluene, 1:1; XIII chloroform–methanol, 49:1; XIV. benzene–ethanol, 19:1. The solvent front was 15 cm above the origin in all cases.

1971). Tlc of aglycones in Systems I through III has generally been supplemented with chromatography in another solvent system in which 5,6-dihydro-5,6-dihydroxyaglycones have greater R_f values, such as System IV (Leeling and Casida, 1966), System VII (Baron and Locke, 1970; Locke and Baron, 1971), or System V (Kuhr and Casida, 1967). *N*-Hydroxycarbaryl and carbaryl cochromatographed in all of these solvent systems (Table I).

N-Hydroxycarbaryl and carbaryl would also be poorly resolved by the two-dimensional tlc technique employed by Leeling and Casida (1966), in which System III was used for development in one direction and System IV for development in a second direction. *N*-Hydroxycarbaryl and carbaryl cochromatographed in both of these systems (Table I). On the other hand, *N*-hydroxycarbaryl and carbaryl would be resolved by the two-dimensional tlc technique utilized by Dorough and Casida (1964), in which development in System II in one direction is followed by development in System X in another. Although *N*-hydroxycarbaryl cochromatographs with carbaryl in System II, it cochromatographs with 5-hydroxycarbaryl and not with carbaryl in System X.

Reported N conjugates of carbaryl, in which the identity of the aglycone was primarily based upon cochromatography with carbaryl in Systems I through III together with cochromatography in any one of Systems IV through VIII, should be regarded as possible *N–O* conjugates of *N*-hydroxycarbaryl. The *N* conjugates of carbaryl reported in tobacco cells incubated in medium containing ^{14}C-carbaryl (Locke and Baron, 1971) are therefore being reinvestigated for the presence of *N–O* conjugates of *N*-hydroxycarbaryl. The *N* glycosides of carbaryl reported in bean plants injected with ^{14}C-carbaryl (Kuhr and Casida, 1967) might also represent *N–O* glycosides of *N*-hydroxycarbaryl, in view of the tlc solvent systems utilized for the tentative identification of carbaryl as the aglycone (Systems II, IV, and V).

In an effort to distinguish *N*-hydroxycarbaryl from carbaryl and carbaryl metabolite aglycones, tlc Systems VI, VII, XIII, and XIV utilized by Radomski and Brill (1970) for the separation of the *N*-hydroxy derivative of 1-naphthylamine from 1-naphthylamine were investigated. In Systems VI and VII, *N*-hydroxycarbaryl cochromatographed with carbaryl (Table I). System VII may prove useful, however, in the tlc identification of 5,6-dihydro-5,6-dihydroxycarbaryl metabolite aglycones. Systems XIII and XIV (Radomski and Brill, 1970) provided an adequate separation of *N*-hydroxycarbaryl from carbaryl, 5-hydroxycarbaryl, 4-hydroxycarbaryl, *N*-hydroxymethylcarbaryl, α-naphthol, 5,6-dihydro-5,6-dihydroxycarbaryl, and 5,6-dihydro-5,6-dihydroxynaphthol (Table I). The greatest separation of carbaryl metabolite aglycones was achieved in both Systems XIII and XIV by developing the plate immediately after solvent preparation (unsaturated tank atmosphere). Almost no edge effect was observed with System XIV under these conditions, whereas the effect was more apparent with System XIII.

System XIV has been utilized in the preliminary investigation of the metabolites produced from $^{14}C_1$-naphthyl-labeled carbaryl by 1-mm cubes of liver, obtained from an 80-g male Osborne-Mendel rat (28-days-old). The cubes were incubated for 22 hr in Trowell T-8 culture medium (Locke, 1971); the culture conditions, radioactive dose, subsequent treatment of culture fractions, and column chromatography of the culture medium after the incubation period have been previously described (Sullivan *et al.*, 1971). Recovery of administered radioactivity was 95%; more than 94% of the radioactivity recovered represented water-soluble metabolites of carbaryl. The radioactivity contained in culture medium incubated with radio-labeled carbaryl in the absence of liver consisted primarily of unchanged carbaryl, together with a small amount of α-naphthol. System XIV was employed with known standards in the tlc of methylene chloride extracts of 0.1 *N* HCl hydrolysates (1 hr at 100°C) of each of the radioactive fractions obtained by column chromatography of the culture medium. Some of the hydrolysates contained radioactivity which cochromatographed with authentic *N*-hydroxycarbaryl in System XIV; this radioactivity accounted for approximately 3% of that recovered from column chromatography of the culture medium. Thus, 3% of the metabolites produced from $^{14}C_1$-naphthyl-labeled carbaryl by rat liver in culture may represent *N–O* conjugates of *N*-hydroxycarbaryl. This preliminary finding must await confirmation of the 1-naphthyl *N*-hydroxy,*N*-methylcarbamate aglycone by infrared and mass spectrometric techniques.

The stability of *N*-hydroxycarbaryl to acid hydrolysis was investigated by hydrolyzing 50 μg of authentic standard for 1 hr at 100°C in a total volume of 1 ml of 0.1 *N*, 1.0 *N*, or 4.0 *N* HCl. The hydrolysates were extracted with methylene chloride and aliquots of the concentrated extracts were chromatographed on tlc plates with authentic *N*-hydroxycarbaryl and its hydrolysis product, α-naphthol. *N*-Hydroxycarbaryl was hydrolyzed to the extent of approximately 5% with 0.1 *N* HCl, 50% with 1.0 *N* HCl, and 100% with 4.0 *N* HCl.

System XIV has been employed in the preliminary investi-

gation of the hydroxylated derivatives produced from carbaryl by an *in vitro* chemical hydroxylation system, originally developed by Udenfriend *et al.* (1954), with some minor modifications (Mayer, 1971). With oxygen bubbling the system produced methylene chloride-extractable hydroxylated derivatives from carbaryl which cochromatographed with *N*-hydroxycarbaryl, 5-hydroxycarbaryl, and 4-hydroxycarbaryl together with *N*-hydroxymethylcarbaryl in System XIV. The control, with nitrogen bubbling, contained essentially unchanged carbaryl (Mayer and Locke, 1971). These preliminary results indicated that the *N*-hydroxy derivative of carbaryl had been produced by the modified Udenfriend *in vitro* system. Previous reports on the action of the Udenfriend *in vitro* hydroxylation system upon 2-naphthylamine (Booth *et al.*, 1955) and upon aniline and tyramine (Brodie *et al.*, 1954) revealed no *N* oxidation products. The *N*-hydroxy derivatives of these compounds may be less stable than *N*-hydroxycarbaryl, and may have been destroyed during the isolation and identification procedures employed. Work is in progress to demonstrate the production of *N*-hydroxycarbaryl from carbaryl by the modified Udenfriend hydroxylation system by use of infrared and mass spectrometric techniques.

The chloroform–methanol (System XIII) and benzene–ethanol (System XIV) tlc systems described for the separation of *N*-hydroxycarbaryl from other carbaryl metabolite aglycones may also prove useful in the separation and identification of *N*-hydroxycarbamate aglycones with regard to conjugates produced in biological systems from other carbamates.

NOTE ADDED IN PROOF. Systems XIII and XIV, used in combination, separate *N*-hydroxycarbaryl from the aglycones listed in Table I as well as from the following compounds: 7-hydroxycarbaryl, desmethylcarbaryl(1-naphthyl carbamate), *N*-acetyldesmethylcarbaryl, *N*-acetylcarbaryl(1-naphthyl *N*-acetyl, *N*-methylcarbamate), and the naphthalene-1,3-, 1,4-, 1,5-, 1,6-, and 1,7-diols. Of the compounds tested, only desmethylcarbaryl cochromatographs with *N*-hydroxycarbaryl in System XIII; only naphthalene-1,5-diol cochromatographs in System XIV.

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