Metabolites of Methyl- and Dimethylcarbamate Insecticide Chemicals as Formed by Rat Liver Microsomes

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Critical interpretation of toxicology and residue studies on an insecticide chemical is possible only when the metabolism of the compound is understood. Biologically active metabolites may be formed, particularly if the modification on the molecule occurs at a site other than the toxophoric grouping. In this respect, metabolites of carbamates formed by mechanisms other than initial hydrolysis at the carbamic ester site may be of importance. Mammals, and microsomal enzymes of liver, carry out many types of hydroxylation reactions including, among others, aromatic hydroxylation, aliphatic hydroxylation, N-dealkylation, O-dealkylation, and sulfoxidation (1, 2). Groupings which are potentially susceptible to such hydroxylation reactions are

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present in methylcarbamate insecticide chemicals.

There is some information in regard to the identity of metabolites formed in mammals or by microsomal enzyme systems from carbaryl (1-naphthyl methylcarbamate), Baygon (2-isopropoxyphenyl methylcarbamate), Pyramat (6-methyl-2-propyl-4-pyrimidinyl dimethylcarbamate), and certain dimethylcarbamates which yield N-methyl, N-hydroxymethylcarbamates (3-8). The in vivo fate of the radiocarbon is known for 10 variously C^{14}-labeled methyl- and dimethylcarbamate insecticide chemicals, but, other than $C^{14}O_2$, the metabolites remain, in the most part, to be identified (9). Certain metabolites of carbaryl, as formed in vivo by rabbits and goats, are identical with those produced by liver microsomal systems, and include derivatives formed by hydroxylation of the N-methyl group and the 4- or 5-position of the naphthyl group, as well as the 5,6-dihydro-5,6-dihydroxy analog (4, 5). Not any of these carbaryl metabolites are more active as anticholinesterase agents than carbaryl itself (4, 5). Insecticidal and/or anticholinesterase metabolites are formed from other carbamates after injection of 1-dimethylcarbamoyl-5-methyl-3-pyrazolyl dimethylcarbamate (dimetilan) into insects (10), after injection of 4-methylthio-3,5-xylyl methylcarbamate (Mesurol), 4-dimethylamino-3-cresyl methylcarbamate (Matacil), and 4-dimethylamino-3,5-xylyl

methylcarbamate (Zectran) into bean plants (11, 12), and on incubation of certain methyl- and dimethylcarbamates with rat liver microsomal enzymes, based on a failure to lose anticholinesterase activity while forming metabolites which yield formaldehyde on acid degradation (7).

A series of experiments were made with each of 10 carbamate-carbonyl-C^{14}-labeled methyl- and dimethylcarbamates to determine their metabolic fate in a system containing rat liver microsomes and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$). With emphasis on those with a carbamate structure, the ether-extractable metabolites were separated by thin-layer chromatography (TLC) and the radioactive spots were located by radioautography. The anticholinesterase activity of the metabolites derived from five methylcarbamates was determined, in situ, on the TLC plates. In some cases, the metabolites were tentatively identified by cochromatography with known compounds.

Methods and Materials

Each of the compounds listed in Table I was utilized with a carbamate-carbonyl-C^{14} label, and each had a radiochemical purity greater than 99 per cent. In addition to $NADPH_2$ and its oxidized form (NADP), nicotinamide adenine dinucleotide in both the oxidized (NAD) and reduced ($NADH_2$) forms was used as a cofactor.

Rat liver was homogenized in 0.25M sucrose and the homogenate was fractionated. The "microsome plus soluble" fraction was the supernatant from centrifugation of the homogenate for 30 minutes at 15,000 g; the "microsome" fraction was the sediment obtained from the microsome plus soluble fraction by centrifugation of the supernatant for an additional 30 minutes at 95,000 g. The microsomal pellet was washed twice with sucrose solution and reconstituted with 0.25M sucrose to give a concentration equivalent to the original 20 per cent homogenate.

In the incubation step, each 25-ml. Erlenmeyer flask contained the following: 2 μmoles of labeled substrate; microsome, or microsome plus soluble, fraction equivalent to 200 mg. of liver; not any or 2 μmoles of cofactor; 250 μmoles of sucrose; 50 μmoles of sodium phosphate; water to make to 2 ml. total volume with a pH of 7.4. The flasks were incubated in air, with shaking, for 4 hours at 37°C. Analysis consisted of ether extraction to recover the original carbamate and carbamate metabolites, and TLC (silica gel G from Kensington Scientific Corp., Berkeley, Calif.; 20 x 20 cm. plates of 0.25 mm. thickness) to resolve these materials. Development of the chromatograms in the case of methylcarbamates involved chloroform-acetonitrile mixture (4 to 1) in the first direction and, after solvent evaporation, ether-hexane mixture (4 to 1) in the other direction;

one-dimensional chromatography with ethyl acetate-ethanol mixture (98 to 2) was used for the dimethylcarbamates. The total radiocarbon present in each of the following fractions was determined by scintillation counting and expressed on a percentage basis: the incubation mixture after incubation but before extraction; the ether and water phases following extraction; each resolved product from TLC, as detected by radioautography.

Cochromatography was used for tentative metabolite identification. The ether extract, containing labeled metabolites, was mixed with 10 to 20 μg, of each non-labeled known compound, the mixture was spotted on a TLC plate, and the plate was developed with the solvent systems as mentioned above, or with ether-carbon tetrachloride mixture (5 to 1). The colored spots for the known carbamates, as detected with ninhydrin or other reagents (4, 10, 13), were compared as to position and shape with darkened areas on the radioautograph, produced by the radiolabeled metabolites.

For localization and assay of cholinesterase inhibitors, the TLC plate containing the resolved radioactive metabolites was sprayed with undiluted human plasma until the gel was wet and appeared glossy. A filter paper (Whatman No. 1) dipped in a plasma-dye mixture was then laid over the wet silica gel, taking care to avoid the trapping of air bubbles. (The plasma-dye mixture consisted of 0.2 per cent

(w./v.) water-soluble cresol red in 0.025N aqueous sodium hydroxide, to which was added, just before use, an equal volume of undiluted plasma.) After 30 minutes at 27°C., the paper on the plate was sprayed with a 4.5 per cent (w./v.) aqueous solution of acetylcholine bromide until the paper was wet; the plate was then placed in a closed moist chamber at 27°C. Within 30 minutes, red spots appeared on a yellow background when cholinesterase inhibitors were present; these colored spots were compared, as to location, with the darkened areas on the radioautograph. Ether (spectro quality reagent) extracts of incubation mixtures containing the microsomes but not any added cofactor, where little metabolism occurred, were compared to corresponding extracts from incubation mixtures containing microsomes plus $NADPH_2$, where more extensive metabolism was always evident; thus, the "microsomes alone" sample served as a control for the "microsomes plus $NADPH_2$" sample. The results were expressed as the minimum amount of compound in micrograms, based on radioactivity, needed to yield a spot due to the inhibition of pseudocholinesterase.

The methods and materials are described in greater detail in reference number 13.

Results

Table I lists the number of carbamate metabolites, both

TABLE I

Metabolites of Methyl- and Dimethylcarbamate Insecticide Chemicals Formed by Rat Liver Microsomal Enzymes in Greater than One Per Cent Yield

Insecticide Chemicals	Metabolites Tentatively Identified[1]	Unidentified Metabolites
1-Naphthyl methylcarbamate (carbaryl)	1-naphthyl N-hydroxymethylcarbamate[a] 4-hydroxy-1-naphthyl methylcarbamate[b] 5-hydroxy-1-naphthyl methylcarbamate[a] 5,6-dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate[2]	none
2-Isopropoxyphenyl methylcarbamate (Baygon)	2-isopropoxyphenyl N-hydroxymethylcarbamate[a] 2-hydroxyphenyl methylcarbamate[a]	two
3-Isopropylphenyl methylcarbamate (UC 10854)	none	five
3,5-Diisopropylphenyl methylcarbamate (HRS-1422)	none	six
2-Chloro-4,5-xylyl methylcarbamate (Banol)	2-chloro-4,5-xylyl N-hydroxymethylcarbamate[a]	two
4-Methylthio-3;5-xylyl methylcarbamate (Mesurol)	4-methylsulfinyl-3,5-xylyl methylcarbamate[c] 4-methylsulfonyl-3,5-xylyl methylcarbamate[c],[3]	one
4-Dimethylamino-3-cresyl methylcarbamate (Matacil)	4-dimethylamino-3-cresyl N-hydroxymethylcarbamate[a] 4-methylamino-3-cresyl methylcarbamate[d] 4-amino-3-cresyl methylcarbamate[d],[3]	none
4-Dimethylamino-3,5-xylyl methylcarbamate (Zectran)	4-dimethylamino-3,5-xylyl N-hydroxymethylcarbamate[a] 4-methylformamido-3,5-xylyl methylcarbamate[d] 4-methylamino-3,5-xylyl methylcarbamate[d] 4-amino-3,5-xylyl methylcarbamate[d]	three
1-Dimethylcarbamoyl-5-methyl-3-pyrazolyl dimethylcarbamate (dimetilan)	1-methylcarbamoyl-5-methyl-3-pyrazolyl dimethylcarbamate[e],[3]	two
1-Isopropyl-3-methyl-5-pyrazolyl dimethylcarbamate (Isolan)	none	one

1) Sources for known compounds: a) M. H. Balba, Division of Entomology and Acarology, University of California, Berkeley; b) J. B. Knaak, Mellon Institute, Pittsburgh, Pa.; c) C. A. Anderson, Chemagro Corp., Kansas City, Mo.; d) A. M. Abdel-Wahab, Division of Entomology and Acarology, University of California, Berkeley and/or T. R. Norton, Dow Chemical Co., Midland, Mich.; e) E. Knusli, J. R. Geigy S. A., Basle, Switzerland.

2) Previously reported (5).

3) Metabolite yield less than 1.0 per cent.

unidentified and tentatively identified, formed in greater than one per cent yield by the microsomes plus $NADPH_2$ system from 10 methyl- and dimethylcarbamate insecticide chemicals. All of the 10 chemicals gave at least one carbamate metabolite, and half of them gave 4 or more carbamate metabolites. The table does not list metabolites formed by hydrolysis at the carbamic ester site nor those at the origin of the TLC plates.

The extent of metabolism of the carbonyl-C^{14}-labeled carbamates generally increased in the following order, based on the components of the liver microsomal system: 1) microsomes alone; 2) microsomes plus soluble alone; 3) microsomes fortified with $NADH_2$; 4) microsomes plus soluble fortified with NAD; 5) microsomes fortified with $NADPH_2$; 6) microsomes plus soluble fortified with NADP. The substrate-specificity for the microsomes fortified with $NADPH_2$ and the microsomes plus soluble fortified with NADP systems fell in the following categories, based on recovery of the original compound: extensively degraded - Banol, Zectran and Isolan; intermediate stability - carbaryl, Mesurol and dimetilan; most stable - Baygon, UC 10854, HRS-1422, and Matacil. Hydrolysis of the parent compound or its carbamate metabolites was greatest with Isolan (26%), Banol (19%), carbaryl (8%) and Mesurol (8%); water-soluble products were greatest with Banol (46%), Mesurol (43%) and Isolan (43%).

The number of metabolites found to be more potent than the original compound as pseudocholinesterase inhibitors was as follows: carbaryl - not any; Baygon - two unidentified carbamate analogs; Banol - one unidentified carbamate analog; Mesurol - the sulfoxide analog; Zectran - the 4-methylamino and 4-amino analogs.

Discussion

The methyl- and dimethylcarbamate groups are frequently more resistant to metabolism, by microsomal enzymes, than are other groupings in the molecule. Groupings found to be susceptible to oxidation or hydroxylation by microsomal enzymes are as follows:

1. N-Methyl, which is converted to N-hydroxymethyl (carbaryl, Baygon, Banol, Matacil, Zectran), N-formamide (Zectran), or is demethylated (Matacil, Zectran, dimetilan);
2. O-Alkyl, which is dealkylated (Baygon isopropoxy group);
3. S-Alkyl, which is converted to sulfoxide and sulfone analogs (Mesurol);
4. Aromatic ring, which is hydroxylated (carbaryl).

The formation of the additional, unidentified carbamate metabolites can be most easily explained on the basis of hydroxylation at different sites on the ring as well as on ring

substituents. There is not any evidence for N-hydroxylation or N-demethylation of the methylcarbamate grouping in Banol, confirming a similar previous study with carbaryl and Baygon (4).

The finding of potent anticholinesterase agents among the metabolites of carbamate insecticide chemicals does not necessarily indicate that these metabolites contribute to the insecticidal activity or to the toxicity to mammals, or that they constitute a hazard as potential residues. This is particularly true of the present findings because pseudocholinesterase of plasma rather than true cholinesterase of nervous tissue was used in the assay of metabolites. In addition, it is not known whether these active metabolites are formed and/or persist under *in vivo* situations in mammals.

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