Oxidation of Methyl- and Dimethylcarbamate Insecticide Chemicals by Microsomal Enzymes and Anticholinesterase Activity of the Metabolites

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Each of the 33 methyl- and dimethylcarbamate insecticide chemicals and related compounds studied is metabolized by the rat liver microsome-reduced nicotinamide–adenine dinucleotide phosphate ($NADPH_2$) system, producing one or more carbamate metabolites in each case. In this system, $NADPH_2$ is required for each of the following types of enzymatic reactions, as demonstrated with the listed carbamates: *N*-demethylation (dimetilan, Matacil, Zectran, and 1-naphthyl dimethylcarbamate); conversion of *N*-methyl to *N*-formamide (Zectran) and to *N*-hydroxymethyl groups (Banol, Baygon, carbaryl, HRS-1422, Matacil, UC 10854, and Zectran); aromatic ring hydroxylation (Baygon and carbaryl) or formation of a dihydrodihydroxy derivative (carbaryl); *O*-dealkylation (Baygon); alkyl hydroxylation of an aralkyl substituent (UC 10854); sulfoxidation (Mesurol and Temik). Carbaryl is produced on *N*-demethylation of 1-naphthyl dimethylcarbamate by the microsome-$NADPH_2$ system, and on spontaneous or enzymatic hydrolysis of 1-naphthyl *N*-acetyl-*N*-methylcarbamate. The microsome-$NADPH_2$ system usually forms one or more anticholinesterase metabolites from methyl- and dimethylcarbamate insecticide chemicals, and certain of these metabolites are more potent inhibitors than the carbamate from which they are derived. Although hydrolysis of the carbamate ester group is not a major reaction in this enzyme system, certain of the products formed in its presence are hydrolyzed, spontaneously or by enzymatic action. Carbamoylation of microsomal proteins probably occurs with certain of the methylcarbamates, their carbamate metabolites, and metabolites of the dimethylcarbamates.

Methyl- and dimethylcarbamates of substituted phenols, pyrazolones, and oximes are useful insecticide chemicals. Therefore, knowledge of the chemical nature, mechanism of formation, and biological activity of the metabolites and degradation products of these chemicals is needed in evaluating their safe use. Much information of this type is now available as a result of in vivo studies on metabolism in mammals (Baron, 1966; Dorough, 1967; Dorough and Casida, 1964; Dorough *et al.*, 1963; Hassan *et al.*, 1966; Knaak *et al.*, 1965; Knaak *et al.*, 1966; Krishna and Casida, 1966; Leeling and Casida, 1966; Williams *et al.*, 1964b), plants (Abdel-Wahab *et al.*, 1966; Kuhr and Casida, 1967; Metcalf *et al.*, 1966a; Mostafa *et al.*, 1966; Williams *et al.*, 1964a), and insects (Dorough and Casida, 1964; Metcalf *et al.*, 1967; Shrivastava, 1967; Zayed *et al.*, 1966; Zubairi and Casida, 1965), and of in vitro studies with enzyme systems prepared from mammals and insects (Dorough and Casida, 1964; Hodgson and Casida, 1960, 1961; Hook and Smith, 1967; Knaak *et al.*, 1965; Leeling and Casida, 1966; Matthews and Hodgson, 1966; Metcalf *et al.*, 1966b; Tsukamoto and Casida, 1967 a and b). Of particular interest here is the variety of metabolites formed by the rat liver microsome enzyme system, because these metabolites are similar in many respects to those formed initially in the detoxication reactions in mammals, insects, and plants. Some pertinent data are found in a preliminary report (Oonnithan and Casida, 1966).

This paper presents data obtained in work done on the metabolism of 10 C^{14}-labeled methyl- and dimethylcarbamates, by enzyme systems from rat liver, in order to determine the nature of the rat liver enzymes involved in the metabolism reactions, the number of carbamate metabolites formed, the chemical nature of the metabolic products, the anticholinesterase (antiChE) activity of the metabolites relative to the original insecticide, and the extent of carbamoylation of microsomal proteins by methyl- and dimethylcarbamates, in the presence and absence of added cofactor. Also, it includes results obtained by the microsomal oxidation of 20 nonlabeled carbamates, and gives the antiChE activity (if any) of the metabolic products derived from these compounds.

MATERIALS, APPARATUS, AND TEST CONDITIONS

The following C^{14}-labeled compounds were obtained from the sources previously mentioned: Banol, Baygon, carbaryl, dimetilan, HRS-1422, Isolan, Matacil, Mesurol, UC 10854, and Zectran, each labeled in the carbamate-carbonyl position; Banol, Baygon, and carbaryl, each labeled in the *N*-methyl position; carbaryl labeled in the 1-naphthyl position; Baygon labeled in the isoprop-1,3-oxy position; sodium carbonate; methylamine hydrochloride (Abdel-Wahab *et al.*, 1966; Krishna and Casida, 1966; Krishna *et al.*, 1962). The 4-methyl-C^{14}-Banol used was purified, by preparative-scale thin-layer chromatography (TLC), from material supplied by The Upjohn Co., Kalamazoo, Mich. Each of the radioactive compounds had a radiochemical purity of 99% or better and an adjusted specific activity of 1.0 mc. per mmole. The nonlabeled carbamates and their hydrolysis products used were those listed in Tables IV and V, which also give their sources. The sample of 1-naphthyl dimethylcarbamate was purified before use to remove certain impurities which are antiChE agents. This was accomplished by stirring the material in a 0.5*N* solution of sodium hydroxide in 10% ethanol for 18 hours at approximately 25° C., ex-

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tracting with ether, drying the ether extract with anhydrous sodium sulfate, and evaporating the ether under a gentle stream of nitrogen. Nicotinamide-adenine dinucleotide (NAD), its reduced form ($NADH_2$), nicotinamide-adenine dinucleotide phosphate (NADP), its reduced form ($NADPH_2$), and acetylcholine were obtained from Calbiochem., Los Angeles, Calif. Pooled, outdated human blood plasma was used as the enzyme source for the anti-ChE tests. The sources of materials and methods for TLC and for detection and measurement of radioactivity were those already reported (Abdel-Wahab *et al.*, 1966; Oonnithan, 1966; Oonnithan and Casida, 1966). Male albino rats, weighing 150 to 160 grams, were obtained from the Berkeley Pacific Laboratories, Berkeley, Calif. The rat liver homogenates were separated into different fractions in a Servall refrigerated centrifuge (Ivan Sorvall, Norwalk, Conn.) and in a Beckman ultracentrifuge (Model L, Beckman Instruments, Menlo Park, Calif.). The enzyme systems containing the substrates were incubated in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill.).

Separation, Detection, and Characterization of Metabolic Products. Thin-layer chromatography was used to separate the carbamates and their metabolic products. For routine analysis, 20 × 20 cm. glass plates coated with silica gel G in 0.25-mm. thickness were employed; however, for preparative scale TLC, the coatings were 0.5 mm. thick. Solvent systems used for TLC were (Figure 1):

Compounds	First Direction (Shown Vertical)	Second Direction (Shown Horizontal)
Substituted-phenyl methylcarbamates (Compounds I to VIII)	Chloroform-acetonitrile (4 to 1)	Anhydrous ether–hexane (4 to 1)
Substituted-pyrazolyl dimethylcarbamates (Compounds IX and X)	Chloroform-acetonitrile (4 to 1)	Ethyl acetate–ethanol (49 to 1)

Chromogenic reagents used for the detection of methyl- and dimethylcarbamates and their metabolites on the developed TLC plates included ninhydrin, Gibb's (*N*, 2,6-trichloro-*p*-benzoquinoneimine) and others; they were prepared as previously reported (Abdel-Wahab *et al.*, 1966; Dorough and Casida, 1964; Krishna *et al.*, 1962; Leeling and Casida, 1966; Metcalf *et al.*, 1966a; Zubairi and Casida, 1965). The localization and assay of cholinesterase-inhibiting spots on TLC plates were accomplished by the method of Oonnithan and Casida (1966). The radioactive areas on thin-layer plates were located by radioautography, using No-Screen x-ray film. The spots produced by the chromogenic reagents and the anti-ChE test were compared with those found by radioautography, noting the degree of match as to shape and position. In the cochromatography experiments, 10 to 20 μg. of a known nonlabeled compound were added to a solution containing the radiolabeled unknown material(s), the mixture was spotted on TLC plates and developed in the normal manner, and the spots were visualized by chromogenic, radioautographic, or antiChE procedures, and compared in the above described manner.

The metabolic products were usually tentatively characterized by cochromatography of the products, as recovered, or of derivatives made from these products (prior to cochromatography). In the latter instances, 1 ml. of an ether solution of the labeled metabolites and/or of known nonlabeled compounds were reacted for 18 hours at 25° C. with one of two reagents: 1 ml. of 2% diazomethane solution in ether, to form the *O*-methyl ether derivatives; 1 ml. of methylisocyanate plus 2 drops of triethylamine, to form bis(*N*-methylcarbamate) derivatives. The products from these reactions were cochromatographed with known compounds under TLC conditions, using ninhydrin to detect nonlabeled compounds and radioautography to detect the labeled ones. To test the presence of benzylic hydroxyl groups among the metabolites of Banol and UC 10854, the radioactive metabolites were resolved by preparative scale TLC, dissolved in isopropyl alcohol, and hydrogenated for 30 minutes at 25° C. and atmospheric pressure in the presence of palladium on charcoal. At the end of the hydrogenation period, the reaction mixture was filtered, the filtrate was evaporated to dryness, and the products were cochromatographed with Banol or UC 10854 (the logical products of the hydrogenation if the only modification on formation of the metabolite was introduction of a benzylic hydroxyl group).

METHODS

Fractionation of Rat Liver Homogenate and Enzyme Incubation Conditions. Immediately on removal, the livers were immersed and held in 0.25*M* sucrose at 5° C. for 5 minutes. After removing any adhering tissues, each liver was rinsed twice with chilled sucrose solution, cut into pieces, and homogenized in 0.25*M* sucrose for 1 minute at 5° C., with a glass-Teflon homogenizer, to yield a 20% (w./v.) homogenate, on a fresh liver weight basis. Filtration through glass wool, and centrifugation at 15,000 G for 30 minutes at −1° C. yielded a supernatant, or microsome-plus-soluble fraction, which was used directly as an enzyme source or was further centrifuged at 95,000 G for 30 minutes at 2° C. to sediment the microsome fraction; in the latter case, the sediment was washed twice by resuspension in 0.25*M* sucrose and resedimentation prior to final resuspension in 0.25*M* sucrose at a concentration equivalent to the original 20% homogenate.

An aliquot containing 100,000 c.p.m. (approximately 0.2 μmole) of each radiolabeled carbamate or 20 to 50 μg. of nonlabeled carbamate, in 0.5 ml. of hexane or chloroform solution, was pipetted into the bottom of a 25-ml. Erlenmeyer flask, and the solvent was evaporated with a gentle stream of nitrogen to yield a fairly uniform substrate deposit on the bottom of the flask. Alternatively, immediately prior to incubation, 0.25 μmole of sodium carbonate-C^{14} or methyl-C^{14}-amine was added to the flask containing all other reaction constituents, as described later. To each flask containing one of the labeled carbamates were added 0.5 ml. of 0.1*M* sodium phosphate buffer (pH 7.4), 1 ml. of enzyme preparation (microsome or microsome-plus-soluble fraction in 0.25*M* sucrose)

equivalent to 200 mg. of rat liver, and 0.5 ml. of one of the following solutions: 0.01*M* sodium phosphate buffer (pH 7.4); 2 μmoles of reduced cofactor ($NADH_2$ or $NADPH_2$) in this buffer; deionized, distilled water; or 2 μmoles of oxidized cofactor (NAD or NADP) in deionized, distilled water, to make a total volume of 2 ml. In each experiment, fresh enzyme preparations and fresh cofactor solutions were used. All reaction constituents were mixed at 5° C., and the reaction mixtures were then incubated in air for 4 hours at 37° C. In some cases, the resulting incubated mixtures were frozen and stored for not more than 24 hours at −15° C.

Quantitative comparisons of substrate metabolism and products formed were made with enzyme fractions and cofactor combinations as follows: microsome-plus-soluble fraction without added cofactor, microsome-plus-soluble fraction with added NAD, microsome-plus-soluble fraction with added NADP, microsome fraction without added cofactor, microsome fraction with added $NADH_2$, and microsome fraction with added $NADPH_2$. For routine preparation of metabolites (for characterization and bioassay purposes), the microsome system without cofactor fortification was used as a control and the microsome-$NADPH_2$ system was used to form the metabolic products.

Analysis of Metabolites. The incubation mixtures were extracted with anhydrous ether to yield aqueous and ether fractions, and components of the ether extract were resolved by TLC; alternatively, protein-bound radiocarbon in the mixtures was determined. Immediately after incubation or after thawing of the frozen samples, incubated mixtures were extracted with two 5-ml. portions of ether and the ether extract was dried over anhydrous sodium sulfate. The radiocarbon content of the aqueous fraction and the ether extract was determined by counting 50-μl. aliquots of each. One half of the ether extract was used for TLC resolution and quantitation of the radioactive components; the radioactive regions of silica gel were scraped into scintillation vials and counted. Each result, for every metabolite component from the individual labeled compound, was related, on a percentage basis, to the total radiocarbon recovered from reaction mixtures containing the particular compound and the microsome fraction without added cofactor. The 100% recovery value was taken as that immediately following the incubation period for the carbamates and that immediately before the incubation period for sodium carbonate-C^{14} and methyl-C^{14}-amine; these determinations were made on 10-μl. aliquots of the incubation mixtures. Two or more experiments were run with each compound and each set of metabolism conditions, and the results were averaged. In studies on protein-bound radiocarbon, each of the carbamates-C^{14}, labeled in the carbonyl, *N*-methyl, ring, or ring-substituent positions, and sodium carbonate-C^{14} and methyl-C^{14}-amine were incubated with individual reaction mixtures of microsome fraction alone or microsome fraction fortified with $NADPH_2$, as described above. After incubation, the protein in each reaction mixture was precipitated by adding 2 ml. of 10% (w./v.) trichloroacetic acid (TCA) solution at 5° C., filtering through Whatman No. 1 filter paper, and washing the precipitate with 1 ml. of TCA solution at 5° C. and with 2 ml. of acetone (to remove organo soluble materials). After drying the precipitate over silica gel desiccant for 18 hours, the protein was burned, and the radiocarbon content was determined (Krishna and Casida, 1966). The amount of radiocarbon incorporated into the microsomal protein was expressed, on a percentage basis, as the amount of radiocarbon in the TCA precipitate relative to the total amount present in the flask containing the microsome fraction without added cofactor (see above). Two or more experiments, each with a fresh enzyme preparation, were made and the results were averaged.

AntiChE Activity of Carbamates and Their Metabolites. The in situ method was used to assay the antiChE activity of each of the experimental compounds, metabolites of nonlabeled carbamates, and the metabolites of carbonyl-C^{14}-labeled carbamates, after they had been resolved by one- or two-dimensional TLC, as previously described (Oonnithan, 1966; Oonnithan and Casida, 1966). Metabolites recovered from the microsome system without cofactor fortification were compared with those recovered from the microsome and $NADPH_2$ system; incubation and extraction conditions were as previously described, except that spectro quality reagent ether was used to minimize antiChE impurities resulting from the evaporation of other grades or purities of ether. The antiChE activity of radioactive compounds or individual radiolabeled metabolites was expressed as the minimum detectable level (MDL), calculated in terms of micrograms of the original compound having an equivalent radiocarbon content. With nonlabeled substrates, a metabolite was considered to be as active as or more active than the original compound when the metabolite from the microsome-$NADPH_2$ reaction mixture was detected, as an antiChE agent, at a dilution equal to or greater than the concentration at which the original compound is detected. Occasionally, the antiChE assay was used in cochromatography studies; in this case, a metabolite of unknown structure was mixed with a radioactive carbamate of known structure and, following cochromatography, the position of the unknown compound, detected by antiChE assay, was compared with that of the known compound, detected by radioautography.

RESULTS

Nature, Substrate Specificity, and Extent of Metabolism by Liver Microsome Systems. Table I presents the distribution of radioactivity in various metabolite fractions following incubation of 10 variously C^{14}-labeled methyl- and dimethylcarbamate insecticide chemicals with rat liver microsome-cofactor systems. In general, the metabolism of each of the carbamate-C^{14} insecticide chemicals tested increases in the following order, based on the number and concentration for components in the incubation mixture: microsome fraction alone, microsome-plus-soluble fraction alone, microsome fraction fortified with $NADH_2$, microsome-plus-soluble fraction fortified with NAD, microsome fraction fortified with $NADPH_2$, and microsome-plus-soluble fraction fortified with NADP. Although most of the apparent metabolites are formed by cofactor-dependent enzymatic reactions, this is not always the case, as with certain carbamoylation and hydrolysis products discussed later. Metabolism by microsomes in-

creases on fortification with $NADH_2$ and, even more so, on fortification with $NADPH_2$; this same relationship also occurs with the microsome-plus-soluble fraction fortified with NAD and NADP, the soluble fraction serving to increase the extent of metabolism. Although the data in Table I do not show it, the number and amount of metabolites recovered from each of the carbamates usually increase as the level of the original compound is decreased, depending on the nature of the cofactor and the presence or absence of the soluble fraction (Oonnithan, 1966).

As shown in Table I, the extent of metabolism under given conditions varies greatly with the substrate. Based on the recovery of the original compound following incubation with the microsome fraction fortified with $NADPH_2$ or the microsome-plus-soluble fraction fortified with NADP, the substrate specificity falls into the following categories: most extensively degraded—Banol, Zectran, and Isolan; intermediate stability—carbaryl, Mesurol, and dimetilan; most stable—Baygon, UC 10854, HRS-1422, and Matacil.

Nature and Extent of Radiocarbon Losses. The total recovery of radiocarbon from carbaryl-carbonyl-C^{14}, after incubation with liver microsome-cofactor systems, varies with the reaction constituents. On the basis of a preliminary experiment, the recovery is highest in reaction mixtures containing the microsomes without added cofactor and these reactions produce little, if any, ether-extractable metabolites, so that only the original compound is recovered on extraction with ether. Therefore, based on this finding with carbaryl, the total amount of radiocarbon found, after incubation in the flasks containing the microsome fraction alone (without cofactor), for each compound is taken as the measure of the radiocarbon added initially and, for calculation purposes, is taken as the 100% value. Losses of radiocarbon occur, with some of the compounds, as shown in Table I, during incubation, extraction, and analysis. The extraction loss averages approximately 9%, ranging from 0 to 25%, for all compounds and this loss does not appear to be related to the compound or to the extent of metabolism prior to extraction. Losses averaging approximately 19%, ranging from 6 to 31%, also result during TLC resolution and quantitation of the radioactive components of the ether extract.

The percentage of the radiocarbon lost during incubation and remaining in the aqueous fraction after extraction with ether varies with the substrate (Table I). Under similar conditions, rapid and almost complete loss of radiocarbon results from incubation of sodium carbonate-C^{14}; therefore, hydrolysis of the carbamate-carbonyl-C^{14} compounds, or their metabolites, to carbonate ion probably results in rapid radiocarbon loss from the incubation mixture. As shown in Table I, loss of radiocarbon during incubation of the carbonyl-C^{14}-carbamates is greatest with Isolan when incubated either with the microsome or microsome-plus-soluble fractions and fortified with $NADPH_2$ or NADP, respectively. Banol, and to a lesser degree carbaryl and Mesurol, shows a loss on incubation in the enzyme systems containing NADP and $NADPH_2$. There is some radiocarbon loss, during incubation, from isopropoxy-C^{14}-Baygon and from 4-methyl-C^{14}-Banol. (The nature of the loss from isopropoxy-C^{14}-Baygon is discussed elsewhere in this paper, but the reason for the radiocarbon loss from 4-methyl-C^{14}-Banol is not known.)

Radiocarbon in the Aqueous Fraction. With each of the carbamate substrates listed in Table I, radioactivity in the aqueous fraction is greater when the microsome system is fortified with $NADPH_2$ instead of with $NADH_2$ or when a cofactor is not added to the system; the same result is encountered with the microsome-plus-soluble fraction when fortified with NADP, as compared with the same system with added NAD or without any added cofactor. The presence of the soluble fraction along with the microsomes has the greatest effect in forming large amounts of water-soluble products with Isolan, dimetilan, and carbaryl. Water-soluble products also account for up to half of the metabolites with Banol and Mesurol whereas, with other compounds, radioactivity in the aqueous fraction accounts for 18% or less of the total radiocarbon.

The *N*-methyl C^{14} label yields more radioactivity in the aqueous fraction than the carbonyl-C^{14} label with Baygon and Banol, but not with carbaryl (Table I). Radiocarbon loss on incubation occurs with Baygon-*N*-methyl-C^{14}, but the nature of this loss is not known. Methyl-C^{14}-amine gives little, if any, radiocarbon in the ether extract or loss of radiocarbon during incubation. As shown in Table I, the ring-C^{14} or ring-substituent-C^{14} label yields about the same amount of radiocarbon in the aqueous fraction with carbaryl and Baygon, and yields a significantly lesser amount with Banol, when compared with their respective carbonyl-C^{14}-labeled samples.

Ether-Extractable Metabolites. CARBARYL. Essentially the same metabolites are formed from carbaryl (Compound I) with carbonyl-C^{14}, *N*-methyl-C^{14}, and naphthyl-1-C^{14} preparations (Table II and Figure 1), each forming three major metabolites (I-b, I-d, and I-e) and three minor ones (I-a, I-c, and I-f). The naphthyl-1-C^{14} sample yields, in addition to the six metabolites with the intact-C-O-C(O)-N-C-moiety, several other products, which are derivatives of 1-naphthol lacking the carbamoyl grouping (I-g through I-m). Metabolite I-m cochromatographs with 1-naphthol. Some of the other products which lack the carbamoyl grouping (I-i through I-l) appear to result from hydrolysis of the major metabolites and further degradation during chromatography; this possibility is in agreement with their chromatographic positions (Figure 1). Metabolites I-b, I-d, I-e, and I-f are tentatively identified (Table III; Dorough and Casida, 1964; Leeling and Casida, 1966); their assigned structures are further confirmed by the reaction characteristics of the radioactive metabolites, compared with those of respective known compounds, with diazomethane. Radioactive metabolites I-e and I-f react with diazomethane to give labeled derivatives with increased R_f values, and these radioactive derivatives cochromatograph with the diazomethane reaction products of nonlabeled 4-hydroxy- and 5-hydroxy-1-naphthyl methylcarbamates, respectively, the derivatives being assumed to be 4-methoxy- and 5-methoxy-1-naphthyl methylcarbamates, respectively. Metabolite I-d, 1-naphthyl *N*-hydroxymethylcarbamate, and metabolite I-b, 5,6-dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate, do not react with diazomethane. Absent among the metabolites are 1-naphthyl carbamate and 1-naphthyl *N*-hydroxy-*N*-methylcarbamate. None of the metabolites

formed by the liver microsome-$NADPH_2$ system is significantly more potent than the original compound as an inhibitor of plasma cholinesterase (Tables II and III).

BAYGON. Preparations of Baygon (Compound II) C^{14}-labeled in the carbonyl, *N*-methyl, and isopropoxy positions give rise to the metabolites indicated in Table II and Figure 1. Carbonyl-C^{14} and *N*-methyl-C^{14} preparations yield several identical metabolites (II-b through II-h), in addition to the material(s) at the origin (II-a). The isopropoxy-C^{14} sample yields each of these metabolites except the one designated as II-d and, in addition, gives three phenolic compounds lacking the carbamate grouping (II-i, II-j, and II-k). Metabolite II-k cochromatographs with 2-isopropoxyphenol. Metabolite II-d, which lacks the isopropyl moiety, contains a phenolic hydroxyl group (as indicated by its reaction with methylisocyanate and diazomethane), and it cochromatographs with 2-hydroxyphenyl methylcarbamate; the loss of the isopropyl group on its formation probably explains the greater radiocarbon losses on incubation with the isopropoxy-C^{14}-labeled sample than with the carbonyl- or *N*-methyl-C^{14} samples. Metabolite II-e contains the 2-[C-(C)C-O]-phenyl-O-C(O)-N-C- moiety intact, and reacts with diazomethane and methylisocyanate, indicating that it contains a phenolic hydroxyl group; this metabolite does not cochromatograph with 2-isopropoxy-4-hydroxyphenyl methylcarbamate but it does cochromatograph with 2-isopropoxy-5-hydroxyphenyl methylcarbamate; this iden-

Table I. Activity of Liver Enzyme Systems and Cofactors for Metabolism of Ten Variously C^{14}-Labeled

No.	Compound	Metabolite Fraction	Per Cent of Initial Radioactivity[a] Found with Indicated Enzyme Fraction and Cofactor					
			Microsome plus Soluble			Microsome Only		
			None	NAD	NADP	None	$NADH_2$	$NADPH_2$
I	Carbaryl-carbonyl-C^{14}	Ether fraction						
		Carbaryl	76	51	9	80	75	35
		Metabolites	0	7	16	0	5	23
		Aqueous fraction	13	25	62	13	12	27
		Loss on extraction	6	12	5	7	8	8
		Other losses	5	5	8	0	0	7
I	Carbaryl-*N*-methyl-C^{14}	Ether fraction						
		Carbaryl	74	66	24	81	74	49
		Metabolites	0	5	17	0	3	18
		Aqueous fraction	14	24	53	13	11	21
		Loss on extraction	8	10	7	6	12	9
		Other losses	4	0	0	0	0	3
I	Carbaryl-naphthyl-1-C^{14}	Ether fraction						
		Carbaryl	79	65	17	82	79	46
		Metabolites	9	10	24	14	14	30
		Aqueous fraction	6	21	52	1	3	17
		Loss on extraction	6	6	11	3	1	5
		Other losses	0	0	0	0	3	2
II	Baygon-carbonyl-C^{14}	Ether fraction						
		Baygon	98	77	28	91	91	47
		Metabolites	0	7	48	0	7	37
		Aqueous fraction	3	4	12	4	4	10
		Loss on extraction	0	10	7	5	0	2
		Other losses	0	2	5	0	0	4
II	Baygon *N*-methyl-C^{14}	Ether fraction						
		Baygon	74	63	21	90	72	36
		Metabolites	0	8	41	0	11	33
		Aqueous fraction	10	11	22	9	9	17
		Loss on extraction	10	9	7	1	2	6
		Other losses	6	9	9	0	6	8
II	Baygon-isopropoxy-C^{14}	Ether fraction						
		Baygon	77	52	16	76	74	29
		Metabolites	18	21	48	12	24	38
		Aqueous fraction	7	9	16	7	3	10
		Loss on extraction	6	15	3	5	3	2
		Other losses	0	3	17	0	0	21
III	Banol-carbonyl-C^{14}	Ether fraction						
		Banol	68	52	1	68	55	5
		Metabolites	1	14	19	0	8	18
		Aqueous fraction	27	27	50	24	21	46
		Loss on extraction	9	12	12	8	18	12
		Other losses	0	0	18	0	0	19
III	Banol-*N*-methyl-C^{14}	Ether fraction						
		Banol	47	23	1	46	33	2
		Metabolites	1	15	11	0	12	13
		Aqueous fraction	58	67	77	46	57	83
		Loss on extraction	6	10	12	8	10	2

[a] Radioactivity level, after incubation in flasks containing microsome fraction alone (no cofactor), taken as the initial amount of radioactivity.

tification finds additional support in that 2-isopropoxyphenyl 1,5-bis(*N*-methylcarbamate) cochromatographs with the product resulting from the reaction of metabolite II-e with methylisocyanate. Metabolite II-f is tentatively identified as 2-isopropoxyphenyl *N*-hydroxymethylcarbamate; however, the *N*-hydroxymethylcarbamate group is not further degraded readily by the microsome-$NADPH_2$ system because no more than trace amounts, if any, of 2-isopropoxyphenyl carbamate are detected after incubation in this system. At least one of the unidentified metabolites and 2-isopropoxy-5-hydroxyphenyl methylcarbamate are more potent cholinesterase inhibitors than Baygon (Tables II and III). The antiChE activity of one of the labeled metabolites, II-e, is only about one seventh of that obtained for authentic 2-isopropoxy-5-hydroxyphenyl methylcarbamate, with which it cochromatographs. This difference possibly results from inadequate resolution and, thereby, contamination with metabolites II-d and II-f, both of which are less active inhibitors, thus reducing the apparent antiChE activity.

BANOL. Table II and Figure 1 give the metabolites found with carbonyl-, *N*-methyl- and 4-methyl-C^{14} preparations of Banol (Compound III); the carbonyl- and *N*-methyl-C^{14} preparations give the same ether-extractable metabolites (III-a through III-f). The 4-methyl-C^{14} compound yields, in addition to the six carbamate metabolites, several other metabolites (III-g through III-m) which are present in small quantities and

Methyl- and Dimethylcarbamate Insecticide Chemicals and the Proximate Fate of Radioactive Metabolites

No.	Compound	Metabolite Fraction	Per Cent of Initial Radioactivity[a] Found with Indicated Enzyme Fraction and Cofactor					
			Microsome plus Soluble			Microsome Only		
			None	NAD	NADP	None	NADH	$NADPH_2$
		Other losses	0	0	0	0	0	0
III	Banol-4-methyl-C^{14}	Ether fraction						
		Banol	45	15	2	64	36	3
		Metabolites	16	39	35	17	32	57
		Aqueous fraction	10	22	43	3	3	12
		Loss on extraction	18	10	11	16	18	17
		Other losses	11	14	9	0	11	11
IV	UC 10854-carbonyl-C^{14}	Ether fraction						
		UC 10854	85	74	20	88	77	42
		Metabolites	1	7	50	0	9	37
		Aqueous fraction	3	2	16	2	2	7
		Loss on extraction	9	19	9	10	13	13
		Other losses	2	0	5	0	0	1
V	HRS-1422-carbonyl-C^{14}	Ether fraction						
		HRS-1422	83	70	15	95	77	62
		Metabolites	1	10	70	1	13	31
		Aqueous fraction	8	6	11	2	2	3
		Loss on extraction	12	8	0	2	8	7
		Other losses	0	6	4	0	0	0
VI	Mesurol-carbonyl-C^{14}	Ether fraction						
		Mesurol	76	67	8	85	64	11
		Metabolites	6	8	15	3	10	14
		Aqueous fraction	12	16	50	6	14	43
		Loss on extraction	10	11	15	6	12	25
		Other losses	0	0	12	0	0	7
VII	Matacil-carbonyl-C^{14}	Ether fraction						
		Matacil	82	65	20	89	80	59
		Metabolites	4	23	58	1	8	27
		Aqueous fraction	4	5	9	4	3	5
		Loss on extraction	13	13	10	6	20	15
		Other losses	0	0	3	0	0	0
VIII	Zectran-carbonyl-C	Ether fraction						
		Zectran	59	34	2	83	61	9
		Metabolites	28	52	67	9	24	70
		Aqueous fraction	8	7	17	5	5	14
		Loss on extraction	9	13	10	3	3	8
		Other losses	0	0	4	0	7	0
IX	Dimetilan-carbonyl-C^{14}	Ether fraction						
		Dimetilan	71	58	36	71	69	59
		Metabolites	3	7	17	2	4	7
		Aqueous fraction	16	22	40	15	17	25
		Loss on extraction	10	7	10	12	3	6
		Other losses	0	6	0	0	7	3
X	Isolan-carbonyl-C^{14}	Ether fraction						
		Isolan	76	30	3	85	51	9
		Metabolites	2	15	24	3	12	17
		Aqueous fraction	7	32	40	4	18	35
		Loss on extraction	10	8	8	8	7	9
		Other losses	5	15	25	0	12	30

Table II. Number, Amount, and AntiChE Activity of Radioactive Metabolites of Variously C^{14}-Labeled Preparations of Carbaryl, Baygon, and Banol, as Formed by the Liver Microsome-$NADPH_2$ System

Component[a]	Per Cent of Initial Radioactivity of Component Found with Indicated C^{14}-Preparation			AntiChE Activity, MDL,[b] μg.	
	Carbonyl-C^{14}	*N*-Methyl-C^{14}	Ring or ring-substituent-C^{14}	Carbonyl-C^{14}	Known compounds[c]
	1-Naphthyl Methylcarbamate (Carbaryl, Compound I)				
I	35.3	48.7	46.0	0.15	0.2
I-a	1.2	0.5	1.4	>0.03	...
I-b	7.5	4.6	5.8	0.2	...
I-c	<0.1	<0.1	1.6	>0.1	...
I-d	8.4	5.7	7.0	>0.3	2.0
I-e	3.9	5.5	3.9	>0.1	0.4
I-f	1.5	1.6	0.9	>0.1	0.1
I-g	...	...	<0.1	...	...
I-h	...	...	<0.1	...	...
I-i	...	...	0.7	...	...
I-j	...	...	1.9	...	...
I-k	...	...	0.8	...	...
I-l	...	...	3.1	...	...
I-m	...	...	2.7	...	...
	2-Isopropoxyphenyl Methylcarbamate (Baygon, Compound II)				
II	46.5	35.6	29.0	0.6	0.5
II-a	<0.1	<0.1	<0.1	>0.05	...
II-b + c	1.1	1.0	1.7	0.2	...
II-d	9.1	11.8	...	>2.0	3.0
II-e	5.5	5.3	6.5	0.4	0.06
II-f	18.6	14.8	15.5	2.0	2.0
II-g	1.2	<0.1	1.1	>0.1	...
II-h	1.7	<0.1	<0.1	...	...
II-i	...	...	1.1	...	...
II-j	...	...	8.5	...	...
II-k	...	...	3.3	...	...
	2-Chloro-4,5-xylyl Methylcarbamate (Banol, Compound III)				
III	5.0	2.2	2.9	0.02	0.03
III-a	<0.1	0.5	2.0	0.03	...
III-b	0.8	1.0	0.9	0.03	...
III-c	<0.1	<0.1	2.0	>0.1	...
III-d	12.6	8.1	9.0	0.1	...
III-e	2.7	2.0	18.8	0.005	...
III-f	1.5	0.8	15.7	0.2	0.15
III-g	...	...	0.8	...	...
III-h	...	...	2.0	...	...
III-i	...	...	1.7	...	...
III-j	...	...	0.4	...	...
III-k	...	...	1.7	...	...
III-l	...	...	1.1	...	...
III-m	...	...	0.7	...	...

[a] Number and letter designations refer to TLC spots for compounds and metabolites as shown in Figure 1.
[b] Minimum detectable level in terms of micrograms of original compound having an equivalent radiocarbon content.
[c] Known compounds are nonlabeled carbamates from synthesis which cochromatograph with respective radioactive components (Table III).

are devoid of the carbamate grouping. Metabolites III-f and III-m cochromatograph with 2-chloro-4,5-xylyl *N*-hydroxymethylcarbamate and 2-chloro-4,5-xylenol, respectively (Table III). Metabolite III-d is the major ether-soluble metabolite containing the carbamate grouping. Metabolite regions III-c, III-e, and III-f are represented by more radiocarbon from the 4-methyl-C^{14} sample than from the carbonyl- or *N*-methyl-C^{14}-labeled samples, indicating that these regions represent mixtures of carbamates and noncarbamates (phenols), with the latter predominating. Reaction of the carbonyl-C^{14} metabolites with diazomethane does not yield new carbamate products; in fact, the *N*-hydroxymethyl derivative decomposes with the loss of its C^{14} label, probably as a result of hydrolysis of the carbamoyl group under the conditions of the reaction. The apparent absence of carbamate metabolites with phenolic hydroxyl groups suggests that hydroxylation of the ring-methyl substituents occurs during metabolism. (This was tested by hydrogenating the metabolite mixture in the ether extract, after removal of residual Banol by TLC purification, and cochromatographing the reaction products with nonlabeled Banol; no radioactive Banol was recovered, but most of the carbonyl- and 4-methyl-C^{14}-labeled metabolites were decomposed during the reaction.) Neither 2-chloro-4,5-xylyl carbamate nor 2-chloro-4,5-xylyl *N*-hydroxy-*N*-methylcarbamate is present as a metabolite (Table III). Metabolite III-d is a less potent inhibitor of plasma cholinesterase than Banol, while metab-

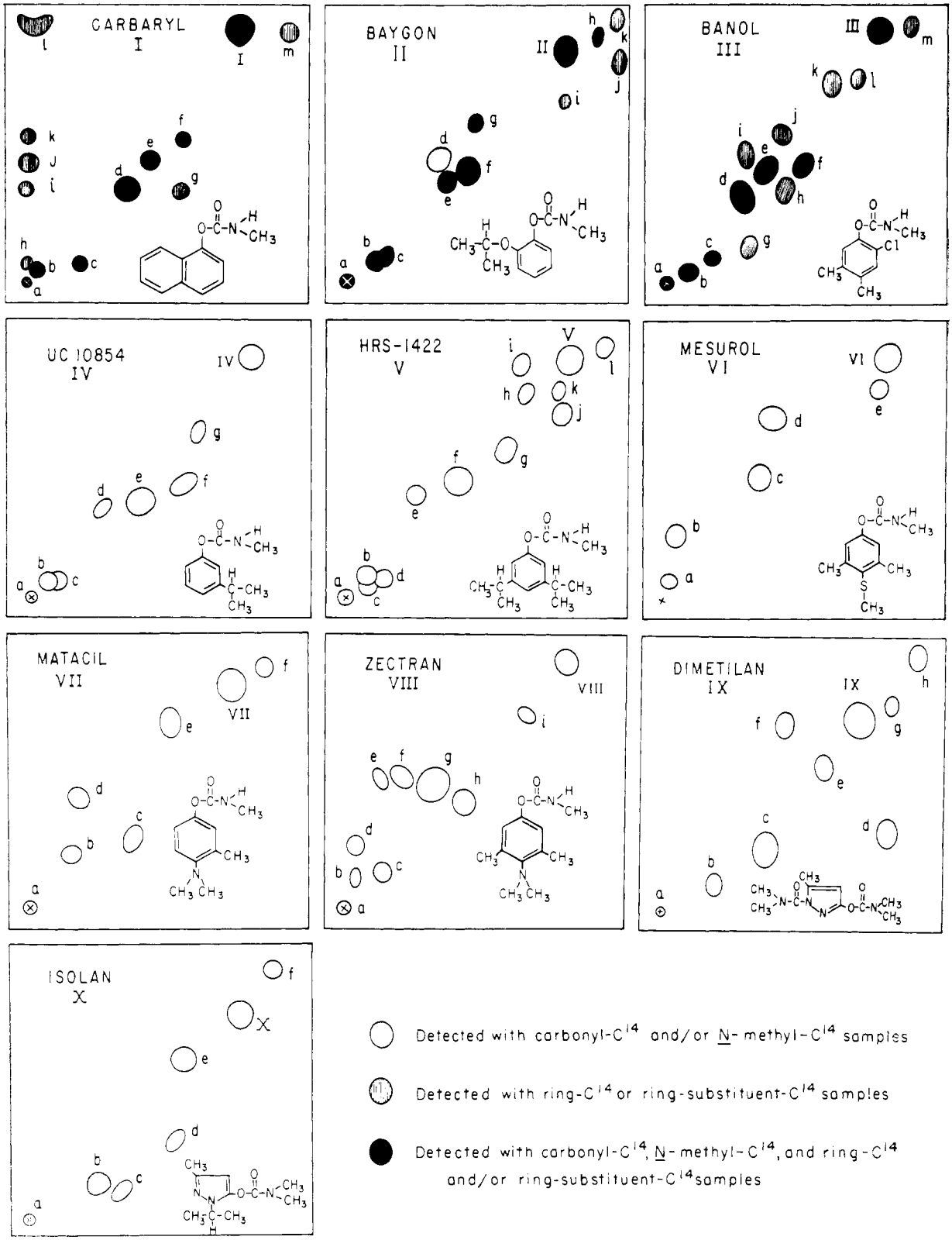


Figure 1. Thin-layer chromatographic patterns of metabolites present in ether extracts of incubation mixtures containing liver microsome-$NADPH_2$ enzyme system and one of 10 variously C^{14}-labeled methyl- and dimethylcarbamate insecticide chemicals, as detected by radioautography

Roman numerals designate the original, unmetabolized compound, and letters designate metabolites

Table III. The AntiChE Activity of 10 Methyl- and

	Compound[a]	AntiChE Activity, MDL,[b] μg.	Metabolite Designation[c]
	Carbaryl and Analogs		
1	4-Hydroxy-1-naphthyl methylcarbamate	0.4	I-e
2	5-Hydroxy-1-naphthyl methylcarbamate	0.1	I-f
3	1-Naphthol	20	I-m
4	1-Naphthyl *N*-acetyl-*N*-methylcarbamate	10	...
5	1,4-Naphthyl bis(*N*-methylcarbamate)	0.08	...
6	1-Naphthyl carbamate	2.0	...
7	1-Naphthyl dimethylcarbamate	1.0	...
8	1-Naphthyl ethylcarbamate	0.2	...
9	1-Naphthyl *N*-hydroxymethylcarbamate	2.0	I-d
10	1-Naphthyl *N*-hydroxy-*N*-methylcarbamate	0.01	...
11	1-Naphthyl methylcarbamate (carbaryl)	0.2	I
	Baygon and Analogs		
12	2-Hydroxyphenyl methylcarbamate	3.0	II-d
13	2-Isopropoxy-4-hydroxyphenyl methylcarbamate	0.15	...
14	2-Isopropoxy-5-hydroxyphenyl methylcarbamate	0.06	II-e
15	3-Isopropoxy-4-hydroxyphenyl methylcarbamate	1.5	...
16	2-Isopropoxyphenol	50	II-k
17	2-Isopropoxyphenyl 1,4-bis(*N*-methylcarbamate)	0.3	...
18	2-Isopropoxyphenyl carbamate	2.0	...
19	2-Isopropoxyphenyl *N*-hydroxymethylcarbamate	2.0	II-f
20	2-Isopropoxyphenyl methylcarbamate (Baygon)	0.5	II
	Banol and Analogs		
21	2-Chloro-3,4-xylyl methylcarbamate	0.2	...
22	2-Chloro-4,5-xylenol	8.0	III-m
23	2-Chloro-4,5-xylyl *N*-acetyl-*N*-methylcarbamate	2.0	...
24	2-Chloro-4,5-xylyl carbamate	0.4	...
25	2-Chloro-4,5-xylyl *N*-hydroxymethylcarbamate	0.15	III-f
26	2-Chloro-4,5-xylyl *N*-hydroxy-*N*-methylcarbamate	0.01	...
27	2-Chloro-4,5-xylyl methylcarbamate (Banol)	0.03	III
	UC 10854 and Analogs		
28	3-(1-Hydroxy-1-methylethyl)phenyl methylcarbamate	0.004	IV-e
29	3-Hydroxyphenyl methylcarbamate	2.0	...
30	3-Isopropyl-4-hydroxyphenyl methylcarbamate	0.002	...
31	3-Isopropylphenol	5.0	...
32	3-Isopropylphenyl 1,4-bis(*N*-methylcarbamate)	0.002	...
33	3-Isopropylphenyl *N*-hydroxymethylcarbamate	0.04	IV-f
34	3-Isopropylphenyl methylcarbamate (UC 10854)	0.004	IV

[a] Source: compounds 44, 48 to 51, 55, 57, and 58 from A. M. Abdel-Wahab, Division of Entomology, University of California, Berkeley, Calif.; 1, 5, 9, 12 to 15, 17, 19, 25, 28 to 30, 32, 33, 35, 37, 38, 46, 53, 56, and 59 from M. H. M. Balba, Division of Entomology, University of California, Berkeley, Calif.; 16, 18, 20, 40 to 43, 45, and 47 from Chemagro Corp., Kansas City, Mo.; 52 and 54 from Dow Chemical Co., Midland, Mich.; 31 and 34 from Hercules Powder Co., Wilmington, Del.; 36 and 39 from Hooker Chemical Co., Niagara Falls, N.Y.; 2 and 10 from J. B. Knaak, Carnegie-Mellon University, Pittsburgh, Pa.; 7 and 60 to 66 from E. Knusli, J. R. Geigy, Basel, Switzerland; 4, 21 to 24, 26, and 27 from A. J. Lemin, Upjohn Co., Kalamazoo, Mich.; 3, 6, 8, and 11 from Union Carbide Corp., New York, N.Y.
[b] Minimum detectable level, as assayed by ChE inhibition.
[c] Refers to ether-soluble radioactive components based on cochromatography (Figure 1 and Tables II and IV).

olite III-e is a more potent inhibitor than Banol; metabolites III-a and III-b also are active. The other metabolites of Banol have a low antiChE activity.

UC 10854. Carbonyl-C^{14}-labeled UC 10854 (Compound IV) yields several ether-extractable metabolites (IV-a through IV-g), as shown in Figure 1 and Table IV; these include one major metabolite, IV-e, which accounts for 28% of the radioactivity in the microsome-$NADPH_2$ system. None of the metabolites reacts with diazomethane to yield products of altered R_f values; therefore, metabolites containing phenolic hydroxyl groups probably are not present. Metabolite IV-e, recovered in radiochemically pure form from preparative-scale chromatoplates, hydrogenates to yield a product cochromatographing with 3-isopropylphenyl methylcarbamate (UC 10854); this supports the cochromatographic evidence that metabolite IV-e is 3-(1-hydroxy-1-methylethyl)phenyl methylcarbamate. Metabolite IV-f cochromatographs with 3-isopropylphenyl *N*-hydroxymethylcarbamate. Metabolite IV-e is equal to the parent compound in antiChE activity (Tables III and IV). 3-Isopropyl-4-hydroxyphenyl methylcarbamate, which is not obtained in detectable levels from the liver microsome-$NADPH_2$ system, is more potent as an antiChE agent than the nonhydroxylated compound (Table III).

HRS-1422. Carbonyl-C^{14}-labeled HRS-1422 (Compound V) yields three major carbamate metabolites (V-e, V-f, and V-g) as shown in Figure 1 and Table IV. None of the major metabolites reacts with diazomethane, indicating that they do not contain phenolic hydroxyl groups.

Dimethylcarbamates, Their Metabolites, and Other Analogs

	Compound[a]	AntiChE Activity, MDL,[b] μg.	Metabolite Designation[c]
	HRS-1422 and Analogs		
35	3,5-Diisopropyl-4-hydroxyphenyl methylcarbamate	0.001	...
36	3,5-Diisopropylphenol	6.0	...
37	3,5-Diisopropylphenyl 1,4-bis(*N*-methylcarbamate)	0.004	...
38	3,5-Diisopropylphenyl *N*-hydroxymethylcarbamate	0.1	V-g
39	3,5-Diisopropylphenyl methylcarbamate (HRS-1422)	0.03	V
	Mesurol and Analogs		
40	4-Methylsulfinyl-3,5-xylyl methylcarbamate	0.01	VI-b
41	4-Methylsulfonyl-3,5-xylyl methylcarbamate	0.1	VI-d
42	4-Methylthio-3,5-xylenol	20	...
43	4-Methylthio-3,5-xylyl methylcarbamate (Mesurol)	0.1	VI
	Matacil and Analogs		
44	4-Amino-3-cresyl methylcarbamate	0.1	VII-d
45	4-Dimethylamino-3-cresol	20	...
46	4-Dimethylamino-3-cresyl *N*-hydroxymethylcarbamate	1.0	VII-c
47	4-Dimethylamino-3-cresyl methylcarbamate (Matacil)	1.0	VII
48	4-Formamido-3-cresyl methylcarbamate	1.0	...
49	4-Methylamino-3-cresyl methylcarbamate	0.1	VII-e
50	4-Methylformamido-3-cresyl methylcarbamate	1.0	...
	Zectran and Analogs		
51	4-Amino-3,5-xylyl methylcarbamate	0.004	VIII-f
52	4-Dimethylamino-3,5-xylenol	5.0	...
53	4-Dimethylamino-3,5-xylyl *N*-hydroxymethylcarbamate	0.2	VIII-h
54	4-Dimethylamino-3,5-xylyl methylcarbamate (Zectran)	0.02	VIII
55	4-Formamido-3,5-xylyl methylcarbamate	0.1	...
56	4-Hydroxy-3,5-xylyl methylcarbamate	0.08	...
57	4-Methylamino-3,5-xylyl methylcarbamate	0.004	VIII-g
58	4-Methylformamido-3,5-xylyl methylcarbamate	0.2	VIII-e
59	3,5-Xylyl 1,4-bis(*N*-methylcarbamate)	0.8	...
	Dimetilan and Analogs		
60	2-Dimethylcarbamoyl-3-methyl-5-pyrazolone	100	...
61	2-Dimethylcarbamoyl-3-methyl-5-pyrazolyl dimethylcarbamate (dimetilan)	0.2	IX
62	2-Dimethylcarbamoyl-3-methyl-5-pyrazolyl methylcarbamate	2.0	...
63	2-Methylcarbamoyl-3-methyl-5-pyrazolyl dimethylcarbamate	0.2	IX-h
64	3-Methyl-5-pyrazolyl dimethylcarbamate	0.02	...
	Isolan and Analogs		
65	1-Isopropyl-3-methyl-5-pyrazolone	80	...
66	1-Isopropyl-3-methyl-5-pyrazolyl dimethylcarbamate (Isolan)	0.01	X

Metabolite V-g is 3,5-diisopropylphenyl *N*-hydroxymethylcarbamate, based on cochromatography. On analogy with the related compound, UC 10854, it is likely that one of the major metabolites of HRS-1422 (V-f) is a 1-hydroxy-1-methylethyl derivative because of its high antiChE activity and chromatographic position. Although the nature of the minor metabolites is not known, several of them are, as shown in Table IV, more potent than the original compound as inhibitors of plasma cholinesterase. 3,5-Diisopropyl-4-hydroxyphenyl methylcarbamate, which is a possible metabolite of HRS-1422, but which is not obtained in detectable levels in the ether extract, is more potent as an antiChE agent than HRS-1422 (Table III).

MESUROL. Carbonyl-C^{14}-Mesurol (Compound VI) yields two major ether-extractable metabolites (VI-b and VI-c) as shown in Figure 1 and Table IV. Metabolite VI-b cochromatographs with 4-methylsulfinyl-3,5-xylyl methylcarbamate (Mesurol sulfoxide) and VI-d, which is present in trace amounts in the microsome-$NADPH_2$ system, cochromatographs with 4-methylsulfonyl-3,5-xylyl methylcarbamate (Mesurol sulfone). Mesurol sulfoxide is about 10 times more potent than Mesurol or Mesurol sulfone as an antiChE agent (Tables III and IV). The second major metabolite, VI-c, is also active as an inhibitor. Some Mesurol sulfoxide formation apparently occurs on exposure of Mesurol to air during chromatography and radioautography because, as shown in Table IV, the labeled product with the R_f of Mesurol is a more potent inhibitor than nonlabeled Mesurol.

MATACIL. Examination of Figure 1 and Table IV re-

Table IV. Number, Amount, and AntiChE Activity of the Radioactive Metabolites of Seven Carbonyl-C^{14}-Labeled Preparations of Methyl- and Dimethylcarbamates, as Formed by the Liver Microsome-$NADPH_2$ System

Component[a]	Per Cent of Initial Radioactivity of Component Found	AntiChE Activity, MDL,[b] μg. Carbonyl-C^{14}	Known compounds[c]
3-Isopropylphenyl Methylcarbamate (UC 10854, Compound IV)			
IV	42.1	0.006	0.004
IV-a	<0.1	0.03	...
IV-b + c	3.6	0.3	...
IV-d	1.2	0.03	...
IV-e	28.0	0.006	0.004
IV-f	3.7	0.05	0.04
IV-g	1.0	...	...
3,5-Diisopropylphenyl Methylcarbamate (HRS-1422, Compound V)			
V	62.4	0.01	0.03
V-a	<0.1	0.04	...
V-b + c + d	4.0	0.01	...
V-e	2.0	0.004	...
V-f	18.0	0.002	...
V-g	6.5	0.3	0.1
V-h	<0.1	0.01	...
V-i	<0.1	0.006	...
V-j + k	<0.1	0.02	...
V-l	<0.1	>0.02	...
4-Methylthio-3,5-xylyl Methylcarbamate (Mesurol, Compound VI)			
VI	10.6	0.02	0.1
VI-a	<0.1	>0.2	...
VI-b	5.8	0.01	0.01
VI-c	7.2	0.05	...
VI-d	<0.1	0.06	0.1
VI-e	0.6	...	...
4-Dimethylamino-3-cresyl Methylcarbamate (Matacil, Compound VII)			
VII	59.3	0.6	1.0
VII-a	0.2	0.51	...
VII-b	0.3	0.25	...
VII-c	9.7	>0.57	1.0
VII-d	0.2	0.14	0.1
VII-e	16.5	0.12	0.1
VII-f	<0.1	>0.14	...
4-Dimethylamino-3,5-xylyl Methylcarbamate (Zectran, Compound VIII)			
VIII	9.0	0.03	0.02
VIII-a	1.5	0.1	...
VIII-b	<0.1	>0.05	...
VIII-c	6.9	0.3	...
VIII-d	1.2	...	...
VIII-e	1.9	>0.2	0.2
VIII-f	3.8	>0.002	0.004
VIII-g	42.6	0.002	0.004
VIII-h	11.0	>0.05	0.2
VIII-i	1.1	0.05	...
2-Dimethylcarbamoyl-3-methyl-5-pyrazolyl Dimethylcarbamate (dimetilan, Compound IX)			
IX	58.9	0.16	0.2
IX-a	0.1	>0.01	...
IX-b	0.6	0.45	...
IX-c	5.1	0.11	...
IX-d	0.2	>0.5	...
IX-e	<0.1	>0.04	...
IX-f	<0.1	>0.04	...
IX-g	<0.1	0.2	...
IX-h	1.2	>0.06	0.2
1-Isopropyl-3-methyl-5-pyrazolyl Dimethylcarbamate (Isolan, Compound X)			
X	9.4	0.006	0.01
X-a	0.2	>0.02	...
X-b	0.4	>0.1	...
X-c	0.4	>0.04	...
X-d	14.3	0.01	...
X-e	1.6	>0.01	...
X-f	<0.1	>0.004	...

[a] Number and letter designations refer to TLC spots for compounds and metabolites as shown in Figure 1.
[b] Minimum detectable level, in terms of micrograms of original compound having equivalent radiocarbon content.
[c] Known compounds are nonlabeled carbamates from synthesis which cochromatograph with the respective radioactive components (Table III).

veals that metabolism of carbonyl-C^{14}-Matacil (Compound VII) yields two major products (VII-c and VII-e) and four minor ones (VII-a, VII-b, VII-d, and VII-f). Three of these products are tentatively identified as follows: VII-c, 4-dimethylamino-3-cresyl *N*-hydroxymethylcarbamate; VII-d, 4-amino-3-cresyl methylcarbamate; VII-e, 4-methylamino-3-cresyl methylcarbamate. The *N*-hydroxymethyl derivative is as potent as the original compound, and the 4-amino- and 4-methylamino-3-cresyl methylcarbamates are 10 times more potent than Matacil, as inhibitors of plasma cholinesterase (Tables III and IV). Some of the unidentified metabolites are more active than Matacil, but they are less active than the 4-amino- and 4-methylamino analogs as antiChE agents.

ZECTRAN. Figure 1 and Table IV demonstrate that many ether-extractable metabolites are formed from carbonyl-C^{14}-Zectran (Compound VIII). One major metabolite, VIII-g, cochromatographs with 4-methylamino-3,5-xylyl methylcarbamate, and another, VIII-h, with 4-dimethylamino-3,5-xylyl *N*-hydroxymethylcarbamate. Minor metabolites identified by cochromatography are: VIII-e, 4-methylformamido-3,5-xylyl methylcarbamate and VIII-f, 4-amino-3,5-xylyl methylcarbamate. One of the major products, VIII-c, and the minor products, VIII-a, VIII-b, VIII-d, and VIII-i are not identified. Metabolites VIII-f and VIII-g are more potent antiChE agents than Zectran, whereas the other metabolites of this compound are less active as pseudocholinesterase inhibitors (Tables III and IV).

DIMETILAN. Carbamate-carbonyl-C^{14}-labeled dimetilan (Compound IX) gives several ether-extractable metabolites, only one of which (IX-c) is present in amounts greater than 5% (Figure 1 and Table IV). Another metabolite, IX-h, cochromatographs with 2-methylcarbamoyl-3-

methyl-5-pyrazolyl dimethylcarbamate. Neither 2-dimethylcarbamoyl-3-methyl-5-pyrazolyl methylcarbamate nor 3-methyl-5-pyrazolyl dimethylcarbamate appears as a metabolite. The major metabolite (IX-c) and 2-methylcarbamoyl-3-methyl-5-pyrazolyl dimethylcarbamate (IX-h) are as active as dimetilan as antiChE agents (Tables III and IV). Other metabolites have low antiChE activity.

ISOLAN. As seen in Figure 1 and Table IV, carbonyl-C^{14}-labeled Isolan (Compound X) yields one major (X-d) and five minor (X-a, X-b, X-c, X-e, and X-f) products, none of which has been identified. Each of the metabolites is less active than Isolan as a cholinesterase inhibitor.

Metabolism of Carbaryl and Banol Analogs. Formation of carbaryl from 1-naphthyl *N*-acetyl-*N*-methylcarbamate (*N*-acetyl carbaryl) occurs on incubation with microsomes. However, this conversion to carbaryl may be nonenzymatic in nature because it not only occurs in the microsome-$NADPH_2$ system, but also on incubation with each of the following systems: buffer alone, buffer fortified with $NADPH_2$, boiled microsomes alone, and boiled microsomes fortified with $NADPH_2$. Microsomes fortified with $NADPH_2$ yield additional metabolites, detected as antiChE agents, from *N*-acetyl carbaryl, and these products chromatograph in the same position as the ones formed when carbaryl is used as a substrate. These metabolites probably result from microsomal oxidation of carbaryl, initially released on enzymatic or nonenzymatic hydrolysis of the *N*-acetyl analog. A similar reaction occurs in the case of 2-chloro-4,5-xylyl *N*-acetyl-*N*-methylcarbamate (*N*-acetyl Banol), giving rise to Banol and other metabolites which are formed from Banol by the liver microsome-$NADPH_2$ enzyme system.

Metabolism of 1-naphthyl dimethylcarbamate by the liver microsome system, without $NADPH_2$ fortification, gives only traces of one antiChE metabolite and this metabolite is carbaryl, based on cochromatography using antiChE assay to detect the metabolite derived from 1-naphthyl dimethylcarbamate, and radioautography to detect the portion of carbaryl-carbonyl-C^{14} added to the ether extract of the reaction mixture immediately before chromatography. When $NADPH_2$ is added to the microsome incubation mixture, greater amounts of carbaryl and additional cholinesterase-inhibiting metabolites are formed from 1-naphthyl dimethylcarbamate, and these products chromatograph in the position of the carbaryl metabolites which are antiChE agents.

AntiChE Activity of Metabolites and Analogs Related to the Radiolabeled Carbamates. The antiChE activities of known metabolites and chemicals related to the 10 methyl- and dimethylcarbamates studied (Compounds I to X) are given in Table III. Certain of the hypothetical carbamate metabolites, which are either absent or not present in any detectable level in the ether extract of incubated liver microsome-$NADPH_2$ systems, are potent inhibitors of plasma cholinesterase. The *N*-hydroxy-*N*-methylcarbamates (Compounds 10 and 26) of substituted phenols are more potent antiChE agents than the respective *N*-methylcarbamates. With the exception of carbaryl, ring hydroxylation in the 4-position gives very potent cholinesterase inhibitors (Compounds 13, 30, and 35) in the case of Baygon, UC 10854, and HRS-1422, respectively. Carbamoylation of the 4-hydroxy compounds with methylisocyanate to yield 1,4-bis(*N*-methylcarbamate) derivatives (Compounds 17, 32, and 37) possibly decreases the antiChE activity, but the products of this reaction are more potent than their monomethylcarbamate analogs. In the case of carbaryl, the MDL for the 4-hydroxy compound is two times as high as for carbaryl; however, 1,4-naphthyl bis(*N*-methylcarbamate) is about 2.5 times more active than carbaryl as an antiChE agent. The phenols and pyrazolones have low antiChE activity (Table III).

AntiChE Activity of Metabolites of the Nonlabeled Carbamates. The antiChE activities of metabolites of 20 nonlabeled methylcarbamate insecticide chemicals or related compounds, as produced on incubation with rat liver microsomes and $NADPH_2$, are tabulated in Table V In the absence of $NADPH_2$ fortification, the amount of metabolites is, in every case, much less than with $NADPH_2$ fortification. Each of the substrates yields at least one cholinesterase-inhibiting metabolite and the number of antiChE agents is sometimes as high as four. Only 3-(1-ethylpropyl)phenyl methylcarbamate (RE 9659), 3-(1-methylbutyl)phenyl methylcarbamate (RE 9660), and 2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime (Temik) yield metabolites more active as cholinesterase inhibitors than their parent compounds.

Since it was studied in greater detail, there is more information on Temik than on the other compounds in this category, even though the Temik sample contained small amounts of Temik sulfoxide as an impurity. Temik oxidizes to its sulfoxide derivative in the process of chromatography; although this reaction takes place to only a minor extent, it is easily demonstrated because of the high antiChE activity of Temik sulfoxide. [To investigate whether or not the oxidation is a function of microsomal enzymes, Temik was incubated with buffer alone, buffer plus $NADPH_2$, and buffer plus microsomes (equivalent in amount to each of 40 and 200 mg. of rat liver), with and without $NADPH_2$ fortification.] On incubation, Temik partially oxidizes to the sulfoxide derivative in buffer alone, while buffer fortified with $NADPH_2$ completely converts Temik to Temik sulfoxide. When incubated in the presence of a low concentration of microsomes without cofactor addition, Temik oxidizes completely to the sulfoxide and sulfone; in the presence of microsomes plus $NADPH_2$ or when a high concentration of microsomes is used with or without $NADPH_2$ fortification, Temik almost completely metabolizes without accumulation of ether-extractable metabolites that inhibit cholinesterase. Temik sulfoxide, on incubation with microsomes alone, largely yields the original compound, with only small amounts of the sulfone and another unidentified inhibitory metabolite being formed; fortification of the microsomes with $NADPH_2$ does not substantially increase the destruction of the sulfoxide. Microsomes, or microsomes fortified with $NADPH_2$, partially destroy the sulfone without the formation of ether-extractable cholinesterase inhibitors.

The substituted phenols and substituted oximes have low antiChE activity (Table V).

Incorporation of Radiocarbon from Carbamate-C^{14} Insecticide Chemicals into Microsomes. As shown in Table VI, incorporation of radiocarbon into the micro-

Table V. Number and AntiChE Activity of Metabolites Produced by Liver Microsome-NADPH$_2$ Enzyme System from Substituted-Phenyl Methylcarbamates, Their Analogs, and Substituted-Oxime Methylcarbamates

No.	Compound[a]	AntiChE Activity, MDL,[b] μg.	AntiChE Metabolites, Number		TLC Solvent System
			Total	Highly active[c]	
	Substituted-Phenyl Methylcarbamates and Analogs				
1	4-Benzothienyl methylcarbamate (Mobam)	0.08	2–3	0	Ether-hexane (4 to 1)
2	2,3-Dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate (NIA 10242)	0.02	2–3	0	Ether-benzene (2 to 1)
3	2-(1-Ethylpropyl)phenyl methylcarbamate (RE 9661)	0.8	2	0	Ether-hexane (4 to 1)
4	3-(1-Ethylpropyl)phenyl methylcarbamate (RE 9659)	0.1	3–4	2	Ether-hexane (4 to 1)
5	4-(1-Ethylpropyl)phenyl methylcarbamate (RE 9662)	3.0	3	0	Ether-hexane (4 to 1)
6	5-Indanyl dimethylcarbamate (I-3)	4.0	1–2	0	Ether-hexane (4 to 1)
7	4-Indanyl methylcarbamate (I-1)	0.4	1–2	0	Ether-hexane (4 to 1)
8	5-Indanyl methylcarbamate (I-2)	0.6	1–2	0	Ether-hexane (4 to 1)
9	2-(1-Methylbutyl)phenyl methylcarbamate (RE 9663)	1.0	1–2	0	Ether-hexane (4 to 1)
10	3-(1-Methylbutyl)phenyl methylcarbamate (RE 9660)	0.2	2–3	1	Ether-hexane (4 to 1)
11	4-(1-Methylbutyl)phenyl methylcarbamate (RE 9664)	4.0	2–3	0	Ether-hexane (4 to 1)
12	3,4-Methylenedioxyphenyl *N*-butoxyethoxyethylcarbamate (S-16)	3.0	1–2	0	Ether-hexane (4 to 1)
13	3,4-Methylenedioxyphenyl dimethylcarbamate (I-6)	6.0	2–3	0	Ether-hexane (4 to 1)
14	3,4-Methylenedioxyphenyl methylcarbamate (I-5)	1.0	2–3	0	Ether-hexane (4 to 1)
15	8-(2-Methylquinolyl) methylcarbamate (GS 13798)	3.0	1–2	0	Chloroform-acetonitrile (4 to 1)
16	3,4,5-Trimethylphenyl methylcarbamate (SD 8530)	0.06	2–3	0	Ether-hexane (4 to 1)
	Substituted-Oxime Methylcarbamates				
17	3-Chloro-6-cyano-2-norbornanone *O*-(methylcarbamoyl)oxime (Tranid)	0.1	1–2	0	Chloroform-acetonitrile (2 to 1)
18	2-Methyl-2-(methylsulfinyl)propionaldehyde *O*-(methylcarbamoyl)-oxime (Temik sulfoxide)	0.001	1–2	0	Chloroform-acetonitrile (4 to 1)
19	2-Methyl-2-(methylsulfonyl)propionaldehyde *O*-(methylcarbamoyl)oxime (Temik sulfone)	0.005	1–2	0	Chloroform-acetonitrile (4 to 1)
20	2-Methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)-oxime (Temik)	0.02	1–3	1	Chloroform-acetonitrile (4 to 1)
	Substituted Phenols and Substituted Oximes				
21	2,3-Dihydro-2,2-dimethyl-7-benzofuranol (NIA 10272)	10	...	...	Ether-benzene (2 to 1)
22	8-Hydroxyquinaldine (GS 25241)	4.0	...	...	Chloroform-acetonitrile (4 to 1)
23	2-Methyl-2-(methylsulfinyl)propionaldehyde oxime (Temik oxime sulfoxide)	50	...	...	Chloroform-hexane (7 to 1)
24	2-Methyl-2-(methylsulfonyl)propionaldehyde oxime (Temik oxime sulfone)	50	...	...	Chloroform-hexane (7 to 1)
25	2-Methyl-2-(methylthio)propionaldehyde oxime (Temik oxime)	50	...	...	Chloroform-hexane (7 to 1)
26	3,4,5-Trimethylphenol	5.0	...	...	Chloroform-hexane (7 to 1)

[a] Source: compounds 3 to 5 and 9 to 11 from Chevron Chemical Co., Richmond, Calif.; 15 and 22 from J. R. Geigy, S. A., Basel, Switzerland; 6 to 8 and 12 to 14 from Gulf Research and Development Co., Pittsburgh, Pa.; 18, 19, and 23 to 25 from J. B. Knaak, Carnegie-Mellon University, Pittsburgh, Pa.; 1 from Mobil Chemical Co., New York, N.Y.; 2 and 21 from Niagara Chemical Co., Middleport, N.Y.; 16 and 26 from Shell Development Co., Modesto, Calif.; 17 and 20 from Union Carbide Corp., New York, N.Y.
[b] Minimum detectable level, as assayed by ChE inhibition.
[c] Higher AntiChE activity than parent compound.

somal proteins takes place in the liver-microsome system, with and without $NADPH_2$ fortification, when each of the labeled carbamate samples, methyl-C^{14}-amine, and sodium carbonate-C^{14} are used as substrates. There is evidence that a chemical reaction, rather than merely absorption or solubilization of the parent compound in microsomal components, probably takes place because the results vary, in part, with the position of the C^{14}-label in the molecule and with the presence or absence of $NADPH_2$. Extensive incorporation of radiocarbon occurs with several carbamates, but not with sodium carbonate-C^{14} and methyl-C^{14}-amine (Table VI). In the case of Banol, the carbonyl-C^{14} and *N*-methyl-C^{14} samples yield protein-bound radiocarbon to about the same extent, which is almost six-fold greater than the level resulting from the use of 4-methyl-C^{14}-Banol; also, this relationship is not altered by $NADPH_2$ fortification. This indicates that the methylcarbamoyl group reacts with microsomal components to yield a relatively stable methylcarbamoyl protein and the phenolic moiety is the "leaving group." To a lesser degree, this same finding results with the three different preparations of carbaryl and Baygon, indicating some protein carbamoylation but, in these cases, the fortification with $NADPH_2$ results in products which do not act in this manner. Very little radiocarbon is protein-bound, in either the presence or absence of $NADPH_2$ fortification, with the other compounds except for Mesurol, dimetilan, and Isolan and, in each of these cases, fortification with $NADPH_2$ enhances the radiocarbon incorporation.

Table VI. Incorporation of Radiocarbon into Microsomal Proteins from Incubation Mixtures Containing Liver Microsomes, with and without $NADPH_2$, and Variously C^{14}-Labeled Methyl- and Dimethylcarbamate Insecticide Chemicals and Their Hydrolysis Products

			Per Cent of Initial Radiocarbon in TCA-Insoluble Fraction	
	Compounds	Position of C^{14}-Label	No cofactor	With $NADPH_2$
I	Carbaryl	Carbonyl	8	8
		N-Methyl	7	6
		1-Naphthyl	3	11
II	Baygon	Carbonyl	3	4
		N-Methyl	3	4
		Isopropoxy	1	4
III	Banol	Carbonyl	21	20
		N-Methyl	21	19
		4-Methyl	4	3
IV	UC 10854	Carbonyl	2	2
V	HRS-1422	Carbonyl	4	3
VI	Mesurol	Carbonyl	8	17
VII	Matacil	Carbonyl	3	2
VIII	Zectran	Carbonyl	3	3
IX	Dimetilan	Carbamate-carbonyl	1	8
X	Isolan	Carbonyl	2	16
XI	Methylamine·HCl	*N*-Methyl	1	1
XII	Sodium carbonate	Carbonate	0	0

DISCUSSION

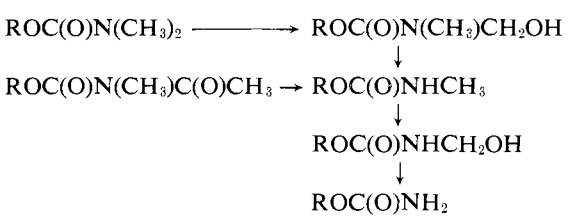
Liver enzymes, particularly those associated with the microsome-$NADPH_2$ system, are active in metabolizing methylcarbamate insecticide chemicals by pathways which are primarily nonhydrolytic in nature (Dorough *et al.*, 1963). The properties of the system, previously studied with carbaryl as the substrate, are similar when each of the other nine methyl- and dimethylcarbamates is used as a substrate (Dorough and Casida, 1964; Dorough *et al.*, 1963; Leeling and Casida, 1966). The great variety of metabolic products encountered with carbamate insecticide chemicals results, in part, from metabolic attack by hydroxylation and oxidation because of the resistance of the methylcarbamate and dimethylcarbamate moieties to enzymatic hydrolysis. The utility of the microsome-$NADPH_2$ reaction mixture as a model in vitro system which gives products similar, in many respects, to those formed in vivo in insects and mammals has been demonstrated (Dorough and Casida, 1964; Dorough *et al.*, 1963; Hodgson and Casida, 1960, 1961; Knaak *et al.*, 1965; Leeling and Casida, 1966; Shrivastava, 1967; Tsukamoto and Casida, 1967, a and b; Zubairi and Casida, 1965). In addition, the metabolites formed in bean plants from carbamates generally are the same as those formed by the liver microsome-$NADPH_2$ system but, in plants, these metabolites quickly conjugate as glycosides (Kuhr and Casida, 1967).

In trying to deduce the types of reactions that can take place at various sites of the methylcarbamate molecule, as a result of incubation with the liver microsome-$NADPH_2$ system, it is important to note that, in this investigation, attempts were made to identify only the ether-soluble metabolites. Also, as seen in Tables II and IV, it is not always possible, with the chromatographic methods used, to resolve the metabolites adequately enough to allow their identification; therefore, some of the TLC areas found probably contain a mixture of components, the following examples being notable: II-b + c, IV-b + c, V-a + b + c, and V-j + k; components remaining at the origin. In addition, there is evidence of inadequate TLC resolution in the case of components I-c, II-g, II-h, III-a, III-c, III-e, and III-f because, in these TLC areas, the results obtained for the variously labeled substrates are inconsistent with the apparent presence of a single, stable compound. Thus, the conclusion is that these TLC areas or regions contain mixtures of compounds, possibly as a result of partial decomposition reactions taking place during chromatography and radioautography.

In the liver microsome system, the formation of ether-soluble metabolites, other than the respective phenols, pyrazolones, or oximes, from methyl- and dimethylcarbamates, generally is dependent on fortification with $NADPH_2$. Also, certain metabolites of such compounds (Tables I, II, and IV) which occur only in trace amounts in the liver microsome-$NADPH_2$ system are present in appreciable amounts after incubation in the microsome-plus-soluble-NADP system (Oonnithan, 1966).

In those instances where comparison compounds are available for cochromatography, all substituted-phenyl methylcarbamates give the respective substituted-phenyl *N*-hydroxymethyl analogs, in amounts ranging up to

approximately 19%, when used as substrates in the liver microsome-$NADPH_2$ system. However, with the same conditions, *N*-hydroxylation to the *N*-hydroxy-*N*-methylcarbamate does not occur and no more than trace amounts, at most, of *N*-dealkylation to the unsubstituted carbamate takes place with the carbaryl, Baygon, and Banol series of methylcarbamates (Dorough and Casida, 1964). Baygon yields trace amounts of 2-isopropoxyphenyl carbamate in the liver enzyme incubation mixtures and this product, again in trace amounts, occurs in the fly enzyme system (Tsukamoto and Casida, 1967b), and in living flies treated with Baygon (Shrivastava, 1967). 1-Naphthyl dimethylcarbamate is *N*-demethylated to give 1-naphthyl methylcarbamate (carbaryl); the intermediate in this case probably is 1-naphthyl *N*-hydroxymethyl-*N*-methylcarbamate. Possibly, certain of the dimetilan and Isolan metabolites also are *N*-hydroxymethyl-*N*-methylcarbamates (Hodgson and Casida, 1960, 1961; Zubairi and Casida, 1965). Substituted-phenyl *N*-acetyl-*N*-methylcarbamates (*N*-acetyl carbaryl and *N*-acetyl Banol) are easily hydrolyzed to carbaryl and Banol, respectively, but this does not necessarily involve an enzymatic reaction. These reactions of the carbamoyl moiety, taken collectively, probably occur in the following sequence, although this complete pathway is not yet established with any one particular substituted-phenyl (R) group:



Evidence in regard to the enzymatic nature of these reactions is available only for the two *N*-methyl hydroxylation steps. *N*-Dealkylation and *N*-formamide formation reactions also occur with the dimethylamino moiety of such 4-dimethylaminoaryl methylcarbamates as Matacil and Zectran (Abdel-Wahab and Casida, 1967; Abdel-Wahab *et al.*, 1966; Kuhr and Casida, 1967; Tsukamoto and Casida, 1967a). In addition, *N*-dealkylation of the dimethylcarbamoyl group in dimetilan is known to occur (Zubairi and Casida, 1965).

Ring hydroxylation occurs with carbaryl in the 4- and 5-positions and with Baygon in the 5-position. Baygon does not hydroxylate in the 4-position or, if it does, this occurs with a very low yield. The 4-hydroxy analogs are not formed at detectable levels from UC 10854 and HRS-1422. Degradation of the 4-dimethylamino group of Zectran does not proceed to the stage of the 4-hydroxy analog with the remaining substituents intact. Carbaryl yields 5,6-dihydro-5,6-dihydroxy carbaryl (Leeling and Casida, 1966), probably through an epoxide intermediate which, in turn, rearranges or hydrolyzes to the 5-hydroxy- or 5,6-dihydro-5,6-dihydroxy analogs, respectively. Probably, one or two of the Baygon metabolites having low R_f values (II-b and II-c) is (or are) such a dihydrodihydroxy derivative(s), but direct evidence in regard to this point is not available.

Isopropyl substituents, either as *O*-alkyl or *C*-alkyl groups, hydroxylate at the tertiary carbon atom; this results in *O*-depropylation of Baygon to 2-hydroxyphenyl methylcarbamate and conversion of UC 10854 to the hydroxypropyl analog. A similar site of hydroxylation possibly leads to certain of the unidentified metabolites of HRS-1422, and of the isoamyl- and *sec*-amylphenyl methylcarbamates (Compounds 3 to 5 and 9 to 11, Table V). No direct evidence is available for oxidation of the aromatic methyl groups of Banol, Mesurol, Matacil, Zectran, and SD 8530 (3,4,5-trimethylphenyl methylcarbamate, Table V), although it is difficult to explain the number of radioactive or cholinesterase-inhibiting carbamate metabolites found for these carbamates unless such an initial site of hydroxylation is postulated.

Sulfoxidation, as a microsome-$NADPH_2$–catalyzed reaction, occurs with Mesurol and probably with Temik, but the subsequent oxidation of the sulfoxide to the sulfone derivatives occurs only to a small extent.

While a definite attempt was made to identify the ether-extractable metabolites formed, the nature of other products can only be interpreted from radiocarbon loss during incubation, resulting from various combinations of cofactor fortification or addition of the soluble fraction to the microsome fraction; from the proportion of radioactivity remaining in the aqueous fraction after extraction with ether; and from the amount of radiocarbon incorporated into microsomal proteins with various labeled preparations. The volatility of the individual carbamates (Abdel-Wahab *et al.*, 1966) is not related to the losses encountered on incubation, extraction, and chromatography of the radiolabeled substrates because the losses do not directly correlate with the volatility and occur only on cofactor fortification. Radiocarbon losses may result from hydrolysis of the carbamates or their metabolites when carbonyl-C^{14} preparations are used, because the radiocarbon of carbonate-C^{14} is lost under the conditions of incubation. (It is assumed that the substituted carbamic acid resulting from hydrolysis of each of the methyl-, dimethyl-, *N*-hydroxymethyl- and *N*-methyl-*N*-hydroxymethylcarbamates will decompose to yield carbonate ion within 4 hours at 37° C. and pH 7.4, the incubation conditions.) In the cases of Isolan and Banol, and to a lesser degree of carbaryl and Mesurol, the loss of radiocarbon with carbonyl-C^{14} samples is cofactor-dependent, indicating that the metabolites formed by the microsome-$NADPH_2$ enzyme system are the products hydrolyzed; the three monomethylcarbamates involved are those that are most susceptible to nonenzymatic hydrolysis (Abdel-Wahab *et al.*, 1966), and certain of their metabolites are expected to be less stable to hydrolysis than comparable metabolites from other methylcarbamates. Radiocarbon loss occurs with Baygon-isopropoxy-C^{14}, and this probably results from enzymatic release of acetone-C^{14} on hydroxylative attack by the microsome-$NADPH_2$ system at the tertiary carbon of the isopropoxy group; loss of the isopropyl moiety as acetone occurs in houseflies injected with Baygon, the acetone volatilizing from the flies (Shrivastava, 1967).

Apart from that portion of radiocarbon which is incorporated into the microsomal protein, radioactivity appearing in the aqueous fraction after incubation of the carbonyl-C^{14}-labeled compounds with liver microsomes and $NADPH_2$ may be present in a form other than car-

bonate ion and yet in a form not extractable into ether. Carbamate metabolites with hydroxyl or other polar groups may be less efficiently extracted and conjugates, such as glucuronides and sulfates, of these materials may also appear almost entirely in the aqueous fraction. While the nature of the materials formed without cofactor fortification is unknown, the additional radioactivity present in the aqueous fraction with added cofactors may represent hydroxylated metabolites and, particularly, their conjugates. *N*-Methyl-C^{14}-labeled carbamates (except Baygon) and methyl-C^{14}-amine do not result in radiocarbon losses during incubation; this is also true for the carbamate labeled in the ring (carbaryl-naphthyl-1-C^{14}). However, such loss does occur, to some extent, with Banol-4-methyl-C^{14}. Methylamine (or its metabolic products) or phenolic products resulting from hydrolysis of the carbamates or their hydroxylation products, or their conjugates, may account for the higher levels of radiocarbon found in the aqueous phase with *N*-methyl-C^{14}-, ring-C^{14}-, or ring-substituent-C^{14}-labeled carbamates than with carbonyl-C^{14}-labeled compounds.

Carbamoylation of microsomal or other proteins, by methylcarbamates or their metabolites, contributes to the radiocarbon retention in the aqueous fraction with carbonyl-C^{14} and *N*-methyl-C^{14} preparations; carbamates most reactive in this respect appear to be the same ones that are most susceptible to nonenzymatic hydrolysis (Abdel-Wahab *et al.*, 1966) and to the loss of radiocarbon from carbonyl-C^{14} compounds (probably as $C^{14}O_2$) from the enzymatic reaction mixtures. The incorporation of radiocarbon from carbonyl-C^{14}-labeled samples of dimetilan, Isolan, and Mesurol into microsomal proteins is increased by fortification with $NADPH_2$; this indicates that reactive metabolites (such as the *N*-methyl-*N*-hydroxymethylcarbamates of dimetilan and Isolan, or the *N*-hydroxymethylcarbamate of Mesurol and/or Mesurol sulfoxide) may be the actual carbamoylating agents. Studies on the in vivo fate of various methyl- and dimethylcarbamates in rats also indicate that carbamoylated proteins are formed and that carbamoylation accounts, in part, for persisting radiocarbon in animals treated with carbonyl- and *N*-methyl-C^{14}-labeled carbamate preparations (Krishna and Casida, 1966). Although the carbamoyl-protein bond formed on the reaction of methyl- and dimethylcarbamates at the esteratic site of cholinesterase is relatively unstable (Wilson *et al.*, 1960), possibly this bond may be more stable with the reactive sites of proteins other than cholinesterase.

The antiChE activity is generally less for *N*-hydroxymethylcarbamates than for *N*-methylcarbamates, with the possible exception of the Matacil series; on the other hand, the *N*-hydroxy-*N*-methylcarbamates are more potent inhibitors than their methylcarbamate analogs in the carbaryl and Banol series. In cases where analogous compounds are available for comparison, the dimethylcarbamates, ethylcarbamate, *N*-acetyl-*N*-methylcarbamates, and unsubstituted carbamates generally are less active and, frequently, are much less active inhibitors than their respective methylcarbamate analogs. The reduced antiChE activity on forming the *N*-acetyl derivatives is consistent with published information (Fraser *et al.*, 1965; Lewis, 1967; Reay and Lewis, 1966). Methylcarbamates with various sites of ring hydroxylation vary in potency, as cholinesterase inhibitors, from moderately active to highly active ones. Certain of the bis(*N*-methylcarbamates), from reaction of the hydroxyphenyl derivatives with methylisocyanate, also are very active inhibitors. In general, hydrolysis of the methylcarbamate or dimethylcarbamate grouping results in a great decrease in activity because the corresponding phenols, pyrazolones, and oximes are relatively very weak antiChE agents compared to the methyl- and dimethylcarbamates.

The possible formation of cholinesterase-inhibiting metabolites of carbamate insecticide chemicals by the liver microsome-$NADPH_2$ system is suggested by the results of Hodgson and Casida (1960, 1961). The results obtained in the present investigation clearly show that some of the carbamate metabolites, so formed, definitely are cholinesterase inhibitors; in fact, certain of them are even more potent than the respective parent compounds. None of the carbaryl metabolites is appreciably more active as a cholinesterase inhibitor than carbaryl itself. [The antiChE activities of carbaryl and its metabolites reported here differ from those found by Leeling and Casida (1966); however, possibly these differences may arise from differences in technique because Leeling and Casida employed alumina plates for cholinesterase inhibition studies whereas silica gel G plates were used in this study.] None of the metabolites of Isolan is a more potent antiChE agent than the parent compound. One metabolite from each of Banol, UC 10854, Mesurol, RE 9660, and Temik, two metabolites from each of Baygon, dimetilan, RE 9659, and Zectran, and more than two metabolites from each of HRS-1422 and Matacil are as active or more active as antiChE agents than their respective parent compounds. Several of these very active antiChE metabolites are tentatively identified. They are: 5-hydroxy Baygon; the hydroxypropyl analog of UC 10854; Mesurol sulfoxide; Temik sulfoxide; and the 4-methylamino- and 4-amino-aryl methylcarbamates from Matacil and Zectran. *N*-Acetyl-*N*-methyl- and *N*,*N*-dimethylcarbamates may yield more active inhibitors, including methylcarbamates, either by nonenzymatic hydrolysis (*N*-acetyl-*N*-methylcarbamates) or enzymatic degradation (dimethylcarbamates); other active metabolites formed from these compounds may include, in part, the same ones formed when a methylcarbamate rather than a precursor for the methylcarbamate is used as the substrate. Additional antiChE metabolites may have gone unnoticed in the present studies (particularly in those experiments involving nonlabeled compounds) because, by the method used, cholinesterase inhibition assays are made on unknown amounts of the metabolites. Major products of low antiChE activity or active compounds present in extremely low amounts may have been overlooked; also, other metabolites, which are not extracted into ether, were not investigated.

Metabolites active as antiChE agents are not necessarily compounds of high toxicity and, in this respect, care is necessary in interpreting the significance of the enzyme-inhibition assays. In addition to the original insecticide chemical, certain active metabolites can contribute to the toxic action in mammals—for instance, 4-methylamino- and 4-amino analogs of Matacil and Zectran are more

toxic to mice than Matacil and Zectran, respectively (Abdel-Wahab and Casida, 1967); the hydroxypropyl analog of UC 10854 is equal to UC 10854 in toxicity to mice (Balba, 1967); the sulfoxides of Mesurol and Temik are more toxic to mice, by intraperitoneal injection, than their corresponding sulfides; methylcarbamates generally are more toxic to mammals than their *N*-acetyl derivatives (Robertson *et al.*, 1965).

As with the insecticidal organophosphorus compounds, metabolism of methylcarbamate insecticide chemicals results in products of both increased and decreased toxicity, depending upon the compound and the metabolizing system. A better understanding of the relative rates of metabolic attack on different substituents in methyl- and dimethylcarbamates may help in arriving at insecticide chemicals of increased selectivity.

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LITERATURE CITED

Abdel-Wahab, A. M., Casida, J. E., J. AGR. FOOD CHEM. **15,** 479 (1967).
Abdel-Wahab, A. M., Kuhr, R. J., Casida, J. E., J. AGR. FOOD CHEM. **14,** 290 (1966).
Balba, M. H. M., Ph.D. dissertation, University of California, Berkeley, Calif., 1967.
Baron, R. L., *Bull. Entomol. Soc. Am.* **12,** 292 (1966).
Dorough, H. W., J. AGR. FOOD CHEM. **15,** 261 (1967).
Dorough, H. W., Casida, J. E., J. AGR. FOOD CHEM. **12,** 294 (1964).
Dorough, H. W., Leeling, N. C., Casida, J. E., *Science* **140,** 170 (1963).
Fraser, J., Clinch, P. G., Reay, R. C., *J. Sci. Food Agr.* **16,** 615 (1965).
Hassan, A., Zayed, S. M. A. D., Abdel-Hamid, F. M., *Biochem. Pharmacol.* **15,** 2045 (1966).
Hodgson, E., Casida, J. E., *Biochim. Biophys. Acta* **42,** 184 (1960).
Hodgson, E., Casida, J. E., *Biochem. Pharmacol.* **8,** 179 (1961).
Hook, G. E. R., Smith, J. N., *Biochem. J.* **102,** 504 (1967).
Knaak, J. B., Tallant, M. J., Bartley, W. J., Sullivan, L. J., J. AGR. FOOD CHEM. **13,** 537 (1965).
Knaak, J. B., Tallant, M. J., Sullivan, L. J., J. AGR. FOOD CHEM. **14,** 573 (1966).
Krishna, J.-G., Casida, J. E., J. AGR. FOOD CHEM. **14,** 98 (1966).
Krishna, J.-G., Dorough, H. W., Casida, J. E., J. AGR. FOOD CHEM. **10,** 462 (1962).
Kuhr, R. J., Casida, J. E., J. AGR. FOOD CHEM. **15,** 814 (1967).
Leeling, N. C., Casida, J. E., J. AGR. FOOD CHEM. **14,** 281 (1966).
Lewis, D. K., *Nature* **213,** 205 (1967).
Matthews, H. B., Hodgson, E., *J. Econ. Entomol.* **59,** 1286 (1966).
Metcalf, R. L., Fukuto, T. R., Collins, C., Borck, K., Burk, J., Reynolds, H. T., Osman, M. F., J. AGR. FOOD CHEM. **14,** 579 (1966a).
Metcalf, R. L., Fukuto, T. R., Wilkinson, C., Fahmy, M. H., Abd El-Aziz, S., Metcalf, E. R., J. AGR. FOOD CHEM. **14,** 555 (1966b).
Metcalf, R. L., Osman, M. F., Fukuto, T. R., *J. Econ. Entomol.* **60,** 445 (1967).
Mostafa, I. Y., Hassan, A., Zayed, S. M. A. D., *Z. Naturforsch. b***21,** 1060 (1966).
Oonnithan, E. S., Ph.D. dissertation, University of California, Berkeley, Calif., 1966.
Oonnithan, E. S., Casida, J. E., *Bull. Environ. Contam. Toxicol.* **1,** 59 (1966).
Reay, R. C., Lewis, D. K., *J. Sci. Food Agr.* **17,** 17 (1966).
Robertson, W. A. H., Fraser, J., Clinch, P. G., Brit. Patent **982,235** (Feb. 3, 1965).
Shrivastava, S. P., Ph.D. dissertation, University of California, Berkeley, Calif., 1967.
Tsukamoto, M., Casida, J. E., *Nature* **213,** 49 (1967a).
Tsukamoto, M., Casida, J. E., *J. Econ. Entomol.* **60,** 617 (1967b).
Williams, E. A., Meikle, R. W., Redemann, C. T., J. AGR. FOOD CHEM. **12,** 453 (1964a).
Williams, E. A., Meikle, R. W., Redemann, C. T., J. AGR. FOOD CHEM. **12,** 457 (1964b).
Wilson, I. B., Hatch, M. A., Ginsburg, S., *J. Biol. Chem.* **235,** 2312 (1960).
Zayed, S. M. A. D., Hassan, A., Hussein, T. M., *Biochem. Pharmacol.* **15,** 2057 (1966).
Zubairi, M. Y., Casida, J. E., *J. Econ. Entomol.* **58,** 403 (1965).

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