Metabolism of Carbamate Insecticide Chemicals in Plants and Insects

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The metabolic fate of a carbamate insecticide chemical in a plant or insect is often very similar. Hydrolysis of the carbamate-ester linkage is usually slow, and oxidation of the aromatic ring, ring substituents, and the carbamate *N*-methyl group is of prime importance. Hydroxylated metabolites are rapidly conjugated *in vivo*, and these conjugates can, in many cases, be cleaved by β-glucosidase. Little is known about the plant enzymes responsible for carbamate metabolism, but in insects a mixed-function oxidase system(s), present in the microsomes, appears to be the main catalyst. Recent studies with cabbage loopers indicate that fat body is the most active carbaryl-metabolizing tissue. This activity is maximum in last instar and early pupal forms, and is dependent on $NADPH_2$ and oxygen. Probable metabolites formed in tissue homogenates include the *N*-hydroxymethyl (major), 5,6-dihydro-5,6-dihydroxy (intermediate), and 4- and 5-hydroxy (minor) derivatives of carbaryl.

During the past 16 years a variety of methyl- and dimethylcarbamates have been shown to be insecticidally active. Some of these compounds have reached commercial production, of which carbaryl has been the most successful. It is presently registered for use on over 100 crops with tolerances on fruit and vegetables ranging from 5 to 12 ppm.

With the decreasing use of chlorinated hydrocarbon insecticides, more carbamates will probably be commercially available in the future. The introduction of new carbamates and the safe and effective use of those carbamates already available demands a thorough knowledge of the metabolism of these compounds in mammals, plants, and insects. This paper presents a condensed review of the published studies concerning the metabolism of carbamate insecticides in plants and insects. More information is available from recent review articles (Casida and Lykken, 1969; Kuhr, 1968a,b; Lykken and Casida, 1969). Results of current work by the author on metabolism of carbaryl in the cabbage looper are also reported.

The chemical names of the carbamates discussed are as follows: 2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime (aldicarb), 4-dimethylamino-*m*-tolyl methylcarbamate (aminocarb), 2-chloro-4,5-xylyl methylcarbamate (carbanolate), 1-naphthyl methylcarbamate (carbaryl), 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate (carbofuran), 1-(dimethylcarbamoyl)-5-methyl-3-pyrazolyl dimethylcarbamate (dimetilan), *m*{[(dimethylamino)methylene]amino}phenyl methylcarbamate (formetanate), 3,5-diisopropylphenyl methylcarbamate (HRS-1422), 75% of 3,4,5- and 18% of 2,3,5-trimethylphenyl methylcarbamate (Landrin), 4-(methylthio)-3,5-xylyl methylcarbamate (methiocarb), *o*-isopropoxyphenyl methylcarbamate (propoxur), *m*-isopropylphenyl methylcarbamate (UC 10854), and 4-dimethylamino-3,5-xylyl methylcarbamate (Zectran).

METABOLISM IN PLANTS

The partial metabolic pathways of a number of carbamate insecticides in certain plant species have been elucidated. The metabolites formed are, in most cases, very similar to those formed in mammals and insects. In general, oxidative metabolism predominates, but there is usually some hydrolysis of the carbamate to its parent phenol, as well as hydrolysis of the oxidative metabolites. Few of the metabolites containing hydroxyl groups are recovered in their free form since conjugation and incorporation into plant constituents is rapid.

Table I illustrates the variety of carbamate oxidations known to occur in plants. Hydroxylation is the primary mode of oxidation. This includes hydroxylation of carbaryl in the 4 and 5 positions of the naphthyl ring, and hydroxylation of propoxur in the 4 and possibly 5 positions of the phenyl ring. There is some evidence for ring hydroxylation of carbanolate and 3-keto carbofuran. Carbaryl also forms the 5,6-dihydro-5,6-dihydroxy derivative which conjugates in the plant. This is analogous to dihydrodihydroxynaphthalene formation in houseflies and rats (Terriere *et al.*, 1961). Hydroxylation of the *N*-methyl group may occur with most aryl methylcarbamates, but this does not seem to be true for the oxime carbamate aldicarb. No free or conjugated *N*-hydroxymethyl or *N*-demethyl aldicarb could be detected in cotton plants treated with aldicarb (Bartley *et al.*, 1970; Coppedge *et al.*, 1967).

Ring substituents are also prime prospects for hydroxylation. The benzylic carbon of carbofuran is hydroxylated, and this metabolite is either conjugated or further oxidized to the keto derivative. Benzylic carbon hydroxylation also occurs on the methyl groups of Landrin, and probably carbanolate, and on the isopropyl group of UC 10854. Similarly, the isopropoxy group of propoxur is hydroxylated to give an unstable intermediate which decomposes to despropyl propoxur. The dimethylamino groups of aminocarb, Zectran, and formetanate are demethylated, presumably through hydroxylation and further oxidation to the formamido derivatives. In broccoli, metabolism of Zectran proceeds through the parent phenol to a variety of oxidative quinones and conjugates with eventual incorporation of ring fragments into lignin (Williams *et al.*, 1964). Finally, oxidation of aldicarb and methiocarb to their respective sulfoxides and sulfones takes place in plants. Of importance is the subsequent metabolism of aldicarb sulfoxide and sulfone to oxime, nitrile, alcohol, amide, and acid derivatives (Bartley *et al.*, 1970).

Hydroxylation of the aromatic ring, aromatic ring substituents, or the carbamate *N*-methyl group provides a "handle" for conjugation with sugars, and possibly amino acids, sulfuric acid, and phosphoric acid. Unlike mammals and insects, plants do not readily eliminate these conjugates and appear to store them for a considerable length of time. In many cases, a large percentage of the conjugates can be cleaved by β-glucosidase action. Usually some of the metabolites are incor-

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Table I. Oxidative Metabolism of Carbamate Insecticide Chemicals in Plants

Type of Oxidation	Compounds Metabolized (References)[a]
Ring hydroxylation	carbanolate[b] (7); carbaryl (6, 9, 12); 3-ketocarbofuran[b] (11); propoxur (9)
N-Methylhydroxylation	carbanolate (9); carbaryl (6, 9); carbofuran (5); Landrin (14); propoxur (9); UC 10854 (9)
Benzylic carbon hydroxylation	carbanolate[b] (7); carbofuran (5, 11); Landrin (14); UC 10854 (9)
O-Dealkylation	propoxur (9)
N-Demethylation	aminocarb (9); formetanate (8); Zectran (9)
Sulfoxidation	aldicarb (2–4, 10, 13); methiocarb (1)

[a] (1) Abdel-Wahab *et al.*, 1966; (2) Bartley *et al.*, 1970; (3) Bull, 1968; (4) Coppedge *et al.*, 1967; (5) Dorough, 1968a; (6) Dorough and Wiggins, 1969; (7) Friedman and Lemin, 1967; (8) Knowles and Sen Gupta, 1970; (9) Kuhr and Casida, 1967; (10) Metcalf *et al.*, 1966a; (11) Metcalf *et al.*, 1968; (12) Mostafa *et al.*, 1966; (13) Skrentny and Ellis, 1970; (14) Slade and Casida, 1970. [b] Partial evidence for these metabolites.

porated into plant tissues and are not extractable with water or organic solvents.

It is important to remember that these results were obtained from experiments on bean, cotton, corn, broccoli plants, and orange seedlings, and do not necessarily represent a detailed picture for all plants. That large differences exist can be seen from the following examples. The half-life of aminocarb injected into bean plants is 2.4 hr, while the half-life of HRS-1422 in the same plant is 3.5 days. In 10 days only 18% of absorbed Zectran is converted to unextractable products in broccoli, while in 6 days 80% of injected Zectran is converted to unextractables in bean plants.

METABOLISM IN INSECTS

Metabolites Formed. As with plants and mammals, carbamate metabolism in insects involves hydrolysis, oxidative attack, and conjugation. Carbaryl has been most widely studied beginning with the work of Eldefrawi and Hoskins (1961). Since ring-labeled-C^{14}-carbaryl or 1-C^{14}-naphthol applied to houseflies, milkweed bugs, and German cockroaches produced the same series of metabolites, it was proposed that hydrolysis of the carbamate ester bond is the first step in insect metabolism of this carbamate. However, the very polar products remaining at the origin of the chromatographs were assumed to be the same for carbaryl and 1-naphthol.

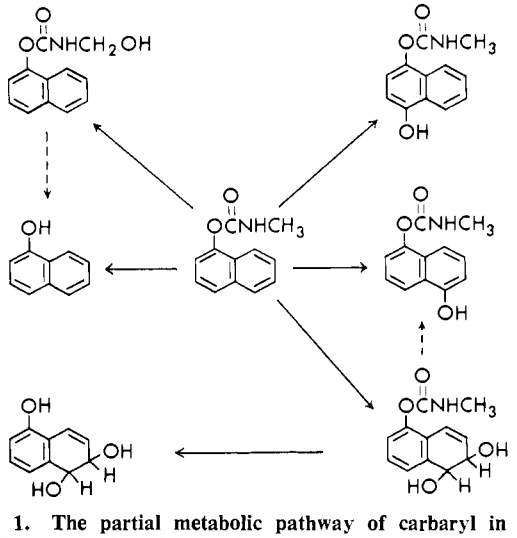

Figure 1. The partial metabolic pathway of carbaryl in insects (after Dorough and Casida, 1964)

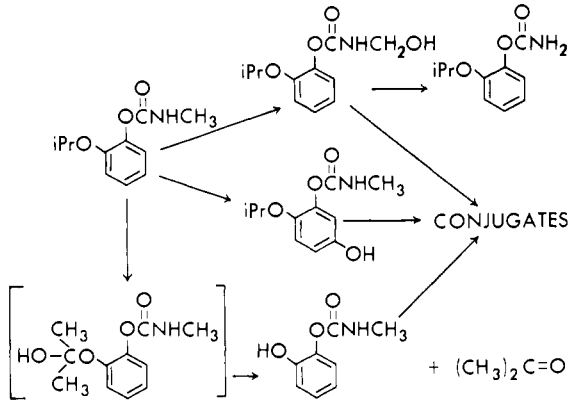

Figure 2. The partial metabolic pathway of Baygon in houseflies (after Shrivastava *et al.*, 1969)

These origin materials may have consisted of a number of conjugated oxidative metabolites. Ku and Bishop (1967) showed that carbaryl is metabolized almost entirely to 1-naphthol and 1-naphthol conjugates in resistant and susceptible strains of the German cockroach.

Other studies reveal that oxidation may be primarily responsible for carbaryl metabolism in insects. American cockroaches metabolize carbaryl to its 4-hydroxy, 5-hydroxy, *N*-hydroxymethyl, and 5,6-dihydro-5,6-dihydroxy derivatives, as illustrated in Figure 1 (Dorough and Casida, 1964). Minor hydrolytic products include 1-hydroxy-5,6-dihydro-5,6-dihydroxy naphthalene and 1-naphthol. Some or all of these metabolites have been detected after topical application of carbaryl to houseflies, American cockroaches, cotton leaf worms, and boll weevils (Andrawes and Dorough, 1967; Dorough and Casida, 1964; Zayed *et al.*, 1966), after injection of carbaryl into bollworm larvae and adults (Andrawes and Dorough, 1967), and after incubation of carbaryl with housefly abdomen homogenates, blowfly fat body, and cabbage looper gut and fat body (Price and Kuhr, 1969; Tsukamoto and Casida, 1967a) or housefly microsomes (Kuhr, 1969; Tsukamoto and Casida, 1967a). In many of these studies a substantial amount of water-soluble metabolites were recovered. In fact, the major excretory product(s) of carbaryl in the housefly is water-soluble (Camp and Arthur, 1967). Since a variety of phenols conjugate as glycosides, phosphates, and sulfates in certain insects (Binning *et al.*, 1967), and since Landrin and propoxur water-soluble metabolites formed in houseflies are cleaved by β-glucosidase, sulfatase, and phosphatase (Shrivastava *et al.*, 1969; Slade and Casida, 1970), it is probable that the carbaryl water solubles are a combination of sulfate, phosphate, and sugar conjugates.

Although metabolites were not identified, evidence for oxidative and hydrolytic degradation of nine insecticidal carbamates in resistant and susceptible strains of houseflies was obtained by Metcalf *et al.* (1967). Expired $C^{14}O_2$ was measured from flies treated with carbamates labeled with C^{14} in various positions of the molecule. Results imply *O*-depropylation of propoxur, *N*-demethylation of methiocarb, UC 10854, carbofuran, aminocarb, and carbaryl, *S*-demethylation of aldicarb, sulfoxidation of aldicarb and methiocarb, and hydrolysis, particularly of carbanolate, methiocarb, and UC 10854.

Very little, if any, hydrolysis of propoxur takes place in resistant and susceptible houseflies (Shrivastava *et al.*, 1969). The major metabolite is 5-hydroxy propoxur with lesser amounts of 2-hydroxy propoxur (depropylation), *N*-hydroxymethyl propoxur, and *N*-desmethyl propoxur (Figure 2).

Most of the hydroxylated metabolites are excreted from the flies as conjugates which are cleaved by β-glucosidase, β-glucuronidase, aryl sulfatase, and phosphatases. However, the cleavage of insect conjugates by β-glucuronidase must be carefully interpreted, since it has been shown that bovine glucuronidase hydrolyzes glucosides (Binning *et al.*, 1967). Some of these metabolites are formed in housefly abdomen homogenates and housefly microsomes (Tsukamoto and Casida, 1967a,b; Tsukamoto *et al.*, 1968) and in American cockroaches (Dorough and Casida, 1964).

Other oxidative metabolites detected in insects include: *N*-hydroxymethyl carbanolate in cockroach (*Blaberus giganteus*) fat body and housefly abdomen homogenates (Gemrich, 1967; Tsukamoto and Casida, 1967a); the *N*-hydroxymethyl, 4-methylamino, 4-amino, 4-methylformamido, and 4-formamido analogs of aminocarb and Zectran in housefly abdomen homogenates (Tsukamoto and Casida, 1967a; Tsukamoto *et al.*, 1968); methiocarb sulfoxide and sulfone in houseflies and housefly abdomen homogenates (Metcalf *et al.*, 1964; Tsukamoto and Casida, 1967a); aldicarb sulfoxide and sulfone in houseflies, boll weevils, bollworms, and tobacco budworms (Bull *et al.*, 1967; Metcalf *et al.*, 1966a); 3-hydroxy, 3-keto, and *N*-hydroxymethyl carbofuran in housefly microsomes and whole houseflies (Dorough, 1968b; Metcalf *et al.*, 1968); *N*-hydroxymethyl derivatives of dimetilan in houseflies, German cockroaches, and American cockroaches (Zubairi and Casida, 1965); and ring-hydroxy, ring-hydroxymethyl, and *N*-hydroxymethyl derivatives of Landrin in houseflies (Slade and Casida, 1970).

Differences in Penetration and Metabolism. Differences between the toxicity of varied carbamates to the same insect, or the same carbamate to several insect strains or species, are well known. Several factors may play important roles in these differences, including: rate of penetration of the carbamate through the insect cuticle (for contact toxicity); rate of metabolism of the carbamate; rate of conjugation of primary metabolites; and rate of excretion of carbamate and/or metabolites.

Nine carbamates are rapidly absorbed by several strains of resistant and susceptible houseflies, with little variation between strains or compounds (Metcalf *et al.*, 1967). However, the excretion of metabolites is appreciably higher by some resistant strains than by susceptible strains with all compounds except carbaryl and carbanolate. This higher excretion rate is probably related to more rapid metabolism in the resistant strains. Marked differences in absorption of UC 10854 by resistant and susceptible houseflies are reported by Georghiou and Metcalf (1961b). In 24 hr, only 50% of applied UC 10854 penetrates susceptible flies. Only 4 hr are required to absorb the same amount by resistant flies, but this absorption rate can be decreased to 15 hr by pretreating with piperonyl butoxide. The penetration rate of propoxur into resistant and susceptible flies is about the same, but a higher proportion of metabolites is present as conjugates in resistant strains (Shrivastava *et al.*, 1969). Pretreatment with synergists does not alter penetration rate, but does reduce the amount of metabolite excretion by resistant strains. A resistant strain of cockroaches absorbs more carbaryl, excretes more carbaryl, and contains more conjugated 1-naphthol than do susceptible strains (Ku and Bishop, 1967). On the other hand, susceptible cockroaches contain more unconjugated 1-naphthol and excrete more 1-naphthol.

Absorption and metabolism of carbaryl varies considerably among houseflies, stable flies, boll weevils, and rice weevils (Camp and Arthur, 1967). The latter absorb only 3% of the applied carbamate in 24 hr, while the stable fly absorbs 57% in the same time period. Boll weevils trapped at a cotton gin (cuticle abraded) absorb carbaryl two to three times faster than field-collected weevils. The two weevil species and the housefly contain the same metabolites internally, but the amount of each metabolite differs with each species.

Differences in the rate of metabolism between susceptible and resistant strains of houseflies are well documented. More rapid metabolism occurs in resistant strains with carbaryl (Eldefrawi and Hoskins, 1961), UC 10854 (Georghiou and Metcalf, 1961b), propoxur (Shrivastava *et al.*, 1969), aldicarb (Metcalf *et al.*, 1966a), Isolan (Plapp *et al.*, 1964), and a variety of carbamates (Tsukamoto and Casida, 1967a). Propoxur is also metabolized faster by a resistant strain of mosquitoes than by a susceptible strain (Georghiou, 1965). More rapid degradation by resistant strains has also been implied from a number of studies involving synergists which slow down metabolism rate (Abd El-Aziz *et al.*, 1969; Eldefrawi and Hoskins, 1961; El-Sebae *et al.*, 1964; Fahmy and Gordon, 1965; Georghiou and Metcalf, 1961a,b; Metcalf and Fukuto, 1965; Metcalf *et al.*, 1960; Metcalf *et al.*, 1967).

These metabolic differences also account for the greater carbamate susceptibility of male houseflies and German cockroaches than females (Abd El-Aziz *et al.*, 1969; Tsukamoto *et al.*, 1968). It appears that metabolic differences are also involved in susceptibility variation with age of flies, but reports differ markedly. Tsukamoto and Casida (1967a) found housefly enzyme preparations that metabolize carbamates differ little in activity from adults 1 to 15 days old. On the other hand, the results of Abd El-Aziz *et al.* (1969) suggest that the activity of the carbamate-metabolizing enzymes in flies decreases as the flies age. Green and Dorough (1968) found adult flies most resistant when 5 days old, with greater susceptibility in younger and older flies. However, variations in mortality were apparently not due to differences in metabolism or total cholinesterase content, but were due to variation in the rate at which carbamates reached the site of action. Finally, the diet can affect metabolism rate. Flies fed on milk are less susceptible to carbamates and contain higher carbamate-metabolizing activity than flies fed on sugar and water (Abd El-Aziz *et al.*, 1969; Tsukamoto and Casida, 1967a).

Differences in metabolism of the same compound in different insects can be appreciated from the following examples. Carbaryl is metabolized to at least six products in the German cockroach, three in the housefly, and only one polar product(s) in the milkweed bug (Eldefrawi and Hoskins, 1961). The ratio of hydrolysis to *O*-depropylation of propoxur is high in American cockroaches, intermediate in spruce budworms, German cockroaches, and yellow mealworms, and low in honeybees, codling moths, blowflies, milkweed bugs, yellowfever mosquitoes, and houseflies (Shrivastava *et al.*, 1969). The major organosoluble metabolite in the budworm larva is *N*-hydroxymethyl propoxur, while the major metabolite in the housefly is 5-hydroxy propoxur.

Localization of Activity. Oxidative detoxication of insecticides in mammals occurs primarily in the liver, specifically the liver microsomes (Brodie *et al.*, 1958; Hodgson, 1968; Shuster, 1964; Terriere, 1968). Less is known about the site of detoxication in insects. Microsomes prepared from whole insect homogenates are often inactive, partly because of the variety of endogenous inhibitors present in these homogenates (Chakraborty *et al.*, 1967; Hook *et al.*, 1968; Krieger and Wilkinson, 1969; Matthews and Hodgson, 1966; Tsukamoto and Casida, 1967a). Another reason for inactivity may be due to the fact that methods used to isolate

insect microsomes frequently emulate mammalian procedures. Recent studies illustrate that homogenization and centrifugation techniques necessary for sedimentation of insect microsomes can differ considerably from those used to obtain mammalian microsomes (Brindley and Dahm, 1970; Cassidy *et al.*, 1969; Price and Kuhr, 1969; Schonbrod and Terriere, 1966). Light and electron microscopic examination of insect subcellular fractions should accompany metabolism studies.

Where active preparations have been realized, evidence supports the theory that carbamates are oxidatively metabolized primarily in the microsome fraction of insects (Gemrich, 1967; Kuhr, 1969; Leeling and Casida, 1966; Shrivastava *et al.*, 1969; Tsukamoto and Casida, 1967a). Associated with insect microsomes is cytochrome P-450, a cytochrome thought to be involved in mammalian liver microsomal oxidations. There is evidence for correlation of P-450 with carbamate metabolism by insect microsomes, but other enzymes may also be involved (Abd El-Aziz *et al.*, 1969; Kuhr, 1969; Matthews *et al.*, 1969; Perry and Buckner, 1970; Price and Kuhr, 1969). A soluble tyrosinase or phenolase prepared from houseflies catalyzes the hydroxylation of certain phenols and carbamates (Abd El-Aziz *et al.*, 1969; Metcalf, 1967; Metcalf *et al.*, 1966b), but it is likely that tyrosinase plays only a minor role in the metabolism of aryl methylcarbamates in insects (Kuhr, 1969; Wilkinson, 1968).

There does not appear to be one insect tissue or organ primarily responsible for carbamate metabolism. However, a few comparative studies indicate that gut and fat body are most involved, as discussed later.

METABOLISM OF CARBARYL BY CABBAGE LOOPER TISSUES

Materials and Methods. Carbaryl-carbonyl-C^{14} (26.4 mc per mmole) was purchased from Amersham/Searle Corp., Des Plaines, Ill. Acetone solutions were made from stock by adding pure unlabeled carbaryl to give 60,000 cpm per 20 μg of carbaryl. Samples of carbaryl and its derivatives were supplied by Union Carbide Corp. Nicotinamide adenine dinucleotide phosphate (NADP) and its reduced form ($NADPH_2$), reduced nicotine adenine dinucleotide ($NADH_2$), reduced glutathione (GSH), flavin adenine dinucleotide (FAD), riboflavin-5-phosphate (FMN), glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase were obtained from Calbiochem, Los Angeles, Calif. The synergists piperonyl butoxide, safrole, and isosafrole were purchased from Pfaltz and Bauer, Inc., Flushing, N.Y.

A Packard liquid scintillation spectrometer (Model 3380) measured radioactivity of samples in vials containing 7 ml of 0.55% 2,5-diphenyloxazole in toluene and 2-methoxyethanol (2 to 1). Thin-layer chromatography (tlc) was accomplished on glass plates coated with 0.25 mm of silica gel G and developed in 4 to 1 ether-hexane. Medical X-ray film (Eastman Kodak Co., Rochester, N.Y.) was used for radioautography and ferric chloride-potassium ferricyanide was used to detect unlabeled compounds for cochromatography experiments (Krishna *et al.*, 1962).

Cabbage looper larvae, *Trichoplusia ni*, were reared on broccoli leaves using a modified procedure of McEwen and Hervey (1960). Although the loopers develop through five instars, the first and second are too small for successful dissection so only larger instars were used in experiments. About 1 day before spinning, the larvae appear lighter in color, probably due to an empty gut, and are rather immobile. This stage was termed the "prepupa." The "spinning pupa" was surrounded by silk but had not yet darkened in color.

A looper was placed in a Petri dish containing ice-cold 1.15% (w/v) KCl and its head was removed. While holding the abdomen tip with forceps, the contents were gently squeezed from the cuticle with a flat-tipped spatula. The tissues were spread apart and separated into fat body, gut, Malpighian tubules, and silk glands. The head, cuticle shell, and testes constituted the carcass. The gut was slit in several places and the gut contents removed with forceps. Each tissue was washed in ice-cold 1.15% KCl and held at 0° C until used shortly thereafter. Tissue homogenates were prepared at 0° C using a hand-operated Potter-Elvehjem tissue grinder and cold 1.15% KCl to give two fat bodies or four guts per ml. When younger larvae or other tissues were used, larger amounts of materials were homogenized to give approximately the same protein concentration. Unless indicated otherwise, the homogenate was centrifuged at 800 G for 15 min at 0° C and the supernatant was used as the enzyme source (800 G homogenate).

Aerobic incubations took place at 25° C with shaking for 30 min. Into a 10 ml beaker was placed 20 μl (20 μg) of the carbaryl-C^{14} solution. The acetone was evaporated and a reaction mixture was added to contain the following: phosphate buffer (5.0×10^{-2}M), G-6-P (2.4×10^{-3}M), G-6-P dehydrogenase (1 unit); NADP (1.18×10^{-4}M); and water to give a final volume of 1.5 ml. Reactions were initiated upon addition of 0.5 ml tissue homogenate or addition of whole tissues plus 0.5 ml KCl (3.86×10^{-3}M). Gut homogenates were incubated at pH 6.9 and fat body homogenates at pH 7.9. Protein concentration of homogenates was determined by a modified Biuret method (Cleland and Slater, 1953). In some experiments, air was replaced by nitrogen or 80% carbon monoxide–20% oxygen. Synergists were added as acetone solutions and the solvent evaporated before addition of carbaryl.

After incubation, the beaker contents were poured into a 15 ml centrifuge tube and the beaker was washed with 2 ml of water, followed by 5 ml of anhydrous ether. Both washes were combined with the reaction mixture and mixed thoroughly. The ether fraction was drawn off and the water extracted two more times with 5 ml of ether. A sample of the combined ether extracts and of the remaining water fraction was measured for radioactivity. The ether extract was transferred to a test tube held in a 35° C water bath and evaporated to dryness under vacuum. The residue was dissolved in 200 μl of acetone and spotted on a tlc plate. After development, the plate was placed under film and held at 0° C for 2 to 4 days. Radioactive areas were then scraped and analyzed for radioactivity. Cochromatography was achieved by adding 20 μg of each suspected unlabeled metabolite to the acetone before spotting. Two-dimensional chromatography followed, using the ether-hexane mixture in the first direction and 3 to 1 chloroform-acetonitrile in the second direction.

A small amount of nonenzymic hydrolysis of carbaryl occurs under the stated conditions, and all results were corrected for this. Each experiment was performed at least three times with three different enzyme preparations.

Results and Discussion. The fat body of the cabbage looper is the most active tissue with respect to carbaryl metabolism, but considerable activity is also present in the gut (Figure 3). Almost identical results are obtained with whole tissues as with tissue homogenates (uncentrifuged), indicating no loss of activity on homogenization of the cells. The fat body activity is high not only because of its larger size, but it is also most active on a protein (specific activity) basis. A similar distribution pattern exists in blowfly larvae where fat body is the most

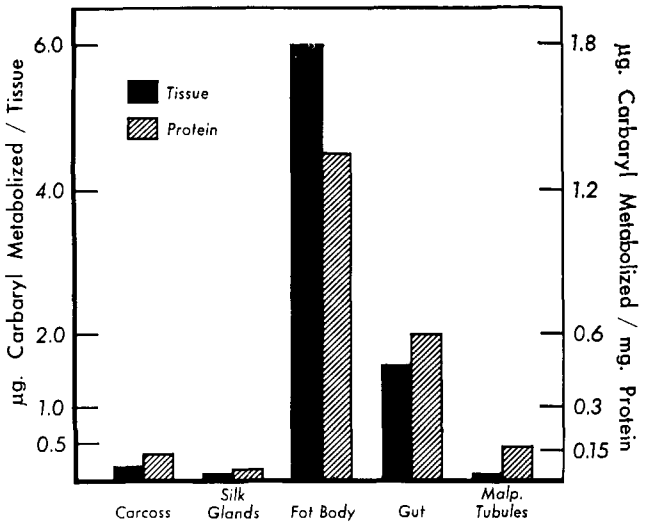

Figure 3. The metabolism of carbaryl by whole tissues and tissue homogenates (uncentrifuged) of fifth instar cabbage looper larvae

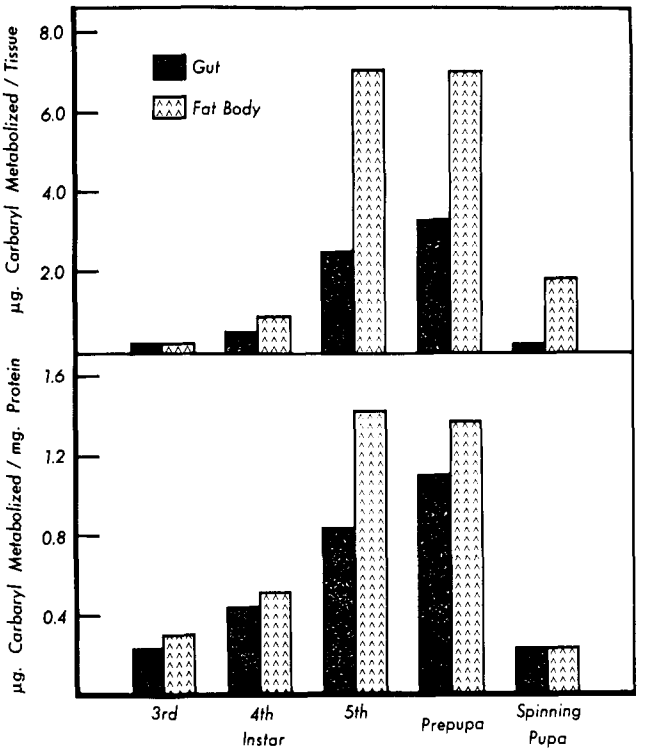

Figure 4. The metabolism of carbaryl by gut and fat body tissues and tissue homogenates (uncentrifuged) from various developmental stages of cabbage looper larvae

effective carbaryl-metabolizing tissue (Price and Kuhr, 1969) Although different tissues were not compared, cockroach fat body has been shown to possess carbanolate-metabolizing activity (Gemrich, 1967). On the other hand, the midgut of adult houseflies is the most active carbamate-metabolizing tissue (Tsukamoto and Casida, 1967a).

Isolated locust fat body is most adept at oxidation of *p*-nitrotoluene, although gastric caecae are also quite active (Chakraborty and Smith, 1964). However, homogenization of any locust tissue results in loss of oxidizing power. The gut tissue from southern armyworm larvae contains considerable aldrin epoxidase activity when compared with other tissues (Krieger and Wilkinson, 1969). Epoxidase distribution is similar in a number of other lepidopterous larvae, although the fat body of loopers possess relatively more epoxidase activity than other lepidopterous fat body (Krieger, 1970). Conversely, the gut of the southern armyworm is more active than the looper gut toward carbaryl. The activation of organophosphate insecticides in the American cockroach occurs in a variety of tissues (Nakatsugawa and Dahm, 1962).

With respect to age of the looper, the most active gut and fat body is present in the last instar and prepupa, with activity falling rapidly in the spinning pupa, or in earlier instars (Figure 4). Again, these differences are not a reflection of size difference in the various developmental stages, since specific activity values parallel tissue values. Armyworm gut epoxidase activity follows a similar pattern with maximum activity present in the last instar (Krieger and Wilkinson, 1969). In the case of blowfly fat body, maximum carbaryl metabolism occurs at about the middle of the larval life, and decreases toward pupation (Price and Kuhr, 1969).

Because the whole homogenates were difficult to extract and chromatograph, the supernatant following 800 G centrifugation was used as the enzyme source (800 G homogenate) for further experiments. This results in about one-third loss of activity with both tissues, but gives consistent results from day to day. Homogenates were used immediately after preparation, since inactivation results upon storage at 0° C. Fifth instars were used because the fat body of the prepupa form is difficult to collect and the guts are rather mushy.

The pH of the incubation medium was varied from 6.0 to 9.0 using phosphate or tris-HCl buffers. Maximum activity is obtained from the fat body homogenate at pH 7.8–8.2 and from the gut homogenate at pH 6.8–7.0, and phosphate buffer gives more activity with both homogenates than tris-HCl. Little difference in activity occurs when isolation and homogenization takes place in phosphate buffer or 0.25 *M* sucrose, instead of 1.15% KCl, and addition of bovine serum albumin causes slight inhibition of activity which is contrary to the results of Tsukamoto and Casida (1967b) with housefly homogenates. The gut contents inhibit metabolism when added to gut or fat body preparations. The inhibitor is probably similar to the one found by Krieger and Wilkinson (1969) in southern armyworms.

The temperature optimum for both homogenates is between 20 and 25° C. This is lower than that reported for carbanolate metabolism (35° C) in cockroach fat body (Gemrich, 1967) or aldrin epoxidation (30° C) in southern armyworm gut homogenates (Krieger, 1970). The carbamate-metabolizing activity of housefly abdomen homogenates is greater at 30° C than at 37° C (Tsukamoto and Casida, 1967a). These results point out the importance of establishing temperature optima for insect preparations rather than using the standard 37° C from mammalian procedures. Reaction rate is linear with respect to enzyme concentration up to at least 1 ml of added homogenate and, after a rapid burst in the first 1 to 2 min, reaction rate is linear with respect to time for about 40 min.

In an average experiment, 0.5 ml of fortified fat body homogenate (1 fat body equivalent, 3.34 mg of protein) converts 4.22 µg of carbaryl to metabolites in 30 min, 34% of these being water-soluble. The gut homogenate (2 gut equivalents, 3.35 mg of protein) metabolizes 2.76 µg of carbaryl, 23% to water-soluble products. The distribution and tentative identity of the ether-soluble metabolites are shown in Table II. Approximately 4 to 5% of the total radioactivity is present as unknown materials, most of which remain at the

Table II. Tentative Identity of Ether-Soluble Metabolites of Carbaryl Formed in Cabbage Looper Gut and Fat Body Homogenates

Metabolite Formed[a]	Percent of Total Ether-Soluble Metabolites	
	Fat Body	Gut
5,6-Dihydro-5,6-dihydroxy carbaryl	23.8	14.1
N-Hydroxymethyl carbaryl	59.8	76.0
4-Hydroxy carbaryl	5.2	4.3
5-Hydroxy carbaryl	6.3	1.5

[a] The incubation medium consisted of phosphate buffer, KCl, G-6-P, G-6-P dehydrogenase, NADP, 800 G tissue homogenate, and 20 μg of carbaryl, as described in the Materials and Methods section. Incubations took place for 30 min at 25° C.

origin with very small amounts chromatographing between the dihydrodihydroxy derivative and hydroxymethyl carbaryl. Identity of the dihydrodihydroxy derivative is based only on its R_f value. The 4-hydroxy, 5-hydroxy, and hydroxymethyl metabolites cochromatograph with the corresponding unlabeled compounds after two-dimensional chromatography. No desmethyl carbaryl or 7-hydroxy carbaryl could be detected as metabolites.

Although both tissues form the same metabolites, their distribution is slightly different. The gut favors the hydroxymethyl metabolite with lesser amounts of ring-hydroxylated derivatives. In housefly microsomes, 4- and 5-hydroxy carbaryl account for almost 75% of the ether-soluble metabolites (Kuhr, 1969). The large production of the *N*-hydroxymethyl metabolite by the loopers is interesting, since it has been implied that *N*-methyl hydroxylation of carbamates by insects has little general significance as a detoxication mechanism (Wilkinson, 1968). This may be true for the metabolism of certain carbamates in houseflies (Fahmy *et al.*, 1966), but it is not true for carbaryl metabolism in cabbage loopers or, probably, propoxur metabolism in spruce budworm larvae (Shrivastava *et al.*, 1969).

The effect of altering the conditions of the standard incubation medium on activity is shown in Table III. Oxygen is essential for activity. Incomplete removal of oxygen probably accounts for the lack of total inhibition. Both tissue homogenates appear to contain a source of $NADPH_2$, but loss of activity occurs on omission of this cofactor. $NADH_2$ does not substitute for $NADPH_2$, and a generating system for the latter is slightly more effective than adding $NADPH_2$. Although G-6-P dehydrogenase was routinely added to the medium, the tissues contain sufficient endogenous dehydrogenase. The cofactors FAD and FMN inhibit metabolism. The activity of both homogenates is due to enzymic activity, since no metabolism occurs with boiled preparations.

Table III. Effect of Various Materials on the Activity of Cabbage Looper Gut and Fat Body Homogenates Toward Carbaryl

Incubation Medium Additions or Omissions[a]	Percent Activity	
	Fat Body	Gut
None	100.0[b]	100.0[c]
Minus oxygen	20.5	28.5
Minus NADP	70.7	57.3
Minus NADP plus $NADPH_2$	97.1	91.5
Minus NADP plus $NADH_2$	65.3	62.4
Plus FAD (10^{-3}M)	73.7	71.6
Plus FMN (10^{-3}M)	59.3	61.4
Plus EDTA (10^{-3}M)	96.6	94.6
Plus KCN (10^{-3}M)	92.3	97.5
Plus glutathione (10^{-3}M)	99.5	99.0
Plus nicotinamide (10^{-3}M)	55.7	76.8
Plus carbon monoxide (80%)	7.3	16.9

[a] Incubation medium as described in Table II. [b] In 30 min, 1 fat body equivalent (3.34 mg protein) metabolizes 4.22 μg of carbaryl. [c] In 30 min, 2 gut equivalents (3.35 mg protein) metabolize 2.76 μg of carbaryl.

Table IV. The Effect of Metal Ions on the Activity of Cabbage Looper Gut and Fat Body Homogenates Toward Carbaryl

Metal Ion Added to Incubation Medium[a]		Percent Activity[b]	
		Fat Body	Gut
None		100.0	100.0
Mn^{2+}	$10^{-3}M$	107.7	111.2
Mg^{2+}	$10^{-3}M$	102.0	97.5
Ni^{2+}	$10^{-3}M$	76.5	97.5
Co^{2+}	$10^{-3}M$	73.8	108.5
Fe^{3+}	$10^{-3}M$	87.0	105.1
Fe^{2+}	$10^{-3}M$	63.4	114.0
	$10^{-4}M$	95.6	106.3
Cu^{2+}	$10^{-3}M$	2.7	3.8
	$10^{-4}M$	30.8	27.4
	$10^{-5}M$	89.9	96.7
Cu^{+}	$10^{-4}M$	80.6	81.1
	$10^{-5}M$	92.2	92.2

[a] Incubation medium as described in Table II. [b] Activity as described in Table III.

Nicotinamide, often added to mammalian microsome preparations to inhibit pyridine nucleotidases, inhibits carbaryl metabolism, as it does in housefly microsomes (Kuhr, 1969). It appears that, in many cases, nicotinamide serves no useful purpose when added to insect homogenates or microsomes (Chakraborty and Smith, 1964; Chakraborty *et al.*, 1967; Fenwick, 1958; Krieger and Wilkinson, 1969; Schonbrod *et al.*, 1965). Reduced glutathione, KCN, and EDTA at 10^{-3}M have little effect on metabolism.

The inhibition of metabolism by carbon monoxide suggests that cytochrome P-450 may be involved. Difference spectra of the tissue homogenates reveal the presence of a peak at 450 mμ, which shifts to 420 mμ on addition of deoxycholate. However, turbidity and sedimentation problems made quantitation impossible.

The only metal ion that causes severe inhibition of metabolism by the homogenates is cupric ion (Table IV). This ion also markedly inhibits epoxidase activity in southern armyworm gut microsomes (Krieger and Wilkinson, 1969). Of particular interest is the difference in the effect of Ni^{2+}, Co^{2+}, Fe^{2+}, and Fe^{3+} on gut and fat body. This variation is not a result of pH differences of the two incubation mediums because similar results are obtained when the buffers are reversed. Coupled with the different pH optima and distribution of metabolites, these ion differences suggest that perhaps a different enzymic mechanism is operating in the two tissues. Of course, endogenous materials in the two preparations may be responsible for these differences. The fact that both require oxygen, both are stimulated on addition of $NADPH_2$, and both are inhibited by methylenedioxyphenyl synergists (Table V) suggests that both contain a mixed-function oxidase.

Preliminary experiments on subcellular fractionation of gut homogenates indicate that activity lies predominantly in the microsome fraction. However, successful fractionation of the fat body has not been achieved. Work is continuing on microsome preparations. With a "purer" source of enzyme, some of the differences between the tissues may be resolved. *In vivo* degradation of carbaryl by the loopers is rapid, and the water-soluble metabolites are presently being characterized.

Table V. The Effect of Synergists on the Activity of Cabbage Looper Gut and Fat Body Homogenates Toward Carbaryl

Inhibitor Added to Incubation Medium[a]	Inhibition (molar I_{50})	
	Fat Body	Gut
Safrole	8.0×10^{-5}	8.0×10^{-5}
Isosafrole	3.6×10^{-5}	7.5×10^{-6}
Piperonyl butoxide	1.0×10^{-6}	5.0×10^{-7}

[a] Incubation medium as described in Table II.

CONCLUSIONS

The persistence of carbamate insecticide chemicals in plants and insects is often limited by the oxidative enzyme systems present within the organism. Hydrolysis of the carbamate-ester bond is usually slow and oxidation, particularly hydroxylation, of the aromatic ring, ring substituents, and the carbamate *N*-methyl group converts the lipophilic toxicant to primary metabolites which, in most instances, are readily conjugated to water-soluble derivatives. Although the metabolites formed in plants, insects, and mammals are similar, differences exist in their rate of formation, their conjugating moieties, and their ultimate fate within the organism.

Very little is known about the plant enzymes responsible for carbamate metabolism, principally because no satisfactory *in vitro* enzyme system has been discovered. Peroxidases and tyrosinases seem likely candidates, but Kuhr and Casida (1967) found little metabolism after incubating carbamates with horse radish peroxidase or mushroom tyrosinase. Aldrin epoxidase activity in pea plants appears to be present in the "soluble" fraction rather than the "microsome" fraction and differs from that described in animal tissues (Lichtenstein and Corbett, 1969). Whether real differences exist in the carbamate-metabolizing enzymes of plants and animals awaits further study.

Although many of the carbamate water-soluble conjugates formed in plants are cleaved by β-glucosidase, positive identification of the intact conjugates is lacking. In only one study has the chemical structure of a plant conjugate been characterized, namely, the glucoside of the phenol of 3-ketocarbofuran (Metcalf *et al.*, 1968). Information on the fate of these conjugates after ingestion by mammals is also lacking. Dorough and Wiggins (1969) found that hydroxylated-carbaryl conjugates are rapidly eliminated from rats.

In the case of insects, more is known about possible enzymes catalyzing carbamate metabolism. As yet, not enough data is available to indicate general localization of detoxication enzymes in certain insect tissues or organs, but fat body and gut may prove to be the most important metabolic sites. Subcellularly, a microsomal mixed-function oxidase, perhaps involving cytochrome P-450, appears to be of primary importance. However, *in vitro* preparations from different insects, or even from different tissues of the same insect, vary considerably in pH optima, temperature optima, metal ion requirements, and metabolite formation, which seems to imply that a complex of enzyme systems is involved.

Recent studies show that *in vitro* preparations, similar in activity to mammalian liver microsomes, can be obtained from insects by avoiding the use of whole animals. Homogenates and/or microsomes from individual tissues or parts of insects, such as abdomens, rapidly metabolize a wide range of carbamates. These *in vitro* systems permit the characterization of primary oxidative metabolites which exist *in vivo* only as conjugates. Although microsomal preparations provide a "purer" source of enzyme, tissue homogenates can be quite active. The latter negate the use of large numbers of insects and save considerable time and effort in fractionation of subcellular particles. *In vitro* methods allow careful study, not only of metabolite formation, but facilitate studies on structure-activity relationships, action of synergists, biochemical genetics of resistance, and selective toxicity. However, it is important to compare *in vivo* and *in vitro* results, a step which is often neglected. The chemical identification of insect conjugates and the mechanisms involved in their formation are other areas that should receive further attention.

The biochemical changes that take place within an insect during its lifetime, particularly during molting and pupation, greatly alter the effectiveness with which the insect can detoxify carbamates. Sex, diet, and chemical exposure also contribute to the myriad of factors that govern metabolism. These factors must be seriously considered when determining the fate of any compound within an insect. As more is learned about these biochemical changes, practical applications of this knowledge may contribute significantly to field control of insect pests.

Thus, although a considerable amount of information exists on the metabolism of carbamates in plants and insects, there is a great deal more to be done. Such studies are not only needed for the continued effective use of carbamate insecticides and the development of new carbamates, but also aid in the development of any new insecticide for the control of insects foraging on food and fiber crops throughout the world.

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