in the chicken and rat. However, the tissue levels of radioactivity in the rabbit, representing clopidol and metabolites, were lower than those in either the chicken or rat, and were below the limits of detection in all tissue 32 hr after administration of the last dose.

The nature of the radioactivity excreted by the rat and chicken has not been investigated, but this study has shown that in the rabbit, the major pathway of metabolism involves hydroxylation and then glucuronidation of the resulting alcohol.

ACKNOWLEDGMENT

The authors are grateful to Freddy Brown of Dow Chemical Co., Kings Lynn, for his help, advice, and encouragement.

LITERATURE CITED

Chasseaud, L. F., Hawkins, D. R., Cameron, B. D., Fry, B. J., Saggers, V. H., *Xenobiotica* **2**, 269 (1972).
Dobbs, H. E., *Anal. Chem.* **35**, 783 (1963).
Fujimoto, J. M., Haarstad, V. B., *J. Pharmacol.* **165**, 45 (1969).
Lewis, J. D., *Int. J. Appl. Radiat. Isotopes* **22**, 39 (1972).
Patterson, M. S., Greene, R. C., *Anal. Chem.* **37**, 854 (1965).
Ryley, J. F., Betts, M. J., *Advan. Pharmacol. Chemother.* **2** (1973).
Smith, G. N., *Poultry Sci.* **48**, 420 (1969).
Smith, G. N., Watson, B. L., *Poultry Sci.* **48**, 437 (1969).
Stevenson, G. T., U.S. Patent 3,206,358 (1965).
Stock, B. L., Stevenson, G. T., Hymas, T. A., *Poultry Sci.* **46**, 485 (1967).

Received for review June 24, 1974. Accepted October 29, 1974.

Carbaryl Penetration into and Metabolism by Alfalfa Leafcutting Bees, *Megachile pacifica*

Guirguis N. Guirguis and William A. Brindley*

Carbaryl metabolism by and penetration into adult alfalfa leafcutting bees, *Megachile pacifica* (Panzer) (= *rotundata* Fabr.), were studied in relation to sex, age, and exposure to piperonyl butoxide. Unchanged carbaryl was a principal compound isolated from the bees after 4 hr, being more prominent in 4-day-old males (48% of extractable metabolites) and less prominent in 4-day-old females (22%) and 1-day-old males (26%). 1-Naphthol, 5,6-dihydro-5,6-dihydroxycarbaryl, 5-hydroxycarbaryl, and carbaryl were recovered from acid hydrolysis of water-soluble conjugates. Two minor organosoluble metabolites were not identified as were none of the conjugating moieties. Carbaryl persistence in the bees was closely related to carbaryl susceptibility. The results demonstrated that the differences in carbaryl LD_{50} values with and without piperonyl butoxide treatment are more meaningful than the quotient of these values.

Lee and Brindley (1975) have reported that adults of the alfalfa leafcutting bee, *Megachile pacifica* (Panzer) (= *rotundata*), are particularly tolerant to carbaryl. The carbaryl tolerance was related to sex and age. Dose–mortality studies showed the carbaryl LD_{50} to be similar for 1-day-old males and 1- and 4-day-old females (240, 245, and 262 μg/g, respectively). Four-day-old males, however, were much more susceptible having an LD_{50} value of 51 μg/g.

Estimations of carbaryl–piperonyl butoxide synergist ratios did not correlate well with the LD_{50} data. Brattsten and Metcalf (1970, 1973) have used the quotient of lethal dose concentrations of carbaryl alone divided by the lethal dose concentrations of carbaryl with the synergist piperonyl butoxide to provide carbaryl–piperonyl butoxide synergist ratios. These ratios provide convenient values for estimating and ranking microsomal oxidase activity in various insects. Lee and Brindley (1975), however, have suggested that use of such a synergist ratio can provide misleading results, and that the difference in the lethal dose levels may be a better *in vivo* estimate of carbaryl detoxication potential.

Treatment of alfalfa leafcutting bees with piperonyl butoxide prior to carbaryl treatment reduced the LD_{50} values such that the quotients (synergist ratios) were 12, 53, and 15 for 1-day-old males, 4-day-old males, and 4-day-old females, respectively (Lee and Brindley, 1975).

Therefore, this study was designed to study carbaryl metabolism and penetration in adult male (1 and 4 days old) and female (4 days old) alfalfa leafcutting bees and to elucidate the effects of piperonyl butoxide on the metabolism of carbaryl.

The alfalfa leafcutting bee is of great importance and efficiency in pollination of alfalfa for seed. Alfalfa seed acreage in 1972 was 378,100 acres, with an estimated seed value of more than $46,000,000 (United States Department of Agriculture, 1973). Idaho, Utah, Nevada, Washington, Oregon, and California had only 48% of this acreage but more than 85% of the revenues and average yields of 480 pounds per acre. The remaining seven states listed as alfalfa seed production areas averaged 90 pounds of seed per acre. The yield difference is due to several factors, but especially due to the presence of effective alfalfa leafcutting bee management.

MATERIALS AND METHODS

Insect and chemical sources, insect maintenance, and chemical applications were as described by Lee and Brindley (1975) except that carbaryl doses were made at 1 μg/bee under light CO_2 anaesthesia in all cases.

1-Naphthyl *N*-methyl-^{14}C-carbamate (methyl labeled) with a specific activity of 26.4 mCi/mmol and 1-naphthol-*1*-^{14}C with a specific activity of 15.2 mCi/mmol were purchased from the Amersham–Searle Corp., Arlington Heights, Ill. Both had radiochemical purities of more than 98%, constituted one spot in our tlc system, and were not further purified. The 1-naphthol-*1*-^{14}C was synthesized to ring-labeled carbaryl by reaction with an excess of methyl isocyanate (Dorough and Casida, 1964). Thin-layer chromatography (tlc) plates coated with a 1-mm thick silica

Department of Biology, Utah State Agricultural Experiment Station, Utah State University, Logan, Utah 84322.

gel G layer (Brinkmann Instruments Inc.) were used to purify the ring labeled material. Nonradioactive carbaryl (99%) was used to adjust the final specific activities to 1 μCi/mmol for *N*-carbaryl-*methyl*-^{14}C and 0.4 μCi/mmol for naphthyl-^{14}C-carbaryl.

The radioactivity of collected samples was determined with a Packard Tri-Carb liquid scintillation counter and counts per minute were corrected for geometry and efficiency (generally 85%) with both internal and external standardization. At least two and often more than two replicates were used for each experiment. Counting solutions (PPO, POPOP, toluene, and Cab-O-Sil) were purchased from the Packard Instrument Co., Inc., Downer's Grove, Ill.

Radioactive fractions in an aqueous acid trap and carbon dioxide trap were collected in a metabolism apparatus as described by Casida *et al.* (1968). Groups of ten treated bees were pooled for each time interval and were placed in the metabolic chamber at 30° with an air flow of about 4–6 ml/min. Insects which were kept for 4 hr or more were provided with a 25% sucrose solution by placing a large BEEM (=1001, Better Equipment for Electron Microscopy, Bronx, N.Y.) capsule containing sucrose-soaked cigarette filter material in the metabolism chamber.

Carbaryl penetration was estimated at times of 0, 5, 10, 20, and 30 min and 1, 2, 4, and 8 hr after carbaryl application. Each replicate of insects was transferred from the holding flask and was rinsed three times with 5-ml portions of cold acetone. The washing was combined in a scintillation vial and evaporated to near dryness with a gentle air stream, and the radioactivity was measured in a mixture of 4 g of PPO and 0.05 g of POPOP per liter of toluene. Insects for 0 time were treated topically and immediately dipped into 5 ml of acetone and then processed as above.

Insects used for determination of metabolite distribution and identity were rinsed externally with acetone as described above, and this washing was analyzed separately. Any excreta in the metabolism chamber were removed by rinsing three times each with 3-ml portions of water and 2-ml portions of diethyl ether. The rinsed insects and vial rinses were combined and macerated for 2 min with an Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.) and the homogenate centrifuged for 5 min to facilitate filtration. The supernatant was filtered with a Büchner funnel and vacuum through a coarse, ashless filter paper and the pelleted material in the centrifuge tube was again homogenized with 6 ml of diethyl ether for 2 min and then filtered.

The ether was poured into a separatory funnel and thoroughly washed several times with water. The water washing and residues collected on the filter papers were rewashed twice with 5-ml portions of chloroform. The chloroform and ether extracts were combined to make the organosoluble fraction and the water extracts constituted the water-soluble fraction.

The ether-soluble and organosoluble extracts were evaporated to near dryness with a gentle air stream at room temperature and redissolved in acetone for tlc or counted in a scintillation mixture containing 3.29 g of PPO, 0.08 g of POPOP, and 40 g of Cab-O-Sil per liter of toluene.

The ^{14}C in the insoluble residues on filter papers was determined by combustion using an oxygen flask combustion technique (Davidson and Oliverio, 1967). A combustion flask and infrared ignition lamp from Thomas Scientific Co., Philadelphia, Pa., were used. After combustion to $^{14}CO_2$, 10 ml of a mixture from 270 ml of phenethylamine, 270 ml of methanol, 5 g of PPO, 100 mg of POPOP, and 460 ml of toluene was added to the flask and swirled over the surfaces for 5 min. After transfer to a scintillation vial, the flask surfaces were rinsed twice more with 3-ml each portions of the phenylethylamine scintillation mixture and counted at efficiencies of about 50–55%.

Thin-layer chromatography was conducted in two dimensions on 20 × 20 cm plates with silica gel G (Brinkmann Inc., Cantiague, N.Y.) prepared in our laboratory at 0.3-mm layer thickness. The plates were activated for 1 hr at 110° and developed first in a sealed chamber with a solvent-saturated atmosphere with 4:1 (v/v) diethyl ether–*n*-hexane. Immediately after drying, the plates were developed in the second direction to minimize decomposition (Dorough and Casida, 1964) in 4:1 (v/v) methylene chloride–acetonitrile. In the case of organosoluble fractions, nonradioactive interfering materials were cleared from the plate by development in benzene.

The developed and dried tlc plates were wrapped with one layer of cellophane and kept in the dark for 1 month at room temperature while pressed against Kodak "No-Screen" medical X-ray film (Eastman Kodak, Rochester, N.Y.). The films were developed according to the manufacturer's directions and the corresponding areas were scraped from the plates and counted with 3.93 g of PPO, 0.09 g of POPOP, and 40 g of Cab-O-Sil per liter of toluene as for the organosoluble and water-soluble fractions.

The R_f values were compared with those from literature references and authentic standards of carbaryl, 4-hydroxy-1-naphthyl *N*-methylcarbamate, 5-hydroxy-1-naphthyl *N*-methylcarbamate, and 1-naphthyl-*N*-hydroxyl methylcarbamate. Other detection systems included both long and short ultraviolet irradiation, iodine vapors, and a variety of chromogenic reagents described by Krishna *et al.* (1962) and Dorough and Casida (1964). These included 15% aqueous KOH, 15% aqueous KOH followed by Gibb's reagent (0.1% *N*-2,6-trichloro-*p*-benzoquinone imine in acetone), 1% ninhydrin in pyridine followed by heat at 100° for 30 min, and 15% aqueous KOH followed by 1 *M* acetic acid in methanol and 0.1% methanolic *p*-nitrobenzenediazonium fluoroborate applied after drying.

Water-soluble metabolites were hydrolyzed (Dorough and Wiggins, 1969) in 0.9 *M* HCl for 1 hr in a boiling water bath and were extracted after cooling with dichloromethane. The hydrolysate was then analyzed by tlc as above. Insects or samples awaiting processing or analysis were frozen and stored at −20° for not more than 30 days. Scintillation mixtures of samples were kept for not more than 5 days at room temperature.

RESULTS

Carbaryl Penetration. The per cent of carbaryl absorption (penetration) was calculated from the difference between the total radioactivity of the applied dose and the radioactivity of the surface washing (Table I).

Graphs of the log of the per cent of carbaryl not penetrated (y) *vs.* the time after application (t) revealed three phases of linear absorption from 0 to 5, 5 to 20, and 30 to 480 min. Applicable equations, derived by least squares and regression analysis, for $\log y = c \log e - Kt \log e$, permitted calculation of times for half-absorption ($t_{1/2}$), the per cent unabsorbed at time 0 (a), and the slope or rate constant of the absorption phase (K) (Table II). These calculations were based on the assumption that the rate of absorption was inversely proportional to the per cent of carbaryl which was unabsorbed ($y' = -Ky$) as a function of time and that these are related by $y = ae^{-Kt}$. Correlation coefficients for the regression curves were −0.94 to −1.00. These calculations assumed that the amounts of radioactivity lost by CO_2 evolution, volatile materials, and that absorbed in or on the cuticle and not removable by solvent, were negligible. Evidence that this is a valid assumption appears in Table III showing the potential error to be not greater than 5%.

By the end of 8 hr, more carbaryl had penetrated into the more susceptible 4-day-old males due to a more rapid

Table I. Percentage of Carbaryl-^{14}C Penetrated and Penetration Rates in Alfalfa Leafcutting Bees[a]

Time after treatment, min	Penetrated %: 1-Day-old males	4-Day-old males	4-Day-old females
0	0.55	0.28	1.80
5	14.43	20.21	13.09
10	17.93	23.45	15.59
20	19.95	26.15	18.00
30	22.12	27.55	19.30
60	24.26	29.51	21.49
120	27.76	31.82	25.02
240	32.87	34.75	31.25
480	41.66	42.44	39.96
240 P.B. treatment	33.22	35.32	31.98

[a] At various times following topical treatment at a dosage of 1 μg/bee.

rate of penetration during the first 20 min after application. The most tolerant bees, the 4-day-old females, had accumulated less of the dose in 8 hr than the 1-day-old males due to less rapid absorption, although a simple t test for differences showed these to be not significantly different at the 5% level. The effect of piperonyl butoxide upon penetration after 4 hr was not significant (Table I).

Radioactivity Recovery. Radioactive carbaryl or metabolites were measured in external rinses, a CO_2 trap, a trap for volatiles, an organic solvent fracion, an aqueous fraction, and the insoluble combusted residue (Table III). Recoveries ranged from 92.48 to 99.23% of the dose applied. Because of the high recovery rates and their relative consistency, the data reported hereafter have been corrected to 100% recovery.

Evolution of $^{14}CO_2$ was generally higher and more related to time after application for the methyl-labeled carbaryl (Table III). The most tolerant insects (4-day-old females) evolved more $^{14}CO_2$ than the less tolerant (1-day-old males) and the least tolerant (4-day-old males), which suggests a possibly higher metabolic rate associated with tolerance. Entrapment of other volatiles from either labeled carbaryl was similar and not related to either time or susceptibility.

Treatment with piperonyl butoxide had no effect upon $^{14}CO_2$ recovery from methyl-labeled carbaryl but did decrease the recovery slightly from ring labeled carbaryl. The recovery of other volatile products was either unchanged or slightly reduced by piperonyl butoxide treatment (Table III).

More impressive changes related to time after treatment and the susceptibility of insects were discovered in the organic solvent and aqueous fractions of homogenized bees (Table III). Radioactivity in the organic solvent fraction decreased with time in the more tolerant insects (1-day-old males or 4-day-old females) while the radioactivity in the aqueous fraction increased. In the 4-day-old females, the decrease in organic soluble radioactive compounds was slightly greater than in the similar but less tolerant 1-day-old males. The residual activity which could not be extracted and was determined by combustion was generally less in the 4-day-old females while the 4-day-old males were intermediate in this aspect.

Table II. Times for Half-Absorption ($t_{1/2}$), the Per Cent (y) Unabsorbed at $t = 0$ (a), and the Absorption Rate Constant (K)[a]

Time after application (t), min	1-Day-old male: $t_{1/2}$	a	K	4-Day-old male: $t_{1/2}$	a	K	4-Day-old female: $t_{1/2}$	a	K
0–5	22.79	99.5	0.0302	15.46	99.8	0.0447	25.04	99.8	0.0276
5–20	134.25	86.7	0.0041	95.32	81.3	0.0051	153.11	88.1	0.0037
30–480	675.09	80.2	0.0007	709.94	71.3	0.0005	717.20	82.6	0.0007

[a] Where $y = ae^{-Kt}$ for three specific absorption phases after applying 1 μg of carbaryl to alfalfa leafcutting bees.

Table III. Distribution of ^{14}C Recovered in Fractions (% of Total Applied) of Adult Alfalfa Leafcutting Bees[a]

Sex, age	hr	% ^{14}C recovered in: External rinse	CO_2 trap: N-CH_3	CO_2 trap: Naphthyl	Volatile trap: N-CH_3	Volatile trap: Naphthyl	Org solvents fraction	Aq fraction	Combusted residues	Total recovery
Male, 1 day	1	76.74	0.13	0.06	0.05	0.13	10.99	7.16	0.90	95.97
	2	72.24	0.24	0.08	0.05	0.14	9.26	15.37	1.97	99.13
	4	67.13	0.53	0.10	0.07	0.17	8.44	19.76	3.20	99.13
	8	58.34	0.98	0.10	0.08	0.19	7.65	23.90	3.98	94.93
Piperonyl butoxide	4	66.78	0.47	0.04	0.02	0.02	18.70	10.62	3.18	99.77
Male, 4 days	1	70.49	0.08	0.09	0.04	0.09	15.57	9.88	1.18	97.24
	2	68.18	0.14	0.07	0.05	0.11	18.45	10.34	1.53	98.69
	4	65.25	0.36	0.09	0.05	0.11	18.29	10.53	2.88	97.36
	8	58.56	0.74	0.11	0.06	0.14	18.87	12.73	3.52	94.48
Piperonyl butoxide	4	64.68	0.36	0.02	0.02	0.02	18.20	6.58	3.03	92.87
Female, 4 days	1	78.51	0.15	0.08	0.04	0.06	9.85	9.01	0.61	98.17
	2	74.98	0.39	0.08	0.10	0.07	7.18	13.79	2.38	97.72
	4	69.75	0.90	0.10	0.11	0.13	6.46	19.35	2.66	99.23
	8	60.04	1.28	0.08	0.19	0.14	5.01	23.46	3.31	95.00
Piperonyl butoxide	4	68.02	0.90	0.04	0.11	0.11	17.22	7.30	2.38	95.93

[a] At varying intervals (hr) after topical treatment with 1 μg/bee of labeled carbaryl.

Table IV. Metabolic Products of Radiolabeled Carbaryl Recovered in the Organosoluble Fraction of Leafcutting Bees[a]

Product	% recovd of	% recovered: 1-Day-old males 1 hr	2 hr	4 hr	4 hr, PB	4-Day-old males 1 hr	2 hr	4 hr	4 hr, PB	4-Day-old females 1 hr	2 hr	4 hr	4 hr, PB
5,6-Dihydro-	a	0.14	0.11	0.09		0.46	1.15	0.98		0.15	0.13	0.09	
5,6-di-	m	0.74	0.44	0.32		1.76	3.94	3.31		0.78	0.61	0.23	
hydroxy-													
carbaryl													
N-Hydroxy-	a			0.12			0.04	0.15		0.06	0.08	0.12	
methylcar-	m			0.42			0.14	0.51		0.31	0.37	0.46	
baryl													
4-Hydroxy-	a	0.07	0.06	0.05		0.19	0.22	0.25		0.11	0.05	0.04	
carbaryl		0.37	0.24	0.18		0.73	0.75	0.84		0.57	0.23	0.15	
5-Hydroxy-	a	0.42	0.34	0.28		0.43	0.70	1.01	0.01	0.53	0.09	0.05	
carbaryl	m	2.22	1.37	0.98		1.64	2.40	3.41	0.04	2.76	0.42	0.19	
Carbaryl	a	10.35	8.41	7.47	18.74	14.25	15.16	14.11	19.27	8.54	6.48	5.60	17.95
	m	54.73	33.84	26.26	63.76	54.45	51.97	47.67	72.09	44.46	30.20	21.54	70.23
N-Hydroxy-	a	0.17	0.15	0.09		0.17	0.43	1.04	0.04	0.31	0.20	0.17	
carbaryl	m	0.90	0.61	0.32		0.65	1.47	3.51	0.15	1.61	0.93	0.65	
1-Naphthol	a	0.30	0.28	0.28		0.51	0.90	1.11	0.02	0.33	0.32	0.35	
	m	1.59	1.13	0.98		1.95	3.09	3.75	0.07	1.72	1.49	1.35	
Unknown A	a			0.04			0.04	0.07					
	m			0.14			0.14	0.24					
Unknown B	a			0.09			0.05	0.07				0.12	
	m			0.31			0.17	0.24				0.46	

[a] On percentage basis of total ^{14}C applied (a) and total metabolites recovered (m).

Table III shows the 4-day-old males to have, at least quantitatively, much less ability to decrease the organosoluble carbaryl and metabolites and to transfer these into an aqueous soluble fraction. The level of radioactivity in the organosoluble fraction was essentially stable after 2 hr, probably reflecting a reduced metabolic capability or loss of ability to conjugate hydroxylated metabolites. This time period also is similar to the end of rapid absorption of the carbaryl dose and beginning of a subsequent phase of steady absorption (Table II).

Treatment of the insects with piperonyl butoxide (Table III) markedly increased the radioactivity in the organosoluble fraction and decreased radioactivity in the aqueous fraction for 1-day-old males and 4-day-old females. There were no changes in the levels of activity in the organosoluble fraction in the susceptible 4-day-old males and less change in the aqueous fraction as compared to more tolerant insects. Even with piperonyl butoxide treatment, significant amounts of radioactivity appeared in the water-soluble fraction of all of the insects.

The unextractable residue, analyzed by combustion, contained relatively little (less than 4% of the total dose) radioactivity but this accumulation was related to the time of exposure (Table III). Piperonyl butoxide treatment had little effect.

Organosoluble Metabolites. Table IV lists the metabolites and their quantities isolated 1, 2, and 4 hr after treatment of the bees. Six hydroxylated metabolites (5,6-dihydro-5,6-dihydroxycarbaryl; 4-hydroxycarbaryl; 5-hydroxycarbaryl; *N*-hydroxycarbaryl; *N*-hydroxymethylcarbaryl; 1-naphthol) accumulated with time in 4-day-old males and decreased with time in 4-day-old females and 1-day-old males. Carbaryl itself decreased with time in the more tolerant insects, but its concentration was essentially stable in the susceptible 4-day-old males.

N-Hydroxymethylcarbaryl and two unidentified metabolites appeared in small amounts in the later phases of the experiment. These increased slightly with time in each of the insects studied (Table IV).

Table IV does not provide a basis for identifying the most important metabolites due to the rapid transfer of radioactivity into the aqueous fraction. It is clear, however, that carbaryl persistance is closely related to susceptibility as the tolerant insects (1-day-old males, 4-day-old females) reduced the carbaryl concentration to slightly more than 20% in the first 4 hr. Susceptible (4-day-old) males could not as effectively decrease the carbaryl concentration.

Piperonyl butoxide treatment (Table IV), which essentially prevented the appearance of any of the organosoluble metabolites, raised carbaryl concentrations by about two- to threefold in the tolerant insects. Clearly, carbaryl persistance was most potentiated in the tolerant insects which show the lowest piperonyl butoxide carbaryl synergist ratios (12, 15) as compared to the high ratio (53) for 4-day-old males (Lee and Brindley, 1975).

Water-Soluble Metabolites. Five water-soluble metabolites were recovered (Table V) from all three insect groups. None were identified. It was presumed they were conjugated in some fashion. These were combined and hydrolyzed in acid, and the hydrolysate was subjected to tlc as for the organosoluble fractions. The hydrolysate contained carbaryl, 1-naphthol, *N*-hydroxycarbaryl, 5-hydroxycarbaryl, and 5,6-dihydro-5,6-dihydroxycarbaryl. There was no evidence for the presence of the other organosoluble metabolites listed in Table IV.

The water-soluble metabolites increased with time in each insect group and the order of concentration was the same (A $\gg$ D > E > C > B) (Table V). Metabolite A was the most predominant and, like metabolites C, D, and E, was isolated from *N*-methyl-labeled carbaryl. Metabolite B was insolated from ring-labeled carbaryl. Metabolites A, C, D, and E contained both the naphthyl ring and *N*-methylcarbamic acid moiety, whereas metabolite B con-

Table V. Metabolic Products of Radiolabeled Carbaryl Recovered in the Water-Soluble Fraction of Leafcutting Bees[a]

Products	% of	% recovery												R_f	
		1-Day-old males				4-Day-old males				4-Day-old females					
		1 hr	2 hr	4 hr	4 hr, PB	1 hr	2 hr	4 hr	4 hr, PB	1 hr	2 hr	4 hr	4 hr, PB	*b*	*c*
A	a	3.64	7.69	9.04	10.65	4.05	4.09	4.16	7.39	3.87	5.59	9.18	7.61	0.59	0.85
	m	19.25	30.95	31.78	36.24	15.38	14.02	14.02	27.65	20.15	26.05	35.29	29.77		
B	a	0.60	1.24	1.64		0.93	1.04	1.11		0.83	1.46	1.64		0.63	0.74
	m	3.17	4.99	5.76		3.55	3.57	3.75		4.32	6.80	6.31			
C	a	0.92	1.85	2.66		1.06	1.10	1.18		1.27	1.83	2.43		0.51	0.6
	m	4.87	7.44	9.35		4.05	3.77	3.98		6.61	8.53	8.19			
D	a	1.16	2.38	3.34		2.14	2.21	2.26		1.68	2.74	3.38		0.45	0.5
	m	6.13	9.58	11.74		8.18	7.58	7.64		8.74	12.77	12.99			
E	a	1.14	2.34	3.26		1.98	2.04	2.10		1.53	2.49	3.17		0.39	0.50
		6.03	9.42	11.46		7.56	6.99	7.10		7.97	11.60	12.19			

[a] On the basis of 100% recovered of the total ^{14}C applied (a) and the total metabolites recovered (m). [b] First dimension solvent system, diethyl ether–*n*-hexane (4:1). [c] Second dimension solvent system: methylene chloride–acetonitrile (4:1).

tained only the naphthyl ring. No attempt to identify the conjugating moiety was made.

Piperonyl butoxide treatment (Table V) prevented the appearance of metabolites B, C, D, and E. Metabolite A was variously affected being greatly increased in susceptible males (4-days old) and increased slightly in tolerant males (1-day old). The piperonyl butoxide treatment decreased metabolite A concentrations in the female bees.

DISCUSSION

The results suggest that carbaryl persistence in the adult leafcutting bee is the dominant factor in its response to the insecticide. Penetration effects became significant only when compared to insects with similar metabolic potential.

The carbaryl metabolites isolated were products of mixed function oxidase activity as evidenced by preventing their appearance by piperonyl butoxide and by their hydroxylated nature. In the more tolerant insects (1-day-old males, 4-day-old females) these were rapidly conjugated and moved to an aqueous fraction of the bee and, hence, out of toxicological effect. Less tolerant insects (4-day-old males) accumulated these metabolites with time indicating that conjugation mechanisms had deteriorated with age.

Carbaryl persistence was also extended in the 4-day-old males and, hence, not only conjugative metabolism but also oxidative metabolism had declined in importance. Hydrolytic metabolism of carbaryl or its hydroxylation products of the *N*-methylcarbamic acid moiety may have also occurred as evidenced by 1-naphthol formation. Because of its accumulation in 4-day-old male bees and its rather similar 1-hr accumulation in all groups of treated insects, we might guess hydrolysis, if it occurs, to be a more stable metabolic mechanism in relation to age. However, until the identity of the carbaryl-derived portion of the water-soluble metabolites is established, it is difficult to assess hydrolysis as an important mechanism.

The inhibition by piperonyl butoxide of 1-naphthol formation suggests that mixed function oxidases, rather than hydrolytic esterases, may metabolize carbaryl to 1-naphthol. Douch *et al.* (1971) concluded that mixed function oxidases were involved in the production of phenolic metabolites from carbamate insecticides.

Some hydroxylated carbaryl metabolites (as 5-hydroxycarbaryl) (Dorough, 1970) have been reported as being more toxic to some animals than carbaryl itself. These undoubtedly have no significance in the leafcutting bee's response, however, as both appear to be less accumulated in any of the insects studied and the high correlation between toxicity and carbaryl persistence weakens any such argument. In addition, further studies with the drugs phenobarbital, chlorcyclizine, and aminopyrine (Lee and Brindley, 1975; Guirguis, 1973) suggest that carbaryl "intoxication" is of little importance in the leafcutting bee.

The metabolic study also shows the data of synergist ratio calculations do not correctly identify the insects with the most mixed function oxidase activities. Because the ratio depends upon the quotient of unsynergized and synergized LD_{50} doses, one would expect the larger values to show a greater contribution by piperonyl butoxide sensitive enzymes, to the safety of the insect (Brattsten and Metcalf, 1973). Susceptible 4-day-old male bees had a synergist ratio of 53 which suggests greater carbaryl metabolism than either 1-day-old males or 4-day-old females with synergist ratios of 12 and 15, respectively (Lee and Brindley, 1975). Our results, however, show that carbaryl metabolism was greater in the 1-day-old males and 4-day-old females.

A better approach, in this case, is to examine the difference, but not the quotient, of the unsynergized and synergized LD_{50} doses. The 1-day-old males and 4-day-old females had similar differences in these LD_{50} doses and similar carbaryl persistence. These were 220 $\mu g/g$ and 26.26% persistence and 247 $\mu g/g$, 21.54% persistence, respectively. The most sensitive insect, the 4-day-old males, had the smallest difference in LD_{50} values and had the greatest carbaryl persistence (*ca.* 50 $\mu g/g$, and 47.6%).

The model substrate EPN employed earlier (Lee and Brindley, 1975) also did not correctly identify potential carbaryl persistence as it predicted similar detoxication activity in 4-day-old males and females. There was good correspondence, however, between EPN detoxication and LD_{50} if male and female bees were considered separately. Of course the conditions for these were somewhat arbitrary and the substrate molecule was quite different in the *in vitro* (EPN) and *in vivo* (carbaryl) tests.

Lipid measurements (Lee and Brindley, 1975) also appear not to be casually related with carbaryl toxicity or persistence in the leafcutting bee, the decrease being comparable in both sexes with age. Cholinesterase sensitivity or successful penetration to the active sites suggested by Winteringham (1969) was not tested by these experiments but also seemed relatively unrelated to the basis for carbaryl interactions in leafcutting bees.

Carbaryl toxicity, therefore, appears to be controlled in alfalfa leafcutting bees by the activity of mixed function oxidase or microsomal enzymes. This important and gen-

eral detoxication system varies in importance with the age and sex of the bees apparently resulting in significantly different carbaryl persistence which, in turn, leads to clear differences in carbaryl toxicity.

N-Hydroxycarbaryl was identified with R_f values in a two-dimensional thin-layer chromatography system. Locke (1972) has shown *N*-hydroxycarbaryl could be confused with carbaryl in one direction (ether–hexane) and with 5-hydroxycarbaryl in the other (methylene chloride–acetonitrile). Until spectral and synthetic confirmation is obtained, therefore, the finding of *N*-hydroxycarbaryl should be viewed with caution.

ACKNOWLEDGMENT

We are grateful to the Union Carbide Corporation for the donation of technical carbaryl and samples of carbaryl metabolites.

LITERATURE CITED

Brattsten, L. B., Metcalf, R. L., *J. Econ. Entomol.* **63,** 101 (1970).
Brattsten, L. B., Metcalf, R. L., *J. Econ. Entomol.* **66,** 1347 (1973).
Casida, J. E., Shrivastava, S. P., Essac, E. G., *J. Econ. Entomol.* **53,** 205 (1968).
Davidson, J. D., Oliverio, V. T., *Atomlight* **60,** 1 (1967).
Dorough, H. W., *J. Agr. Food Chem.* **18,** 1015 (1970).
Dorough, H. W., Casida, J. E., *J. Agr. Food Chem.* **12,** 294 (1964).
Dorough, H. W., Wiggins, O. G., *J. Econ. Entomol.* **62,** 49 (1969).
Douch, P. G. C., Smith, J. N., Turner, J. C., *Life Sci.* **10** (Pt II), 1327 (1971).
Guirguis, G. N., Ph.D. Dissertation, Utah State University, Logan, Utah, 1973.
Krishna, J. G., Dorough, H. W., Casida, J. E., *J. Agr. Food Chem.* **10,** 462 (1962).
Lee, R. M.-S., Brindley, W. A., *Environ. Entomol.*, in press (1975).
Locke, R. K., *J. Agr. Food Chem.* **20,** 1078 (1972).
United States Department of Agriculture, *Agr. Statistics 1973,* 272 (1973).
Winteringham, F. P. W., *Annu. Rev. Entomol.* **14,** 409 (1969).

Received for review June 3, 1974. Accepted November 6, 1974. Contribution from the Utah State Agricultural Experiment Station, Journal Paper No. 1860, and the Department of Biology, College of Science, Utah State University. This research was supported, in part, by Utah Agricultural Experiment Station Projects 696 and 764, and U.S. Department of Agriculture Contract 12-13-100-10,635 (33).

Degradation of 4-Aminopyridine-^{14}C in Corn and Sorghum Plants

Robert I. Starr* and Donald J. Cunningham

Acetone-soluble radiolabeled metabolites, as determined by thin-layer chromatographic procedures, were not detected in roots and shoots of young corn plants (*Zea mays*) cultured 7 days in nutrient solutions containing 10 ppm of 4-aminopyridine-^{14}C. Autoradiograms of thin-layer plates containing acetone extracts of young sorghum plants (*Sorghum vulgare*), 2 weeks after treatment, showed some degradation of the labeled compound, with three similar major metabolites detected in both roots and shoots. The majority of ^{14}C extracted from these tissues was suggested to be present as the parent chemical, or possibly as 4-aminopyridine released during hydrolysis of sugar and/or amino acid conjugates. Less radioactivity was extracted from sorghum tissues maintained 7–14 days in nutrient solutions than from those kept in cultures 1 hr to 7 days, suggesting that some of the radiolabeled parent compound was bound or incorporated into the tissues of those plants cultured the longer periods. Although these data indicate that additional studies are necessary, the work suggests that 4-aminopyridine absorbed and translocated by corn and sorghum plants is not degraded in appreciable quantities to extractable, nonconjugated products, potentially more toxic than the parent chemical.

The avian frightening agent, 4-aminopyridine, has proved effective for reducing blackbird damage to ripening corn (De Grazio *et al.*, 1971); cracked corn treated with this compound and broadcast in fields (De Grazio *et al.*, 1972) causes birds that ingest treated baits to fly erratically and emit distress calls, thereby inducing other members of the flock to abandon the area. Previous studies with ^{14}C-labeled 4-aminopyridine, as described in a recent paper by Starr and Cunningham (1974), have shown that corn and sorghum plants absorb and translocate the chemical and/or its metabolites from treated nutrient solutions, that the degree of uptake is dependent upon plant age, and that the majority of the radioactivity is present in the roots and lower vegetative shoot tissues of the plants.

When an organic compound is translocated to storage areas within a plant, it may be subjected to degradation processes that can detoxify the material or, in some instances, increase its toxicity (Mitchell *et al.*, 1960). This study was conducted to evaluate the extent of breakdown of absorbed and translocated 4-aminopyridine-^{14}C in young corn and sorghum plants grown for short periods in treated nutrient cultures.

U.S. Bureau of Sport Fisheries and Wildlife, Wildlife Research Center, Federal Center, Denver, Colorado 80225.

MATERIALS AND METHODS

Chemicals and Reagents. Analytical grade 4-aminopyridine was either furnished by the Phillips Petroleum Co. or purchased from the J. T. Baker Laboratories and was usually recrystallized from an acetonitrile solution before use. α-Labeled 4-aminopyridine-^{14}C was purchased from the International Chemical and Nuclear Corporation. The sample was recrystallized with unlabeled 4-aminopyridine before use and was determined to be sufficiently radiopure by thin-layer chromatography–autoradiography, with a specific activity of 0.15 mCi/mmol. All reagents used were of analytical grade quality unless otherwise specified.

Culture and Processing of Plants. Test plants were seedlings of corn (*Zea mays,* Pioneer 3956 hybrid) and sorghum (*Sorghum vulgare,* Northrup King mini milo 50A hybrid) retained from the earlier translocation study (Starr and Cunningham, 1974). The seedlings were maintained in foil-covered pint jars containing modified Hoag-