THE METABOLISM OF CARBARYL* IN THE CATTLE TICK *BOOPHILUS MICROPLUS* (CANESTRINI)

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Summary

The metabolism of carbaryl has been studied in the engorged adult female cattle tick, *B. microplus* using ^{14}C-labelled compounds. Forty-eight hours after injection of [carbonyl-^{14}C]carbaryl only 6% of the radioactivity could be recovered in an aqueous acetone extract while 90% was recoverable after an equivalent injection of [1-^{14}C]carbaryl. Hydrolysis of the carbamate ester, hydroxylation, and conjugation were the major metabolic pathways. The radioactivity of the extract was completely accounted for as 1-naphthol (55%) and 1,5-dihydroxynaphthalene (45%) after acid hydrolysis. 1,4-Dihydroxynaphthalene appears to be absent. Evidence was also obtained for some hydroxylation of the *N*-methyl group. No unchanged carbaryl could be detected.

I. Introduction

Carbaryl is a broad-spectrum insecticide effective for the control of the cattle tick *Boophilus microplus* (Roulston and Wilson 1964). Metabolic studies with carbaryl in mammals and insects are numerous. Metabolites identified in mammals usually as conjugates include 1-naphthol, 4- and 5-hydroxycarbaryl, 1-naphthyl *N*-hydroxymethylcarbamate, 5,6-dihydro-5,6-dihydroxycarbaryl, and 5,6-dihydro-1,5,6-trihydroxynaphthalene (Dorough and Casida 1964; Knaak *et al.* 1965, 1968; Leeling and Casida 1966). Studies in various insects have led to the identification of several of the same metabolites (Dorough and Casida 1964; Andrawes and Dorough 1967; Camp and Arthur 1967).

In none of the foregoing studies was a tick or mite used as the experimental animal. Interest in the metabolism of carbaryl in the cattle tick was stimulated by the appearance of a strain showing cross resistance to carbaryl (Roulston and Wharton 1967). This paper describes the metabolism of [^{14}C]carbaryl in engorged adult female *B. microplus*.

II. Materials and Methods

(a) Chemicals

[1-^{14}C]Carbaryl, specific activity $1 \cdot 15 \times 10^7$ disintegrations per minute per milligram, was synthesized from [1-^{14}C]naphthol by the method of Skraba and Young (1959). [carbonyl-^{14}C]-Carbaryl, specific activity $2 \cdot 64 \times 10^8$ disintegrations per minute per milligram, was purchased from the Radiochemical Centre, Amersham, Bucks. Solutions for injection were freshly prepared

* Recommended common name for 1-naphthyl *N*-methylcarbamate.

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immediately prior to use. The carbaryl samples gave single radioactive spots on thin-layer chromatography. 5-Hydroxycarbaryl, m.p. 160–163°C, was prepared from methyl isocyanate and the dihydroxynaphthalene (Knaak *et al.* 1965). *N*-Hydroxymethylurea, m.p. 110°C, was prepared according to Einhorn and Hamberger (1908).

(*b*) *Ticks*

Engorged adult female ticks of the Wollongbar strain were obtained from the Department of Agriculture Cattle Tick Research Station, Wollongbar, N.S.W. This strain is maintained as a standard susceptible strain.

Ticks were injected in the abdomen through the ventral surface on the fourth day after dropping from the host animal. A dimethyl sulphoxide solution containing [1-^{14}C]carbaryl (0·5–0·7 μg) or [carbonyl-^{14}C]carbaryl (0·2 μg) in 1–3 μl was used for each tick. The radioactivity injected per tick was determined by carbon-14 counting of 10 vials each containing the dose volume of injected solution and scintillation fluid (10 ml). A freshly prepared solution of carbaryl was used for each experiment.

The ticks were placed in waxed paper containers in batches of 10–50 immediately after injection, and incubated at 30°C and 90% relative humidity for 48 hr. In all experiments there was no mortality at 48 hr and oviposition continued throughout incubation. The ticks were killed by freezing and stored at −20°C until required.

(*c*) *Radiochemical Determinations*

All carbon-14 determinations were carried out by liquid scintillation spectrometry using a Packard 3314 Tri-Carb liquid scintillation spectrometer with external standard. The scintillation fluid contained 0·15% 2,5-diphenyloxazole and 0·005% 1,4-bis(4-methyl-5-phenyloxazol-2-yl)-benzene in thiophene-free toluene, and up to 2 ml absolute alcohol where required for homogeneity, or Cab-O-Sil (0·4 g, Packard Instrument Co. Inc.) for suspension.

(*d*) *Extraction of Ticks and Estimation of Metabolites*

One container of ticks was used for each extraction. The contents including eggs were homogenized in 10% aqueous acetone in a Servall omni-mixer. Centrifugation removed the precipitated protein and debris, which was subsequently washed with further 10% aqueous acetone. The original supernatant and washings were combined with washings of the holding container, filtered through glass wool, and adjusted to a known volume. Aliquots were removed to determine the total radioactivity extracted.

For reverse isotope dilution analyses weighed portions of unlabelled 1-naphthol, 1,4-dihydroxynaphthalene, 1,5-dihydroxynaphthalene, 5-hydroxycarbaryl, or carbaryl were added to aliquots of the aqueous acetone tick extract. The acetone was removed under reduced pressure and the concentrate was extracted with peroxide-free ether or treated by one of the following methods:

(1) Chemical hydrolysis was effected by heating with hydrochloric acid (1·0N) in a nitrogen atmosphere at 95°C for 6–10 hr. The mixture was then cooled, saturated with potassium chloride, and extracted with peroxide-free ether. The ether extract was dried with anhydrous sodium sulphate and then evaporated to dryness under reduced pressure. The residue was chromatographed on Florisil columns to isolate the diluent(s).

(2) Enzymatic hydrolyses were carried out with Glusulase (Endo Laboratories Inc.) containing 100,000 units β-glucuronidase activity and 50,000 units arylsulphatase activity per millilitre. The concentrated aqueous tick extract (5–10 ml) was adjusted to pH 5·0 with 0·2M acetic acid–sodium acetate buffer. The pH choice made is intermediate between optimal values for β-glucuronidase activity and arylsulphatase activity. Enzyme (0·1 ml) was added and the mixture incubated under nitrogen for 12 hr at 37°C. The mixture was then saturated with potassium chloride, extracted with peroxide-free ether, and worked up.

(3) Mild acid treatment was effected with hydrochloric acid, 1·0N for 2 hr at 37°C or 0·5N for 24 hr at 37°C.

Derivatives of the isolated compounds, except for carbaryl, were prepared before recrystallization to constant specific activity. 1-Naphthol was converted to its *p*-nitrobenzoate ester, m.p. 145°C; 1,4- and 1,5-dihydroxynaphthalene to their diacetates, m.p. 129–130°C and 161–162°C respectively; 5-hydroxycarbaryl to its *N*,*O*-diacetate (Sullivan, Eldridge, and Knaak 1967), m.p. 114–115°C.

Silica gel G (E. Merck AG) was used for thin-layer chromatography. For carbamates, the hydroxynaphthalenes, and their derivatives ether–hexane (4 : 1 v/v) was used for development, and for the methylureas ammonia–water–isopropanol (1 : 2 : 7 v/v) or ammonia–methanol–ethyl acetate (1 : 1 : 3 v/v) was used. For column chromatography 60/100 mesh Florisil (Floridin Co.) was used. 1-Naphthol was eluted with light petroleum (b.p. 60–80°), and 1,5-dihydroxynaphthalene with light petroleum–ether (3 : 1 v/v).

III. Results

The recovery of radiolabel in the aqueous acetone extract of ticks 48 hr after dosing with carbaryl was considerably greater when the label was in the aromatic ring than when it was in the carbonyl group of the ester. For [1-^{14}C]carbaryl recovery of radioactivity was 90±4% while for [carbonyl-^{14}C]carbaryl it was 6·1±0·7%. Thus after 48 hr the bulk of the carbamate ester bond of the injected carbaryl had been cleaved.

Table 1

REVERSE ISOTOPE DILUTION ANALYSES ON THE AQUEOUS ACETONE EXTRACT OF TICKS DOSED WITH [1-^{14}C]CARBARYL

Expt. No.	Hydrolytic Treatment	% of Extracted Radioactivity Recovered as		
		Carbaryl	1-Naphthol	1,5-Dihydroxy-naphthalene
1	None	n.d.*	—	—
2	0·5N HCl, 37°C, 24 hr	1·3	—	—
3	None	—	1·9	—
4	1·0N HCl, 95°C, 6 hr	—	55·6	44·8
5	1·0N HCl, 95°C, 6 hr	—	55·2	44·8
6	Glusulase, 37°C, 12 hr	—	37·3	6·4
7a	Glusulase, 37°C, 4 hr	—	—	12·1
7b†	Glusulase, 37°C, 4 hr then 1·0N HCl, 37°C, 2 hr	—	—	19·9

* Not detectable. † 5-Hydroxycarbaryl not detected.

The reverse isotope dilution experiment (Table 1) and the following experiments were all conducted with [1-^{14}C]carbaryl. All percentages are expressed as fractions of the activity extracted into aqueous acetone. In a peroxide-free ether extraction of the aqueous acetone extract after removal of the acetone only 4·4% of the radioactivity passed into the ethereal layer. When the tick extract was first subjected to Glusulase hydrolysis, and then extracted with ether, 38·4% of the radioactivity was recovered in the ethereal phase.

In separate experiments the tick extract was examined for free 1-naphthol (isolated as its *p*-nitrobenzoyl ester) and carbaryl. The latter was not detected, but 1·9% of the radioactivity could be accounted for as 1-naphthol. A second isotope

dilution analysis for carbaryl after mild acid hydrolysis (0·5N HCl at 37°C for 24 hr) indicated 1·3% of the radioactivity was carbaryl. A control experiment showed that no hydrolysis of carbaryl to 1-naphthol occurs under these conditions.

The tick extract was hydrolysed with 1N hydrochloric acid at 95°C, extracted with ether, and thin-layer chromatograms run on the ether extract. Autoradiography of the plates indicated the presence of only two radioactive spots of approximately equal intensity. These co-chromatographed with 1-naphthol and 1,5-dihydroxynaphthalene respectively. Reverse isotope dilution experiments allowed 45% of the radioactivity to be accounted for as 1,5-dihydroxynaphthalene and 55% as 1-naphthol. Thus the total radioactivity of the tick extract was accounted for (100%). To confirm that there had been no oxidative attack on carbon-4 of carbaryl, further reverse isotope dilution analyses were performed with 1,4-dihydroxynaphthalene using hot acid hydrolysis. Preparative thin-layer chromatography and multiple recrystallization of the resultant 1,4-diacetoxynaphthalene failed to give a constant specific radioactivity. The specific radioactivity fell continuously, the final levels being 6 and 10% of the extracted radioactivity in two separate experiments. Attempts to quantitatively separate 1,4- and 1,5-dihydroxynaphthalene as the phenols or acetates to enable concurrent reverse isotope dilution analyses to be carried out were unsuccessful.

Recovery of radioactivity from the tick extract as 1-naphthol and the 1,5-dihydroxynaphthalene derivative was considerably reduced (compared to results after hot acid hydrolysis) when the hydrolysis was effected with Glusulase. Recoveries were 37·3% as 1-naphthol and 6·4–12·2% as 1,5-dihydroxynaphthalene. When the enzyme hydrolysate was ajdusted with hydrochloric acid to 1·0N and allowed to stand at 37°C for a further 2 hr, the recovery of radioactivity as 1,5-diacetoxynaphthalene was increased from 12·2 to 19·9%. In an experiment using enzyme followed by mild acid treatment no label could be recovered as 5-hydroxycarbaryl-*N*,*O*-diacetate.

In order to demonstrate the presence of the *N*-hydroxymethyl derivative of carbaryl or similar metabolites the aqueous acetone extract of ticks which had received [carbonyl-^{14}C]carbaryl was evaporated to one-tenth of its volume and treated with ammonia, sp. gr. 0·880 (Friedman and Lemin 1967). The reaction mixture was concentrated and applied to thin-layer chromatography plates. After development with ammonia–water–isopropanol (1 : 2 : 7 v/v) the chromatogram was divided into 1-cm bands and the radioactivity of each portion of silica gel determined. Radioactive peaks were observed in regions with the same R_F values as *N*-hydroxymethylurea and *N*-methylurea (0·46 and 0·67 respectively).

In a second experiment the region corresponding to hydroxymethylurea was eluted from the silica gel with acetone, and re-applied to a second plate after removal of the bulk of the solvent. Development in ammonia–methanol–ethyl acetate gave two radioactive spots each giving a positive test with Ehrlich's reagent. The minor spot, R_F value 0·39, corresponded to hydroxymethylurea, while the major at R_F value 0·60 was probably the methyl ether of hydroxymethylurea formed by reaction with methanol under silica gel acid catalysis (Walker 1953). This reaction of *N*-hydroxymethylurea on the chromoplate was demonstrated using two-dimensional chromatography. The R_F value of *N*-methylurea in this solvent system is 0·49.

IV. Discussion

The importance of hydrolysis of the carbamate ester bond of carbaryl by the cattle tick is indicated by recoveries of radiolabel in the aqueous acetone tick extracts following administration of ring-labelled and carbonyl-labelled carbaryl. About 90% of the ring label was recovered compared with 6% of the carbonyl label. Experiments with [carbonyl-^{14}C]carbaryl indicated that only 50% of the injected radioactivity could be recovered in the extract 2 hr after dosing. The hydrolytic step appeared to be virtually complete 21 hr after dosing.

This hydrolysis may be caused in part by the mammalian blood protein ingested by the feeding tick. Casida and Augustinsson (1959) demonstrated that bovine plasma has carbamatase activity towards carbaryl. Although respiratory $^{14}CO_2$ was not measured, it is probable that the bulk of the injected carbonyl label not accounted for in the extract was lost this way. There was no significant contribution to this loss by protein binding of the toxicant or metabolites having an intact carbamate ester group. This is shown by the high recoveries of radiolabel in experiments using ring-labelled carbaryl. Loss of carbonyl label may also occur through carbamylation of free amino and hydroxyl groups of tick components, particularly proteins. Except where the hydroxyl group is that of the active site serine of acetylcholinesterase, such a transcarbamylation is an effective detoxication step.

The importance of hydrolysis of carbaryl in metabolism by the cattle tick agrees with results of Eldefrawi and Hoskins (1961) obtained for other insects. In contrast, non-hydrolytic pathways predominate in the cotton leaf worm *Prodenia litura* (Zayed, Hassan, and Hussein 1966), cockroach *Periplaneta americana* (Dorough and Casida 1964), and rat (Knaak *et al.* 1965; Krishna and Casida 1966).

Of the metabolites extracted from [carbonyl-^{14}C]carbaryl-dosed ticks a carbaryl conjugate accounts for about 20%. Since the presence of 5-hydroxycarbaryl could not be demonstrated in the extract following consecutive Glusulase hydrolysis and mild acid treatment, Glusulase-labile conjugates of 5-hydroxycarbaryl and 5,6-dihydro-5,6-dihydroxycarbaryl must be absent. The remaining radioactivity must be present as Glusulase-stable conjugates of 5-hydroxycarbaryl and 5,6-dihydro-5,6-dihydroxycarbaryl, or as *N*-hydroxymethylated derivatives of carbaryl. The presence of the latter was shown by the identification of *N*-hydroxymethylurea in ammonia-treated tick extracts.

A general scheme for the metabolism of carbaryl in engorged adult female *B. microplus* is shown in Figure 1. The radioactivity of the aqueous acetone extract of ticks dosed with [1-^{14}C]carbaryl was completely accounted for as 1-naphthol and 1,5-dihydroxynaphthalene. Although reverse isotope dilution analyses for 1,4-dihydroxynaphthalene were not completely satisfactory, probably due to difficulty in removing traces of the 1,5-isomer during repeated purification, it was concluded that the 1,4-isomer was not present in the extract. The increased recovery of radioactivity as 1,5-dihydroxynaphthalene after consecutive Glusulase hydrolysis and mild acid treatment indicates the presence of Glusulase-labile conjugates of 5,6-dihydro-1,5,6-trihydroxynaphthalene. On mild acid treatment the dihydrotriol would be expected to eliminate a hydroxyl group as water from the carbon-6 position, becoming fully

aromatic by analogy with 1,2-dihydro-1,2-dihydroxynaphthalene (Booth and Boyland 1949).

The lack of oxidative attack at carbon-4 of the aromatic ring contrasts with results obtained with rat liver preparations, the cockroach, housefly (Dorough and Casida 1964), and rabbit (Leeling and Casida 1966). Conjugates of 4-hydroxycarbaryl

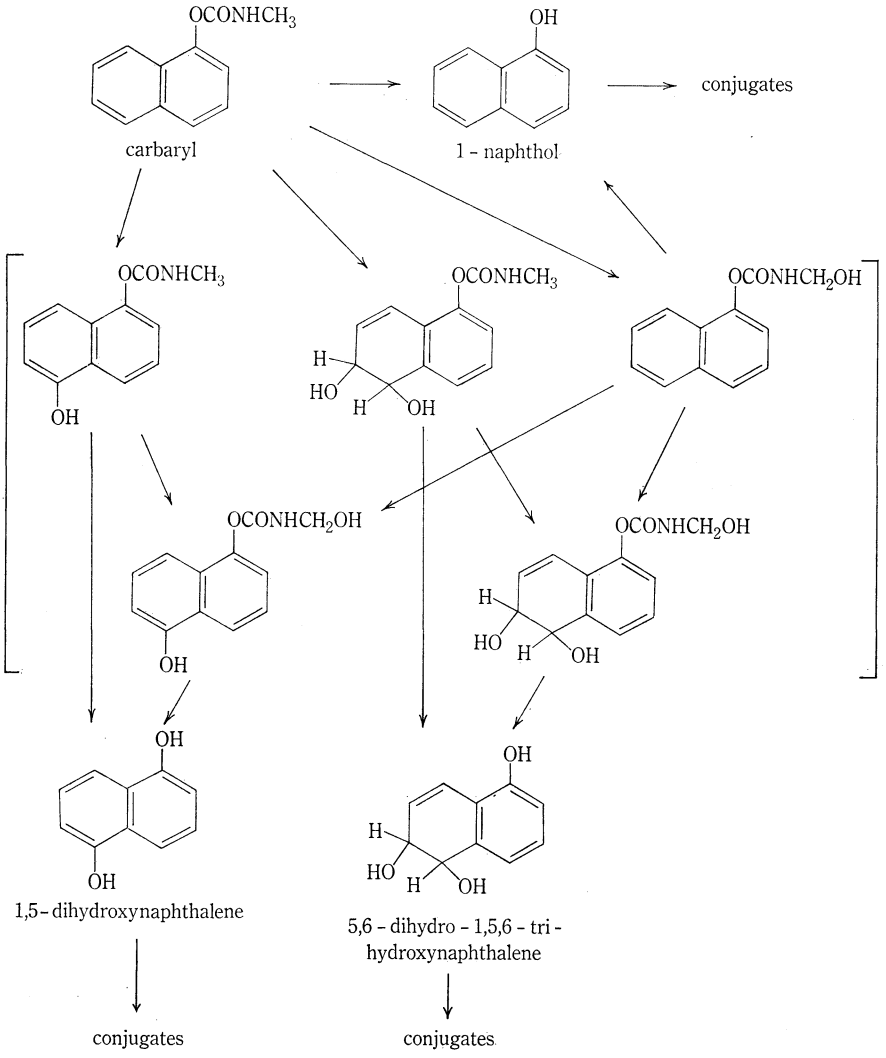

Fig. 1.

were the only oxidation products identified by Knaak *et al.* (1965, 1968) in examination of urine of mammals dosed with carbaryl. However, with bollworms, *Heliothis zea*, and boll weevils, *Anthonomus grandis*, metabolites arising by oxidation of the aromatic ring only at carbon-5 were identified (Andrawes and Dorough 1967). No 4-hydroxylation was reported. In the work of Dorough and Casida (1964) and Andrawes and Dorough (1967) with insects, however, considerable amounts of water-soluble

metabolites were formed which were not examined. The foregoing metabolic differences may reflect differing orientating effects on enzyme binding in the various species studied (Daly, Jerina, and Witkop 1968).

The nature of the conjugates present in the tick extracts remains unknown. The small amount of carbaryl conjugate present is possibly a glycoside analogous to the *N*- and *O*-glucuronides of carbaryl described by Knaak *et al.* (1965). Glusulase is a snail gut enzyme and was chosen for its wide range of hydrolytic activity. It is active towards β-glucuronides, sulphates, other glycosides, phosphates, and phenolic esters (Ryan, unpublished data). All such conjugates of carbaryl metabolites would be expected to be hydrolysed under the conditions used. It is apparent, however, that conjugates are present which are cleaved only by the hot acid hydrolysis. It is also surprising that glutathione conjugates appear to be absent. Such conjugates would not be cleaved by the acid treatment used, and it would not be possible to account for all the extracted radioactivity after acid hydrolysis as 1-naphthol and 1,5-dihydroxynaphthalene in their presence. Glutathione conjugates are formed on incubation of rat liver slices with naphthalene (Booth, Boyland, and Sims 1960), and since glutathione-conjugating ability for aryl and benzyl halides has been demonstrated for *B. decolaratus* (Hitchcock and Smith 1963) the formation of such conjugates with carbaryl would not have been unexpected.

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