PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY 11, 159–165 (1979)

The Possible Role of Carnitine in the Selective Toxicity of the Miticide Cycloprate

GARY B. QUISTAD,* LUANA E. STAIGER,* DAVID A. SCHOOLEY,*
THOMAS C. SPARKS,†,[1] AND BRUCE D. HAMMOCK†

*Biochemistry Department, Zoecon Corporation, 975 California Avenue, Palo Alto, California 94304
†Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, California 92521

Received November 23, 1978; accepted January 19, 1979

The titers of free and total carnitine were determined in selected developmental stages of mites (*Tetranychus pacificus*) and several insects. In general, insects contained more total carnitine than mites, and mite eggs less than other mite stages. In contrast to mammals, these arthropods contain little free carnitine, with most carnitine present as the acetate ester. Comparing metabolism of cycloprate (hexadecyl cyclopropanecarboxylate) in different developmental stages of mites indicates that selective ovicidal toxicity may result from the production of cyclopropanecarboxylic acid and its subsequent conjugation with carnitine. Thus, *O*-(cyclopropylcarbonyl)carnitine is a significant metabolite in mite eggs, but not in other stages. We believe that the selective ovicidal activity of cycloprate in mites may result from sequestration of carnitine as this metabolite leading to an inability to transport long-chain fatty acids into the mitochondria causing a lethal disruption of lipid utilization.

INTRODUCTION

Cycloprate (hexadecyl cyclopropanecarboxylate, trademark Zardex) is presently under development by Zoecon Corporation for commercial use as a miticide (1). The compound exhibits a rather remarkable specificity of toxic action, i.e., cycloprate has predominantly ovicidal activity against phytophagous mites (2, 3). The eggs of insects, as well as the motile stages of both mites and insects, are less sensitive to cycloprate. Limited data (4) further suggest that cycloprate is less toxic to eggs of predaceous mites than to those of phytophagous mites.

Carnitine is a widely distributed, essential cofactor for normal long-chain fatty acid degradation (5). The mitochondrial membrane encloses the enzymes for β-oxidation and excludes long-chain fatty acids and their CoA esters. However, acylcarnitines are transported across the mitochondrial membrane, thereby transporting fatty acids for subsequent utilization as energy sources. We have shown in previous mammalian metabolism studies (6–9) that carnitine can be sequestered as a conjugate by a cycloprate metabolite, cyclopropanecarboxylic acid (CPCA).[2] We have postulated that this conjugate, *O*-(cyclopropylcarbonyl)carnitine (i.e., CPCA-carnitine), may play a fundamental role in explaining species differences in mammalian toxicity (9). We now report data which support the involvement of carnitine in the selective miticidal toxicity of cycloprate.

METHODS AND MATERIALS

Carnitine titer. The following insects which were reared at Zoecon were assayed for carnitine: tobacco hornworm (*Manduca sexta*), housefly (*Musca domestica*), vagrant grasshopper (*Schistocerca nitens*), and tobacco budworm (*Heliothis virescens*).

[1] Present address: Department of Entomology, Louisiana State University, Baton Rouge, La. 70803.

[2] Abbreviations used: lc, liquid chromatography; lsc, liquid scintillation counting; CPCA, cyclopropanecarboxylic acid; CPCA-carnitine, *O*-(cyclopropylcarbonyl)carnitine.

0048-3575/79/040159-07$02.00/0

Also assayed were mealworms (*Tenebrio molitor*) which were purchased from Sure-live Mealworm (Paramount, Calif.). Lima beans (*Phaseolus limensis*) and mites (*Tetranychus pacificus*) were obtained from H. Johnson and J. A. McMurtry (U. C. Riverside). Both free and total carnitine were determined at the picomole level by the enzymatic method of Parvin and Pande (10). According to this method, the tissue is initially homogenized with ethanol. Carnitine in the 2.5% ethanolic tissue extract is subsequently reacted with [^{14}C]acetyl CoA to form [^{14}C]acetylcarnitine which is selectively separated from [^{14}C]acetyl CoA by adsorption of the latter from solution with charcoal. The resultant [^{14}C]acetylcarnitine is quantitated by liquid scintillation counting (lsc) in Insta-gel (Packard). A single ethanolic extract of most insects and lima beans was assayed in duplicate for both free and total carnitine. Two separate extracts of all mite stages and some insects (i.e. *Manduca* adults, *Musca* eggs, and *Schistocerca* eggs) were prepared on different days for carnitine analysis in duplicate. Comparative carnitine titers for rats utilized five females analyzed in duplicate.

The presence of acetylcarnitine in both *Manduca* adults and *Tetranychus* eggs was determined by reversed-phase liquid chromatography (lc). Authentic standards of acetyl- and propionylcarnitine were prepared from the corresponding acyl halide and carnitine hydrochloride in trifluoroacetic acid (cf. (11)). A portion of the ethanolic extracts of *Manduca* and *Tetranychus* was separately resolved into acetyl- and propionylcarnitine by lc. The expected elution volumes for these acylcarnitines were determined from a *prior* injection of standards. No standards were coinjected with extracts. Fractions coincident with the expected elution volumes of acetyl- and propionylcarnitine were collected, lyophilized, and assayed for total carnitine (10).

Mite metabolism of cycloprate. The surface of a glass vial (40 cm^2 area) was coated with [*carboxyl*-^{14}C]cycloprate (0.1–0.3 μCi, 54.1 mCi/mmol, 98.4% radiochemical purity by lc (μ-Bondapak C_{18}, methanol:water 90:10)). Female adult mites and mite eggs (ca. 100 mg each) were added to separate vials and gently rotated to insure good contact with the cycloprate-coated glass. After 24 or 48 hr, the mites were removed and rinsed with petroleum ether. The vial was rinsed with acetone and the mites were homogenized in ethanol. The radioactivity in each fraction was quantitated by lsc (Packard Model 2425). Metabolites were resolved by thin layer chromatography of aliquots on silica gel GF (Analtech, hexane:ether, 5:1) and radioactivity was detected by a radiochromatogram scanner (Packard Model 7201). The identity of cyclopropanecarboxylic acid was confirmed by derivatization to its *p*-phenylphenacyl ester which cochromatographed with an authentic standard upon lc as described previously (12). The structure of *O*-(cyclopropylcarbonyl)carnitine (CPCA - carnitine) was verified by elution behavior of labeled metabolite vs synthetic standard on lc (Fig. 1). The radiolabeled zone corresponding to CPCA-carnitine from lc was saponified and assayed for carnitine (10). There was sufficient carnitine in the zone to account for all of the radiolabeled metabolite.

RESULTS AND DISCUSSION

Insects have played a fundamental role in the history of carnitine biochemistry. Indeed, carnitine was originally called vitamin B_T (T = *Tenebrio*) and the *Tenebrio* bioassay was used to determine carnitine titers in numerous other organisms (13). The physiological function of carnitine as mediator of fatty acid oxidation in mitochondria is well documented in mammals (14) and insects (15). More recently Bieber and Choi (16) have proposed that carnitine participates in amino acid metabolism since branched-chain acids can be isolated from mammalian tissue as acylcarnitines. Robinson and Goldsworthy (17) have suggested also that the adipokinetic hormone of locust flight muscle acts on carnitine acyltransferase.

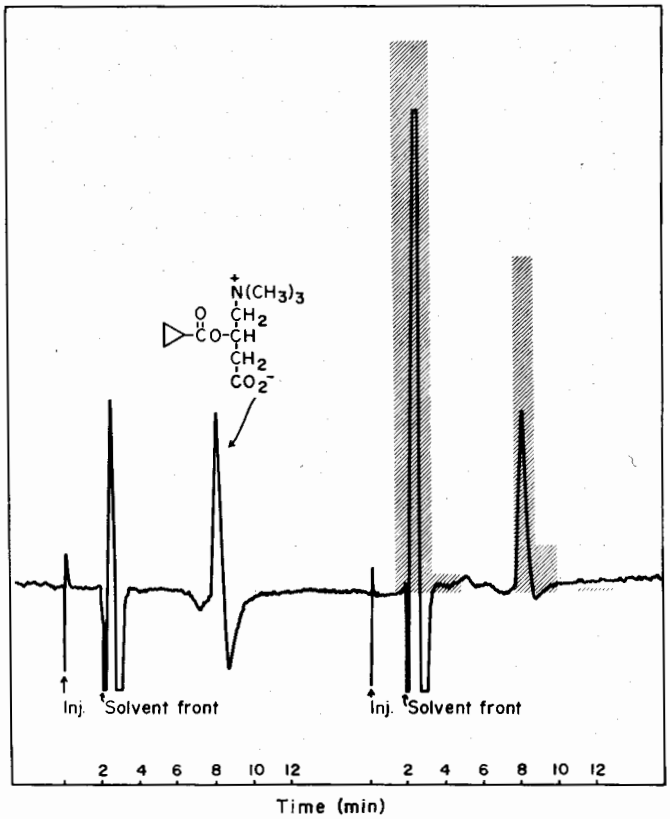

FIG. 1. *Elution of metabolically produced O-(cyclopropylcarbonyl)carnitine during liquid chromatography (lc): Waters M-6000 pump, Valco loop injector, Waters R-401 refractive index detector, LiChrosorb RP-8 column (25 × 0.46 cm), 0.01 M NH_4HCO_3, 1.6 ml/min. The solid line represents refractive index detector response to added synthetic standard and hatched bars show distribution of eluted radiolabel in metabolite fractions of mite extract.*

The purpose of this work is to explore the reasons for the higher toxicity of cycloprate to mite eggs as compared to motile mites or insects. Since our previous metabolic studies in rats (6) and dogs (8) indicated a possible correlation between toxicity and the quantitative abundance of the cyclopropanecarboxylic acid conjugate of carnitine (CPCA-carnitine), we investigated the potential importance of this conjugate in mites and insects. We approached the question of selective toxicity from two directions. First, we determined the actual titer of both free and bound carnitine in various insect and mite developmental stages. Such titer determinations in insects have been reported previously (13), but the method for carnitine assay given recently by Parvin and Pande (10) rendered older methods obsolete by greatly improving sensitivity to the picomole level. Secondly, we determined the relative abundance of CPCA-carnitine by following the metabolic fate of [^{14}C]-cycloprate in mite adults and eggs.

Insect and Mite Carnitine

In mammals, carnitine exists in equilibrium with its acetylated form (5). Smaller quantities of longer-chain acylcarnitines and even branched-chain acylcarnitines also occur in mammals, but the most abundant state of carnitine is free. When mites and insects were assayed for carnitine, to our surpirse very little free carnitine was

TABLE 1

Titers of Free and Total Carnitine in Mites, Insects, Lima Beans, and Rats

	Wet weight (μg/g)		Dry weight (μg/g)	
	Free	Total	Free	Total
Mites				
Adult female	0.11	12.0	0.42	46.1
Adult male	0.14	13.1	0.59	56.8
Immature	0.19	12.5	0.86	56.4
Eggs	0.22	13.0	0.42	20.5[a]
Lima bean (mite host)	0.07	1.3	0.5	8.7
Insects				
Manduca egg	0.05	53.0	0.06	61.1
larva	0.81	9.4	5.1	59.1
adult	0.44	166	0.67	253[b]
Tenebrio larva	0.09	47.3	0.22	115
adult	0.12	65.2	0.27	149
Musca egg	0.06	102	0.20	331[c]
larva	0.08	23.8	0.28	83.4
Schistocera egg	0.06	3.9	0.11	6.9[d]
Heliothis larva	0.04	21.6	0.12	66.5
Rats (female)				
Heart	145	175		
Liver	46	57		

[a] Range for separate extracts = 16.4–25.2.
[b] Range for separate extracts = 250–255.
[c] Range for separate extracts = 320–340.
[d] Range for separate extracts = 6.0–7.8.

found (Table 1). However, saponification of extracts released substantial amounts of carnitine (reflected as *total carnitine*). By analogy to the mammalian biochemistry of carnitine we expected that the "bound" carnitine in arthropods would be the acylation product of carnitine and fatty acids. Methods for the qualitative and quantitative analysis of the acyl moieties of acylcarnitines in rat tissue are available (18), but these are rather prolonged procedures. Therefore, portions of the ethanolic extracts from *Manduca* adults and *Tetranychus* eggs were resolved preparatively by lc into fractions corresponding to acetyl- and propionylcarnitine (Fig. 2). Saponification of these fractions released free carnitine which was then enzymatically assayed. For extracts of both *Manduca* adults and mite eggs, the preponderance of the total carnitine was present as acetylcarnitine, 90 and 95% respectively. Propionylcarnitine contributed only 2 and 3% respectively. The apparent abundance of acetylcarnitine in both insects and mites is intriguing since the role of acetylcarnitine in mammals is still ambiguous (14).

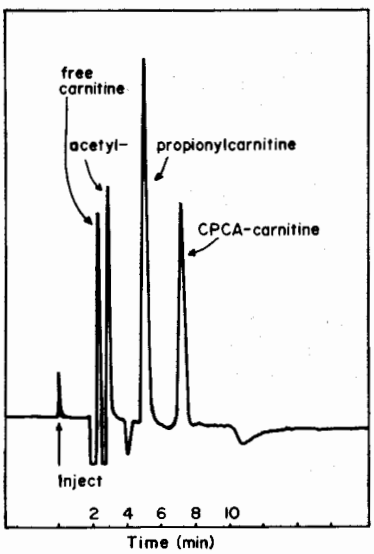

FIG. 2. *Resolution of acylcarnitine standards by lc. Same conditions as Fig. 1.*

Comparison of the total carnitine titers in mites and insects revealed several suggestive trends (Table 1). In general, insects contained substantially more total carnitine than mites although *Schistocerca* eggs provided a notable exception. For insects there seemed to be no general correlation between development stage and amount of total carnitine. However, mite eggs had a comparatively lower total carnitine titer than immature or adult mites on a dry-weight basis and generally less carnitine than most insects.

Mite Metabolism of Cycloprate

In a further attempt to rationalize the greater toxicity of cycloprate to mite eggs than to motile mite forms, we investigated the metabolic fate of [^{14}C]cycloprate in female adults and eggs (Table 2). The application rate of cycloprate was approximately equal to that necessary to induce a 50% mortality in mite eggs (cf. (3)). While the apparent rates of penetration into eggs and adults appeared comparable, there was a striking difference in metabolic profile. Recovered radiolabel from adults consisted almost entirely of unmetabolized cycloprate. In contrast, mite eggs degraded cycloprate extensively to CPCA and CPCA-carnitine and both of these metabolites increased in abundance with time (Fig. 3). At 24 hr posttreatment, CPCA-carnitine was at least 30-fold more plentiful in (sensitive) mite eggs as compared to (insensitive) adults. From our data (Tables 1 and 2). we calculate that 2 and 4% of the total initial carnitine pool in mite eggs was sequestered as CPCA-carnitine at 24 and 48 hr, respectively. It is an open question whether or not removal of carnitine at these rather low levels would elicit a toxic response in mite eggs since only about 2% of the total carnitine in mite eggs is free (in contrast to about half in mammals) and the biodynamics of carnitine in mites (or insects) is unknown. It is also possible that the accumulation of CPCA-carnitine occurred at the expense of acetyl- or propionylcarnitine, rather than free carnitine.

We attempted to further validate the role of CPCA-carnitine in the toxic mode of action for cycloprate by simultaneous application of cycloprate and the potential antidotes, carnitine or linoleoylcarnitine. Re-

TABLE 2

Metabolism of [carboxyl-^{14}C]Cycloprate by Mites

	% Total recovered ^{14}C		
	Animal wash	Vial wash	Animal extract
Eggs[a]			
24 hr	6	32	61
Cycloprate	5.9	28	26
CPCA	—	3	16
CPCA-carnitine	—	—	3
48 hr	6	31	63
Cycloprate	5.6	25	16
CPCA	—	4	26
CPCA-carnitine	—	—	5
Adult females[b]			
24 hr	22	39	39
cycloprate	21.6	38.6	37.1
CPCA	—	—	<0.3
CPCA-carnitine	—	—	<0.1

[a] 94.3 and 83.9% of the applied ^{14}C was recovered for 24 and 48 hr eggs, respectively.
[b] 94.8% of the applied ^{14}C was recovered.

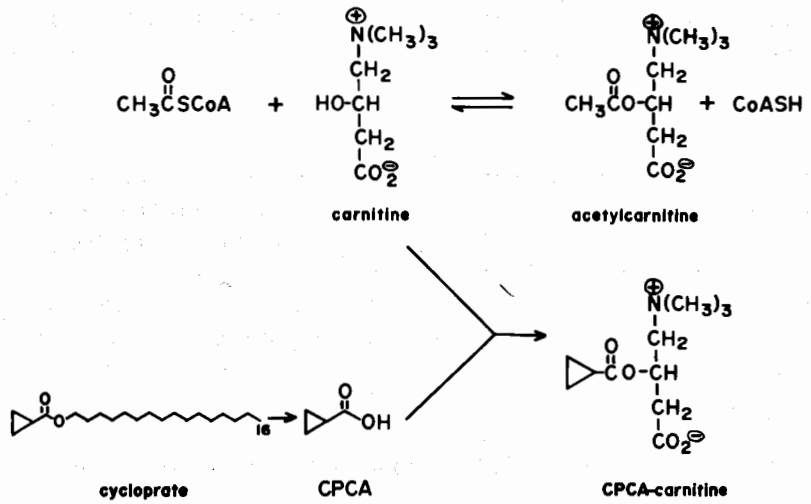

FIG. 3. *Formation of acetylcarnitine and its relationship to metabolism of cycloprate by mite eggs.*

versibility of toxicity was not observed, but the highly polar carnitine "rescue agents" may not penetrate the chorion of mite eggs.

From structure–activity relationships for cycloprate analogs (i.e., esters of CPCA, cyclopropylmethanol, and ω-cyclopropyl alkanoic acids), Henrick *et al.* (3) contend that the biological activity of these compounds results from conversion to an activated metabolite. Our data reveal that such an activated metabolite is very likely CPCA or its carnitine ester. By analogy with mammalian biochemical toxicology of cycloprate (8), the formation of CPCA-carnitine suggests that production of this conjugate can disrupt lipid metabolism (cf. 19). Our data cannot exclude alternative explanations of selective toxicity based solely on the presence of CPCA. For example, in mammals CPCA also disrupts gluconeogenesis (20) and ketone body formation ((8) and references therein), so it is possible that several enzymes may be affected in mites as well. CPCA could also disrupt fatty acid oxidation by sequestering CoA as an ester.

CONCLUSIONS

Our data are consistent with the proposed hypothesis that cycloprate is selectively toxic to phytophagous mite eggs because of rapid metabolism to cyclopropanecarboxylic acid, which sequesters carnitine, already present in relatively low amounts in such eggs. Thus, utilization of lipid, the principal energy source in eggs, may be disrupted. The relatively low order of toxicity of cycloprate in other mite stages appears to be due to metabolic stability of cycloprate. This work also suggests that CPCA might be a useful probe in further explorations of carnitine's physiological functions in mites and insects.

ACKNOWLEDGMENTS

We thank D. C. Cerf and G. B. Staal for bioassay of cycloprate with carnitine. We particularly thank H. Johnson and J. A. McMurtry (Division of Biological Control, U.C. Riverside) for a generous supply of mites. Contribution No. 72 from Zoecon Research Laboratories.

REFERENCES

1. C. A. Henrick and G. B. Staal, U.S. Patent 3,925,460, December 9, 1975.
2. G. B. Staal, G. F. Ludvik, S. G. Nassar, C. A. Henrick, and W. E. Willy, A novel group of miticides containing the cyclopropane moiety, *J. Econ. Entomol.* 68, 91 (1975).
3. C. A. Henrick, W. E. Willy, G. B. Staal, and G. F. Ludvik, Ovicidal activity and its relation to chemical structure for the two-spotted spider mite (*Tetranychus urticae* Koch) in a new class of miticides containing the cyclopropyl group, *J. Agr. Food Chem.* 24, 1023 (1976).

4. W. H. Palmer and D. G. Koslucher, Field evaluations of a new class of mite ovicides, *Proc. North Cent. Branch Entomol. Soc. Amer.* **30,** 55 (1975).
5. R. Bressler, *in* "Lipid Metabolism" (S. J. Wakil, Ed.), p. 49, Academic Press, New York, 1970.
6. G. B. Quistad, L. E. Staiger, and D. A. Schooley, Environmental degradation of the miticide cycloprate (hexadecyl cyclopropanecarboxylate). 1. Rat metabolism, *J. Agr. Food Chem.* **26,** 60 (1978).
7. G. B. Quistad, L. E. Staiger, and D. A. Schooley, Environmental degradation of the miticide cycloprate (hexadecyl cyclopropanecarboxylate). 3. Bovine metabolism, *J. Agr. Food Chem.* **26,** 71 (1978).
8. G. B. Quistad, L. E. Staiger, and D. A. Schooley, Environmental degradation of the miticide cycloprate (hexadecyl cyclopropanecarboxylate). 4. Beagle dog metabolism, *J. Agr. Food Chem.* **26,** 76 (1978).
9. G. B. Quistad, L. E. Staiger, and D. A. Schooley, "The Miticide Cycloprate (Hexadecyl Cyclopropanecarboxylate); Investigations of its Selective Toxicity," 175th National Meeting of the American Chemical Soc., Anaheim, Calif., March 1978, Abstract PEST-39.
10. R. Parvin and S. V. Pande, Microdetermination of (-)carnitine and carnitine acetyltransferase activity, *Anal. Biochem.* **79,** 190 (1977).
11. C. C. Guilbert and A. E. Chung, Metabolism of cyclopropanecarboxylic acid: A new role for carnitine, *J. Biol. Chem.* **249,** 1026 (1974).
12. G. B. Quistad, L. E. Staiger, and D. A. Schooley, Environmental degradation of the miticide cycloprate. 2. Metabolism by apples and oranges, *J. Agr. Food Chem.* **26,** 66 (1978).
13. G. Fraenkel and S. Friedman, Carnitine, *in* "Vitamins and Hormones" (R. S. Harris, G. F. Marian, and K. V. Thimann, Eds.), p. 73, Academic Press, New York, 1957.
14. J. Bremer, Carnitine and its role in fatty acid metabolism, *Trends Biochem. Sci.* **2,** 207 (1977).
15. A. M. T. Beenakkers, The influence of carnitine on fatty acid oxidation in insect muscle, *Arch. Neérl. Zool.* **16,** 535 (1966).
16. L. L. Bieber and Y. R. Choi, Isolation and identification of aliphatic short-chain acylcarnitines from beef heart: Possible role for carnitine in branched-chain amino acid metabolism *Proc. Nat. Acad. Sci. USA* **74,** 2795 (1977).
17. N. L. Robinson and G. J. Goldsworthy, A possible site of action for adipokinetic hormone on the flight muscle of locusts, *J. Insect Physiol.* **23,** 153 (1977).
18. Y. R. Choi, P. J. Fogle, P. R. H. Clarke, and L. L. Bieber, Quantitation of water-soluble acylcarnitines and carnitine acyltransferases in rat tissues, *J. Biol. Chem.* **252,** 7930 (1977).
19. C. C. Corredor, K. Brendel, and R. Bressler, Studies on the mechanism of the hypoglycemic action of 4-pentenoic acid, *Proc. Nat. Acad. Sci. (USA)* **58,** 2299 (1967).
20. J. J. Bahl, E. Shrago, K. Brendel, and R. Bressler, Inhibition of liver gluconeogenesis and CO_2 production in both heart and liver by cyclopropanecarboxylic acid *in vitro*, *Proc. West. Pharmacol. Soc.* **21,** 229 (1978).