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Mechanisms of Resistance to Bromophos-ethyl in Two Strains of the Cattle Tick *Boophilus microplus*

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Abstract

B. microplus larvae of the organophosphorus-resistant Biarra and Mt Alford strains were respectively 8× and 35× resistant to bromophos-ethyl compared with larvae of the standard organophosphorus-susceptible Yeerongpilly strain. Both resistant strains had acetylcholinesterase with decreased sensitivity to inhibition by the oxon of bromophos-ethyl *in vitro*; this was the only resistance mechanism apparent in the Biarra strain but Mt Alford larvae were protected additionally by increased metabolism of the oxon *in vivo* to water-soluble products. Total degradation rates for the parent chemical were similar in all strains and relatively slow. Both bromophos-ethyl and its oxon were potent inhibitors of mixed-function oxidase *in vivo* and it seemed that the slow oxidative metabolism of bromophos-ethyl (the major pathway) could be attributed to substrate and/or product inhibition. No phenolic metabolites were detected and the major water-soluble metabolite was identified electrophoretically as 2,5-dichlorophenyl-*o*-ethyl phosphate. Some debrominated oxon was detected in all samples of larvae after dosage with bromophos-ethyl or with its oxon, indicating that oxidation of bromophos-ethyl to the oxon, debromination of the oxon followed by deethylation was a major degradative sequence.

Introduction

The acaricide bromophos-ethyl, 4-bromo-2,5-dichlorophenyl diethyl phosphorothionate gave satisfactory, control of a number of organophosphorus-resistant strains of the cattle tick *Boophilus microplus*, but had to be used at increased concentrations to control the Biarra strain and was virtually ineffective against the Mt Alford strain (Schnitzerling *et al.* 1974). Resistance factors of 8× (Biarra) and 23× (Mt Alford) obtained by the larval immersion technique were more consistent with the field behaviour of these strains of ticks in response to bromophos-ethyl than were the factors of 3·9× and 5× obtained by the impregnated packet technique (Roulston 1973). Although the Biarra strain (Roulston *et al.* 1968) and the Mt Alford strain (Schnitzerling *et al.* 1974) have been characterized previously, neither has been examined extensively with respect to bromophos-ethyl resistance. We undertook such a study using well established methods for determining acetylcholinesterase activity *in vitro* and *in vivo* and ^{3}H ring-labelled bromophos-ethyl for acaricide metabolism studies in the Biarra, Mt Alford and the reference, organophosphorus-susceptible Yeerongpilly strains of tick larvae.

Materials and Methods

Radioactive Acaricide

Bromophos-ethyl, labelled with ^{3}H in the phenyl group, had a specific activity of 54·6 mCi/mmol and was a gift from Celamerck, Ingelheim/Rhein. This material was purified by preparative paper-

partition chromatography using polyethylene glycol 400 as stationary phase and n-hexane–benzene–methanol (65:24:5, v/v) as solvent.

Radioactive Oxon

The oxon of [^{3}H]bromophos-ethyl was prepared by bromine oxidation of bromophos-ethyl in 80% (v/v) aqueous ethanol solution for 5 h at 30°C and purified by preparative paper chromatography. It gave a single radioactive spot when chromatographed in the silicone–aqueous methyl cyanide system used for metabolism studies.

Non-radioactive Reference Compounds

Unlabelled bromophos-ethyl, its oxon and their deethyl derivatives together with the Celamerck compound S4747 (2,5-dichlorophenyl diethyl phosphorothionate) and the corresponding derivatives were obtained from Celamerck or prepared by the methods indicated in Table 1. Identity of preparations was checked by ultraviolet and infrared spectroscopy and by microanalysis.

Table 1. Reference compounds used for chromatographic or electrophoretic identification of ^{3}H compounds extracted from larvae dosed with [^{3}H]bromophos-ethyl or its oxon

Asterisks indicate identified compounds

Bromophos-ethyl and derivatives (X = 4-bromo-2,5-dichlorphenyl)		S4747 and derivatives (X = 2,5-dichlorophenyl)
*Bromophos-ethyl[A]	X–P(S) $(OEt)_2$	S4747[A]
*Bromophos-ethyl oxon[B]	X–P(O) $(OEt)_2$	*S4747 oxon[B]
*Bromophos-ethyl, deethyl[A]	X–P(S) (OEt)OH	S4747, deethyl[E]
Bromophos-ethyl oxon, deethyl[A]	X–P(O) (OEt)OH	*S4747 oxon, deethyl[A]
*Bromophos-ethyl, bis-deethyl[C]	X–P(S) $(OH)_2$	S4747 bis-deethyl[C]
Bromophos-ethyl oxon, bis-deethyl[C]	X–P(O) $(OH)_2$	S4747 oxon, bis-deethyl[C]
Bromophos-ethyl, phenol derivative[D]	X–H	S4747, phenol derivative[D]

[A] From Celamerck.
[B] Bromine oxidation of thio parent (Roulston *et al.* 1966).
[C] pH 11 bis-deethylation of parent (Stenersen 1969).
[D] NaOH hydrolysis of parent in 80% (v/v) aqueous methanol.
[E] NaI deethylation of parent in acetone (Spencer *et al.* 1958).

Strains of Tick Larvae

Acaricide susceptible larvae were from the unselected Yeerongpilly (Y) reference strain which has been cultured in isolation for about 28 years. Biarra (B) strain larvae, resistant to organophosphorus and carbamate acaricides because of an acetylcholinesterase (EC 3.1.1.7) (AChE) with a greatly decreased sensitivity to inhibitors, had normal detoxication systems (Roulston *et al.* 1968). Mt Alford (MA) strain larvae, in addition to AChE of decreased sensitivity to inhibitors which was virtually identical to that of B larvae, had increased ability to detoxify organophosphates (Schnitzerling *et al.* 1974). Ticks were cultured as described (Schuntner and Thompson 1978) and the larval progeny were used 14 days after hatching. At this age there are about 35 000 larvae in a 1-g sample.

Dosage of Larvae

Larvae were dosed with labelled or unlabelled chemicals by immersion in aqueous suspensions of chemical for 5 min (Roulston *et al.* 1966). Duplicate samples weighing 0·2 g were used for metabolism studies and for the determination of AChE, while duplicate samples of *c.* 100 larvae were subsampled, after dosage, into paper packets and incubated for 24 h at 30°C and 90% R.H. to determine mortality. Duplicate results were averaged and LC_{50} values determined from best lines through points obtained by plotting log concentration of bromophos-ethyl against probit mortality.

AChE in vivo *and* in vitro

The bimolecular rate constant k for the inhibition of AChE *in vitro* and the effects of acaricidal dosage of larvae on AChE *in vivo* were determined as described previously (Roulston *et al.* 1968; Schnitzerling *et al.* 1974).

Metabolism of Radiochemicals in Larvae

Larvae which had been dosed with radiochemicals were incubated at 30°C and 90% R.H. until required for assay, except where anaerobic conditions were maintained by passing humidified N_2 through samples of larvae in cotton-wool plugged tubes. Incubated samples were washed twice with 3 ml acetone (external rinse) and extracted twice by homogenization in 2 ml 30% (v/v) methanol in acetone using an all-glass homogenizer cooled in ice-water. The combined extracts were centrifuged and an aliquot of the supernatant equivalent to 10 mg larvae was used for thin-layer separation of metabolites. Aliquots of external rinse equivalent to 20 mg larvae were also examined by thin-layer chromatography.

Separation and Identification of Metabolites

Extracts of larvae which had been treated with [^{3}H]bromophos-ethyl were chromatographed in a reversed-phase partition system on Eastman t.l.c. cellulose sheets which contained fluorescent indicator. The sheets were prepared by dipping them in a 4% (v/v) solution of silicone 550 in n-hexane down to but clear of the sample zone. The sample zone and the area below it were left uncoated to avoid lipophilic overload due to sample extractives. The sample, equivalent to 10 mg of larvae, was applied to the dried, coated sheets as a 2-cm streak. Ascending development with 40% (v/v) methyl cyanide in water was carried out to 16 cm. Reference compounds (200 μg) were applied with samples, usually one at a time, and chromatograms containing reference compound only were run alongside the samples. The heavy reference loads and parallel chromatograms were necessitated by the quenching effect of the silicone on the subsequent visualization. The reference spots were located under short-wave ultraviolet light, marked with pencil and the ^{3}H chromatogram was sectioned below, through and above the reference spot for the determination of the radioactive profile by scintillation counting. Bromophos-ethyl and S4747 were only partially resolved in the above system but separation was achieved by chromatography on the silicone-coated cellulose with 75% (v/v) methyl cyanide in water.

Location of Inhibitors on Paper Chromatograms

Samples of Y, B and MA larvae were dosed with 0·01% bromophos-ethyl, incubated for 16 h, rinsed and extracted. Aliquots of extracts equivalent to 100 mg of larvae were chromatographed on Whatman 3 MM paper in the silicone–aqueous methyl cyanide system. Sections of the developed chromatograms, 1 cm long, were eluted with 70% (v/v) methyl cyanide in water and aliquots of these extracts were tested for AChE inhibitor using bovine erythrocyte AChE as enzyme source in a colorimetric assay (Ellman *et al.* 1961). Similarly prepared extracts from chromatograms of untreated larvae were taken as control samples.

Electrophoresis of Water-soluble Fractions

The water-soluble fractions of extracts of [^{3}H]bromophos-ethyl dosed larvae were eluted from chromatograms with 90% (v/v) aqueous methanol. Each extract was cleaned up by two extractions with n-hexane and aliquots were electrophoresed on Schleicher & Schuell 2040B paper in a high-voltage apparatus. A current of 70 mA was maintained by 4000 V across 40 cm using 0·03 M sodium acetate buffer, pH 7·0. Reference compounds were applied with the samples near the cathode end in sufficient amounts (*c.* 40 μg) to allow ultraviolet visualization.

Routine Analysis of Extracts

Extracts were chromatographed in the silicone–40% (v/v) methyl cyanide system as above except that reference compounds were not added. The dried developed chromatograms were cut into 1-cm sections and the latter eluted with 1·5 ml methanol in counting vials for *c.* 10 min. The vial contents were counted after adding 10 ml Instagel.

Inhibition of Mixed-function Oxidase

This was determined using [^{14}C]carbaryl as substrate by the method described by Schuntner and Thompson (1976).

Results and Discussion

Resistance and AChE

The 24-h LC_{50} values determined for Y and B larvae, Table 2*a*, were the same as those reported by Schnitzerling *et al.* (1974). However, our resistance factor of 35 for MA larvae was higher than their reported factor of 23.

Table 2. (*a*) 24-h LC_{50} values and resistance factors for Y, B and MA larvae, and bimolecular rate constant *k* for inhibition of AChE of the three strains *in vitro* by bromophos-ethyl oxon. (*b*) Residual AChE activity as percentage of control activity *in vivo* following dosage of Y, B, and MA larvae with aqueous suspensions of bromophos-ethyl at the respective LC_{50} concentration, 8, 16 and 24 h after treatment

(*a*)

Strain	LC_{50} (% concn)	Resistance factor	*k* (litre mol^{-1} min^{-1})
Y	0·0012	1	$1·7 \times 10^5$
B	0·010	8	$2·3 \times 10^4$
MA	0·044	35	$2·3 \times 10^4$

(*b*)

Strain	Concn of bromophos-ethyl	Residual AChE (% of control) 8 h	16 h	24 h
Y	0·0012	25·3	2·7	2·4
B	0·010	21·2	1·4	1·8
MA	0·044	19·3	1·9	0·5
Mean range (±)		1·3	0·4	0·1
Extreme range (±)		1·4	0·6	0·1

The bimolecular rate constants for inhibition of AChE of B and MA larvae were the same while the value of Y was higher by a factor of 7·4. This supports the conclusion that AChEs in B and MA larvae are identical. The two enzymes have previously shown similarities in their responses to oxons of diazinon and chlorpyrifos (Schnitzerling *et al.* 1974), and coumaphos (H. J. Schnitzerling, unpublished data). All of our *in vitro* inhibition studies have been done with oxons of thiophosphate acaricides as the acaricides themselves are not AChE inhibitors.

The inhibitory effects of bromophos-ethyl on AChE of Y, B and MA larvae dosed at their respective 24-h LC_{50} concentrations are shown in Table 2*b*. Eight hours after dosage only 19–25% of AChE activity remained in all strains while at 16 and 24 h less than 2·7% of the control enzyme activities were determined. The similarity in responses indicates a correlation of AChE inhibition and lethal effect, supporting earlier observations on toxicity of coumaphos in *B. microplus* larvae (Roulston *et al.* 1966).

Separation and Identification of Metabolites

Thin-layer chromatograms of extracts of [^{3}H]bromophos-ethyl-treated larvae yielded major radioactive peaks coinciding with the parent (I), its oxon (II) and the 'water-soluble' region (IV) (Fig. 1). A minor peak coincided with the debrominated oxon (III) while no activity was detected in the region of the phenol derivatives between III and IV.

The identity of the radioactive peak coinciding with I was in doubt because of overlap with S4747. This was further investigated by chromatography of extracts using the 75% (v/v) methyl cyanide system. The radioactive peak, previously near the origin, migrated wholly with bromophos-ethyl to R_F 0·67. There was no radioactive compound coincident with S4747 at R_F 0·86. Tests for AChE inhibitors in

preparative paper chromatograms of extracts of [^{3}H]bromophos-ethyl-treated larvae were strongly positive at the II (oxon) position for Y and B larvae, but a barely significant response resulted for MA larvae. High-voltage electrophoresis of the water-soluble fraction IV resolved it into a major radioactive peak which migrated with 2,5-dichlorophenyl-*o*-ethyl phosphate, while two minor peaks migrated with mono- and bis-deethyl bromophos-ethyl. Larvae were also treated with the ^{3}H-labelled oxon of bromophos-ethyl and the resulting water-soluble fraction yielded the same major peak after electrophoresis.

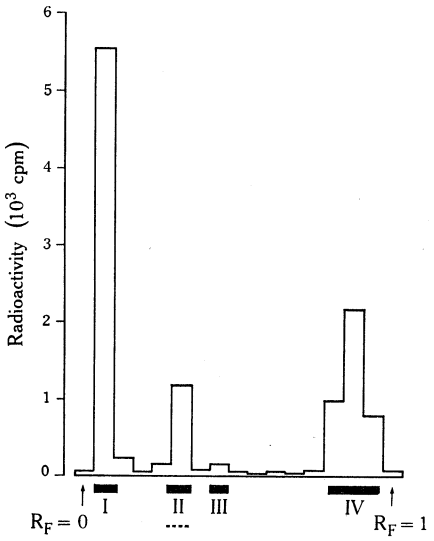

Fig. 1. Thin-layer chromatographic separation of [^{3}H]bromophos-ethyl and metabolites extracted from larvae 24 h after dosage with 0·001 % radiochemical in aqueous suspension. Reference compounds visualized by short-wave ultraviolet light (solid bars) were I, bromophos-ethyl; II, bromophos-ethyl oxon; III, S4747 oxon; IV, deethyl derivatives (water-soluble fraction). – – – Location of acetylcholinesterase inhibitor.

Penetration and Metabolism of [^{3}H]Bromophos-ethyl

Samples of larvae were analysed 3, 6 and 24 h after dosage with 0·001 % (w/v) [^{3}H]bromophos-ethyl. The amounts of ^{3}H external, and ^{3}H extractable internal material together with unextractable residues are shown in the first section of Table 3,

Table 3. Total external, and extractable or unextractable internal ^{3}H material, and metabolic distribution of ^{3}H label in extracts of larvae 3, 6 and 24 h after dosage with 0·001 % [^{3}H]bromophos-ethyl

Strain	Incubation time (h)	Total ^{3}H material (μg-equiv. of [^{3}H]bromophos-ethyl)			Chromatographically separated metabolites (% of total extracted ^{3}H material)			
		External	Internal		Bromophos-ethyl	Oxon	Debromo-oxon	Water-soluble
			Residue	Extract				
Y	3	16·6	0·6	19·5	85·5	5·6	0·7	8·2
B	3	17·9	0·6	16·3	86·6	4·6	0·5	8·3
MA	3	18·7	0·6	16·9	88·3	3·6	0·5	7·6
Y	6	10·4	0·7	21·8	81·5	7·9	0·5	10·1
B	6	10·3	0·6	20·4	80·9	7·1	0·8	11·2
MA	6	13·3	0·8	22·5	81·1	4·8	0·7	13·4
Y	24	9·8	0·8	22·3	53·6	11·0	1·0	34·4
B	24	5·8	1·1	23·3	50·9	14·0	1·4	33·7
MA	24	5·7	1·5	27·2	54·5	4·2	0·6	40·7
Mean range (±)		0·6	0·1	0·6	0·9	0·2	0·1	0·9
Extreme range (±)		1·2	0·1	1·0	2·1	0·5	0·2	2·0

while the second section shows the results of chromatographic separation of metabolites in these extracts as the percentage of the total extract. The external rinses were also chromatographed but contained only parent material at 3 and 6 h. Small amounts of metabolites (<2% of the total wash) were found in 24-h samples. These 'external' metabolites were not included in the table and do not change the comparative results. Relatively little of the penetrated ^{3}H material became unextractable, the greatest residue (5·2% of the total internal material) occurring in MA larvae at 24 h.

Chromatography of the larval extracts showed that bromophos-ethyl was degraded slowly, at about the same rate in all strains. The toxicologically significant difference was in the levels of the oxon of bromophos-ethyl (the active AChE inhibitor) particularly at 24 h when MA larvae contained considerably less toxicant than Y or B larvae. This was complemented by larger amounts of the water-soluble fraction, thus maintaining a total degradation rate similar to Y and B strains. This could result if most metabolism occurred via the oxidation to oxon at a similar rate in all strains followed by a somewhat faster degradation of the oxon to the water-soluble fraction in the MA strain. The continuing low levels of the debrominated oxon and the steadily increasing amounts of the water-soluble fraction (largely debrominated) suggested that much of the oxon was first debrominated and the product then rapidly deethylated to water-soluble products leaving only a small amount of the intermediate debromo oxon.

Inhibition of Bromophos-ethyl Metabolism

The results in Table 4*a* show that metabolism of bromophos-ethyl was substantially inhibited by piperonyl butoxide in all strains by about the same percentage, the greatest decrease being in the percentage of oxon. The mixed-function oxidase system is probably responsible for the initial oxidation of the acaricide to oxon and this appears to be the major metabolic route.

Inhibition of Bromophos-ethyl Oxon Metabolism

The results of studies of inhibition of bromophos-ethyl oxon metabolism are given in Table 4*b*. The control samples, ^{3}H-labelled oxon alone, showed that MA larvae were most successful in detoxifying the oxon and this explains the low levels found in bromophos-ethyl-treated MA larvae. Piperonyl butoxide caused relatively little but variable inhibition of oxon metabolism in the three strains of larvae. Because of this inconclusive result the effects of anoxic incubation conditions on oxon metabolism were determined. Substantial inhibition of metabolism in all strains resulted from incubations of larvae in a nitrogen atmosphere. Hence, regardless of the ineffectiveness of piperonyl butoxide, detoxication of the oxon appears to be oxygen dependent.

Inhibition of Mixed-function Oxidase by Bromophos-ethyl and Derivatives

Oxidation of [^{14}C]carbaryl to water-soluble metabolites in *B. microplus* larvae is inhibited by recognized mixed-function oxidase inhibitors and by anaerobic incubation (Schuntner and Thompson 1976), and thus appears to be catalysed by the mixed-function oxidase system. Bromophos-ethyl, its oxon and its phenol were compared with piperonyl butoxide as inhibitors of the enzyme *in vivo* (Table 5). At a concen-

tration of 0·001%, bromophos-ethyl and the oxon were as effective as piperonyl butoxide while the phenol was ineffective. This raises the possibility that the two active compounds have a high affinity for the enzyme and that the oxon may successfully compete not only with piperonyl butoxide but with bromophos-ethyl. This would explain the slow metabolism of the latter and possibly the lack of effect of piperonyl butoxide on oxon metabolism.

Table 4. (*a*) Metabolism of [^{3}H]bromophos-ethyl in larvae 6 h after dosage with 0·001% [^{3}H]bromophos-ethyl alone or with 0·02% piperonyl butoxide. (*b*) Metabolism of [^{3}H]bromophos-ethyl oxon in larvae 6 h after dosage with 0·001% radiochemical (incubated in air or nitrogen) and 6 h after dosage with 0·001% radiochemical and 0·02% piperonyl butoxide

(*a*)

Strain	Treatment	Composition of extract (% of total ^{3}H material)[A]			Inhibition of bromophos-ethyl metabolism (%)
		Bromophos-ethyl	Oxon	Water-soluble	
Y	Bromophos-ethyl	82·0	7·6	10·4	
Y	Bromophos-ethyl + piperonyl butoxide	95·4	0·7	3·9	74·4
B	Bromophos-ethyl	81·7	7·0	11·3	
B	Bromophos-ethyl + piperonyl butoxide	96·4	0·4	3·2	80·3
MA	Bromophos-ethyl	82·4	4·5	13·1	
MA	Bromophos-ethyl + piperonyl butoxide	96·4	0·4	3·2	79·5
Mean range (±)		0·2	0·1	0·2	
Extreme range (±)		0·5	0·5	0·5	

(*b*)

Strain	Treatment	Composition of extract (% of total ^{3}H material)[B]		Inhibition of oxon metabolism (%)
		Oxon	Water-soluble	
Y	Oxon, air	75·4	24·6	
Y	Oxon, N_2	91·1	8·9	63·8
Y	Oxon + piperonyl butoxide	79·3	20·7	15·9
B	Oxon, air	69·1	30·9	
B	Oxon, N_2	91·1	8·9	71·2
B	Oxon + piperonyl butoxide	77·8	22·2	28·2
MA	Oxon, air	35·2	64·8	
MA	Oxon, N_2	80·5	19·5	69·9
MA	Oxon + piperonyl butoxide	36·5	63·5	2·1
Mean range (±)		0·4	0·4	
Extreme range (±)		0·8	0·8	

[A] Extracts contained ^{3}H material equivalent to 20·2±1·6 μg [^{3}H]bromophos-ethyl/g larvae.
[B] Extracts contained ^{3}H material equivalent to 8·7±0·8 μg [^{3}H]bromophos-ethyl oxon/g larvae.

Metabolic Pathways

Our data indicate the metabolic pathways for bromophos-ethyl as shown in Fig. 2. The predominant route appears to be through the mixed-function oxidase-catalysed oxidation to oxon. This is indicated by the effectiveness of piperonyl butoxide in

preventing metabolism of the acaricide to both oxon and water-soluble material. As piperonyl butoxide had little effect on degradation of the oxon the small amount of water-soluble material produced from bromophos-ethyl in the presence of piperonyl butoxide doubtless was formed from oxon. Further evidence for this route lies in the small but consistent amount of debrominated oxon present in bromophos-ethyl-dosed larvae and in the major water-soluble 'end product', co-electrophoretically

Table 5. Inhibition of mixed-function oxidase oxidation of [^{14}C]carbaryl in Biarra larvae 4 h after dosage with 0·001% [^{14}C]carbaryl alone or with 0·001% bromophos-ethyl or oxon or 4-bromo-2,5-dichlorophenol or piperonyl butoxide

Treatment	^{14}C water-soluble material (% of total extracted ^{14}C material)[A]	Inhibition of oxidation of [^{14}C]carbaryl (%)
Carbaryl	54·5	
Carbaryl + bromophos-ethyl	14·4	73·6
Carbaryl + oxon	12·5	77·1
Carbaryl + 4-bromo-2,5-dichlorophenol	54·6	0
Carbaryl + piperonyl butoxide	13·1	76·0
Mean range (±)	1·1	
Extreme range (±)	1·7	

[A] Extracts contained ^{14}C material equivalent to 0·61 ± 0·05 μg [^{14}C]carbaryl/g larvae.

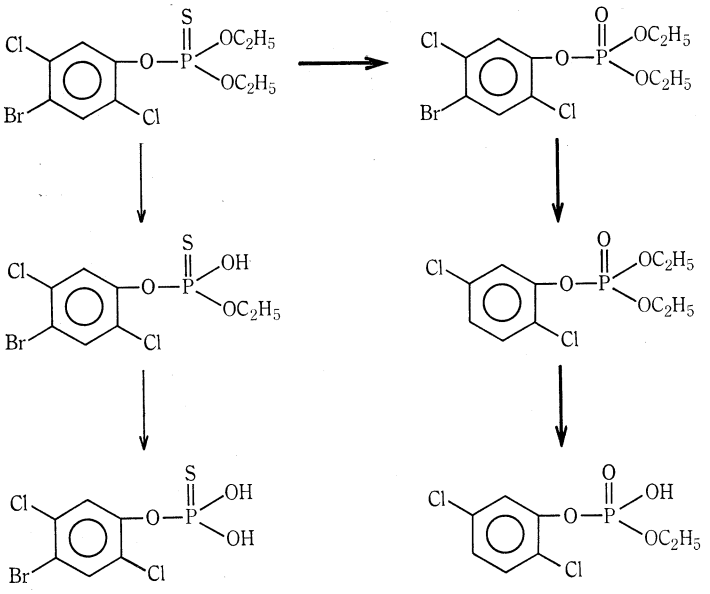

Fig. 2. Indicated metabolic pathways of bromophos-ethyl in *B. microplus* larvae. The predominant route is shown by the heavier arrows.

identified as the debromo-deethyl derivative of the oxon. Although debromination has been reported as a metabolic process in tomato (Stiasni *et al.* 1969), this was unexpected as a major process in *B. microplus*. The presence in the water-soluble fraction of small amounts of mono- and bis-deethyl bromophos-ethyl suggested that there was little direct deethylation of the parent, and the complete absence of phenolic derivatives indicated that there was no cleavage of the aryl-phosphate bond. Although

piperonyl butoxide had little effect on metabolism of bromophos-ethyl oxon the oxygen dependency of this metabolism suggests that it might still be catalysed by mixed-function oxidase.

Resistance Mechanisms

On our evidence, resistance of B larvae to bromophos-ethyl can be attributed only to the insensitivity of its AChE to inhibition by bromophos-ethyl oxon while MA larvae, in addition to this mechanism, were protected by their increased ability to detoxify the oxon. The relatively small difference in k values for the Y and B type AChE, 7·4×, is somewhat incongruous when compared with differences found for other organophosphate inhibitors, namely 10^2–10^3× (Schnitzerling *et al.* 1974). An additional mechanism which could enhance small resistances to bromophos-ethyl is suggested by the results of a preliminary experiment in which oxon levels of B and MA larvae were determined 16 h after their dosage with 0·03% [^{3}H]bromophos-ethyl. This dose, 30× our standard 0·001% dose, produced only a 7× increase in concentration of oxon in B larvae and a 4× increase in MA larvae. This indicates that the toxicity of the acaricide at near the field concentration (0·05%, w/v) cannot be increased greatly by increasing the dose. We conclude that it would be worthwhile studying metabolism of prospective acaricides at elevated doses in normal and in resistant ticks to determine the likely toxic reserves.

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