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SPECIFICITY OF ETHIRIMOL IN RELATION TO INHIBITION OF THE ENZYME ADENOSINE DEAMINASE

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Summary Ethirimol specifically inhibits powdery mildews, but these may differ in their sensitivity to the fungicide, and genotypes within a particular mildew species may also vary. Evidence is presented which suggests that such specificity towards mildews may be related to the presence of an unusual form of the enzyme adenosine deaminase, which is absent from other organisms. Differences in either the amount of this enzyme present, or its sensitivity to ethirimol may explain why fungicide activity differs between mildew species. In contrast, no differences were detected between adenosine deaminases from several Erysiphe graminis genotypes, although they differed widely in sensitivity to ethirimol, indicating that changes in this enzyme are not the cause of ethirimol resistance in this species.

INTRODUCTION

Ethirimol (2-ethylamino-4-hydroxy-5-n-butyl-6-methyl pyrimidine; Milstem*) acts only against some powdery mildews. It prevents appressoria formation by Erysiphe graminis during primary infection of barley (Bent, 1970; Hollomon, 1977), although other phases of disease development are also affected (Hollomon 1977). The evidence available suggests that ethirimol interferes in some way with adenine metabolism (Slade et al, 1972; Bent, 1970; Hollomon, 1979). However during appressoria formation purines are not synthesised de novo (Hollomon, 1979), and any adenine needed in nucleic acid synthesis etc. must be obtained by salvage from existing adenine nucleotides. The action of ethirimol may, therefore, be associated with this aspect of adenine metabolism.

In many organisms, hydrolytic deamination of adenosine by adenosine deaminase (E.C. 3.5.4.4.) to yield inosine is an important step in purine salvage. This paper examines the effect of ethirimol on adenosine deaminase (ADA-ase) from various sources.

METHODS AND MATERIALS

Fungal material was obtained from diseased plants in the field (Uromyces fabae, Puccinia obtegens), in the greenhouse (Erysiphe graminis, E. pisi, E. polygoni, Sphaerotheca fuliginea, Uromyces apendiculatus), or from laboratory cultures grown in appropriate liquid media. Myzus persicae was maintained on detached potato leaves, and barley plants (cv. Proctor) grown in a greenhouse. Purified ADA-ase from calf intestinal mucosa was purchased from Sigma Chemical Co. (London).

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Tissues were broken as appropriate and extracted in 0.1M phosphate buffer, pH 7.8. Extracts were centrifuged (8,700 x g for 1 min) to remove unbroken cells and larger debris, and dialysed overnight at 4°C against 1,000 volumes of buffer before use. Enzyme assays were performed at 25°C for 10 min using, where indicated, either [2-^{3}H] adenosine (55mCi/mmol; ADA-ase) or [G-$^3H_3$]deoxyadenosine (50mCi/mmol; Deoxy ADA-ase) as substrates. The products, [2-^{3}H] inosine or [G-^{3}H] deoxyinosine, were separated by TLC on Silica Gel 60F_{254} using n-butanol: acetone: water: 0.880 ammonia, 75:60:22.5:4.5, as solvents. Spots were located under u.v. light, scraped from the plates, and any radioactivity eluted from the Silica Gel with water before counting in Toluene (1 l.), Triton X-100 (500 ml), PPO (7g), POPOP (0.6g) in an Inter-technique liquid scintillation spectrometer. Enzyme activity is expressed in pmol substrate converted/mg protein/10 min at 25°C. A more detailed account of the assay procedures is to be published elsewhere (Hollomon, manuscript in preparation).

Mildew bioassays were conducted according to Hollomon (1977). The effects of ethirimol on rusts were assessed from spore germination tests in distilled water, and for other fungi from colony growth on appropriate agar media.

Fungicides were obtained from the relevant manufacturers for which thanks are gratefully acknowledged.

RESULTS

Enzymic activity with some of the properties of adenosine deaminase (no requirement for co-factors; a broad pH optimum between pH 5.5 - 8.5; deamination of both adenosine and deoxyadenosine, but not the corresponding purine bases or nucleotides) was readily detected in dialysed extracts from barley powdery mildew conidia. This activity was inhibited by ethirimol (Table 1). Inhibition, pH dependent, was greatest at pH 7.8 (Fig. 1). Lineweaver-Burke plots of enzyme activity at pH 7.8, and at different ethirimol concentrations, indicated that the fungicide was a non-competitive inhibitor of ADA-ase. The K_I for ethirimol with deoxyadenosine as substrate was 2.32×10^{-5}M (Fig. 2) and with adenosine as substrate 9.5×10^{-6}M. ADA-ase from E. pisi was also inhibited by ethirimol, whereas the same enzyme from E. polygoni and Sphaerotheca fuliginea was less affected. Bioassays showed these two mildews to be about 1000 times less sensitive to ethirimol than was the ethirimol sensitive genotype of barley powdery mildew used in Table 1. Most other fungi contained ADA-ase but even at high concentrations ethirimol had no effect, paralleling the lack of fungicidal activity of ethirimol to them. Purified ADA-ase from calf-intestine was also insensitive to ethirimol. No ADA-ase was detected in Myzus persicae or in healthy 10-day-old seedling leaves of barley. But extracts from mildewed leaves, 7-days after inoculation, and with extensive surface mycelium, contained ADA-ase, although these preparations were less sensitive to ethirimol than were those from barley mildew conidia (Table 1).

Genotypes of barley powdery mildew may differ in their sensitivity to ethirimol, although the magnitude of these differences depends somewhat on the type of bioassay used (Hollomon, 1975). Nevertheless, the K_I for ethirimol against deoxy-ADA-ase from conidia of several different genotypes was almost constant, despite their wide range in sensitivity by bioassay to ethirimol (Table 2). Other parameters such as V_{max} and K_m were also not correlated with ED_{50} values.

Many fungicides inhibit powdery mildews, although only the hydroxy-pyrimidines prevent appressoria formation (Schlüter and Weltzien 1971). Even at high concentrations, none of the fungicides examined so far inhibits ADA-ase from barley powdery mildew conidia (Table 3).

DISCUSSION

Without de novo purine biosynthesis, the salvage of adenine from existing nucleotides is probably essential if primary infection of mildews is to succeed. Whilst salvage may occur by several routes, in barley powdery mildew deamination of adenosine seems important, and its inhibition by ethirimol is likely, therefore, to soon affect development.

The fact that only adenosine deaminases from powdery mildews were inhibited by ethirimol suggests that they contain a form of the enzyme not found elsewhere, which may account for the specificity of ethirimol towards these diseases. Although permeability to the fungicide, and the ability to detoxify it may vary, differences between mildew species in their sensitivity to ethirimol may also be due to differences either in the amount of ADA-ase present, or in the sensitivity of the enzyme to ethirimol. Differences within a particular species cannot be explained in this way, however, for the ADA-ases from all the E. graminis genotypes examined so far were similar, despite a wide range of ED_{50} values.

Since ethirimol is a non-competitive inhibitor of ADA-ase it probably binds to a regulatory site on the enzyme. In eukaryotic organisms, regulation of a particular metabolic sequence is often achieved by a single metabolite which binds to some site on several enzymes in that pathway. Structurally similar inhibitors which bind to such a regulatory site may affect more than one enzyme, and this frequently is the case with nucleoside antibiotics (Suhadolnik, 1979). Although examination of a few other enzymes involved in purine salvage in barley powdery mildew has not yet revealed any inhibition by ethirimol, it is possible that it may inhibit more than just ADA-ase. If so, resistance to ethirimol might require similar mutational change in several enzymes, explaining why ethirimol resistance is not yet serious.

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Table 1

Effect of ethirimol on ADA-ase from different sources

	ADA-ase activity+ No Ethirimol	ADA-ase activity+ 200 µg/ml Ethirimol	% inhibition	Ethirimol sensitivity ED_{50} µg/ml
Powdery mildews				
E. graminis conidia	48.0	0.8	98	0.019
E. pisi conidia and mycelium	6.7	0.4	94	0.45
E. polygoni conidia	60.7	38.1	37	29.17
Sphaerotheca fuliginea conidia	8.9	4.5	42	10.35
Other fungi				
Streptomyces scabies	7.9	8.5	None	No effect
Penicillium sp. spores	2.0	2.1	None	
mycelium	76.9	72.2	6	No effect
Cladosporium cucumerinum spores & mycelium	11.4	14.8	None	No effect
Fusarium sp. mycelium	34.9	33.5	4	No effect
Uromyces apendiculatus spores	2.3	2.4	None	
germlings	4.4	4.4	None	No effect
Uromyces fabae germlings	8.5	7.5	12	No effect
Puccinia obtegens spores	*	*	*	
germlings	4.3	4.7	None	No effect
Saccharomyces cerevisiae	0.8	0.7	12	No effect
Plants				
Barley (cv. Proctor) healthy	*	*	*	
mildew infected	4.8	2.8	42	
Others				
Calf intestine (purified)	54,100	48,300	11	
Myzus persicae	*	*	*	

*ADA-ase activity not detected.

+Adenosine concentration in all assays was 3.6×10^{-4}M.

Table 2

Effect of ethirimol on deoxy ADA-ase from conidia of different barley powdery mildew genotypes

Genotype	Ethirimol sensitivity ED_{50} µg/ml	K_I ethirimol
23D5	0.019	2.32×10^{-5}M
DH22	0.47	2.59×10^{-5}M
JS21	0.51	1.09×10^{-5}M
32B5	2.01	1.69×10^{-5}M
DH9	3.35	1.14×10^{-5}M
DH14	17.80	2.67×10^{-5}M

Table 3

Effect of several fungicides on ADA-ase from E. graminis

Fungicide	ADA-ase* activity	% Inhibition	Bioassay Inhibition of appressoria formation	Bioassay Inhibition of colony growth
None	48.0			
Ethirimol 20 µg/ml	10.0	79	1.2×10^{-6}M	3.0×10^{-5}M
Dimethirimol 20 µg/ml	25.3	47	1.2×10^{-6}M	5.4×10^{-4}M
"Persulon" (a.i. fluotrimazole) 1 mg/ml	45.8	5	No effect	slight effect
Methyl benzimidazol-2-yl-carbamate 5.8 µg/ml	48.6	None	No effect	3.6×10^{-5}M
Triadimefon 250 µg/ml	51.3	None	No effect	1.0×10^{-3}M
Chloraniformethan 250 µg/ml	49.3	None	No effect	3.0×10^{-5}M
Pyrazophos 333 µg/ml	48.5	None	No effect	4.4×10^{-4}M
Tridemorph 250 µg/ml	48.7	None	No effect	5.3×10^{-5}M

*Adenosine concentration in all assays was 3.6×10^{-4}M

Fig. 1. Effect of pH on inhibition of ADA-ase by ethirimol

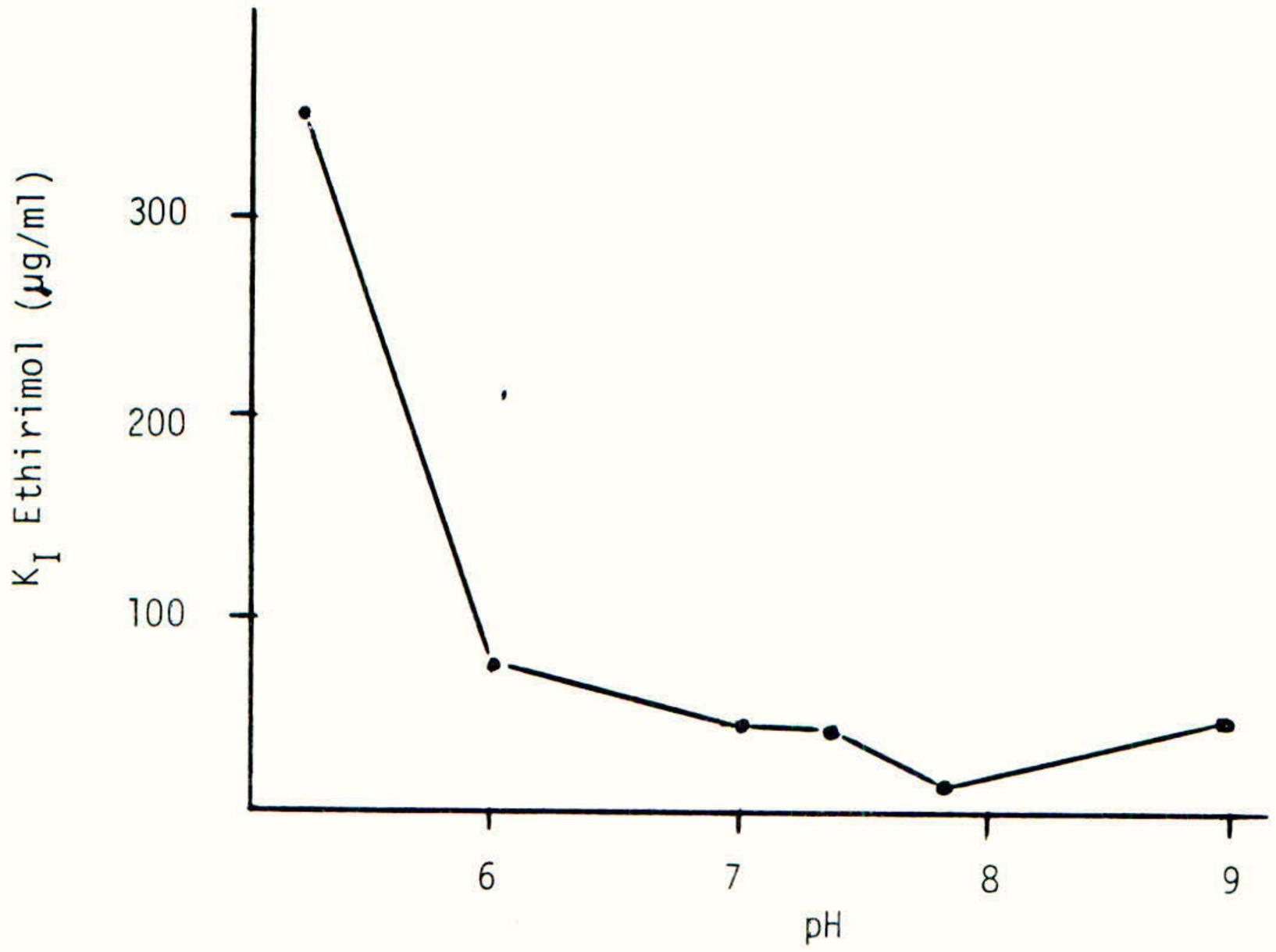
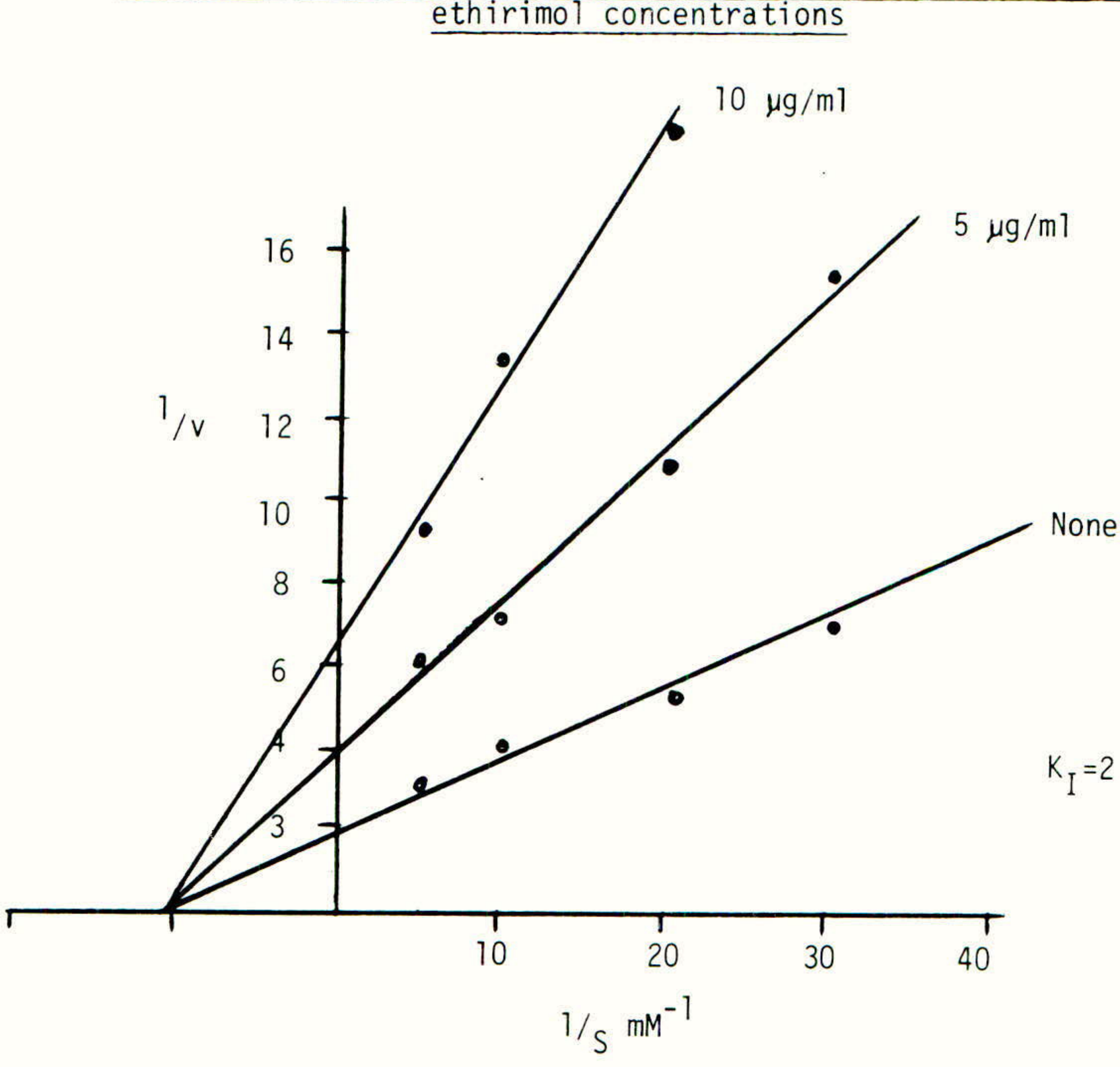


Fig. 2. Lineweaver-Burke plots of deoxy ADA-ase activity at different ethirimol concentrations



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THE METABOLISM OF TWO METHYLENEDIOXYPHENYL COMPOUNDS IN SUSCEPTIBLE AND RESISTANT STRAINS OF Tribolium castaneum

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Summary Piperonyl butoxide and a juvenile hormone mimic (compound 2b of W.S. Bowers) were both metabolised more rapidly in resistant than susceptible Tribolium castaneum adults. The main site of attack on both compounds in the resistant strain was the methylene group of the methylenedioxyphenyl moiety, but in the susceptible strain other sites on the molecules were of greater importance. Developing larvae of the resistant strain were resistant to the hormonomimetic effects of compound 2b and the toxic effects of high doses of piperonyl butoxide. This resistance and the metabolic differences between the two strains should be considered when assessing the synergistic effects of piperonyl butoxide.

INTRODUCTION

CHAMP and CAMPBELL-BROWN (1970) showed that a malathion-resistant strain (CTC 12) of Tribolium castaneum (Herbst) from Australia was resistant to lindane, DDT, pyrethrum, carbamates and many organophosphorus insecticides besides malathion. The strain is also resistant to compounds with juvenile hormone activity, synthetic pyrethroids and diflubenzuron (DYTE, 1972; DYTE et al., 1975; CHAMP and DYTE, 1976. This wide spectrum of cross resistance suggests one non-specific resistance mechanism or several mechanisms.

Our previous studies suggested that resistance to organophosphorus compounds in this strain involves detoxication (DYTE et al., 1970). The best known non-specific detoxication processes in insects are those involving mixed function oxidases. These enzymes can often be inhibited by methylenedioxyphenyl (MDP) compounds such as piperonyl butoxide, which are known to act as insecticide synergists and may have enhanced activity against resistant strains (CASIDA, 1970). Since we found that MDP synergists did not overcome the resistance of this strain of T. castaneum to organophosphorothioates (P = S compounds) or organophosphates (P = O compounds) we decided to examine the metabolism of MDP compounds in both the susceptible and the resistant strains. The two compounds studied were the synergist, piperonyl butoxide (Fig. 1) and one of the juvenile hormone mimics (compound 2b of BOWERS, 1969) (Fig. 2) to which this strain of T. castaneum is resistant.

MATERIALS AND METHODS

Chemicals

Piperonyl butoxide (I), labelled ^{14}C in the methylene moiety, was prepared by methylation of the corresponding catechol (VI) with 0.2 mCi of ^{14}C-diiodomethane, by using the method of KUWATSUKA and CASIDA (1965). Yield based on radioactivity was 54 per cent. The following unlabelled "reference metabolites", α-6-propylpiperonylic acid (II); 6-propylpiperonyl alcohol (III); α-(hydroxyethoxy-4,5-

methylenedioxy-2-propyl toluene (IV) and α-[2-(2-hydroxy ethoxy)ethoxy-4,5-methylenedioxy-2-propyltoluene] were synthesised by the methods of ESAAC and CASIDA (1969).

Bowers' compound 2b, labelled ^{14}C in the methylene moiety, was prepared similarly: ^{14}C-sesamol was produced by peracetic acid oxidation of ^{14}C-piperonal (BOESEKEN and GREUP, 1939) formed by ring closure of protocatechualdehyde with ^{14}C-diiodomethane. By the method of BOWERS (1969), 1-bromo-3,7-dimethyl-2,6-nonadiene was prepared from ethyl-1-bromo-3-pentene. This was then converted to 3,7-dimethyl-1-(4,5-methylenedioxy)-2,6-nonadiene by reaction with ^{14}C-sesamol and sodium hydroxide, and finally to the 6,7-epoxide (compound 2b) by oxidation with perbenzoic acid. The final yield of chromatographically pure radioactive product was 15% of the initial 0.5 mCi diiodomethane. For both radiolabelled compounds, feasibility and accuracy of synthesis was confirmed by NMR spectroscopy of unlabelled pilot runs and the 'model' 2b compound was checked by the method of DYTE (1972) to confirm that it had satisfactory juvenile hormone activity compared with a reference sample provided by Roche Products Ltd.

Reaction of unlabelled 2b with perchloric acid in aqueous tetrahydrofuran (WHITE, 1972) yielded the 6,7-diol derivative in 65 per cent yield. All other compounds used were either available commercially or had been prepared during earlier studies.

Treatment and exposure of adult insects

Adults of two strains of Tribolium castaneum (Herbst), 3-5 weeks old, which had been cultured on wholewheat flour at 25°C and 70% r.h. were used. They were the susceptible strain 1 of DYTE and BLACKMAN (1970) and the resistant strain CTC 12 of CHAMP and CAMPBELL-BROWN (1970), and are the same as those used previously by DYTE (1972).

Synergist or hormone was dissolved in cyclohexanone and applied topically with a 0.05 µl capillary, as described by DYTE and BLACKMAN (1967). Batches of 30 insects were used for the studies with piperonyl butoxide and of 20 insects for the work with 2b. In both cases the total dosage of each compound per batch was 1.5 µg and the specific activity was adjusted by adding unlabelled material to standardise the total ^{14}C activity at 3,000 cpm.

After treatment, each group of insects was confined, in a room maintained at 25°C and 70 percent r.h., without food, by a glass ring standing on a glass plate covered with Whatman no. 1 filter paper. Each of these rings (6 cm diam. x 2 cm high) was put in an empty desiccator which was then sealed with vaseline and continually flushed with a current of dry air. The air from each desiccator was passed through two Dreschel bottles containing 5% (satd) barium hydroxide solution, to trap evolved $^{14}CO_2$.

At appropriate intervals after treatment the insects, together with rings, plates and papers, were put into the icebox of a domestic refrigerator for a maximum of 1 day to await homogenisation.

Separation and assay of metabolites

Procedures were similar for both compounds studied. Each batch of insects together with the paper on which it had been confined, was macerated with 20 ml of diethyl ether and the extract centrifuged. The rings and plates were washed with ether and the rinsings were added to the supernatant, after centrifugation of the homogenate, to form extract A. The precipitated insect and paper debris were then homogenised with 20 ml aqueous sodium acetate buffer, pH 5, and centrifuged. The supernatant was extracted with ether and after separation, the ether was dried and added to extract A; 10 mg β-glucosidase (Koch-Light Ltd) was added to the remaining

aqueous portion and incubated overnight (15 h) at 38°C. This was then extracted with 10 ml ether which was dried after separation (extract B). The aqueous portion was made alkaline with sodium hydroxide and incubated for 3 h at 60°C before re-extraction with ether (extract C). The aqueous layer was finally evaporated to dryness under reduced pressure and residual ^{14}C activity measured. The ether extracts (A,B,C) were analysed by thin-layer chromatography (TLC), and the areas corresponding to known metabolites (Table 1) removed and their radioactivity assessed by liquid scintillation counting. Unlabelled derivatives (the catechols etc) were identified but not quantified by TLC followed by electron-capture gas-liquid chromatography (GLC) of their trimethylsilyl (TMS) ether derivatives on a glass column 1 metre x 2 mm i.d. packed with 80/100 mesh Chromosorb W coated with 4 per cent Apiezon (Perkin-Elmer Ltd) and nitrogen gas flow of 100 ml per min.

RESULTS

Piperonyl butoxide

Extraction of insect homogenates followed by TLC yielded seven radiolabelled components, 4 ether-soluble (I, II, III, IV) and 3 water-soluble (VII, IX, X). Two of the latter yielded the same ether-soluble compound (II) after incubating with glucosidase and/or alkaline digestion, together with two unlabelled products. Compounds tentatively identified by co-chromatography were unchanged piperonyl butoxide, 6-propylpiperonylic acid, 6-propylpiperonyl alcohol, the alcohol IV which was present in trace amounts and (unlabelled) the catechol derivative of I. From the digests, the ether-soluble compound co-chromatographed with 6-propylpiperonylic acid and left a water-soluble compound (V) after alkaline treatment that yielded CO_2 on reaction with silver nitrate suggesting a formate-formaldehyde derivative. In essence the aqueous portion containing water-soluble compound (V) was neutralised with dilute nitric acid and 0.1 M silver nitrate added with warming until a black precipitate of silver formed. Evolved CO_2 was trapped as barium carbonate. The remaining radioactivity was accounted for by CO_2 also trapped as barium carbonate.

There were no qualitative differences in the labelled metabolites from the two strains but considerable quantitative differences (Fig. 1, Table 2). Metabolism of the synergist was much more rapid in the resistant strain with predominant attack at the labelled methylenedioxy group to yield the catechol and $^{14}CO_2$, and the unidentified formate/formaldehyde derivative V. In susceptible beetles the polyether was attacked preferentially (Fig. 3).

Compound 2b

Metabolism of compound 2b was also qualitatively similar in the two strains but much more rapid in the resistant beetles (Fig. 2, Table 3). Whereas hydration of the epoxy group was the main metabolic route in the susceptible beetles, the main attack was again at the methylenedioxy group in the resistant strain.

Ether-soluble compounds from both strains included intact 2b, the catechol resulting from attack at the methylenedioxy carbon, and the free 6,7-diol produced by hydration of the epoxide group. Water-soluble products yielded the 6,7-diol after glucosidase incubation, as well as traces of the unlabelled catechol. At least one unidentified compound was found in each strain (Table 3) though these were apparently different compounds. From the resistant strain, the unknown was a conjugate yielding on glucasidase treatment a ^{14}C- water-soluble compound that could be readily oxidised to CO_2. In the susceptible insects, the unknown(s) was unaffected by glucosidase treatment and yielded only traces of CO_2 on oxidation by $AgNO_3$. It may be relatively more important than the unknown(s) from the resistant beetles since it accounted for 25% of the radioactivity recovered at 48 h. Attack at the methylenedioxy group seemed to be relatively unimportant in the susceptible strain (Fig. 4).

DISCUSSION

Previous work (DYTE et al., 1975) has shown that the CTC 12 strain is resistant to the hormonomimetic effects of compound 2b, and it is also resistant to the toxicity of relatively high doses of piperonyl butoxide (J.P. EDWARDS, unpublished). Both compounds were metabolised more rapidly in the resistant than in the susceptible beetles. It seems very likely that this is the main cause of the resistance to methylenedioxyphenyl compounds, particularly since the methylenedioxy group is the major site of attack in the resistant but not the susceptible beetles (Tables 2 and 3).

The preferential attack on the methylenedioxy group by the resistant beetles is of particular interest because this is the part of the molecule essential for synergistic activity. The methylenedioxyphenyl synergists enhance insecticide toxicity by inhibiting the mixed function oxidase system of microsomes (CASIDA, 1970). In the resistant strain it is possible that the affinity of enzyme and inhibitor has become an affinity of enzyme and substrate. Our failure to overcome the insecticide resistance of the CTC 12 strain of T. castaneum by using methylenedioxyphenyl synergists is not therefore surprising since the strain is resistant to these compounds. It cannot be taken to indicate that mixed function oxidases are not involved in the resistance.

Acknowledgements

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Table 1

Thin-layer chromatography of piperonyl butoxide, compound 2b and their metabolites

Compound	R_f (X 100) in solvent systems* 1	2	3
Piperonyl butoxide (I)	21	45	76
Piperonyl alcohol	6	32	69
Piperonal	13	27	61
Piperonylic acid	0	12	59
Catechol deriv. of (I), (IV)	17	49	55
Catechol	16	54	64
6-propyl piperonylic acid (II)	34	66	77
6-propyl piperonyl alcohol (III)	31	51	75
Alcohol (IV)	25	42	49
Formic Acid	0	0	-
Formaldehyde	0	0	-
6-propyl piperonal	50	68	-
Compound 2b	25	-	82
Catechol deriv. of 2b	8	-	75
Diol deriv. of 2b	0	-	62
Sesamol	20	-	80

* 1 = ether : hexane (1 : 4); 2 = benzene : methanol (3 : 1); 3 = methanol : chloroform (1 : 4). Chromogens were iodine and chromotropic acid, and chromatography was on Kodak K301R sheets activated at 100°C for 30 min.

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Table 2 and Fig. 1

Labelled metabolites of piperonyl butoxide from susceptible (S) and resistant (R) strains of Tribolium castaneum

Metabolite	Strain	^{14}C activity (%) at various times (h) after treatment			
		10	15	24	48
Intact piperonyl butoxide (I)	S	92.6	86.7	60.8	47.6
	R	52.8	30.8	11.9	nil
6-propyl piperonylic acid (II)	S	4.8	8.6	18.8	19.8
	R	11.7	14.7	11.6	9.9
6-propyl piperonyl alcohol (III)	S	0.8	1.9	4.7	7.5
	R	2.0	2.1	5.3	5.2
Compound VII (glucoside of II)	S	-	-	3.6	10.6
	R	1.0	1.0	7.7	11.9
Compound VIII (conjugate of II?)	S	-	-	1.0	1.7
	R	-	-	-	-
Compounds IX and X	S	-	-	3.1	3.8
	R	1.0	6.8	11.8	18.8
Compound V (formate yielding $^{14}CO_2$)	S	-	-	2.1	2.0
	R	20.7	31.7	36.9	37.7
Carbon dioxide	S	-	1.7	3.9	4.9
	R	7.1	12.2	11.7	15.1
Total activity recovered	S	98.2	98.9	98.0	97.9
	R	96.3	99.3	96.9	98.6

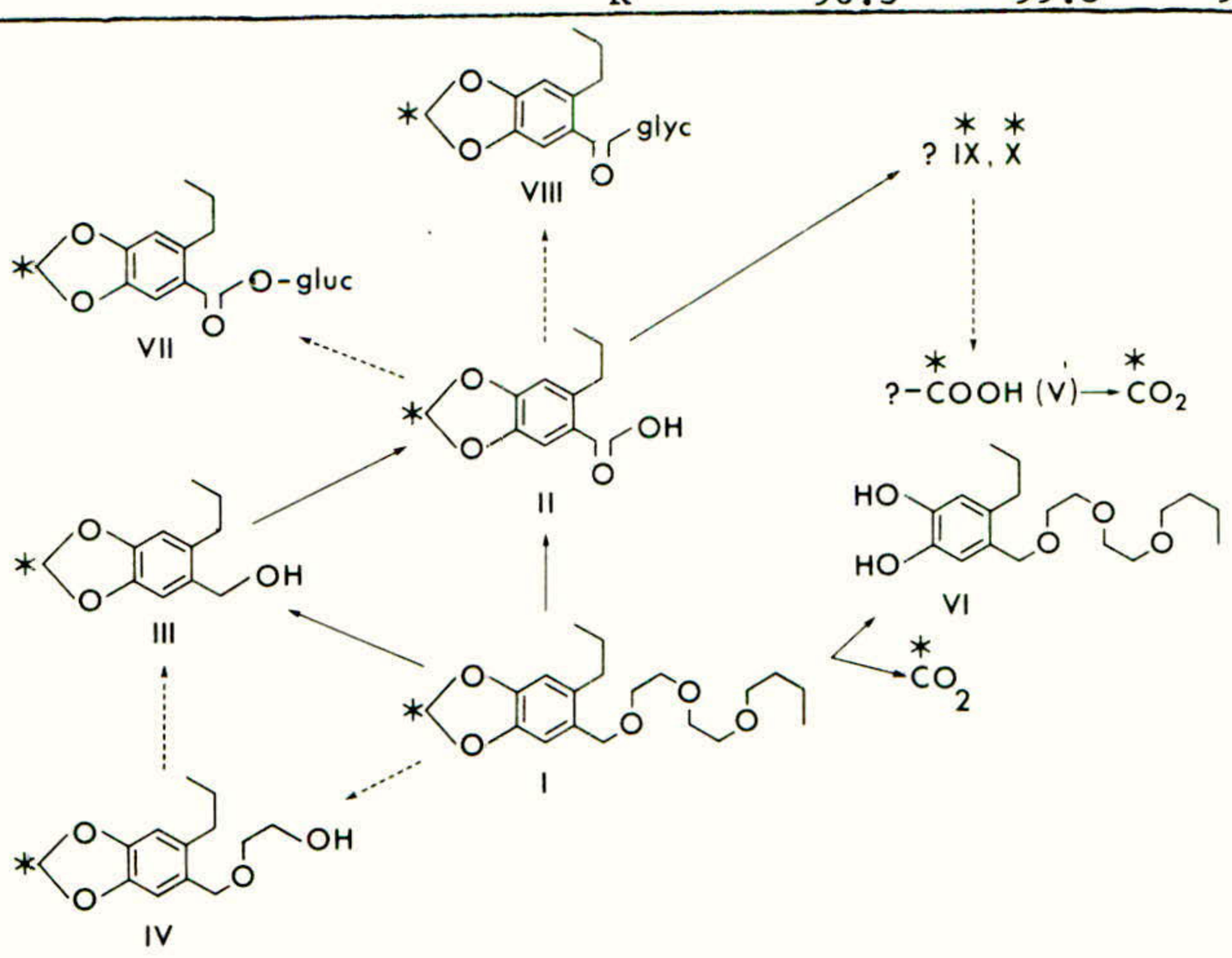


Table 3

Labelled metabolites of Bowers' compound 2b from susceptible (S) and resistant (R) strains of Tribolium castaneum

Metabolite	Strain	^{14}C activity (%) at various times (h) after treatment				
		1	5	15	24	48
Intact 2b	S	98.2	98.3	82.4	73.6	32.1
	R	91.3	75.0	30.3	9.4	nil
Diol derivative (free)	S	nil	nil	7.1	10.3	23.9
	R	nil	2.3	5.7	13.1	20.4
" " (conjugated)	S	nil	nil	3.1	5.3	19.8
	R	nil	nil	1.4	5.2	5.7
" " (total)	S	nil	nil	10.2	15.6	43.7
	R	nil	2.3	7.1	18.3	26.1
Carbon dioxide	S	nil	nil	2.6	2.4	3.1
	R	5.2	13.4	51.7	56.3	51.8
Unknown*	S	nil	nil	3.7	4.8	17.3
	R	1.0	4.2	6.7	12.1	17.7
Total activity recovered	S	98.2	98.3	98.9	96.4	96.2
	R	97.5	94.9	95.8	96.1	96.6

* There were differences in the labelled unknowns recovered from the two strains (see text).

Fig. 2

Pathways of metabolism of compound 2b

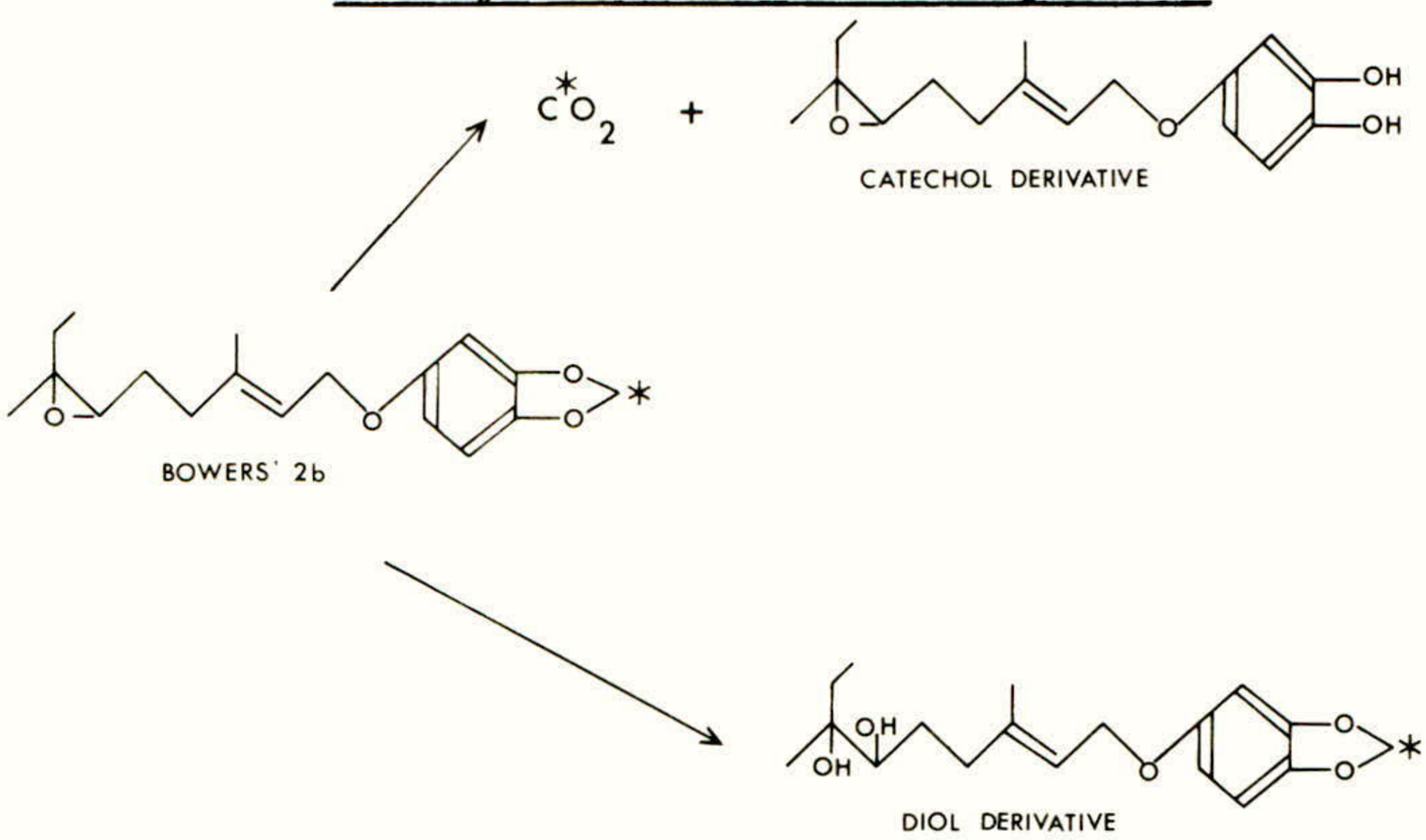


Fig. 3

Loss of piperonyl butoxide and production of labelled carbon dioxide (and formate) during metabolism by susceptible (S) and resistant (R) Tribolium castaneum

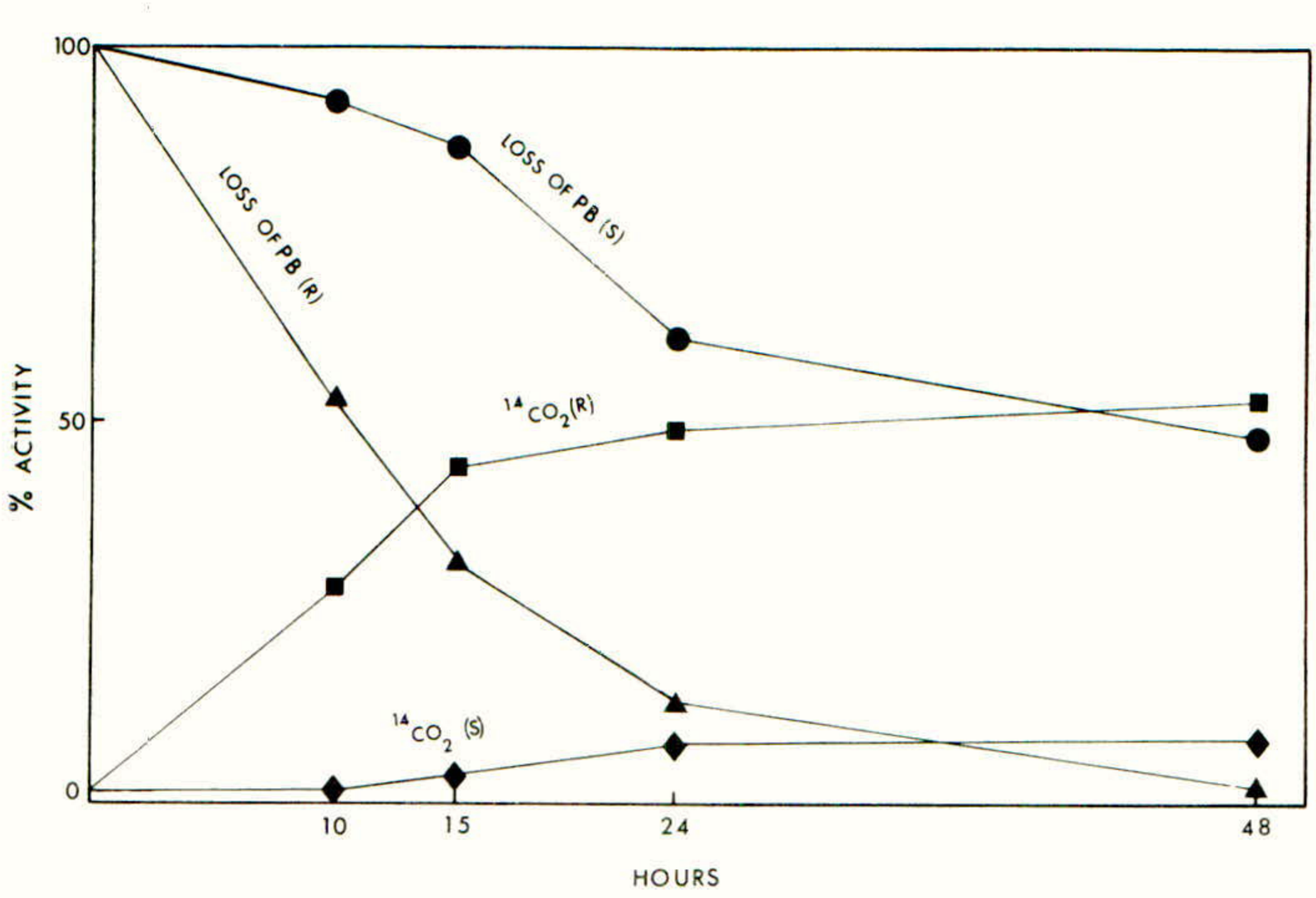
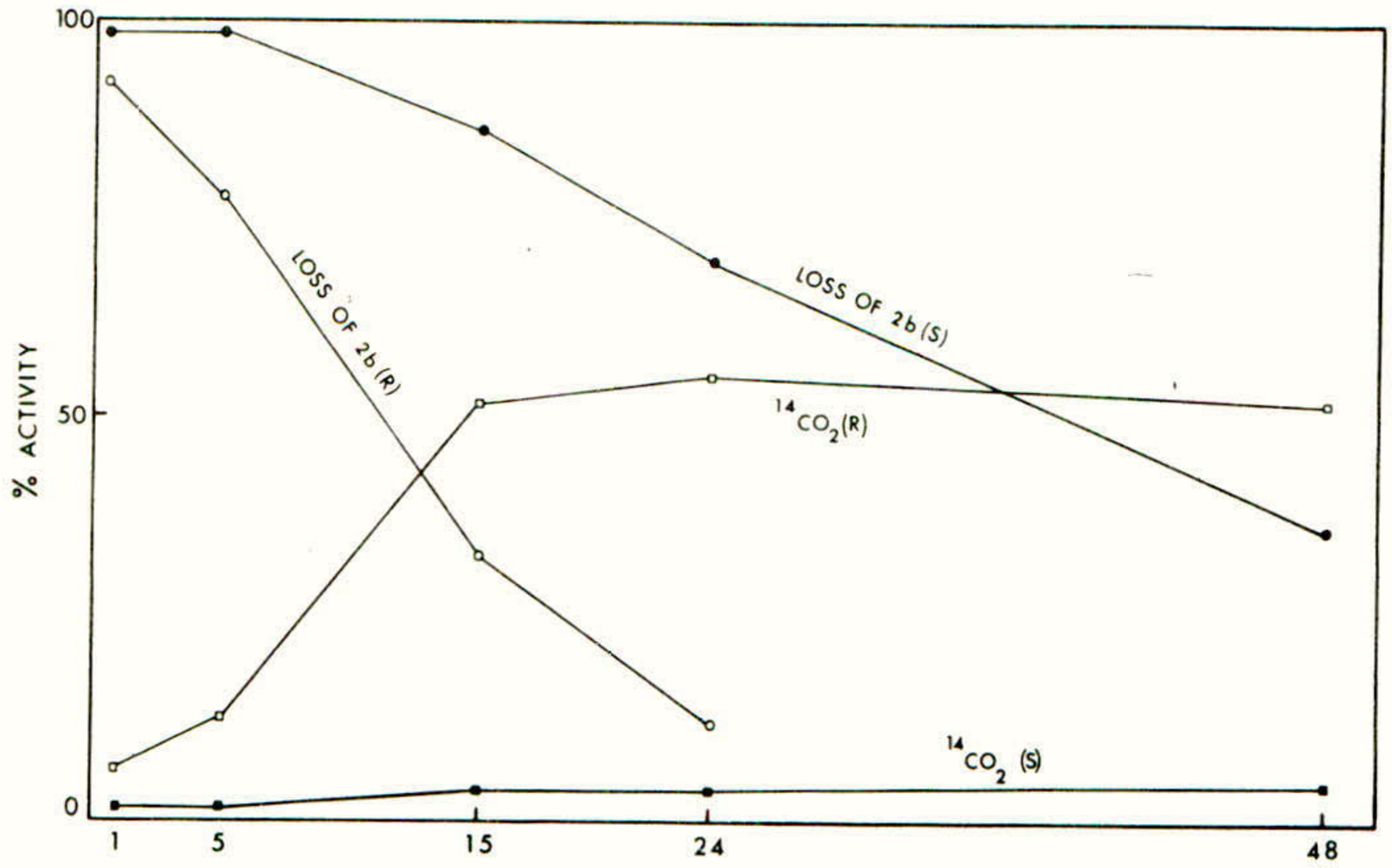


Fig. 4

Loss of compound 2b and production of labelled carbon dioxide during metabolism of 2b in susceptible (S) and resistant (R) Tribolium castaneum



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THE EFFECTS OF AMITRAZ ON INSECTS

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Summary Amitraz has been shown to kill the eggs and neonate larvae of a very wide range of lepidopterous pests in the field, and is particularly useful in repeat spray programmes since it controls caterpillars before they damage the crop. Besides its ovicidal effect the value of the compound is enhanced by its effects on large larvae and adults. Fecundity and egg laying behaviour of treated moths are affected and the feeding behaviour of larvae is disrupted, reducing the egg laying potential of adults and the crop damage caused by larvae. Since the eggs, larvae and adults of many beneficial insects, particularly lepidopteran parasites, are unaffected by amitraz, it should be useful in integrated control programmes.

Résumé On a démontré que l'amitraz tue les oeufs et larves neonates d'un très grand nombre de lépidoptères. Ce produit est particulierement intéressant lorsqu'on l'utilise selon un programme de traitements répétés étant donne qu'il tue les chenilles avant qu'elles n'endommagent la culture. En outre la valeur de ce produit tient à ce que son activité ovicide est renforcée par ses effets sur larves âgées et adultes. La fécondité et la ponte des lépidoptères traités sont affectées, leurs larves ne se nourrissent plus. Ceci conduit à une réduction des pontes et des dommages causés par les larves. Enfin étant donne que les oeufs, les larves et les adultes de nombreux insectes auxiliaires notamment parasites des lépidoptères ne sont pas affectés par l'amitraz, on peut l'inclure dans les programmes de lutte integrée.

INTRODUCTION

Amitraz has been known for some time to possess selective insecticidal properties in addition to its strong acaricidal activity (Weighton et al, 1973). However, during trials on aphids and mites in top fruit it was evident that amitraz gave considerable control of some Lepidoptera, as had been observed in laboratory studies, and this provided the stimulus for extensive trials with amitraz for the control of Lepidoptera in a number of crops. Field observations suggested that amitraz killed the eggs and neonate larvae of lepidopterous pests but not later larvae, nor did it significantly reduce the numbers of natural insect predators or parasites present in trials. This paper reviews the effects of amitraz on lepidopterous pests and their parasites/predators in the field and records the results of laboratory experiments to define the effects of amitraz giving the observed field responses.

FIELD RESPONSES

Early observations on amitraz performance in the field showed that one of its most important effect on Lepidoptera was a direct lethal action on eggs. Table 1 shows the percentage hatch of egg batches of Spodoptera littoralis during field trials in Egypt. Egg batches in tests plots were marked and, after treatment, examined at daily intervals.

Table 1

Effect of amitraz on Spodoptera egg masses in cotton trials - 1977

Amitraz Rate g a.i./ha	Hatch	No. of Egg Masses	Response % Total
720	complete	20	15
	partial	9	7
	none	107	79
480	complete	33	21
	partial	10	7
	none	111	72
0	complete	84	90
	partial	1	1
	none	8	9

Amitraz clearly exerts a considerable effect on the egg masses of Spodoptera. In a later section it will be shown that amitraz kills both eggs and neonate larvae of Lepidoptera as well as having a number of non-lethal effects which may also be significant under field conditions. In field trials this ovicidal/neonate larvicidal action is apparent from a few days after treatment (Table 2).

Table 2

Control of Heliothis larvae on cotton by amitraz and permethrin treatments, Turkey, 1977

Days Post Spray		Amitraz 400 g a.i./ha	Permethrin 125 g a.i./ha	Untreated
Prespray	Larval no.*	80	24	54
2	Larval no.	31	1	34
	% Mortality	39	93	-
6	Larval no.	13	1	23
	% Mortality	62	90	-
13	Larval no.	10	81	39
	% Mortality	83	55	-

*Larval numbers per 150 cotton plants. Percentage mortalities corrected for untreated variations using Abbotts formula (Abbott 1925).

This example serves to illustrate some important points on the action of amitraz. Two days after spraying larval numbers decreased slightly in the amitraz treated plots, which may have been caused by death of neonate larvae or a repellent effect. Larval numbers progressively decrease from two days after amitraz treatment.

This is thought to result from the ovicidal activity of amitraz preventing the emergence of new larvae from treated eggs. The large larvae pupate but no new larvae hatch, consequently reducing populations.

This effect of amitraz on eggs and neonate larvae is more apparent when the compound is used in a repeated programme of sprays as shown in Table 3.

Table 3

Control of Spodoptera during a repeat spray programme in Egypt, 1977

Date sprayed	Date of count	Larval numbers: Amitraz 480 g a.i./ha	Larval numbers: Untreated
2.7 + 12.7	13.7	1	72
	20.7	0	4
22.7	28.7	0	0
1.8	3.8	0	188
	10.8	7	86
11.8	17.8	0	37
21.8	28.8	0	0
	9.9	0	78

Amitraz has been shown to be safe to insect predators (Coulon 1978, Evenson 1979), confirming the value of amitraz in integrated pest management programmes. Coulon (1978) concluded that amitraz used at 0.14-0.28 kg a.i./ha did not significantly reduce populations of beneficial arthropods sampled by sweeping, other than a reduction in the population of Chrysopa larvae. Figure 1 shows an example of the population dynamics of parasitic Hymenoptera in treated and untreated plots.

Hence, it has been shown that amitraz effectively controls the eggs and neonate larvae of insects from the order Lepidoptera. Excellent control of Spodoptera and Heliothis has been achieved in the field, especially when used in spray programmes. In addition, amitraz has little effect on many beneficial arthropods and is therefore suitable for integrated pest management programmes.

However, other non-lethal effects of amitraz have been noted in the laboratory which might contribute to effective insect control in the field.

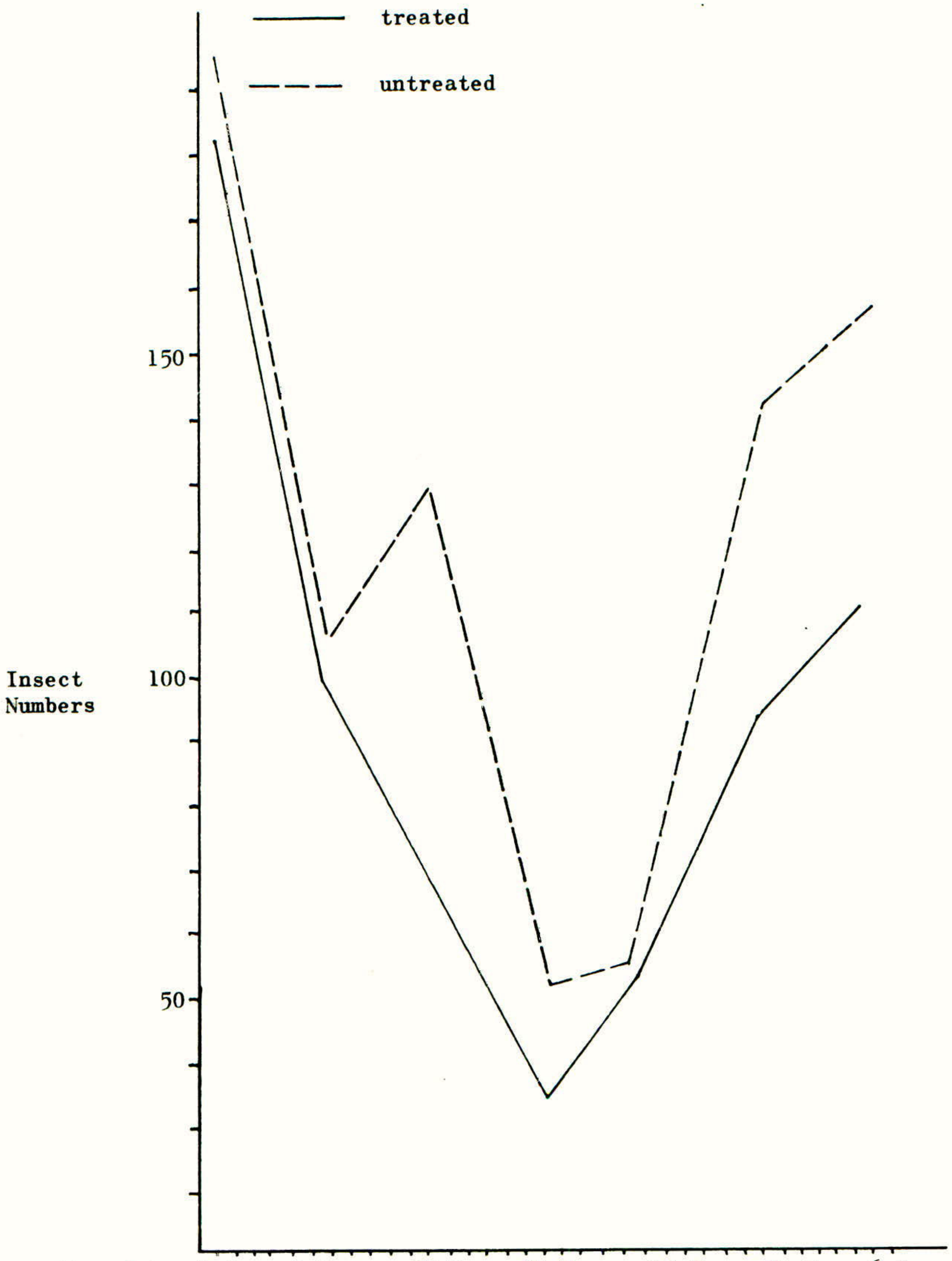

Fig. 1 Mean numbers of parasitic Hymenoptera per 400 sweep in amitraz study, Chambers, Louisiana, 1977. Fran Coulon (1978).

LABORATORY STUDIES OF THE NON-LETHAL EFFECTS OF AMITRAZ

1. Reduction in the fecundity of adult Lepidoptera

When larvae of Pieris brassicae are reared on cabbage leaves treated with amitraz at 300 or 600 ppm the adults of this treated generation show a considerable decrease in fecundity. There is a similar effect on the Fl generation adults (Fl generation larvae being reared on untreated cabbage), although this is less marked than that on treated generation adults (Table 4).

Table 4

Fecundity of adult Lepidoptera following treatment of larvae with amitraz

	Pieris brassicae+ Treated generation adults (1)	Pieris brassicae Fl generation adults (1)	Spodoptera littoralis Treated generation adults (2)
Untreated	55	63	156
Amitraz 50 ppm	-	-	133
500 ppm	22	-	-
600 ppm	20	52	-

+Figures in columns are the numbers of eggs laid/female/day, observed over a period of (1) 16 days or (2) 7 days.

Table 5 shows that direct application of amitraz to adult Lepidoptera significantly reduces the number of eggs laid per female. In addition, there are suggestions that the reduction in fecundity of adult Lepidoptera after amitraz treatment may result from an alteration of their behavioural pattern, since untreated Pieris lay eggs only on leaf surfaces but amitraz treated butterflies also laid eggs on the sides of the laying cage (constructed from cheese cloth stretched over wooden frames) or on plastic plant pots present in the cage.

Table 5

Effect of direct spray application of amitraz on the fecundity of adult Lepidoptera

Treatment	number of eggs laid/female/day Pieris brassicae (1)	Heliothis armigera (2)
Untreated	14.4	51.1
Amitraz 100 ppm	11.0	31.7
500 ppm	3.2	4.4
1000 ppm	5.6	-

(1) Observed over a period of 15 days
(2) Observed over a period of 16 days

Amitraz also reduced the longevity of adult Pieris treated by direct spray, (Table 6), thus further decreasing the number of eggs laid per female per generation. At high temperatures (34°C) amitraz exerts a direct lethal action on adult Lepidoptera (Table 6).

Table 6

Survival of amitraz-treated adult Pieris at two temperatures

Temp.	Control[+]	Amitraz 100 ppm
	Days survived after treatment	
27°C	14.3 ± 1.16	11.3 ± 2.87
34°C	8.5 ± 1.97	1.33 ± 0.52

[+]Controls were sprayed with the formulation blank without active ingredient

Thus, amitraz affects the fecundity of adult Lepidoptera in three ways. Firstly, larvae fed on sub-lethal concentrations produce adults with reduced fecundity. In a spray programme any surviving larvae would be exposed to low levels of amitraz and would consequently produce fewer eggs as adults. Secondly, the oviposition behaviour of treated adults is disrupted and this may significantly affect the survival of the eggs and young larvae. Thirdly, adult moths treated with large doses of amitraz are killed and with sub-lethal doses have a shorter life span. All these factors may affect the laying capability of populations in the field and may significantly reduce the level of insect attack. In repeated spray programmes these effects would be even more dramatic.

2. Antifeeding action

Amitraz inhibits lepidopterous larvae from feeding by both repellency and a direct anorectic effect.

To detect repellency, cabbages were sprayed at the 4 true leaf stage with various chemicals, and then kept in the glasshouse. After 3 days, sections of the leaves were placed in round petri dishes (14 cm diameter) together with untreated pieces of cabbage leaves. Ten 4th instar larvae of Plutella xylostella were introduced into the petri dish and the larvae on treated and untreated leaves counted after 4 h. The results from six replicate experiments are shown in Table 7. The control figure shows the random distribution of caterpillars on two pieces of untreated cabbage.

Table 7

Numbers of Plutella larvae on treated and untreated leaves after 4 h in choice tests

Treatments	Mean number of caterpillars on cabbage leaves	
	Treated*	Untreated* 95% C.L.
Amitraz 500 ppm	1.33	8.67 ± 1.10
Di-n-butyl phthalate 2000 ppm	2.67	7.33 ± 1.40
Di-n-butyl phthalate 500 ppm	3.83	6.17 ± 1.86
Formulation minus amitraz 2000 ppm (equivalent)	3.67	6.33 ± 2.18
Control	4.67	5.33 ± 1.40

*Each value is the mean of six determinations with 95% confidence limits shown.

In a second experiment, not involving choice testing, ten 4th instar Plutella larvae were placed on two inch squares of either treated or untreated cabbage, and the area eaten by the larvae after 24 hours measured by use of a leaf area meter (Table 8). This showed that amitraz alters the feeding behaviour of Plutella larvae although the significance of this effect on behaviour to the control of pests in the field has not been established.

Table 8

Percentage damage by Plutella larvae of cabbage leaf squares treated with amitraz

Treatment	Percentage damage
Control	58 ± 2.8
Amitraz 500 ppm	51 ± 2.7

SPECIFICITY OF AMITRAZ

Amitraz specificity is of two types; it is specific to certain species of insects - SPECIES SPECIFICITY, and it is also specific to certain stages in the life cycle of some of the susceptible species - LIFE CYCLE STAGE SPECIFICITY. Both types contribute to the survival of beneficial insects under field conditions.

1. Life cycle stage specificity

Amitraz kills Lepidoptera at only two stages of their life cycle, eggs and neonate larvae. In many cases, particularly when the dose applied is in the range LD_{10}-LD_{95}, larvae may emerge from treated eggs and die as many as 2-3 days later. During this phase of 'slow death' the larvae appear to behave differently, and their lack of feeding causes little damage to the host plant. This delayed death after emergence from the egg often results in characteristic groups of young larvae arranged in halos around groups of eggs and is particularly noticeable when large egg masses, such as those of Spodoptera, are treated with amitraz. As well as the ovicidal action, neonate larvae are killed by amitraz for several hours after hatching.

The unusual symptoms of amitraz poisoning, and in particular the delayed action after treatment of the egg means that significant analysis of this action in laboratory studies requires care. If LD_{50} values are based on direct and obviour mortality of eggs within a short period after treatment, then amitraz will appear to be inactive; this point will be discussed more fully in a future paper.

2. Species specificity

Coulon (1978) reported that with the exception of Chrysopa larvae amitraz applied to cotton in the U.S.A. did not significantly reduce the numbers of most beneficial insects. Its effects on a number of insect species have been examined at Lenton and it did not kill adult Coleoptera or Hymenoptera. For example, when sprayed on lady-birds (Adalia bipunctata), 0.1% amitraz gave no mortality after 3 days but 0.01% demeton-S-methyl killed all insects in this period. Similarly amitraz did not kill honey bees (Apis mellifera) when applied as a 0.25% spray. Amitraz also has little effect on the eggs of Coleoptera; at concentrations up to 500 ppm it does not kill mustard beetle eggs (Phaedon cochlearidae) nor those of the 2-spot ladybird (Adalia bipunctata). Similarly, treated mustard beetles show no changes in fecundity nor behaviour, suggesting that amitraz only reduces the fecundity of adults of species susceptible to amitraz at certain stages in the life cycle.

CONCLUSION

Amitraz is an effective ovicide controlling many species of Lepidoptera in the field, especially when used in repeated spray programmes. Such cumulative effect is unlikely from ovicidal action alone, and amitraz has now been shown to have subtle effects on both adult moths and large caterpillars. It reduces egg laying ability of moths by decreasing their longevity and fecundity, and treated caterpillars eat less, grow more slowly, are repelled by treated plants and produce adults with reduced egg laying potential.

These effects, coupled with its proven safety to lepidopteran predators and other beneficial insects, mean that amitraz may be used safely and effectively to control caterpillars.

Acknowledgements

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