Effect of piperonyl butoxide on diazinon resistance in field strains of the sheep blowfly, *Lucilia cuprina* (Diptera: Calliphoridae), in New Zealand

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Abstract

Pretreatment of first instar larvae of 28 resistant strains of *Lucilia cuprina* (Wiedemann) with the inhibitor of microsomal oxidases, piperonyl butoxide, resulted in a biphasic response to the phosphorothioate insecticide diazinon. Analysis of the data revealed a complex response in which both synergist-dependent and independent effects occurred. The responses varied markedly from strain to strain. A laboratory susceptible strain and field strains with resistance factors of less than 20-fold exhibited, in the presence of piperonyl butoxide, an increased LC_{50} with respect to diazinon whereas those strains with > 20-fold resistance were synergized by the compound. We conclude tentatively that microsomal mixed-function oxidases play a contributory role in the development of resistance and that the variation in synergist effect from strain to strain may be attributed, at least in part, to the two-fold effect of these enzymes on phosphorothioate insecticides such as diazinon.

Introduction

The sheep blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae), is a pest of economic significance to the sheep meat and wool industries and its development of resistance to insecticides, including those of the organophosphorus class, is a matter of concern. Resistance to many organophosphorus insecticides by *L. cuprina* has been shown to be due, in the first instance, to the action of a mutated carboxyl esterase (the E3 esterase) which acquires the ability to hydrolyse phosphate triester insecticides (Hughes & Raftos, 1985; Parker *et al*., 1991; Newcomb *et al*., 1997). Such a mechanism was first demonstrated in *Musca domestica* Linnaeus (Diptera: Muscidae) (Oppenoorth & Van Asperen, 1960) and the molecular basis of this form of resistance is now widely recognized. However, other studies have produced evidence indicating strong correlations between resistance to diazinon and the activity of glutathione S-transferases (Wilson & Clark, 1996) and microsomal oxidases (Kotze & Sales, 1995) in field strains of the insect. These observations were merely suggestive of a role for these enzymes in the development of resistance and we have sought different approaches to clarify the possible involvement of other resistance mechanisms in this insect.

A useful tool in the elucidation of resistance mechanisms has been the use of synergists (Hughes, 1982; Prabhaker *et al*., 1988; Scott *et al*. 1990; Osman *et al*., 1991; Ishaaya, 1993). Synergists act by inhibiting specific metabolic pathways, possibly involved in detoxification, which may have been altered or amplified in resistant strains. The result of the inhibition is a more-than-additive increase in the toxicity of an insecticide when used in combination with the synergist (Price, 1991). For example, synergism by *S,S,S*-tributylphosphorotrithioate (TBPT or DEF) is an indicator of hydrolytic

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action by esterases (Plapp & Tong, 1966). Synergism by Sesamex® or by piperonyl butoxide (PBO) indicates the involvement of microsomal mixed-function oxidase (MFO) detoxification enzymes. These enzymes are involved in a multitude of oxidative detoxification reactions (Kulkarni & Hodgson, 1984) and elevated activities are thus likely to be associated with the development of resistance. In the present study, we examine the effects of the mixed-function oxidase inhibitor, piperonyl butoxide, on the toxicity of diazinon in *L. cuprina* as an approach to establish the role of the mixed-function oxidase enzymes in resistance in this insect.

Materials and methods

Insects

Laboratory strains

A laboratory susceptible strain (NSW) of *L. cuprina*, with no history of organophosphate insecticide exposure, was obtained from the Biological and Chemical Research Institute, New South Wales Department of Agriculture, Rydalmere, Sydney, Australia. Two strains (K and L) were resistant strains that had been maintained in the laboratory for several years.

Field strains

Lucilia cuprina field strains were derived from cases of flystrike in regions of New Zealand extending from Kaikohe in the north of the North Island to Blenheim, in the north of the South Island. Larvae, obtained from struck sheep, were mailed to Wallaceville Animal Research Centre in 250 ml sample pots containing a small amount of Vermiculite®. They were reared through to adulthood and maintained as single-species strains. The generation on which tests were carried out was determined by the number of eggs produced by females at any one time, as these tests required in excess of 4000 first instar larvae. Generally, the second generation after isolation was employed. Flies were fed sugar and water *ad libitum*, with larvae being reared on a combined diet of processed pet food and minced ox liver.

Bioassay

Parallel toxicological tests were carried out on larvae of the same strain. One test acted as a control and provided an indication of resistance of the field population, and the other measured the effect of the synergist, piperonyl butoxide, on resistance.

In the synergist test, ten 120 × 30 mm strips of Whatmann chromatography paper (3MM) were treated with 1000 ppm piperonyl butoxide (technical grade, 90%, obtained from Aldrich Chemical Company, Inc., USA) in acetone per test. Papers were rolled and placed into 50 × 10 mm glass vials and 200–300 first instar larvae applied to each tube. Each tube was provided with 1 ml of fortified (2% yeast extract and 0.5% monobasic potassium orthophosphate) sheep serum and stoppered with non-absorbent cotton wool. Larvae were incubated for 2 h at 25°C, after which larvae were removed by rinsing papers with distilled water onto a filter paper in a Buchner funnel. A vacuum was applied to remove excess water and then larvae were transferred to the toxicological test developed by Levot (1990), as described by Wilson & Clark (1996). As a further control, the toxicity of piperonyl butoxide alone was determined, in both the laboratory susceptible strain and in the most resistant strain, as above, using concentrations of 1000, 2000, 5000 and 10000 ppm of the compound. The LC_{50} for diazinon was also determined for these two strains in the presence of the above four concentrations of piperonyl butoxide and in its absence.

Statistical analyses

Dose response was analysed by probit regression (Finney, 1971) using a computer program obtained from the Biological and Chemical Research Institute, Rydalmere, Australia, Sydney. Resistance factors (RFs) were calculated by dividing the LC_{50} of the field strain by that of the susceptible strain, in the presence or the absence of piperonyl butoxide. A 'synergist ratio' was calculated by dividing the LC_{50} for larvae treated only with diazinon by the LC_{50} for larvae pre-treated with piperonyl butoxide. Unweighted linear regressions were carried out using the data analysis package in Microsoft Excel™.

Results

Toxicity data for 28 strains of *L. cuprina* larvae (including one susceptible and two laboratory resistant strains) are presented in table 1. The LC_{50} for the susceptible strain, which is used in calculation of all resistance factors was determined in March 1992. Eight LC_{50} values for the susceptible strain, determined at irregular intervals during the period 1992–1997, had a mean value of 0.059 ± 0.011. For the most highly resistant strain from the Takapau area (number 28), the mean LC_{50} value during the period 3/4/95 to 15/10/97 was 2.35 ± 0.26 (3). The toxicological characteristics of these strains, which represent the extremes of resistance, remained essentially unchanged during a period of years.

The toxicity of piperonyl butoxide alone was tested in the susceptible and the most resistant (41-fold) strain from the Takapau area. Mortality was found to be negligible at the concentration routinely employed (0 and 3% for susceptible and resistant, respectively) and was found to reach only moderate levels at ten times this concentration (20% and 13%, respectively). We also tested a range of concentrations of the synergist for their effect on the toxicity of diazinon. Using the two strains above, we have found that increasing the concentration of piperonyl butoxide above 1000 ppm, as high as 10000 ppm, caused no further change in the mortality caused by diazinon, corrected for that caused by piperonyl butoxide at the concentration employed. The averaged, corrected LC_{50}s for diazinon at 1000, 2000, 5000 and 10000 ppm of piperonyl butoxide were 0.127 ± 0.02 and 1.36 ± 0.26 mg l^{-1} for the two strains respectively.

Resistance factors in the field strains, calculated from the LC_{50} data for diazinon (in the absence of piperonyl butoxide) ranged from 8.8 to 41.2 in a strain from Takapau (strain 28). There was a strong correlation between LC_{50} values obtained in the synergized and unsynergized tests ($r = 0.75$, $P < 0.0005$) and a linear regression was carried out between the two sets of data. The datum for the susceptible strain was excluded from this regression. The line of best fit had a gradient of 0.54 ± 0.12 and intercepted on the vertical axis at a value of 0.59 ± 0.14 mg l^{-1} (see fig. 1). A significant, linear relationship was also obtained between the LC_{99} values obtained in the presence and absence of the synergist.

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Table 1. Effect of piperonyl butoxide on the toxicity of diazinon to first instar larvae from strains of *Lucilia cuprina*.

Strain no.	Location	Sample date	Treatment	LC_{50} (95% F.L.)* mg l^{-1}	LC_{99} (95% F.L.) mg l^{-1}	Slope (S.E.)	RF
0	Lab susceptible	–	DZ	0.05 (0.05–0.06)	0.08 (0.07–0.09)	4.70 (0.46)	1.0
0	Lab. susceptible	–	DZ + PBO	0.08 (0.07–0.09)	0.15 (0.11–0.21)	8.55 (2.05)	1.0
1	Leeston	18 Apr. 94	DZ	0.44 (0.37–0.52)	1.32 (0.95–1.86)	4.83 (0.78)	8.8
1	Leeston	18 Apr. 94	DZ + PBO	0.87 (0.69–1.10)	3.43 (1.62–7.28)	3.92 (1.06)	10.9
2	Dorie	20. Apr. 94	DZ	0.46 (0.36–0.59)	1.57 (0.99–2.49)	4.39 (0.97)	9.2
2	Dorie	20. Apr. 94	DZ + PBO	0.74 (0.66–0.83)	2.44 (1.88–3.17)	4.48 (0.50)	9.3
3	Levin	15 Mar. 94	DZ	0.62 (0.55–0.70)	1.42 (1.10–1.83)	6.52 (1.05)	12.4
3	Levin	15 Mar. 94	DZ + PBO	1.14 (1.07–1.22)	2.88 (2.49–3.34)	5.77 (0.46)	14.3
4	Marton	20 Apr. 94	DZ	0.66 (0.54–0.79)	1.91 (1.25–2.92)	5.01 (0.95)	13.2
4	Marton	20 Apr. 94	DZ + PBO	0.76 (0.61–0.95)	2.25 (1.37–3.71)	4.93 (1.17)	9.5
5	Ashburton	17 Apr. 95	DZ	0.66 (0.61–0.72)	1.63 (1.36–1.94)	5.99 (0.41)	13.2
5	Ashburton	17 Apr. 95	DZ + PBO	1.01 (0.95–1.08)	2.08 (1.81–2.39)	7.45 (0.53)	12.6
6	Levin	11 Apr. 94	DZ	0.68 (0.58–0.80)	2.03 (1.37–3.02)	4.89 (0.84)	13.6
6	Levin	11 Apr. 94	DZ + PBO	0.85 (0.71–1.02)	2.98 (2.00–4.45)	4.27 (0.68)	10.6
7	Ashburton	25 Apr. 95	DZ	0.80 (0.67–0.94)	1.93 (1.28–2.90)	6.07 (1.37)	16.0
7	Ashburton	25 Apr. 95	DZ + PBO	1.11 (0.89–1.37)	3.95 (2.28–6.82)	4.21 (0.88)	13.9
8	Lab strain (L)	Lab F34	DZ	0.83 (0.73–0.94)	1.83 (1.33–2.52)	6.75 (1.36)	16.6
8	Lab strain (L)	Lab F34	DZ + PBO	1.13 (0.98–1.30)	2.90 (1.93–4.34)	5.68 (1.16)	14.1
9	Turakina	20 Feb. 95	DZ	0.87 (0.81–0.92)	1.96 (1.69–2.26)	6.58 (0.62)	17.4
9	Turakina	20 Feb. 95	DZ + PBO	0.87 (0.69–1.08)	4.08 (2.33–7.15)	3.46 (0.66)	10.9
10	Turakina	18 May 95	DZ	0.93 (0.76–1.14)	4.66 (2.56–8.48)	3.32 (0.60)	18.6
10	Turakina	18 May 95	DZ + PBO	1.44 (1.27–1.63)	3.91 (2.75–5.56)	5.34 (0.82)	18.0
11	Rakaia	14Feb.94	DZ	0.93 (0.82–1.04)	2.63 (1.94–3.58)	5.13 (0.68)	18.6
11	Rakaia	14Feb.94	DZ + PBO	1.12 (1.06–1.19)	3.58 (3.08–4.15)	4.63 (0.28)	14.0
12	Turakina	10 May 94	DZ	0.95 (0.77–1.19)	4.01 (2.32–6.94)	3.74 (0.70)	19.0
12	Turakina	10 May 94	DZ + PBO	1.08 (1.00–1.17)	3.01 (2.54–3.57)	5.26 (0.53)	13.5
13	Bulls	05 Apr. 94	DZ	0.98 (0.91–1.06)	2.37 (1.96–2.87)	6.10 (0.87)	19.6
13	Bulls	05 Apr. 94	DZ + PBO	0.84 (0.70–1.00)	2.54 (1.71–3.78)	4.83 (0.89)	10.5
14	Hastings	29 Dec. 92	DZ	1.00 (0.93–1.07)	2.70 (2.28–3.19)	5.39 (0.59)	20.0
14	Hastings	29 Dec. 92	DZ + PBO	1.08 (1.00–1.17)	2.54 (2.11–3.05)	6.29 (0.63)	13.5
15	Kihikihi	07 Mar. 94	DZ	1.02 (0.89–1.17)	3.94 (2.80–5.55)	3.97 (0.47)	17.0
15	Kihikihi	07 Mar. 94	DZ + PBO	1.09 (0.93–1.27)	3.62 (2.38–5.50)	4.46 (0.79)	13.6
16	Kamo	19 Apr. 95	DZ	1.04 (0.97–1.11)	2.98 (2.51–3.55)	5.06 (0.45)	20.8
16	Kamo	19 Apr. 95	DZ + PBO	0.78 (0.63–0.97)	3.48 (1.68–7.20)	3.59 (0.83)	9.4
17	Rangiora	04 Jan. 93	DZ	1.11 (1.04–1.19)	1.87 (1.59–2.20)	10.32 (1.50)	22.2
17	Rangiora	04 Jan. 93	DZ + PBO	1.82 (1.68–1.97)	3.08 (2.55–3.72)	10.23 (1.69)	22.8
18	Lab strain (K)	Lab F8	DZ	1.15 (1.09–1.21)	2.67 (2.36–3.02)	6.37 (0.65)	23.0
18	Lab strain (K)	Lab F8	DZ + PBO	1.48 (1.30–1.68)	3.78 (2.66–5.38)	5.69 (1.03)	18.5
19	Dorie	23 Apr. 95	DZ	1.21 (1.14–1.28)	3.55 (3.09–4.07)	4.98 (0.34)	24.2
19	Dorie	23 Apr. 95	DZ + PBO	1.14 (0.89–1.45)	3.83 (2.28–6.44)	4.41 (0.96)	14.3
20	Masterton	2 Mar. 94	DZ	1.23 (0.14–1.32)	2.38 (1.99–2.84)	8.10 (0.98)	24.6
20	Masterton	2 Mar. 94	DZ + PBO	1.36 (1.17–1.58)	2.78 (1.96–3.93)	7.48 (1.62)	17.0
21	Takapau	03 Apr. 95	DZ	1.33 (1.19–1.49)	2.87 (2.24–3.68)	6.99 (1.00)	26.6
21	Takapau	03 Apr. 95	DZ + PBO	1.26 (1.10–1.44)	2.75 (2.03–3.71)	6.85 (1.28)	15.8
22	Blenheim	19 Mar. 95	DZ	1.54 (1.44–1.65)	5.39 (4.68–6.21)	4.28 (0.23)	30.8
22	Blenheim	19 Mar. 95	DZ + PBO	1.16 (1.11–1.21)	2.86 (2.60–3.15)	5.93 (0.26)	14.5
23	Kaikohe	18 Mar. 94	DZ	1.55 (1.25–1.93)	6.32 (3.64–10.98)	3.82 (0.72)	31.0
23	Kaikohe	18 Mar. 94	DZ + PBO	1.38 (0.92–2.06)	4.56 (1.62–12.85)	4.49 (1.93)	17.3
24	Blenheim	15 Mar. 94	DZ	1.56 (1.46–1.66)	4.76 (4.08–5.55)	4.81 (0.28)	31.2
24	Blenheim	15 Mar. 94	DZ + PBO	1.34 (1.25–1.43)	4.04 (3.46–4.71)	4.86 (0.34)	16.8
25	Takapau	03 Mar. 95	DZ	1.74 (1.63–1.85)	5.15 (4.45–5.96)	4.94 (0.26)	34.8
25	Takapau	03 Mar. 95	DZ + PBO	1.05 (1.00–1.11)	2.38 (2.14–2.66)	6.55 (0.53)	13.1
26	Blenheim	25 Apr. 94	DZ	1.82 (1.56–2.13)	7.92 (5.27–11.90)	3.65 (0.43)	36.4
26	Blenheim	25 Apr. 94	DZ + PBO	1.58 (1.47–1.70)	5.40 (4.54–6.43)	4.37 (0.40)	19.8
27	Blenheim	28 Dec. 92	DZ	2.03 (1.87–2.20)	4.41 (3.59–5.42)	6.89 (0.86)	40.6
27	Blenheim	28 Dec. 92	DZ + PBO	2.16 (2.05–2.27)	4.36 (3.81–4.99)	7.61 (0.83)	27.0
28	Takapau	4 Feb. 98	DZ	2.06 (1.9–2.22)	7.04 (5.80–8.55)	5.62 (0.29)	41.2
28	Takapau	4 Feb. 98	DZ + PBO	1.19 (0.81–1.75)	5.97 (2.65–13.48)	4.28 (1.35)	14.9

FL, 95% fiducial limits; RF, resistance factor – the ratio of LC_{50} for the resistant strain, in the presence or absence of synergist, to that for the susceptible strain, in the presence or absence of synergist; DZ, diazinon treatment; DZ + PBO, diazinon + pretreatment with piperonyl butoxide.

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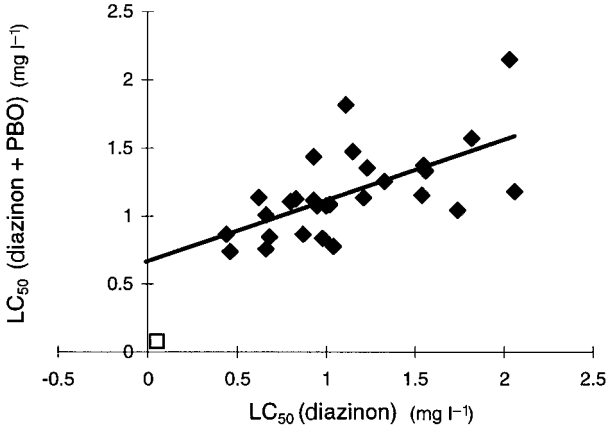

Fig. 1. Correlation between LC_{50} values for diazinon against *Lucilia cuprina*, obtained in the presence and absence of the synergist, piperonyl butoxide. LC_{50} values for first instar larvae of *L. cuprina* were obtained as described in methods. LC_{50} (diazinon) is the LC_{50} value obtained with diazinon alone whereas LC_{50} (diazinon + PBO) is the value obtained after pretreatment with piperonyl butoxide, as described in methods. The solid line is the line of best fit to data points excluding that for the susceptible strain, the values for this strain (open symbol) being included on the graph for the purpose of comparison only.

In order to emphasize trends in the data, the LC_{50} values were recalculated as synergist ratios, as described in the methods. The values ranged from 0.5 to 1.7 denoting both antagonistic (<1) and synergistic (>1) responses. The greatest synergistic response (1.7) occurred in the Takapau strain (strain 28) where the LC_{50} for diazinon changed from 2.06 mg l^{-1} to 1.19 mg l^{-1}. The most pronounced antagonistic response (0.5) occurred in the Leeston (strain 1) and Levin (strain 3) strains. The LC_{50} for the Leeston strain showed an increase from 0.44 to 0.87 mg l^{-1} and that for strain number 3 from 0.62 to 1.14 mg l^{-1}. A ratio of 0.6 was observed with the diazinon-susceptible laboratory strain.

Figure 2 shows that there was a strongly significant, positive correlation between the resistance factor and the synergist ratio ($r = 0.75$; $P < 0.0001$): as resistance factors increased, so too did synergism of diazinon toxicity by piperonyl butoxide. However, about 70% of the strains studied responded to piperonyl butoxide by exhibiting an antagonistic response.

Discussion

As discussed in the introduction, evidence has been published to suggest that resistance to organophosphorus insecticides in *L. cuprina* may depend upon multiple factors. Since the studies presenting this evidence employed model substrates, they can be regarded only as indicative of such dependency and the intention of the present work was to seek additional evidence as to whether the microsomal oxidases were involved in the development of resistance.

Our results suggest, in the first instance, that the microsomal oxidases are probably involved, but that this involvement is complex. Figure 1 shows a strong, apparently linear, relationship between the toxicity of diazinon in the presence of piperonyl butoxide and that in the absence of the synergist. This simple graph contains more information than might at first appear and a detailed evaluation of these data reveals a situation of some complexity. Of note is the fact that the regression line does not pass through the origin, indicating that the toxicological response includes, at a minimum, two factors, one responsive to the synergist and one unaffected by the presence of the compound. Our starting hypothesis was that these factors corresponded to two different biochemical mechanisms, one of which was significantly inhibited by the synergist. For simplicity, and because it is compatible with the observed data, in this hypothesis we proposed that the resistance should be a linear function of the activity of the target enzyme.

In this model, two expressions may be formulated for each strain, depending on whether the synergist (at a fixed dose) is present or not.

These are, for the *i*th strain:

$$LCi_{50d} = \mathbf{a} + x_{i.}\, P_d \quad (1)$$

$$LCi_{50dpb} = \mathbf{a} + x_{i.}\, P_{dpb} \quad (2)$$

The first term **a**, is the synergist-independent term and is assumed to represent a basal level of resistance, common to all strains. The second, variable component of the resistance is related to the activity (x_i in strain i) of the enzyme(s) involved in the resistance mechanism by a proportionality constant, the value of which will be inversely related to the synergist concentration used. This constant is P_d in the case of the untreated larvae, and P_{dpb} in the case of the synergized insects. P_{dpb} will, of course, have the lesser value, since the effect of a synergist is to reduce the value of the $LC_{50.}$

Plotting one against the other, as in fig. 1, gives the linear relationship below, in which the gradient corresponds to the fractional activity in the presence of synergist of the enzymes contributing to the resistance. The vertical intercept may be used to calculate the magnitude of **a**, the synergist-independent component of the toxicological response.

$$LCi_{50dpb} = \mathbf{a}.(1\text{-}P_{dpb}/P_d). + LCi_{50d}.(P_{dpb}/P_d). \quad (3)$$

This equation appears to be compatible with the data shown in fig. 1: a strong, positive, linear correlation exists between the two sets of data ($P < 0.001$) and the gradient and vertical intercept are positive as required by the equation. The value of the gradient suggests, in this model, a 46% inhibition of the enzyme system by the synergist. However, calculation of the value of **a** suggests that our starting hypothesis is overly simple. The value of **a** corresponds to an LC_{50d} of 1.28 mg l^{-1}, higher than 75% of the experimental values, whereas, from equation 1, it should be the lowest of them all.

This conclusion is underscored by recalculating the data as synergist ratios and plotting these against the unsynergized RF values (fig. 2). These show that the effect of the synergist is two-fold. With strains of relatively low resistance, the effect is to antagonize the action of the insecticide. At higher resistance factors, the effect is one of synergism. There appears then to be a continuous variation between the two extreme responses, depending on the degree of resistance.

A further point may be made. As stated above, the gradient of the line in fig. 1 indicates that, if a single enzymatic process is responsible for the varying resistance, it is inhibited by the synergist only to the extent of approximately 50%. (If the inhibition were complete, the line in fig. 1 would have zero gradient.) However, tests with

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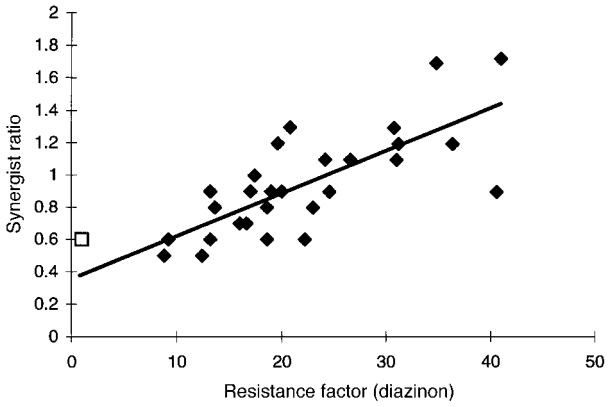

Fig. 2. Relationship between the effect of piperonyl butoxide on the toxicity of diazinon to first instar larvae of *Lucilia cuprina* and their resistance to diazinon. Synergist ratios were calculated as described in methods. Thus the synergist ratio = LC_{50} (diazinon) / LC_{50} (diazinon + PBO), where LC_{50} (diazinon) is the LC_{50} value obtained with diazinon alone and LC_{50} (diazinon + PBO) is the value obtained after pretreatment with piperonyl butoxide. Resistance factors employed in this graph were calculated for the action of diazinon in the absence of piperonyl butoxide. The solid line is the line of best fit calculated for data points obtained for all strains, excluding the susceptible strain. The value for this strain (open symbol) is included on the graph for the purpose of comparison.

varying concentrations of the synergist suggest that the target of the piperonyl butoxide was maximally inhibited. This suggests in turn that there is at least a third factor, which is increased with increasing resistance, but which is unaffected by the synergist. Although the simple two-term expression for LC_{50} above does not explain the detail of this behaviour in its entirety, consideration of the model is justified by the indications of complexity that it reveals.

The qualitative aspects of the data are clear: the data exhibit both synergist-dependent and independent behaviour. As previously (Wilson & Clark, 1996), we tentatively attribute the synergist-independent component, manifested as a non-zero vertical intercept in fig. 1, and which appears to be minimal in the case of the susceptible (NSW) strain, to the mutated E3 esterase.

Our interpretation of the occurrence of both synergistic and antagonistic effects is based on the fact that the action of the mixed-function oxidases on phosphorothioate insecticides is two-fold. Diazinon is converted by the action of mixed-function oxidases to products such as hydroxydiazinon, diazoxon and hydroxydiazoxon, of which the latter two are actively toxic (Shishido *et al*., 1972; Eto, 1974; Pisani-Borg *et al*., 1996). Subsequent inactivation is likely to be the result of both oxidative and hydrolytic reactions. It is suggested that in the less resistant strains, the mixed-function oxidase activity is dominated by the isoforms catalysing the activation of diazinon. The effect of inhibition of the mixed-function oxidases by the synergist is therefore primarily to depress the intoxication process, leading to decreased toxicity. In the more resistant strains, the mixed-function oxidase activity is dominated by isoforms which have been selected for their ability to detoxify the *oxon* forms of the insecticide. Suppression of mixed-function oxidase activity in these strains therefore has the effect of slowing the removal of the toxic forms of the insecticide and thus enhancing toxicity. It is probable also that the two different classes of isoenzyme will differ in their susceptibility to inhibition by piperonyl butoxide.

There is a wealth of evidence in the literature to lend support to this interpretation of these results. The potency of piperonyl butoxide as a synergist has been shown to vary markedly with insecticide, target species (Raffia & Priester, 1985; Silcox *et al*., 1985; Welling & De Vries, 1985; Hagler *et al*., 1988; Prabhaker *et al*., 1988) and, as in the present case, within different strains of the same species (Prabhaker *et al*., 1988; Sparks & Byford, 1988; Scott *et al*., 1990; Bagwell & Plapp, 1992). Comparative studies of resistant and susceptible house fly strains have shown at least six forms of cytochrome P450 and qualitative and quantitative differences in isozyme composition (Yu & Terriere, 1979; Feyereisen, 1983; Ronis *et al*., 1988). It has been suggested that cytochrome P450 in resistant forms may have high catalytic activity due to mutation; or resistant insects may have alterations of regulation in the cytochrome P450 gene which is expressed differently in susceptible forms (Soderlund & Bloomquist, 1990). There is thus ample precedent for the assumption of different mixed-function oxidases, expressed to differing extents and of differing synergist susceptibility.

This interpretation may not constitute the complete explanation of the results. Other mechanisms may play a role. Wahla *et al*. (1976) demonstrated a reduction in penetration of diazinon through the larval cuticle of the large white butterfly *Pieris brassicae* (Linnaeus) (Lepidoptera: Pieridae) by piperonyl butoxide and Martin *et al*. (1997) have demonstrated a similar effect in fifth instar larvae of the tobacco budworm *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae). Other possible effects on insecticide penetration by piperonyl butoxide have been suggested as mechanisms of synergism by this compound in other species such as the cotton boll worm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Kennaugh *et al*., 1993) and the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Forgash, 1985). Clearly, modulation by the synergist of rates of penetration of the insecticide could contribute to the effects reported here.

We find evidence for only modest inhibition (15–25%) of ali-esterase activity, or glutathione S-transferase activity *in vitro* by saturating concentrations of piperonyl butoxide and consider it unlikely that inhibition of these enzyme systems is responsible for the piperonyl butoxide-dependent effects reported here (A.G. Clark and J. Whittaker, unpublished).

In summary, this study finds additional evidence in support of the idea that mixed-function oxidases contribute to increased resistance levels in some field strains of *L. cuprina*. Other publications indicate the existence of other resistance factors which include the hydrolytic mechanism embodied in the E3 esterase, (Hughes & Raftos, 1985; Parker *et al*., 1991; Newcomb *et al*., 1997) which probably constitutes the basal resistance mechanism and, as secondary contributors, the glutathione *S*-transferases (Wilson & Clark, 1996). In view of the probably multifactorial nature of the resistance and the bi-phasic response to piperonyl butoxide, we conclude that the prospects for using such synergists to overcome resistance in the field are not promising.

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