AMPLIFIED ESTERASES A_2 AND B_2. HAS RESISTANCE OCCURRED ONCE OR SEVERAL TIMES?

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Abstract—Elevation of esterase activity is the most common form of organophosphate resistance in the *Culex pipiens* complex. There are several electrophoretically distinguishable forms of esterase which can be elevated. Raymond et al (1991) have put forward the hypothesis that the most common form of elevation, involving esterases A_2 and B_2, has occurred only once and then spread worldwide through migration. Their hypothesis is based on identical restriction fragment length patterns observed in the amplicon containing the B_2 esterase from *Culex* strains worldwide. This does not, however, account for the different resistance patterns observed in the *Culex* strains which contain only this esterase mechanism. A study of five resistant strains, including two collected from the same cities as Raymond's study, and one insecticide susceptible strain, has now revealed that both the A_2 and B_2 esterases interact with a range of insecticides to a similar extent, hence both have a role in resistance. Within the different *Culex* populations the A_2s can be separated on the basis of their physical characteristics and interactions with a range of organophosphates plus the carbamate propoxur. The B_2 esterases are more similar between strains, but some of these are also kinetically distinct. The data suggest that the A_2 and B_2 esterases, as visualised on polyacrylamide or starch gels do not represent a single allelic form of either esterase, but that these both contain allelic variants with similar Rf values. This has already been documented for the B_4/B_5 esterases, and unpublished data suggests that the same is true for the B1 esterase in Californian and Cuban populations of *Culex quinquefasciatus*. We propose that the hypothesis of a single amplification event of the B_2 allele, and its subsequent spread through migration, is too simplistic. Data on the purified esterases from a range of strains suggests that either multiple allelic forms of A_2 and B_2 are present in a single strain or that different allelic forms occur in each strain. The elevated esterases have only been detected over the last thirty years, and their initial appearance in a number of populations is well documented. This time scale is insufficient for variation to occur on the scale demonstrated here, from the amplification of a single allelic form of both the A_2 and B_2.

INTRODUCTION

The common house mosquito *Culex quinquefasciatus* occurs throughout Asia, Africa, the Americas and the Middle East. It is a major biting nuisance and a vector of filariasis in some regions. Extensive use of pesticides, both directly against this mosquito, or indirectly through crop spraying around its larval habitats, has resulted in selection of broad spectrum organophosphate resistance throughout most of its range. Invariably the resistance mechanism selected involves an elevation in the level of general esterase activity, this can also be coupled with an insensitive form of acetylcholinesterase, which increases the levels of organophosphate resistance and extends the cross-resistance spectra to carbamates (Peiris & Hemingway, 1990; Raymond *et al* 1987; Raymond *et al* 1989; Villani *et al* 1983, Bisset *et al* 1990).

Different esterase isozymes have been associated with resistance. Raymond et al (1987) have classified these as A or B esterases with respect to their preference for - or -naphthyl acetate (NA). This classification is independent of that of Aldridge (1953), under which all the esterases concerned are B-type serine esterases. In California and Cuba the B_1 esterase is elevated (Mouches *et al* 1986; Bisset *et al* 1990), but in the majority of its range *Cx quinquefasciatus* has the esterases A_2 and B_2 co-elevated. These show almost complete linkage disequilibrium, the two forms always being elevated together in the same individual. The A_2 and B_2 esterases are, however, immunologically distinct. Antisera raised to native or denatured A_2 cross- reacts with other A esterases, but not the B esterases and vice versa.

The B_1 esterase has been cloned and the mechanism for its elevation shown to be amplification, with up to 250 copies of the gene present in resistant individuals (Mouches *et al* 1989). Elevation of B_2 activity is also based on gene amplification (Raymond et al 1989) and the A_2 and B_2 genes are known to be tightly linked (Wirth *et al* 1990). The amplification unit on which the B_2 gene occurs is significantly larger than the gene itself (Mouches *et al* 1990). On the basis of identical restriction digest patterns of the B_2 amplicon from resistant strains of *Culex quinquefasciatus* from major ports,

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Raymond *et al* (1991) have put forward the hypothesis that this resistance mechanism has occurred only once, and spread worldwide by migration. This has major ramifications for resistance management, which is based on the premise that resistance genes are initially rare (at or around the mutation rate found primarily in their heterozygous state), and in the absence of positive insecticidal selection pressure are at a significant selective disadvantage.

To test the migration hypothesis five resistant strains of *Cx quinquefasciatus* and one susceptible strain have been analysed biochemically and by bioassay.

MATERIALS AND METHODS

Insect colonies

Tanga '85 was collected from Tanga, Tanzania in 1985 and has been maintained in the laboratory under intermittent chlorpyrifos selection pressure.

Dar '91 was collected from Dar es Salaam, Tanzania in 1991. The population has been under selection pressure in the field with fenitrothion and has been maintained in the laboratory without selection since colonisation.

Muheza was collected from Muheza, Tanzania in 1987 from an area where pesticides are not routinely used for *Culex* control. It has been maintained under intermittent chlorpyrifos selection since colonisation.

SPerm was collected from Jeddah, Saudi Arabia in 1989. It was selected for twenty generations with permethrin and subsequently selected intermittently with malathion and temephos in the laboratory.

Pel was collected from Peliyagoda, Sri Lanka in 1984. The population was heterogeneous for organophosphate resistance. Pel SS and Pel RR were both derived from this strain. Pel SS is insecticide susceptible and was obtained by single family selection for low esterase activity. The colony was initially established from at least 20 different low esterase families.

Pel RR was selected from the Pel strain by temephos selection for thirty generations at the 80% mortality level (Peiris & Hemingway 1990).

WHO larval bioassays were undertaken by exposing batches of 25 4th instar larvae to known insecticide concentrations in 250 ml of distilled water. Insecticide solutions were made in alcohol and 1 ml of the alcohol solution added to the water. For each bioassay at least five concentrations giving mortality between 0 and 100% were tested, and four replicates were tested at each concentration. Bioassays were always done with a control of 1 ml of alcohol alone. There were no mortalities in controls. After 24 hrs pesticide exposure at 25±2°C the number of dead larvae were counted. Mortality data were processed by log-dosage probit mortality regression analysis and the lethal concentrations which gave 50% (LC_{50}) and 90% (LC_{90}) mortalities calculated.

The A_2 and B_2 esterases were purified to homogeneity from each strain by sequential column chromatography and preparative electrophoresis.

The A_2 and B_2 esterases were initially both purified by Q-Sepharose and phenyl Sepharose chromatography under the conditions described for A_2 by Ketterman *et al* (1992). They were separated from each other on the phenyl Sepharose column, the A_2 eluting first. The fractions of the two peaks were combined separately and dialysed against dry sucrose. Buffer exchange into the hydroxylapatite buffer was performed on PD-10 columns according to the manufacturer's instructions. The A_2 esterase was applied to a 2.2 × 5.4 cm hydroxylapatite column as described previously, the activity peak was collected and applied onto a 9% acrylamide gel (17 ml) with a 4% acrylamide stacking gel (9 ml) in a Prep-Cell model 491. The gel was run at 15 W constant power and the pure enzyme was eluted with the electrode buffer containing 10 mM DTT.

The B_2 esterase was applied to a hydroxylapatite column (2.2 × 5.4 cm) equilibrated with 10 mM phosphate buffer (pH 6.8) containing 50 mM NaCl and 10 mM dithiothreitol. The esterase activity was eluted with a 5 bed volume gradient of phosphate buffer (10-200 mM pH 6.8) containing no NaCl. Activity eluted as a single peak and the fractions were combined and concentrated in Amicon centriprep 10 concentrator units. Buffer exchange into 0.1 M phosphate buffer containing 10 mM EDTA (pH 7.8) was performed on Nap-5 columns. This sample was applied to a *p*-

chloromercuribenzoate column (1.5 × 5 cm) equilibrated with the same buffer. The esterase activity was eluted with a 5-bed volume gradient of the equilibrating buffer and 20 mM phosphate buffer pH 6.8 containing 30 mM β-mercaptoethanol. During purification, esterase activity was assayed at 405 nm using 1 mM *p*-nitrophenyl acetate in 50 mM phosphate buffer (pH 7.4) at 22°C. Unless otherwise stated, all enzyme assays were performed at 22°C. Specific activities are given in units/mg protein. A unit is equivalent to the hydrolysis of 1 µmol of *p*-nitrophenyl acetate in 1 min under the assay conditions used.

Protein concentrations were estimated by the method of Bradford (1976) using bovine serum albumin as the standard protein.

Electrophoresis of native protein samples was performed in 7.5% acrylamide gels in 0.1M Tris-borate buffer, pH 8.0 by the method of Davis (1964). The gels were stained for esterase activity with 0.04% (w/v) and -naphthyl acetate and 0.1% (w/v) fast blue B in 100 mM phosphate buffer. SDS-PAGE was performed on 4-20% gradient gels with molecular weight markers ranging from M_r 18,500–106,000. Coomassie Blue R250 was used to stain for protein.

For the inhibition kinetics stopped time inhibition assays were performed using *p*-nitrophenyl acetate as the substrate. The purified enzyme was incubated with a series of concentrations of insecticides (or with phosphorothionate oxon analogues), and at various times aliquots were withdrawn and residual activity was determined by measuring the rate of substrate hydrolysis. The chlorpyrifos-oxon and paraoxon k_a's were determined in the presence of substrate by the method of Main & Dauterman (1963). The re-activation rates were measured by incubating the purified enzyme with the insecticide or oxon for 15 mins so that the enzyme was more than 90% inhibited. The unbound inhibitor and enzyme -inhibitor complex were separated on Nick spin columns following the manufacturer's instructions. Aliquots of the enzyme-inhibitor complex were removed at fixed time intervals and residual activity with p-NPA measured. The pseudo-first order inhibition rate constants were calculated and used to determine the various inhibition constants (Aldridge & Reiner 1972).

Pel RR fourth instar larvae (0.5g) were thoroughly ground under liquid nitrogen. The powdered larvae were mixed with 20 volumes of guanidium thiocyanate buffer and sodium lauryl sarcosinate was added to a final concentration of 0.5%. The homogenate was centrifuged for 5,000g for 10 min and the supernatant then sheared through a 23-gauge needle. This was loaded onto a 5.7 M CsCl cushion and centrifuged at 20°C for 20 hrs at 40,000 rpm. The pellet of total RNA was collected, ethanol washed and resuspended in TE. mRNA was isolated from total RNA with the PolyATract mRNA isolation system IV (Promega), cDNA was synthesized with the Riboclone cDNA synthesis system (Promega). Primers were constructed on the basis of knowledge of the B_1 esterase sequence (Mouches et al 1990) and other published esterase sequences. PCR was undertaken by 40 cycles of amplification using a step programme. Genomic DNA from Pel SS and Pel RR was isolated according to the method of Sambrook *et al* (1989). The DNA from both strains was digested to completion with a number of restriction enzymes and then separated by agarose gel electrophoresis. The digests were transferred to Hybond™N+ nucleic acid transfer membranes and probed with a P^{32} labelled cDNA from the PCR. The filters were hybridised overnight, then washed at high stringency.

RESULTS

Table 1 gives the LC_{50} values and resistance ratios for four of the rsistant strains against a range of insecticides. Although resistance occurs to all the pesticides tested in all strains the levels of resistance are clearly different between strains, despite the only resistance mechanism detected in all cases being elevated A_2/B_2.

The A_2 and B_2 elevated esterases account for the majority of the total esterase activity seen in crude homogenates of resistant insects. With the purification procedures followed the A_2 esterase accounted for the majority of the total esterase activity recovered (41%) (see Table 2). The purified B_2 accounted for 3% of total starting activity. These may not, however, represent the starting proportions of the two esterases, as initial experiments showed that B_2 was the more labile of the two enzymes, and although the addition of 25 mM DTT in the early stages of purification greatly improved B_2 stability, it is probable that this enzyme is lost disproportionately to A_2.

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Table 1. LC_{50} values for a range of *Culex quinquefasciatus* strains and the resistance ratios in these strains compared to the insecticide susceptible Pel SS strain

Insecticide	*Culex* strain									
	Pel SS		PelRR		SPerm		Tanga 85		Dar 91	
	LC_{50}	RR	LC_{50}	RR	LC_{50}	RR	LC_{50}	RR	LC_{50}	RR
Chlorpyrifos	0.0003	1	0.011	36	0.027	90	0.05	167	0.047	157
Parathion	0.0013	1	0.026	20	—	—	—	—	—	—
Malathion	0.034	1	0.22	6	0.54	16	0.065	1.9	4.4	130
Fenitrothion	0.02	1	0.075	4	0.12	6	0.03	1.5	0.45	23
Propoxur	0.4	1	0.8	2	2.4	6	0.6	1.5	—	—

Table 2. Purification of the esterases B_2 and A_2
The purification was monitored by the assay of 1 mM *p*-nitrophenyl acetate hydrolysis at 22°C. The two esterases were separated after the phenyl-Sepharose column. PCMB is the *p*- chloromercuribenzoate affinity column.

Step		Specific Activity (Units/mg)	Protein (mg)	Purification	Recovery (%)
10,000 g supernatant		1.03	692.5	—	—
Q-Sepharose		13.15	63.93	12.7	117.5
Phenyl-Sepharose	A_2	33.22	12.67	32.20	58.9
	B_2	21.49	3.98	20.8	12.0
Hydroxylapatite	A_2	204.78	1.63	198.8	46.8
	B_2	47.27	1.45	45.81	9.6
Prep-Cell	A_2	363.15	0.80	352.6	40.7
PCMB	B_2	51.82	0.395	50.2	2.9

Michaelis constants, K_m and V_{max} values were determined for three general esterase substrates for the purified A_2 and B_2 esterases from the Pel RR strain (Table 3). Both esterases showed a higher affinity towards *p*-nitrophenyl caproate than *p*-nitrophenyl acetate. However, the rate of hydrolysis was considerably lower for B_2 than A_2.

Esterase A_2 had a much higher affinity for α-NA than B_2. This is reflected in the substrate preference of A_2 for α-NA, when incubated with both α- and β NA (see Table 3). The Michaelis constants for β-NA could not be determined, as this substrate was insoluble at the concentrations required. The results for the three substrates clearly show that the two enzymes are readily distinguishable from each other kinetically with a range of substrates.

Earlier data on relative pesticide metabolism rates in the Pel SS and Pel RR strains suggested that the sequestration rather than metabolism of insecticides was the primary function of the elevated esterase-based resistance mechanism (Peiris & Hemingway, in press).

The slow rates of dephosphorylation or decarbamylation (k_3) of both the A_2 and B_2 esterases confirmed this. The combined data for the bimolecular rate constants (k_a) and k_3's for the carbamates and oxon analogues of the phosphorothionates show that both the A_2 and B_2 esterases readily bind to these inhibitors, but are only slowly able to metabolize them.

Table 3. Substrate interactions of esterases B_2 and A_2
The rate of substrate hydrolysis was measured at 405 nm for *p*-nitrophenyl acetate and *p*-nitrophenyl caproate, and at 235 nm for α-naphthyl acetate at 22°C. A unit corresponds to the hydrolysis of 1 μmol of substrate in 1 min under the assay conditions used.

	A_2		B_2	
	V_{max} (units/mg)	K_m (μM)	V_{max} (units/mg)	K_m (μM)
p-nitrophenyl acetate	472.0±51.4	145.8±45.0	63.4±0.8	140.00±50.0
p-nitrophenyl caproate	788.2±74.8	35.4±9.7	83.3±9.8	17.0±6.0
α-naphthyl acetate	717.9±48.4	30.5±6.1	200.9±15.0	172.5±40.6

Table 4. Bimolecular rate constants for the interaction of B_2 carboxylesterases with insecticides

Insecticide	$k_a \times 10^{-5}$ (M^{-1} min^{-1})					
	PelSS	PelRR	Dar'91	Tanga'85	Muheza	SPerm
Malaoxon*	0.400[a] ±0.050	0.496[a,b] ±0.168	0.553[a,b] ±0.195	0.513[a,b] ±0.097	0.383[a] ±0.057	0.615[b] ±0.015
Fenitrooxon*	0.328[a] ±0.008	1.604[b] ±0.300	5.668[c] ±0.957	3.080[d] ±0.183	4.059[c,d] ±0.630	5.237[c] ±0.503
Chlorpyrifos oxon*	2.285[a] ±0.811	1545.0[b] ±140.04	1674.9[b] ±269.12	2095.7[b] ±396.98	1750.9[b] ±323.88	2086.9[b] ±523.69
Paraoxon*	1.937[a] ±0.054	169.88[b] ±53.123	180.75[b] ±24.31	139.15[b] ±42.0	170.23[b] ±24.271	154.42[b] ±29.153
Propoxur**	<0.0001[a]	0.0052[b] ±0.0017	0.0074[b] ±0.0014	0.0048[b] ±0.0004	0.0065[b] ±0.0007	0.0061[b] ±0.0018

* -Organophosphate
** -Carbamate
[Values with different superscripts are significantly different from each other]

The k_a values for the formation of the phosphorylated or carbamylated esterases (k_a) for both purified A_2 and B_2 were determined for several insecticides.. There was no significant interaction of either esterase with the phosphorothionates at their maximum solubility limits. However, both esterases interacted readily with the oxon analogues of these organophosphorus insecticides.

Figures 1–4 express the k_a values for A_2 with chlorpyrifos-oxon, malaoxon, fenitroxon and paraoxon in the five resistant strains as percentages of the k_a values for the same oxons in the susceptible Pel SS strain. For chlorpyrifos-oxon the A_2 from all the resistant strains were significantly different from the susceptible, and the esterase from Pel RR was also different from the other esterases (Fig. 1). With fenitrooxon the Tanga 85 A_2 was different from all the other A_2s, it was also significantly different from the Muheza, Pel RR and SPerm A_2s with malaoxon (Fig. 4). Taken together, the data show that the only A_2s which cannot be distinguished on the basis of their interaction with different inhibitors are those from Muheza and SPerm.

The actual k_a values for the purified B_2 esterases are given in Table 4. Differences between the B_2 esterases were less than those for the A_2s from the same strains. The B_2 from Pel SS was significantly different from the B_2 enzymes from the resistant strains, and the inhibitors malaoxon and fenitrooxon were also able to distinguish some of the B_2s from the resistant strains from each other (see Table 4).

The order of increasing reactivity for all B_2s from the resistant strains was propoxur, malaoxon, fenitrooxon, paraoxon, chlorpyrifos-oxon. A similar order of increasing reactivity was observed for the B_2 from the susceptible Pel SS strain, but the levels of cross-reactivity, particularly with chlorpyrifos-oxon, paraoxon and propoxur were much lower. A similar order of increasing reactivity was observed for the A_2s. This is also reflected in the insecticide cross-resistance spectra shown by the resistant strains where, for example in Pel RR, the greatest resistance is shown to chlorpyrifos, with lower resistance to paraoxon, and very little resistance to propoxur (see Table 1).

DISCUSSION

The five insecticide resistant strains of *Culex quinquefasciatus* used in this study all contained the elevated esterases A_2 and B_2 at high frequencies. In four of the strains this was the only resistance mechanism detected (Peiris & Hemingway 1990; Hemingway *et al* 1990). In a strain from Dar es Salaam in 1986 there were indications of a very low frequency of an altered acetylcholinesterase (AChE)-based resistance mechanism, as well as the more common A_2/B_2 mechanism. A sample of ninety six Dar '91 larvae did not contain the altered AChE, although it is still possible that the mechanism is present at a low frequency in the colony as a whole. This may account, in combination

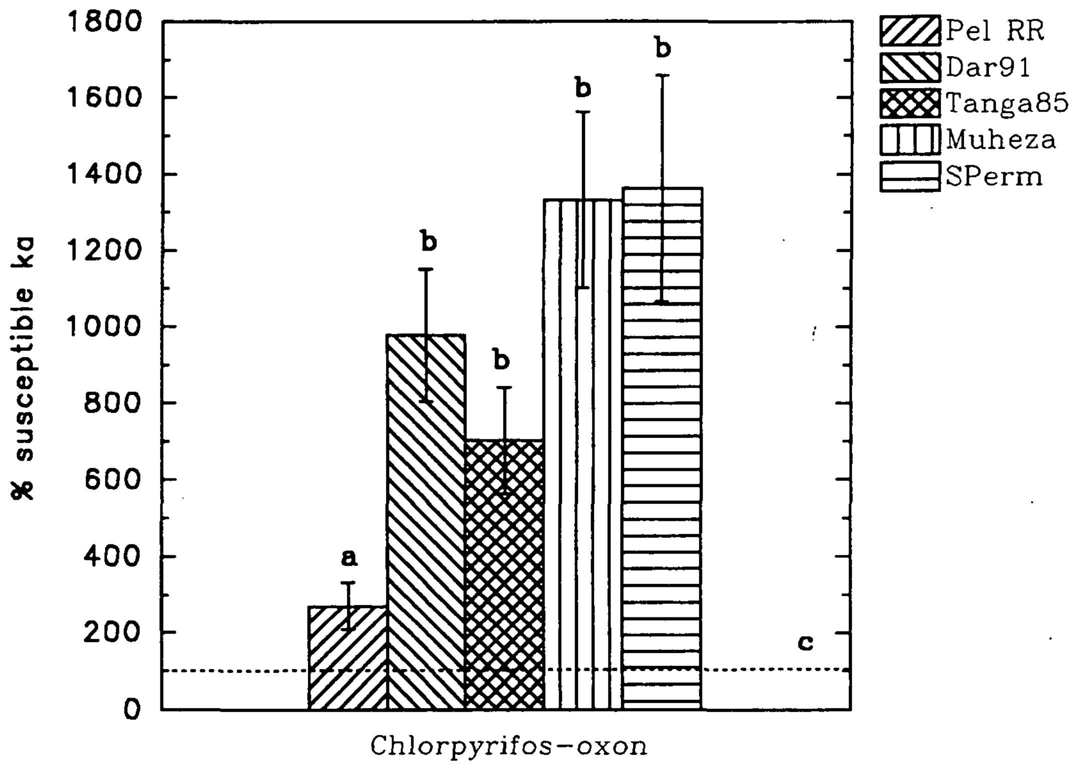

Fig. 1. Bimolecular rate constants (k_a) for chlorpyrifos-oxon with a range of resistant strains of *Culex quinquefasciatus* expressed as a percentage of the value in the insecticide susceptible Pel SS strain.

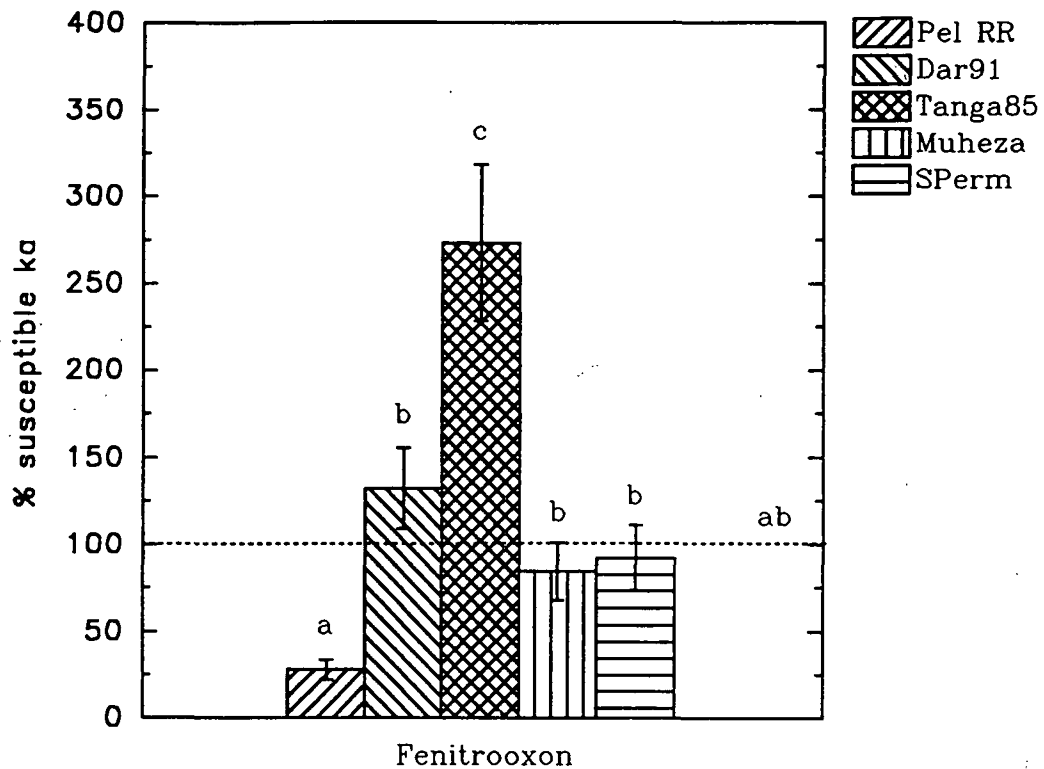

Fig. 2. Bimolecular rate constants (k_a) for fenitrooxon with a range of resistant strains of *Culex quinquefasciatus* expressed as a percentage of the value in the insecticide susceptible Pel SS strain.

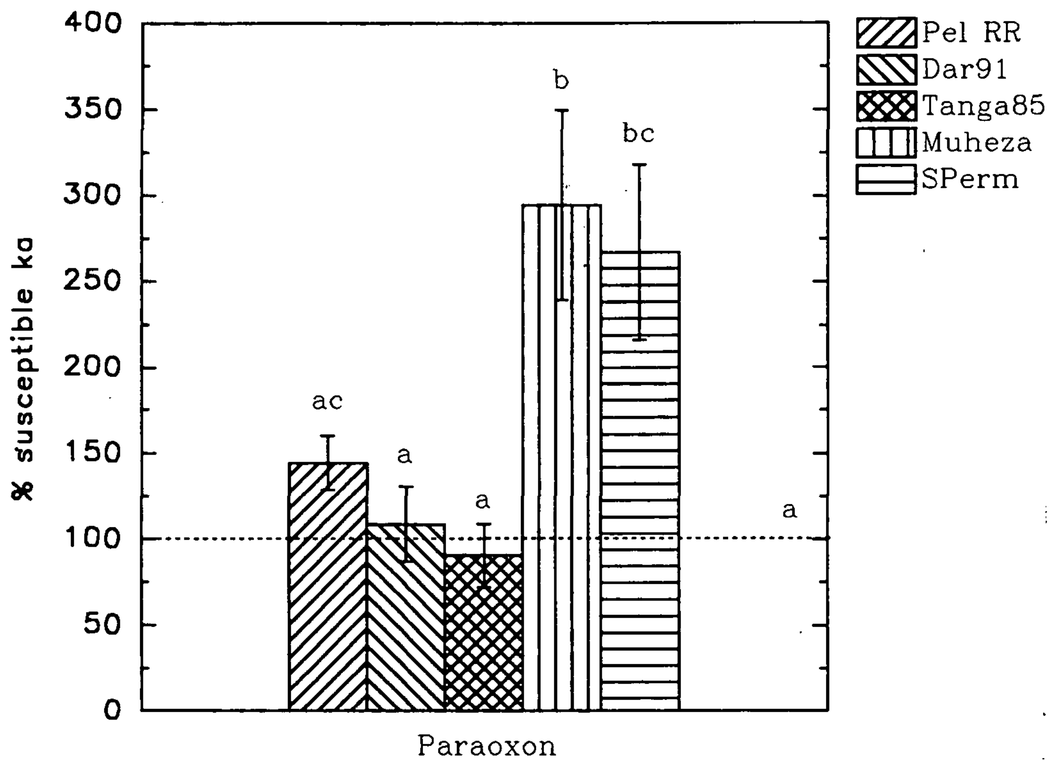

Fig. 3. Bimolecular rate constants (k_a) for paraoxon with a range of resistant strains of *Culex quinquefasciatus* expressed as a percentage of the value in the insecticide susceptible Pel SS strain.

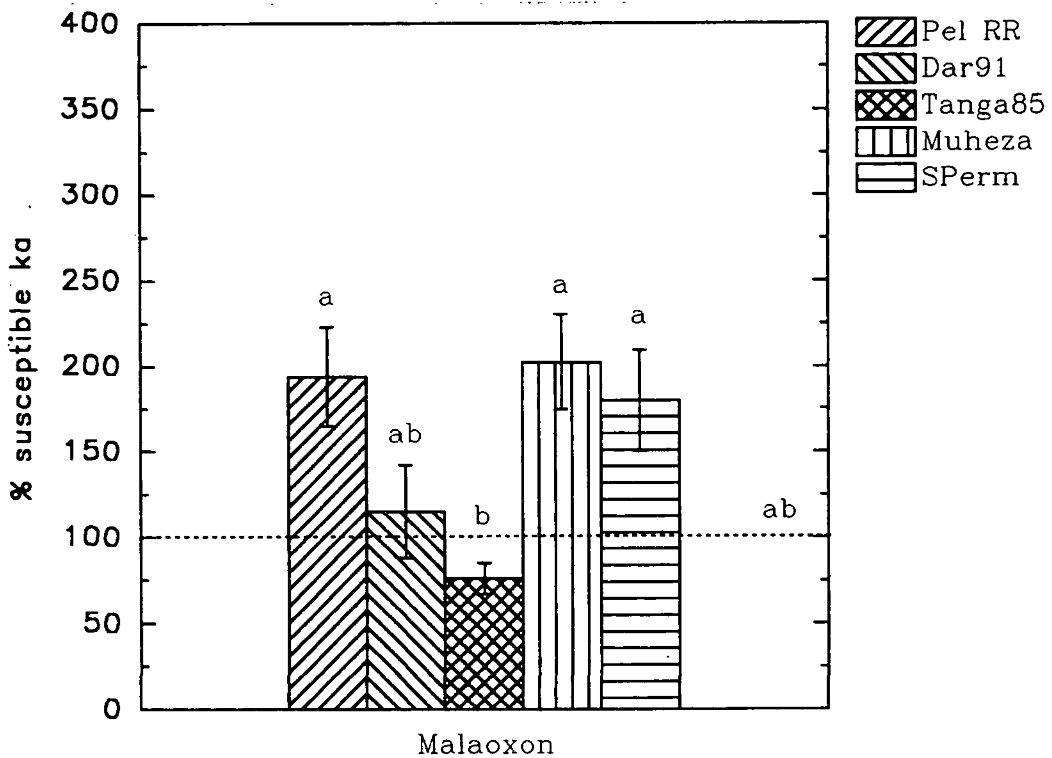

Fig. 4. Bimolecular rate constants (k_a) for malaoxon with a range of resistant strains of *Culex quinquefasciatus* expressed as a percentage of the value in the insecticide susceptible Pel SS strain.

with the elevated esterase mechanism, for the higher levels of resistance seen in this strain compared to the other strains.

The mean total esterase activity measured in crude homogenates of the resistant strains was highest in the Pel RR strain. This does not correlate directly with the observed resistance levels at either the LC_{50} or LC_{90}, as the Pel RR strain had significantly lower levels of resistance to a number of pesticides than the other resistant strains. The order of the resistance ratios between pesticides also differed between strains with apparently identical resistance mechanisms. Therefore, either the A_2 and/or B_2 enzymes are not identical between strains, or the esterases have different specificities for different pesticides and occur in different proportions in the resistant strains. Raymond *et al* (1991) argued, on the basis of identical restriction digest patterns of the flanking regions of the B_2 gene from different populations, that amplification of the B_2 allele of the Est B locus has occurred only once and spread worldwide. Two of the populations they analysed were collected from identical cities to those used in this study. In contrast the elevated B_4 esterase found in *Culex pipiens* in France has a different restriction fragment length polymorphism to that of an esterase with an identical Rf value on starch gels from Cyprus. The B_2 and B_4 esterases also have almost identical mobilities on starch gels, but the B_4 esterase is not associated with the A_2 esterase. They now, therefore, suggest that amplification of the Est B locus has occurred at least four times with three electrophoretically distinguishable allelic forms of the gene (B_1, B_2 and B_4/B_5), but maintain that B_2, as the most common form has spread by migration throughout the majority of the world (Poire *et al* 1992).

We have demonstrated on the basis of differential intensities of hybridisation of a B_2 cDNA from the Pel RR strain to this resistant and the susceptible strain that gene amplification is the likely cause of increased quantities of B_2 in this case. The kinetic differences observed between the Pel SS and Pel RR B_2s may lead to speculation that the differential hybridisation signal is due to sequence differences between the two strains, with a higher specificity towards the Pel RR B_2 being shown under the high stringency conditions used. It has yet to be demonstrated that the elevation of activity with A_2 is due to gene amplification. However we do know that the A_2 and B_2 genes are tightly linked together on the same chromosome and that they occur and are maintained in almost complete linkage disequilibrium. It follows that if the B_2 amplification occurred only once and spread through migration, then the event causing the A_2 elevation must have occurred and spread concurrently. The amplicon on which the B_2 gene occurs is much larger than the gene itself, and the simplest explanation of the A_2 elevation is that the gene sits on the same amplification unit as the B_2 and is co-amplified.

The data presented here clearly show that the 'A_2' and 'B_2' esterases in the susceptible Pel SS strain are physically and kinetically distinct from the enzymes in the resistant strains. There is some evidence from immunoblots that a non-elevated 'A_2' esterase with mobility on native page identical to that in Pel SS occurs in Pel RR, although the close Rf values of these esterases make this difficult to interpret because of the high levels of elevation of the A_2 in the Pel RR. The enzymes in Pel SS probably represent distinct allelic forms of the A_2 and B_2 esterase loci and should be given a different nomenclature from the esterase forms in the resistant strains. The data for physical difference between the A_2 and B_2 esterases in the resistant strains is weaker, although slight consistent differences in the relative mobilities of the purified proteins were found with native PAGE. Kinetic data with propoxur and a range of organophosphates demonstrated that both the A_2 and B_2 esterases have a role in resistance, as both esterases interact with all the inhibitors to a similar extent. The differences between the two enzymes are insufficient to cause the observed differences in resistance between strains due to variations in the proportions of A_2 and B_2, unless there is a big difference in the distribution of the two esterases within the insect which influences their efficacy.

The bimolecular rate constants for phosphorylation and carbamylation of the A_2 and B_2 esterases indicate that both enzymes will act as efficient binding agents for organophosphates and will act more weakly against carbamates. This mirrors the type of cross- resistance spectra invariably seen with this elevated esterase based resistance with higher resistance to organophosphorus than carbamate insecticides. The k_a values for organophosphates such as chlorpyrifos-oxon suggest that the specificity of the A_2 and B_2 esterases are likely to be as high if not greater than the specificity of acetylcholinesterase for this compound. The k3 values for all the inhibitors for both A_2 and B_2 show that neither esterase is effective at metabolising these insecticides, and confirm that the primary route of this resistance mechanism is sequestration rather than metabolism.

There were significant differences in the bimolecular rate constants for the range of inhibitors with both the A_2 and B_2 esterases between the resistant and susceptible strains and between all the resistant strains, although the differences were greater for A_2 than for B_2. These differences were consistent between enzyme preparations, with multiple enzyme preparations of the two enzymes being undertaken for all strains. This suggests that either there are clearly different single allelic forms of both A_2 and B_2 in each of the resistant populations, or that A_2 and B2 are effectively allelic mixtures in each population and that different allelic variants predominate within the mixture in the different resistant strains. The latter hypothesis may be more likely as three of the populations in this study originated from Tanzania and were collected from sites less than 100 miles apart, although the years of their collection differed.

The data presented here are not compatible with the rare amplification and rapid migration model put forward for the amplified esterases by Raymond *et al* (1991). The elevated esterase based resistance mechanism was first detected in *Culex quinquefasciatus* in the early 1970s after extensive field usage of organophosphorus insecticides. If the hypothesis that this mechanism occurred only three or four times from a single esterase B locus and then one of these amplificated enzymes has spread worldwide is correct, then it is unlikely that in the space of twenty years numerous allelic variants of these esterases could have occurred via mutation of one or more of the amplified genes, and have been selected to very different frequencies in the different resistant populations. We believe that the number of amplified alleles of the Est A and B loci are much greater than has been suggested to date, and that the electrophoretic mobility of the esterases on either starch or acrylamide gels is an extremely poor indicator of the actual allelic variant or variants present in a given strain within the A_2 or B_2 classifications. We have not, as yet, determined the restriction fragment length polymorphisms surrounding the B_2 esterases of the strains used in this study. Hence we are unable to comment on whether identical patterns occur and why the conclusions of our biochemical studies and the molecular biological studies of the French group show such disparity.

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