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THE MINIMUM ACETYLCHOLINESTERASE (AChE) FRACTION COMPATIBLE WITH LIFE DERIVED BY AID OF A SIMPLE MODEL EXPLAINING THE DEGREE OF DOMINANCE OF RESISTANCE TO INHIBITORS IN AChE "MUTANTS"

HENDRIK R. SMISSAERT, FATIMA M. ABD EL HAMID* and WILLEM P. J. OVERMEER

Laboratory for Research on Insecticides, Marijkeweg 22, Wageningen and Laboratory of Experimental Entomology, Amsterdam, The Netherlands

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Abstract—Resistance to pesticidal organophosphorus esters in spider mites is due to decreased reactivity of the target enzyme, acetylcholinesterase. The relative tolerances to a series of phosphorus inhibitors of a susceptible strain (S), a homozygous resistant strain (R) and their hybrid (F_1) could be explained quantitatively with a simple model. A 1·6-fold greater tolerance of R compared with the hybrid appeared to be due to the two-fold higher amount of the insensitive enzyme in the homozygous R-mites. This permits the calculation of the minimum fraction of acetylcholinesterase compatible with life (P) in the R-mites, providing $P = 0{\cdot}16$. The relative tolerance of R to S, the degree of resistance, to five inhibitors was found to be consistent with the model which requires that the degree of resistance multiplied with the ratio of enzyme reactivities of R and S be equal to the ratio of log P for R- and S-mites (equation 3). Application of this equation provided a P-value of 0·023 for S-mites which means that normally there is a 40-fold excess.

The function of the enzyme acetylcholinesterase (AChE; EC3.1.1.7) is the rapid hydrolysis of acetylcholine after transmission of a nerve impulse by this ester across the cholinergic synaptic cleft. The primary cause of death by poisoning with AChE-inhibitors is generally accepted to be the accumulation of acetylcholine, causing excessive stimulation followed by total block of impulse transmission. It is widely assumed that there is a considerable excess of AChE relative to what is strictly needed. The minimum fraction of AChE compatible with life (P), i.e. the reciprocal of the degree of excess, is of interest for model studies on AChE inhibition and therapeutic efficiencies of its treatment [1]. Although there is some experimental evidence for AChE excess in mammals [2, 3], the data on insects [4, 5] are conflicting.

There are two main problems. Firstly there is the experimental problem of the determination of the degree of AChE inhibition *in vivo*. Active enzyme separated by some barrier from free inhibitor may be rapidly inhibited as soon as the barrier is destroyed by homogenization necessary to estimate the AChE activity remaining. Addition, before homogenization, of enough substrate to saturate and thus to protect the enzyme from further inhibition improves the reliability of the estimate [4]. A second and more serious problem is the interpretation of the value of P obtained in this way. It provides a mean value of the fraction of all remaining AChE activity of the body or the part of it used for the estimation, while this fraction may vary due to differences in accessibility to the inhibitor [6].

The purpose of this paper is to show that the value of P, in spider mites, can be calculated in a way that is free of these uncertainties. If the model applies, the present value concerns only the AChE of those synapses of which functional deterioration causes the death of a spider mite. The procedure also provides a quantitative explanation of the degree of resistance to a series of organophosphorus inhibitors of a strain with decreased AChE reactivity.

In contrast to vertebrates neuro-muscular transmission in arthropods is not cholinergic [7]. It is not known whether mortality is due to the blocking of a particular part of the nervous system.

The estimate of P essentially depends on the relation between P and the LD_{50}. A reduction of the original normal AChE activity necessarily increases the value of P to hold an identical minimum activity and (other things being equal) lowers the LD_{50}. In this respect it is not important whether the lower original AChE activity is due to a reduction of the amount of enzyme or to lower activity per enzyme molecule. The rational is that a certain minimum activity level is required to hydrolyse acetylcholine at a sufficiently high rate necessary for normal, or non-lethal, impulse transmission. Green [1] treated the therapeutic effect (increase in LD_{50}) of compounds such as atropine as a reduction of P in the presence of the antagonist. An equation developed by Green to deduce the ratio of LD_{50} values in the presence and absence of atropine was used to calculate P from the known ratio of LD_{50}'s and the ratio of P values (equation 4). In another example the differing reactivities of the AChE's of the two strains for the inhibitors had to be taken into account (equation 3).

* Present address: Atomic Energy Establishment, Enshas, Egypt.

MODEL AND EQUATIONS

AChE inhibition by an organophosphorus ester *in vivo* is assumed to follow scheme (1)

$$\begin{array}{l} E + I \xrightarrow{k_i} EI \\ \quad\quad\quad\quad \Big\downarrow k_e \\ \quad\quad\quad\quad I' \end{array} \qquad (1)$$

where E, EI and I represent active enzyme, inhibited enzyme and inhibitor respectively. k_i is the second order rate constant of inhibition and a measure of the reactivity between enzyme and inhibitor, k_e is a first order rate constant representing the sum of all elimination and detoxification reactions. The basic assumption made by Green [1] is that an animal dies once its AChE activity reaches a critical minimum level. The LD_{50} then is the initial amount of inhibitor needed to reach this lethal level when all the inhibitor has been eliminated or reacted. If the sum of all elimination reactions is much faster than the first order rate of inhibition ($k_e \gg k_i \times [I]$), it can be shown [1] that

$$-\ln P = k_i \times LD_{50}/k_e \qquad (2)$$

where P is the minimum AChE activity as defined over the original activity.

The assumption that $k_e \gg k_i \times [I]$ requires at least that the total amount of enzyme is negligible compared to the LD_{50}, both expressed in stoichiometric units. It is convenient to discuss at this point the evidence for this. Eldefrawi [8] using topical application to estimate the LD_{50} of S-females, found the LD_{50} for paraoxon to be about 3 pmole per female. The amount of AChE of strain S was calculated to be maximally 10^{-4} pmole per female from final inhibition experiments with the rapid irreversible inhibitor *O*-ethyl-2-triethylammonium ethylphosphorothiolate iodide*. There is at least, therefore, a 30,000-fold excess of inhibitor.

Equation (2) neglects several obviously important factors such as penetration rate and distribution of inhibitor *in vivo*. Green, however, showed the deductions from the model on relative effects of different forms of treatment to be surprisingly consistent with experimental results. This must mean that these factors are cancelled out if one uses as Green did, the ratio of equation (2) for two different conditions or, as in the present paper, for two differing strains. In the latter case we obtain an equation of the form

$$\ln P'/\ln P = k_i' \times LD_{50}'/k_i \times LD_{50} = Q \qquad (3)$$

where the prime denote the values in another strain.

If the k_e values of the two strains are identical as assumed, k_e from equation (2) disappears in equation (3). k_i indicates the *in vivo* inhibition, while the constant is estimated *in vitro*. Therefore we have to assume that at least the ratio of k_i values for the two enzymes is identical *in vitro* and *in vivo*.

If we compare two strains where the k_i values are identical, equation (3) reduces to

$$\ln P'/\ln P = LD_{50}'/LD_{50} \qquad (4)$$

STRAINS, METHODS AND MATERIALS

Two strains of the spider mite (*Tetranychus urticae* Koch) susceptible (S) and resistant (R) to AChE inhibiting organophosphorus compounds were used. Resistance depends on a single genetical factor [9]. A procedure of backcrossing the R-gene into the S genome is likely to have resulted in identity of other characters of the strain. The R strain, made homozygous for the R-factor, is known as strain Systox Pure (SP) [9]. Further details have been described previously [10].

Resistance has been shown to be due to an abnormal insensitivity of the R-strain AChE to inhibitors [10, 11]. Apart from its low reactivity with inhibitors, the total AChE activity per female (and per weight unit) *in vitro* is lower in the R-strain than in S-strain [10].

The second order rate constants of inhibition (k_i) were estimated by the method of Aldridge [12], incubating enzyme with varying inhibitor concentrations for varying periods before dilution with a buffered substrate solution to determine the remaining activity. Enzyme activity was measured by the method of Ellman [13], using acetylthiocholine as a substrate. Further details have been described previously [11].

The tolerances *in vivo* of the mites to these compounds were estimated by the so called "slide-dip" technique [14]. The LC_{50} values represent the concentrations in ppm of the inhibitor causing 50 per cent mortality on dipping adult females attached on a microscope slide in the solution for 10 sec. The mites were kept at 27° and a relative humidity of 80%. Mortalities were counted after 24 hr. LC_{50}'s were estimated by plotting per cent mortality on a probit scale against log inhibitor concentration.

The inhibitors (Table 2) were at least 95 per cent pure and used as obtained from the supplier. Following the numbering used in Table 2 they are: (1) oxoimidan = *O,O*-dimethyl *S*-(*N*-methylphthalimido) phosphorothiolate (from Stauffer Chem. Co.), (2) *O*-isobutyl *S*-(*N*-methylphthalimido) ethyl-phosphonothiolate (from Stauffer Chem. Co.), (3) oxo-azinphosmethyl = *O,O*-dimethyl *S*-(4-oxo-1,2,3-benzotriazin-3[4H]-yl methyl)-phosphorothiolate (from Bayer), (4) oxo-azinphosethyl = diethyl analogue of compound 3 (from Bayer), (5) omethoate = *O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl)phosphorothiolate (from Bayer). All other chemicals used were commercially available and reagent grade.

CALCULATION OF *P* FOR THE R-STRAIN

This section makes use of data published by Schulten [15]. He provided precise values of the LD_{50} for three organophosphorus pesticides for the same R-strain, an S-strain, different from the one we used, and their hydrid (F_1) (Table 1).

Table 1 shows that the relative LC_{50} of the homozygous R-strain to the hybrid (last column) is constant although the degree of resistance varies from about 10 to nearly 200. Moreover the relative AChE reactivity (k_i) of S to R for the actual inhibitors, the oxo-analogues of azinphosmethyl and parathion, differ considerably, viz. about 10 (Table 2 compound 3) and 600 [11], respectively. Therefore the constancy of the relative LC_{50} of R to the F_1 is difficult to understand if the S-type AChE of the hybrid contributes to its minimum AChE activity, affecting its LC_{50}. The most likely explanation is that essentially all the AChE remaining active after application of the LC_{50} of the F_1 is of the R-type. Additional evidence that this is so can be

* Unpublished experiments.

Table 1. Tolerances of strains S, R, and the hybrid (F_1)

Compound	Strains	LC_{50} (ppm)	LC_{50} of R/ LC_{50} of F_1
Demeton-*S* methyl	S	16·6	
	F_1	139 ± 5	1·50
	R	208 ± 8	
Parathion	S	9·7	
	F_1	1126 ± 52	1·63
	R	1832 ± 98	
Azinphosmethyl	S	13·7	
	F_1	74 ± 4	1·68
	R	124 ± 4	

LC_{50} is the concentration causing 50 per cent mortality. Data from Schulten [15].

obtained by the following calculation (equation 3). If the value of P of the R-type AChE of the hybrid is 0·32 (2 × P of strain R, see below), the remaining activity of the S-enzyme with a 9-times higher k_i (e.g. oxo-azinphosmethyl, Table 2) becomes $3{\cdot}5 \times 10^{-5}$ of its original activity (ln 0·32/ln P_s = 1/9). Thus the fractional activity is nearly 10,000 times higher for the R than for the S enzyme.

As is perhaps to be expected, homozygous R-females appear to contain twice as much of the R-type enzyme as the hybrids [10]. Therefore, the value of P of the R-enzyme in the hybrid (that of S does not contribute) should be twice that in the homozygous strain and we are able to apply equation 3 which then reads

$$\ln P/\ln 2 \times P = LC_{50} \text{ of R}/LC_{50} \text{ of } F_1 = 1{\cdot}6$$

The value 1·6 is the mean for the three compounds of Table 1 and provides a P value of the R strain of 0·16. Thus the R females can just survive when up to 84 per cent of the AChE, of possibly specific synapses, is inhibited.

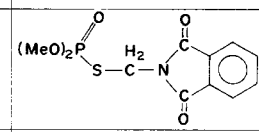
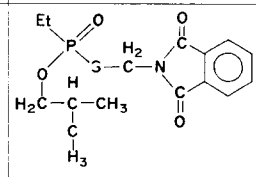
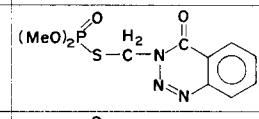
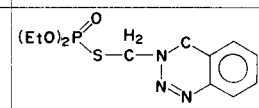
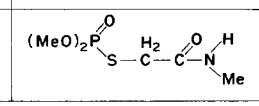
CALCULATION OF *P* FOR THE S-STRAIN

As has been published [10], the AChE activity of R-homogenates to acetylcholine at a concentration of 7×10^{-4} M is about three-fold lower than that of S. When the *in vivo* activity is also lower, other things being equal, the R mites should be more susceptible than S-mites because P of R should be higher. However the k_i values are not equal (the main cause of resistance) and to calculate P, equation 3 has to be used instead of equation 4 as in the previous section.

Table 2 shows the LC_{50} and k_i values of five inhibitors for both strains. The value of $LC_{50} \times k_i$ of R divided by that of S, denoted by Q, is also given. According to equation (3) Q should be a constant thus independent of the inhibitor. The mean Q-value is 0·48 and the standard deviation 0·13. The distribution of Q-values, in this limited series, provides no reason to suppose that Q depends on the inhibitor. This is more significant if one considers that the value of $LC_{50} \times k_i$ varies up to 2000-fold.

If it is accepted that the mean of Q is probably the ratio of log P of R over that of S (equation 3), substitution of P_r = 0·16 and Q = 0·48 gives P_s = 0·023.

Table 2. Potencies to kill ($1/LC_{50}$) and to inhibit (k_i) the AChE of strains S and R

No.	Inhibitor		Potency S	Potency R	Q
1		k_i	$8{\cdot}4 \times 10^6$	$6{\cdot}1 \times 10^5$	0·66
		LC_{50}	0·94	8·5	
2		k_i	$1{\cdot}2 \times 10^6$	$3{\cdot}7 \times 10^4$	0·31
		LC_{50}	22·0	220	
3		k_i	$9{\cdot}2 \times 10^6$	$9{\cdot}5 \times 10^5$	0·43
		LC_{50}	0·6	2·5	
4		k_i	$3{\cdot}6 \times 10^6$	$5{\cdot}5 \times 10^4$	0·55
		LC_{50}	3·5	125	
5		k_i	$1{\cdot}3 \times 10^4$	$2{\cdot}5 \times 10^2$	0·44
		LC_{50}	1·0	23	

LC_{50} is the concentration of inhibitor causing 50 per cent mortality (ppm). k_i is the second order rate constant of inhibition *in vitro* (M^{-1} min^{-1}). $Q = (LC_{50} \times k_i)$ of R/ $(LC_{50} \times k_i)$ of S. The mean of Q-value is 0·48 ± 0·11. Except for compound 5, each Q-value is the average of two independent estimates. The variance ratio (F) of between- over within-inhibitor values of the first four compounds is 1·3 with 3 and 4 degrees of freedom.

DISCUSSION

The conclusions will be discussed in the order of their probable reliability viz., the explanation of the independence of the relative LC_{50} of R to the F_1 of the inhibitor, the value of P for R mites, the explanation of the independence of Q from the inhibitor and finally the value of P for S mites.

Firstly the constancy of the LC_{50} of R over that of the hybrid, at variance with the degree of resistance and the relative reactivities, is quite remarkable and there is little doubt that the S enzyme cannot contribute to the minimum activity of the hybrid in this series (Table 1). The reason of course, is that the S enzyme is more rapidly inhibited than the R enzyme but also requires that the activity of the R enzyme is in excess of the minimum activity. Thus, independent of any model, the P value of R is certainly less than 0·5.

On the reliability of the value of P for R mites the question is whether the factor 1·6 (mean LC_{50} of R over that of F_1) is an estimate of ln P/ln 2 × P (equation 4). One may distinguish two factors; the validity of the model and a possible effect of the method to test mortality, on the value obtained. The first point will be discussed below. Concerning the second, a very similar figure (1·5) for parathion was obtained by Helle [9] who used quite a different mortality test. Therefore there are no reasons to doubt the reliability of the estimate of P for R mites. However, because the error in P is much larger than that in log P, the accuracy of P is probably rather low.

When considering the independence of Q from the inhibitor the large standard deviation of Q must be taken into account. Although the value was calculated from four independent estimates it cannot be proved statistically that Q is independent from the inhibitor.

Evidence for a constant Q-value is provided by the fact that there is no correlation of Q with k_i, the LC_{50}, their product or the structure of the inhibitor in this limited series (Table 2). The constancy of Q, if accepted, means that there is a linear relationship between the degree of resistance and the unreactivity ($1/k_i$) of the AChE's of the two strains (LC_{50} of R/LC_{50} of S = $Q \times k_i$ of R). If for simplicity it is assumed that P is identical for the two strains, making $Q = 1$, the degree of resistance of the mites would be identical to that of their AChE's. Such a simple relationship should not be expected and the relative constancy of Q was surprising. In terms of scheme (1), a minimum model to apply equation (3) (excess of inhibitor with respect to enzyme), a constant Q value requires that all rates affecting the LC_{50} are of first order with respect to the inhibitor concentration and the value of the first order rate constant (k_e, scheme 1) should be identical in S and R mites. The concentrations in R-mites (e.g. the LC_{50}) must be higher than those in S by a factor equal to the relative reactivity of S to R-AChE. The higher the latter value (k_i of S/k_i of R) the more probable it is that the rate of some process in R-mites will level off below these concentration. If so, the value of Q should increase e.g. if penetration of inhibitor is involved, or decrease if some detoxification reaction is concerned. The relative LD_{50} of paraoxon, with a relative k_i of S to R of 600 [11], is only 10 [8], making $Q = 1/60$. The reason why the Q-values obtained (Table 2) do not show too much variance, may partly be incidental but will presumably mainly be due to the relatively low ratio of k_i values of the two enzymes in this series viz. up to about 65. The relative constancy of Q in contradistinction with the up to 2000-fold variance of $LC_{50} \times k_i$, which is proportional to k_e (equation 2), provides evidence that scheme (1) essentially applies and k_e of S and R is identical. It is perhaps worthwhile to notice that a constancy of $k_i \times LC_{50}$ (e.g. k_e) necessarily causes Q to be constant. If so, we have had not the slightest evidence that $Q = \ln P_r/\ln P_s$ because the value of Q could depend on a constant deviation from first order kinetics of the sum of all elimination reactions in the R-mites. This possibility seems quite unlikely in case of the variation in k_e shown in this series.

Returning at this point to the validity of equation (4), it may suffice to mention that the condition and requirements are less rigorous and practically the reverse of those discussed above for equation (3). In the first place, the higher the relative k_i of S to R the less S-type AChE will remain active at the LC_{50} of the hybrid and the better will equation (4) describe the actual situation. Further, because the LC_{50}'s of R over that of the F_1 differs only 1·6-fold, serious changes of k_e are less likely than in the comparison of S and R-mites. Thus equation (4) is much more likely to be valid than (3).

The reliability of P for S-mites may be questionable. Firstly we were using P of R as a reference value and any error in it would affect P of S. Secondly there may be a systematic error in any of the four estimates which make up Q, e.g. the ratio of k_i values *in vivo* may differ from that estimated *in vitro*. The main reason to have confidence in the P-estimate is the fact that the *in vitro* activity of S is higher than that of R and we should therefore expect a lower value of P for S thus a Q-value lower than unity. The P obtained for S, viz. 0·023, suggests that the AChE *in vivo* at the site of action is about 7-fold higher in S than in R-mites. Unfortunately we have no accurate data of activity to acetylcholine *in vitro* for comparison. Apart from the difficulty that one should use crude homogenates for this purpose, the K_m estimations vary too much. At present we are only able to say that the ratio of V_{max}/K_m (which is proportional to the rate of substrate hydrolysis at concentrations much lower than K_m) of S over R *in vitro* is between 2 and 6. Given P of R is 0·16 this corresponds to P-values of S between 0·08 and 0·026 in fair agreement with the value obtained. Therefore the mean of Q is probably a reasonable estimate of $\ln P$ of R/$\ln P$ of S. The accuracy of the P estimate is low due to the relatively high standard error and the fact that log P is involved. Using the 95 per cent confidence limits of the mean of Q (0·37–0·59), those of the mean of P (0·023) are 0·007 and 0·045.

The significance of the present value is of course not that it is more accurate than previous estimates by determination of AChE inhibition *in vivo* [2–5] but merely that it is independent from it, avoiding the difficulties discussed. The value obtained shows that there is roughly a 40-fold excess of AChE in those synapses of which functional disintegration causes the death of a spider mite. The present value for S-mites is strikingly similar to one ($P = 0{\cdot}02$) tentatively suggested as a mean P for mouse whole brain [2]. Most of the P-values reported [3] are 0·1 or higher, which may be explained by varying inhibition in different parts of the brain or tissue examined.

Finally it is of interest to discuss the degree of dominance of resistance according to the model applied. Firstly, since the relative LC_{50} of R to the F_1 is (above certain k_i ratios) a constant, an increase of the degree of resistance should necessarily cause an increase of the degree of dominance. Using log LC_{50}'s to calculate [16] the degree of dominance (D)

$$D = \frac{2 \times \log F_1 - \log R - \log S}{\log R - \log S}$$

where the symbols for the strains denote their LC_{50}'s as in the equations to follow. If we substitute $F_1 = R/1{\cdot}6$ the equation can be rearranged to

$$D = 1 - 0{\cdot}41/\log (R/S) \qquad (5)$$

We may replace R/S by $Q \times k_i$ of S/k_i of R (equation 3.). Substitution of $Q = 0{\cdot}48$ provides

$$D = 1 + 0{\cdot}41/[0{\cdot}32 - \log (k_s/k_r)] \qquad (6)$$

These equations only hold for sufficiently high relative k_i of S to R that the S-enzyme does not contribute to the minimum activity of the F_1 at its LC_{50}.

Secondly, we will discuss the effect of lower relative k_i of S to R on the degree of dominance. Equation (3) shows that at a relative $k_i = \ln P_s/\ln P_r = 1/Q \simeq 2.$, the LC_{50} of S and R, and therefore also of the F_1, are identical. If the relative k_i of S to R is even less than $1/Q$, the order of LC_{50}'s should reverse to R, F_1, S.

Taking into account a 10-fold higher activity of the S-enzyme, it can be shown (equation 3) that this enzyme starts to contribute to the minimum activity of

the F_1 if there is a less than 6-fold difference in k_i. If so, the relative LC_{50} of R to the F_1 is not a constant any more but rapidly decreases with k_i ratios decreasing below 6, causing a further decrease of the degree of dominance. An expression can be derived relating the LC_{50} of S, R and F_1 which reads (symbol for strain denote its LC_{50})

$$F/R = 1 + \ln[2 - P_s \exp(F/S - 1)]/\ln P_r \quad (7)$$

An equation to calculate the relative k_i of S to R necessary to arrive at certain relative LC_{50}'s of F_1 to S can be obtained by substitution of LC_{50} of R from equation (3) into (7) providing

$$k_s/k_r = F_1/S \times Q \times 1/\{1 + \ln[2 - P_s \exp(F/S - 1)]/\ln P_r\} \quad (8)$$

So far, the second point discussed above is of academic interest only, because the lowest relative k_i of a pesticidal organophosphorus inhibitor known to us is that of oxo-azinphosmethyl e.g. 9 (Table 2).

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