SINGLE-MOSQUITO TEST TO DETERMINE GENOTYPES WITH AN ACETYLCHOLINESTERASE INSENSITIVE TO INHIBITION TO PROPOXUR INSECTICIDE

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ABSTRACT. A sensitive technique allowing to identify the three genotypes (Ace^{SS}, Ace^{RR} and Ace^{RS}) of the *Ace* gene existing in natural populations of *Culex pipiens* in southern France is described. The technique is based on the comparison of AChE (acetylcholinesterase) activity in 3 equal aliquots taken from the homogenate of a single mosquito (a) in absence of inhibitor (R_A), (b) in presence of eserine that inhibits the AChE encoded by Ace^S and Ace^R alleles (R_I) and (c) in presence of a concentration of propoxur inhibiting the AChE coded by the Ace^S allele but not by the Ace^R allele (R_G). The mosquito tested is Ace^{SS} when $R_G = R_I$, Ace^{RR} when $R_G = R_A$ and Ace^{RS} when $R_I < R_G < R_A$.

INTRODUCTION

A resistance mechanism involving acetylcholinesterases (AChEs) with reduced sensitivity to inhibition by organophosphates and carbamates has been described as a resistance mechanism in many insect species (Hama 1983). Normal and insensitive AChEs are transmitted as monofactorial characters (*Ace* gene) and several authors have attempted to devise *in vitro* methods to determine the *Ace* genotype of single individuals. Most methods are based on the measurement of activity differences that generally exist between the sensitive and insensitive enzyme forms (Miyata et al. 1980). These activity differences are however often not large enough to avoid ambiguities due to the nature of the *Ace* genotypes or due to manipulations, insect size, physiology, genetic background, etc. (Hemingway and Georghiou 1983). According to Devonshire and Moores (1984), methods based on inhibition differences should be more accurate. We present here a sensitive technique and we show how it allowed a reliable determination of the three *Ace* genotypes existing in populations of *Culex pipiens* Linn. in southern France.

PRINCIPLE OF THE TECHNIQUE

Formal genetic studies have shown that, in southern France, *Cx. pipiens* displays two AChE forms: a "normal" form coded by allele Ace^S and a form insensitive to inhibition by propoxur coded by Ace^R (Raymond et al. 1985). Figure 1 presents the AChE activity recorded in homogenates of Ace^{SS}, Ace^{RS} and Ace^{RR} mosquitoes in the presence of increasing concentrations of propoxur. It can be seen that AChE activity in Ace^{SS} genotypes is almost completely inhibited by 9.09×10^{-5} M propoxur whereas activity of Ace^{RR} genotypes is almost unaffected. This concentration of propoxur is therefore discriminative.

The test involved the comparison of AChE activities in 3 equal aliquots taken from the homogenate of a single mosquito using the method of Ellman et al. (1961). Aliquot 1 serves as the 100% AChE activity reference (or R_A); it contains no inhibitor. Aliquot 2 establishes the AChE 100% inhibition reference (or R_I); it contains 0.01 M eserine sulfate, in order to totally inhibit both AChE forms of *Cx. pipiens* (Raymond et al. 1985). To aliquot 3 a discriminating concentration (9.09×10^{-5} M) of propoxur is added. Thus, if R_G represents the AChE activity observed in the sample containing the discriminating concentration of propoxur: (a) $R_G = R_I$ in Ace^{SS} genotypes; (b) $R_G = R_A$ in Ace^{RR} genotypes; and (c) $R_I < R_G < R_A$ in Ace^{RS} genotypes.

This procedure avoids intrinsic and extrinsic variations in AChE activity that arise between individual mosquitoes as discussed by Hemingway and Georghiou (1983) and allows for precision in *Ace* genotype determinations.

DESCRIPTION OF THE TECHNIQUE

EQUIPMENT. 1.5 ml Eppendorf tubes; 1 ml glass potter homogenizer with a glass pestle; refrigerated centrifuge (10,000 g); Vernon densitometer for microtitration plate readings; microtitration plates; "Pipetman" or "Eppendorf" automatic pipettes to measure the following volumes: 1 ml, 100µl, 90µl and 10µl.

STOCK SOLUTIONS. *Solution A:* 0.1 M sodium phosphate, pH = 8.0. *Solution A':* solution A containing 1% of Nonidet P40 or Triton X100. *Solution B;* 0.1 M eserine sulfate (Sigma E8625) in water. *Solution C:* 10^{-3} M propoxur in water prepared from 0.1 M ethanol solution. *Solution D:* 0.1 M acetylthiocholine in water. *Solution E:*

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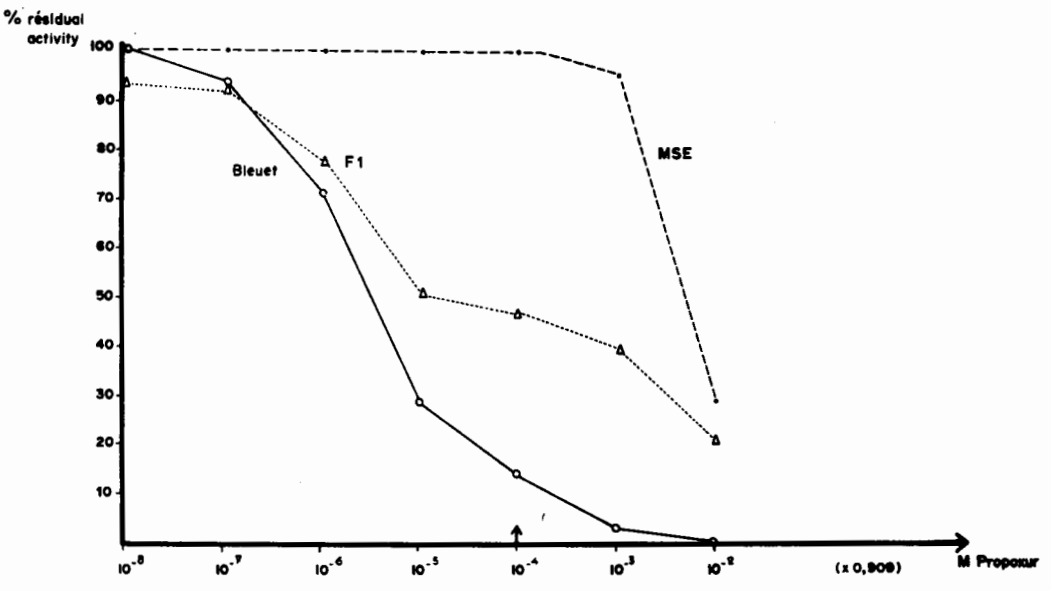

Fig. 1. AChE activity of three single-mosquito homogenates with Ace^{SS} (Bleuet), Ace^{RR} (MSE) and Ace^{RS} (F1: Bleuet × MSE) genotypes, in presence of increasing concentrations of propoxur. The vertical arrow indicates the best propoxur discriminating concentration (9.09×10^{-5} M).

79.3 mg of 5,5-dithiobis(2-nitrobenzoic acid) and 30 mg of sodium bicarbonate in 10 ml of 0.1 M sodium phosphate, pH 7.2.

WORKING SOLUTION. *Solution F:* 5 ml of solution E, 1 ml of solution D and 84 ml of solution A. Solution F is prepared every day as needed and kept in the dark during the experiment.

PREPARATION OF THE MOSQUITOES. Each mosquito (with or without its abdomen) is homogenized at 4°C in the glass potter homogenizer in 1 ml of solution A′. Pupae or fourth instar larvae may be used instead of adults, provided that care is taken to "dry" the insect on filter paper. The homogenate is transferred into an Eppendorf tube and centrifuged at 10,000 g at 4°C for 2 minutes. The supernatant is either used immediately or stored at −20°C.

PROCEDURE. To analyse AChE activity of each mosquito extract, three wells of the microtitration plate are needed. The first (H1) is used to determine R_A, the second (H2) to determine R_I and the third (H3) to estimate R_G. One hundred μl of mosquito extract are introduced in each well. Then, 10μl of water are added to Hl, 10μl of solution B to H2 and 10μl of solution C to H3. The microtitration plate is incubated 30 minutes at 22°C before the addition of 90μl of solution F to each well. Densitometric reading is done after 2 hours of incubation in the dark.

RESULTS AND DISCUSSION

Figure 2 gives an example of the three types of densitometric graphs obtained; they resemble "rockets," the height of which is proportional to the optical density which is negatively correlated to AChE activity. For the first mosquito extract (first three "rockets"), H3 displays the same activity as H1 (i.e., $R_G = R_A$); AChE is not inhibited by the propoxur discriminating concentration; this phenotype corresponds to the Ace^{RR} resistant genotype. The AChE of the second mosquito extract (the three "rockets" in the middle) is completely inhibited by propoxur (H3 displays the same activity as H2: $R_G = R_I$); this phenotype corresponds to the Ace^{SS} genotype. Note that the overall AChE activity (H1) is higher with the second Ace^{SS} mosquito than with the first Ace^{RR}; this is in agreement with the fact that insensitive AChE in our strain of *Cx. pipiens* has a lower activity than the normal one (Raymond et al. 1985). Finally, the third mosquito (three last "rockets") presents an intermediate phenotype: the activity in H3 is between that for H1 and H2 (i.e. $R_I < R_G < R_A$). This corresponds to the heterozygous genotype, Ace^{RS}. As control, one mosquito of each known genotype (Ace^{RR}, Ace^{RS} and Ace^{SS}) was included as reference in every microtitration plate.

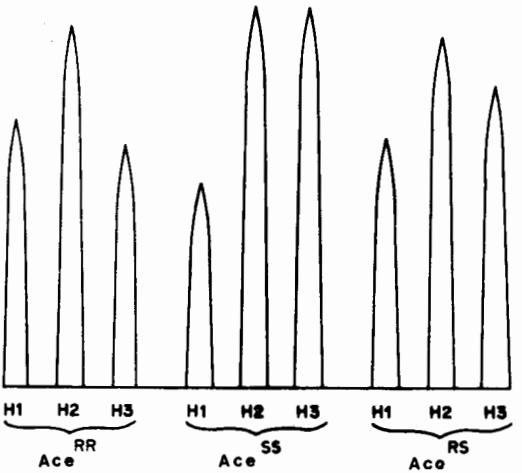

Fig. 2. Densitometric graphs of individual mosquitoes typical for each of the three genotypes. See text for explanations.

This test utilizes only 0.3 ml of the extract prepared from each individual; 0.6 ml is still available to repeat the present test or to perform other enzymatic studies. When *Ace* genotypes are determined on extracts prepared from the head plus thorax, the abdomen can be used to analyze the phenotypes of a highly active esterase using the filter paper test of Pasteur and Georghiou (1981).

The single-mosquito test described here has been used successfully in our laboratory to investigate the linkage relationships between the *Ace* gene and various other genes: *Est-3*, sex and *y* (= yellow larva) (Raymond et al., unpublished data). Its applicability to other insect species, or even to *Culex pipiens* from geographic areas other than southern France, remains to be tested. The most critical point of the method is to find a carbamate or another insecticide with which it is possible to induce the total inhibition of the normal AChE without affecting the insensitive form. The few studies that compare the inhibition characteristics of AChE in susceptible and resistant strains suggest that this should be possible in most cases by testing a large range of concentrations of various carbamates or oxidized organophosphates (P=0). When strains homozygous for each AChE form are not available, one should be able to estimate a concentration that totally inhibits the AChE of Ace^{SS} insects without doing so for the other genotypes; thus the test will separate Ace^{SS} from both Ace^{RR} and Ace^{RS} genotypes.

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