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The genetics of *Dacus oleae*

II. The genetics of two adult esterases

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1. INTRODUCTION

Dacus oleae (Gmel.), a dipteron of the Tephritidae (=Trypetidae) family, is a well known pest of the olive fruit. Because of its economic importance, a vast research program has been planned in order to apply the technique of release of sterile males for its eradication. The need for a better knowledge of the fly's biology and especially a search for genetic markers became urgent. As no morphological mutants were available or found in natural populations, it has been decided to investigate enzyme polymorphisms. After the pioneering work of Wright (1963) on esterases in *Drosophila melanogaster* this field of research proved to be extremely fertile.

Dacus oleae presents many interesting features as an experimental material for genetical and population studies: its ecology is known to a certain extent better than *Drosophila*, it is strictly monophagous and easily trapped, and its size is bigger. Because of its size multiple enzyme detection is facilitated in animals trapped in the wild, which is not easy for *Drosophila*, where enzyme detection is possible only on well fed and bred laboratory flies. On the other hand it presents the disadvantage of not being, until now, easily cultured on an artificial medium.

Nothing is known of its genetics. The cytogenetical data available can be summarized as follows: The fly displays a male heterogamety (XY), its diploid chromosome number is 10, and a crude map of its salivary chromosomes has been drawn (Krimbas, 1963). The present work deals with the description and genetics of two autosomal unlinked esterase genes of the adult fly: an acetylcholinesterase and a pseudocholinesterase or lipase. The great number of active alleles in both genes as well as the existence of silent ones are some of the interesting features disclosed.

2. MATERIALS AND METHODS

The animals were obtained from natural collections in olive-tree orchards, mainly in Attica, but also in Phthiotis, Chania, Messenia and Corfou. Some were bred on artificial medium (Hagen, Santas & Tsecouras, 1963; Santas, 1965). All families described were, however, obtained from culture on fresh olive fruits. Culture conditions of the adults were the ones described by Santas (1965). Adults for electrophoresis were at least 3 days old. The absence of a satisfactory method of culture on artificial media, the short period during which fresh olives are available,

and the fact that *Dacus* flies were introduced in laboratory cultures only 5 years ago made the crosses laborious and the yield of progeny in some families small.

The esterases of adult flies were studied by starch gel electrophoresis (starch from Connaught Medical Laboratories). Two different discontinuous buffer systems gave clear pictures, those described by Ashton & Braden (1961) and by Poulik (1957). Poulik's discontinuous system has been mostly used.

Individuals were ground in two drops of distilled water. Then a small piece of Kleenex paper was put on the homogenate in order to retain chitin fragments when the solution was absorbed by a Whatman no. 3 paper (dimensions $1{\cdot}7 \times 0{\cdot}4$ cm). Attempts to increase the enzyme yield using saccharose, deoxycholate, Tween 80, EDTA solutions alone or in combinations instead of water or breaking cells by sonication (20 kc/s) and centrifuging were unsuccessful.

A continuous water spray at the lower surface of the gel and a piece of ice on top of it ensured a low temperature during electrophoresis. 600–700 V were applied for about 2 h (180–200 mA, a field of 18 V/cm). Electrophoresis was over when the brown front had migrated to a distance of 12 cm from the origin.

The gel was sliced and incubated for 15 min. in a phosphate buffer, pH = 6·4. A solution of 20 mg of α-naphthyl acetate, first dissolved in a small amount of alcohol and then in 100 c.c. of phosphate buffer, was added to the gel together with a solution of 100 mg of Fast Blue BB salt in 100 c.c. of phosphate buffer. After 10 min of incubation, even at room temperature, the enzyme bands appeared. When the gel was well stained it was cleared and kept in a solution of water, methanol and glacial acetic acid (5:5:1 V/V).

3. THE CHARACTERIZATION OF THE ESTERASES

It will be shown in the next section that the biochemical phenotypes of the individuals revealed by electrophoresis are controlled by two genes, named *Est A* and *Est B*. From now on we shall abbreviate these symbols as *A* and *B*. The enzymes synthesized by these two genes differ in their biochemical properties *in vitro*. Cholinesterase and arylesterase from human serum were run simultaneously in order to compare the esterases of systems A and B with well-known ones. The gels were then stained with different substrates. In Table 1, the number of + signs indicates the intensity of staining, depending on the capacity of the enzyme to hydrolyse different substrates. The − sign means no staining, no hydrolysis. Also, while the gel was stained with the usual substrate, α-naphthyl acetate, substances considered as activators were added. The + sign indicates that activation took place, while the − indicates no activation. Finally, substances considered as inhibitors were tested. Plus signs, in this case, measure the inhibition power.

Many classifications of esterases have been proposed. We shall follow the one of Augustinsson (1961), which is considered the most complete. However, it should be mentioned that these classifications are sometimes artificial, and each esterase, perhaps each isoenzyme, differs in a unique way from every other and the different classes of esterase enzymes overlap.

Esterase A is strongly inhibited by Rogor, an organic phosphate compound used as insecticide for this fly. We used Rogor solution from Montecatini having 94–96 % active substance. Parathion and eserine sulphate also inhibit it strongly. The enzyme is slightly activated by some bivalent cations. These observations as well as the fact that Esterase A fails to hydrolyse α-naphtyl butyrate while it can hydrolyse esters of higher fatty acids suggest that it is an *acetylcholinesterase* (Allen, 1960).

Table 1. *Biochemical properties of the esterases detected by stain reaction on the gel. Further explanation in the text*

	Serum cholin-esterase	Serum aryl-esterase	Esterase A	Esterase B
Substrate hydrolysed				
α-Naphthyl acetate	+ + +	+ + +	+ + +	+ + +
α-Naphthyl propionate	.	.	+ + +	+ + +
α-Naphthyl butyrate	+ +	+ +	−	+ +
α-Naphthyl laurate	−	−	+	+ +
Naphthol-AS-acetate	−	+ +	−	+ +
Naphthol-AS-nonanoate	.	.	−	+ +
p-Nitrophenylacetate	−	−	−	−
Benzoylcholine chloride	−	−	−	+
Activators				
Zn^{2+}, Cu^{2+}, Hg^{2+}	−	−	+	−
Sodium taurocholate	.	.	−	−
Acetylcholine	−	−	−	−
Inhibitors				
Rogor: 0·5 %	−	−	+ + +	−
1 %	−	−	+ + +	+
2 %	−	−	+ + +	+ + +
Parathion 10^{-3} M	.	.	+ + +	+ + +
Eserine sulphate: 10^{-5} M	.	.	+	−
10^{-4} M	+	−	+ +	+
$2{\cdot}5 \times 10^{-4}$ M	+ +	−	+ + +	+ +
5×10^{-4} M	+ +	−	+ + +	+ + +
8×10^{-4} M	+ + +	−	+ + +	+ + +
EDTA Na_2 10^{-2} M	−	+	−	−
Mn^{2+}, Cu^{2+}, Hg^{2+}	.	.	−	+ +
Sodium taurocholate	.	.	−	−

Esterase B is less inhibited by Rogor. This differential inhibition provided a method for reading, on the gel, esterase B alone. The upper slice of the gel was incubated with 1·5 % Rogor and was stained for esterase B only, while the lower slice was stained for both A and B. This proved to be critical for the exact reading of gels when it was discovered that many isozyme zones of A and B overlapped in their respective positions. Eserine sulphate inhibits the B system at a concentration of 5×10^{-4} M, after pre-incubation for 30 min. The above properties, together with the fact that EDTA is not an inhibitor, prove that the enzyme is not an arylesterase. The fact that esterase B hydrolyses esters of fatty acids suggests that

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it is a *lipase*. However, it is inhibited by eserine sulphate and hydrolyses benzoylcholine. According to these observations esterase B could be considered as a *non-specific cholinesterase* or *pseudocholinesterase* (Oki, Oliver & Funnel, 1964).

4. THE CROSSES

Virgin females were crossed singly to one male each and their progeny collected. Electrophoresis was applied to the parents, as well as the progeny. The results of thirty-four families are indicated in Tables 2–4.

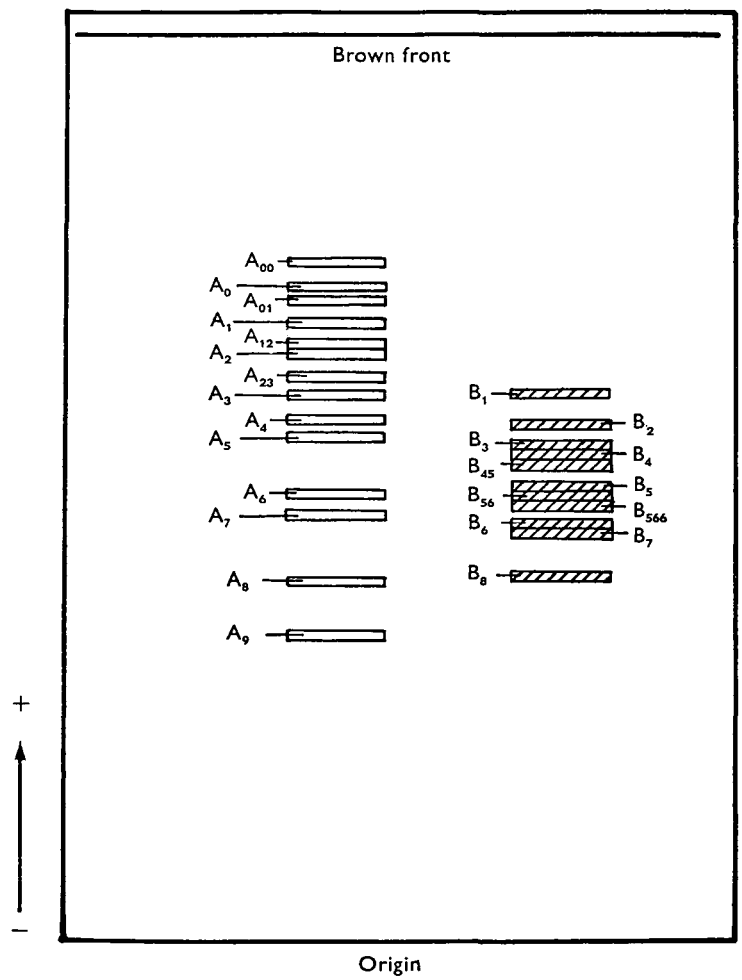

Fig. 1. The respective positions on the gel of the isozymes of the alleles of genes *A* and *B*.

Each fly disclosed a number of zones varying from one to five. Until now we have not observed individuals without any esterase of the systems studied. It became evident that the biochemical phenotypes of all the flies could be explained by the

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existence of the two previously mentioned genes. Gene *A* is the one that controlled the synthesis of isozyme bands, of which the most frequent run faster. The bands of each gene were named according to their distance from the origin. For example A_1 runs faster than A_2. Later on, intermediate bands were found and in order not to change the nomenclature they have been designated as follows: A_{12} runs between A_1 and A_2, A_{23} between A_2 and A_3, and so on. Figure 1 shows the

Table 2. *Number of progenies tested in crosses for which the phenotypes of both parents were known*

A *locus excluding 'silent' alleles*

Genotype of male parent	Genotype of female parent							
	A_1A_2	A_1A_7	A_1A_8	A_2A_2	A_2A_3	A_2A_4	A_2A_7	A_2A_8
A_1A_2	34	.	19	13	.	.	5	.
A_1A_7	.	.	.	7	14	.	.	.
A_2A_2	.	23	.	58	7	.	12	5
A_2A_3	.	.	.	.	17	.	.	.
A_2A_7	30	.	.	22	.	5	.	10
A_3A_3	.	.	.	5	.	.	.	.
A_6A_8	.	.	.	.	.	.	17	.

Test of deviation from expectation among segregating progeny.
Total $\chi^2 = 2{\cdot}65$, D.F. $= 10$, $P = 0{\cdot}98$–$0{\cdot}99$.

Table 3. *Number of progenies tested in crosses for which the phenotypes of both parents were known*

B *locus excluding 'silent' alleles*

Genotype of male parent	Genotype of female parent					
	B_2B_4	B_3B_4	B_4B_4	B_4B_5	B_4B_6	B_4B_7
B_2B_4	.	.	13	.	18	.
B_2B_6	.	.	.	.	7	.
B_4B_4	36	10	78	45	24	22
B_4B_5	19	.	.	.	.	.
B_4B_6	.	.	23	19	35	.
B_4B_7	.	.	26	.	.	.
B_6B_6	.	.	7	.	.	.

Test of deviation from expectation among segregating progeny.
Total $\chi^2 = 7{\cdot}66$, D.F. $= 10$, $P = 0{\cdot}50$–$0{\cdot}70$.

respective positions of the bands of systems A and B. Certain zones of systems A and B run at the same distance from the origin: A_3 with B_1, A_5 with B_3, A_6 with B_{56}, and A_7 with B_6. Fortunately there is a slight difference in colour intensity between A and B zones, and this together with the Rogor inhibition permitted a clear reading of the gels.

In these thirty-four families seven zones for system A, namely A_1, A_2, A_3, A_4, A_6, A_7 and A_8, and six zones for system B, namely B_2, B_3, B_4, B_5, B_6 and B_7 were studied. The results of most of these families have been consolidated for gene *A* in Table 2

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and for gene B in Table 3. It is clear that the results could be explained by the existence of two autosomal genes with multiple active alleles. Each active allele controls the synthesis of a specific isozyme. The segregations conform to these simple mendelian hypotheses.

Individuals homozygous for one active allele of gene A (or B) show only one band of the A (or B) system. Individuals heterozygous for two active alleles of gene A show two bands. On the contrary individuals heterozygous for two active alleles of gene B show three bands, two expected from the respective alleles and a third nearly in the middle, a hybrid enzyme. Schwartz (1960) first studied such a case in the E_1 esterase locus of *Zea maïs*. We conclude that enzyme B acts as a dimer and the hybrid enzyme is composed of two different polypeptides, synthesized by each of the two alleles. Shaw (1965) mentioned that nineteen such

Table 4. *Segregation data in families in which both parents are of known phenotype and in which silent alleles segregate*

Parents' genotypes ♀	♂	Progeny
(1) A_2A_S	A_2A_S	$9(A_2-)$, $4(A_SA_S)$
(2) A_2A_8	A_SA_S	$10(A_2A_S)$, $8(A_8A_S)$
(3) A_1A_2	A_SA_S	$5(A_1A_S)$, $2(A_2A_5)$
(4) A_2A_S	A_2A_8	$3(A_2-)$, $3(A_2A_8)$, $2(A_8A_S)$
(5) A_2A_3	A_7A_S	$3(A_2A_7)$, $1(A_2A_S)$, $4(A_3A_7)$, $3(A_3A_S)$
(6) A_2A_7	A_7A_S	$2(A_2A_S)$, $1(A_2A_7)$, $2(A_7-)$
(7) A_3A_S	A_1A_8	$1(A_1A_S)$, $2(A_1A_3)$, $1(A_3A_8)$, $1(A_8A_S)$
(8) A_1A_S	A_2A_2	$3(A_1A_2)$, $3A_2A_S)$
(9) B_4B_7	B_6B_S	$5(B_4B_6)$, $2(B_6B_7)$, $2(B_4B_S)$, $5(B_7B_S)$

Test of deviation from expectation among segregating progeny.
Total $\chi^2 = 2{\cdot}14$, D.F. $= 2$, $P = 0{\cdot}30$–$0{\cdot}50$.

cases of hybrid enzymes have been studied in different organisms. Numerous other cases have been reported since then. The staining of the three bands in heterozygotes for two active alleles of gene B was not the same. The first two bands showed a stronger staining reaction than the third.

One family (Table 4, row 1) had both parents showing only an A_2 zone. Nine of the progeny had an A_2 and four did not show a band for the A system. In row 2 of the same table the mother was A_2A_8 but the father did not show a band for the A system. Ten of the progeny showed an A_2 band only and eight an A_8 band only. These families are explained if we accept the existence of a 'silent allele' or 'alleles', in the gene A. The existence of a silent allele also explains the families of rows 3, 4, 5, 6, 7 and 8 of the same table. The silent allele acts as a recessive: individuals A_2A_2 and A_2A_S (A_s for A silent) bear the same phenotype. Individuals homozygous for the silent allele do not produce any enzyme zone in the A system.

A silent allele exists also in the B system as the family of row 9 in Table 4 shows. The female parent was B_4B_7, having the three characteristic zones, and the male parent showed only a B_6 zone. Seven progeny were B_4B_6 or B_6B_7 but also seven progeny showed either only B_4 or only B_7. This family could be explained by the

existence of a silent allele at the locus *B*. The frequency of this allele is rather small but in the following paper we shall mention the existence of individuals homozygous for B_s in a population study.

The data of the thirty-four families were used in two other ways.

In ten of the thirty-four families it was possible to detect from the offspring the male or the female gametes produced by the parents and test the hypothesis of independent segregation. No family showed evidence of linkage. Data are presented in Table 5. These tests indicate that the hypothesis of independent segregation is not disproved.

Table 5. *Results of tests for linkage between genes* A *and* B. *The chi-square tests the probability of independent segregation*

	No. of gametes tested	χ^2	D.F.	*P*
Female gametes	137	9·37	13	0·70–0·80
Male gametes	57	4·19	6	0·50–0·70

Table 6. *Results of tests of the hypothesis that genes A and B are not partially sex-linked*

	No. of progeny	χ^2	D.F.	*P*
Gene *A*	124	8·84	11	0·50–0·70
Gene *B*	110	10·14	9	0·30–0·50

That the two genes are not completely sex-linked is shown by the existence of males heterozygous for genes *A* and *B*. The hypothesis that they are partially sex-linked, in a possible common part of the *X* and *Y* sex chromosomes has also been tested in nine families for gene *A* and in seven families for gene *B* (in total twelve out of the thirty-four families) where it was possible to detect in the progeny the origin of each allele (from father or mother). Knowing the sex of the progeny, we tested the hypothesis of independent segregation from sex. In no family was any linkage found. We can conclude that neither gene *A* nor gene *B* is partially sex-linked. The consolidated chi-square tests for both genes are presented in Table 6.

5. DISCUSSION

The polymorphism of genes *Est A* and *Est B* in adult *Dacus oleae* is impressive. At the time of discovery it was a uniquely rich case of enzyme polymorphism. A considerable number of families (some of which are reported in a subsequent paper) were analysed in order to be certain that all isozymes found in the gels were controlled by alleles of only two genes. Meanwhile a short report on a rich isozyme polymorphism fo the esterase locus *E* in the butterfly *Colias eurytheme* was discovered (Burns & Johnson, 1967).

The question, however, remains as to how frequent are those rich polymorphisms

and whether they are a feature of only a few enzyme systems, e.g. esterases. Lewontin & Hubby (1966) and Hubby & Lewontin (1966) estimated that an average population of *Drosophila pseudoobscura* is polymorphic for 30% of its genes and each individual is heterozygotic on average for 12% of them. Their samples were small but the most polymorphic genes were an esterase with six active alleles, a malic dehydrogenase, a leucine aminopeptidase and a larval protein with four active alleles each.

It is possible that the esterase genes in insects disclose an especially rich polymorphism: two Diptera, *Dacus* and *Drosophila*, and a Lepidopteron, *Colias*, seem to follow this rule. Unpublished observations of Mr S. Tsakas on *Dacus oleae* reveal that a few other genes are polymorphic (an amylase, a leucine aminopeptidase and an alkaline phosphatase) but their polymorphism is poorer by far than that of the esterases of the adult flies. S. Tsakas found a third esterase gene in pupae, with a rather rich polymorphism. But most of the systems studied were monomorphic.

It is possible that the enzyme structure of esterases permits many amino acid changes without the enzyme being destroyed and without affecting the fitness of the individuals that bear it. An alternative hypothesis could be formulated: In the last decades most of the insecticides used are inhibitors at least of some esterases. It is possible that a part of this polymorphism is related to this fact.

The existence of silent alleles raises the problem whether these alleles control the synthesis of any kind of polypeptide. That silent alleles are not always really silent is shown by the work of Johnson (1966) on a larval alkaline phosphatase of *Drosophila melanogaster*. Two active alleles *F* and *S* and a silent *O* have been discovered. *FO* individuals show only the F band but *SO* individuals display two bands, one of S and another considered as a hybrid enzyme between a polypeptide synthesized by the *S* allele and another by the *O*, the silent allele. The heterozygotes *FS* show a hybrid enzyme.

SUMMARY

The genetics of two esterase systems in adults of the olive fruit fly *Dacus oleae* (Gmel.) have been studied by starch gel electrophoresis. Two unlinked autosomal genes, each with multiple alleles, control the synthesis of a number of isozymes. Gene *A* controls the synthesis of an acetylcholinesterase, and gene *B* that of a pseudocholinesterase or lipase. Gene *B* produces hybrid enzyme in heterozygotes for active alleles. In crosses the segregation of seven active and one silent allele in gene *A*, and of six active and one silent allele in gene *B*, have been studied. Six more active alleles for gene *A* and five active alleles of gene *B* have not been studied in this paper.

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