HOMOLOGOUS ESTERASES IN THREE SPECIES OF THE VIRILIS GROUP OF DROSOPHILA¹

MAUREEN SIMS McREYNOLDS²

Department of Zoology, The University of Chicago, Chicago, Illinois 60635

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AN analysis of biochemical similarities and differences between species can provide two kinds of information. On the one hand, such an analysis may be regarded as simply providing additional taxonomic characters, and thus, may be important in helping to distinguish between taxonomic groups, particularly where morphological similarity is great. In addition, and more important, such analysis provides a means of evaluating the amount and kind of genetic change that occurs at single loci during evolution—something morphological information alone can never provide. According to current theories of gene action, the proteins are essentially direct gene products. The colinearity of the genetic material with the amino acid sequence of a protein specified by that material has been demonstrated by YANOFSKY and his co-workers (1964). A comparison of the structure of several proteins in taxonomic groups can thus provide direct information on the amount of change in these genes within and between the groups and help to solve some current questions in evolutionary theory.

The technique of histochemical staining for enzymes after gel electrophoresis affords a simple method for discriminating a large proportion of alleles at a number of different genetic loci (see HUBBY and LEWONTIN 1966), and, furthermore, these techniques can be used to compare directly homologous genes in different species. In order to examine the evolution of genetic systems more closely it is necessary to compare related species at the gene level. Questions concerning both intrapopulation genetic structure and the evolution of new species can be approached by this technique. For example, are the same loci polymorphic and monomorphic in different but closely related species? WRIGHT and MACINTYRE (1963) found that two sibling species, *D. melanogaster* and *D. simulans*, shared a polymorphism for two Esterase-6 alleles. Are the alleles found in related species generally the same or different? If different species have different forms of enzymes, can these be correlated with the proposed phylogenetic relationships of the species? The work to be described was undertaken to obtain information on such questions.

Three species of the virilis group of *Drosophila*, *D. virilis*, *D. americana* and *D. novamexicana*, were used in the present study. The nine members of this

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² Present address: Genetics Foundation, Department of Zoology, The University of Texas, Austin, Texas 78712.

species group are essentially sibling species. Their relationships to one another have been studied extensively by PATTERSON, STONE and their co-workers (PATTERSON and STONE 1952; STONE, GUEST and WILSON 1960). The species used in the present study differ ecologically, but are genetically closely related. Non-specific esterases of the three species were examined and the variation found in two of these esterases is reported in the present paper. What appear to be the same two esterase loci often had several alleles in each of the three species, but usually different alleles were found in the different species. The degree of heterogeneity observed varied with the species and the enzyme.

TABLE 1
Drosophila strains used in this study

Univ. Texas Collection No.	Species	Collection date	Locality
.....	<i>virilis</i>		New York (Pasadena strain)
.....	<i>virilis</i>		Truckee, California
.....	<i>virilis</i>		Japan
.....	<i>virilis</i>		Brazil
1736.6	<i>virilis</i>	Sept. 1947	Hang Chow, China
1801.1	<i>virilis</i>	Sept. 1947	Texmelucan, Mexico
1999	<i>virilis</i>	May 1950	Argentina
2375.8	<i>virilis</i>	(rec'd 1956)	Chile
Eastern strains			
.....	<i>a. americana</i>		Anderson, Indiana
.....	<i>a. americana</i>		Independence, Ohio
1893.10	<i>a. americana</i>	July 1947	South Hero, Vermont
1901-5a*	<i>a. americana</i>	July 1948	Jackson, Michigan
3013.1	<i>a. americana</i>	(rec'd 1961)	South Carolina and Georgia
Western strains			
821.12*	<i>a. americana</i>	Aug. 1940	Georgetown, Texas
1773.4e*	<i>a. americana</i>	Aug. 1947	Chadron, Nebraska
1760.8i*	<i>a. americana</i>	July 1947	Poplar, Montana
1760.8q*	<i>a. americana</i>	July 1947	Poplar, Montana
2067.1n*	<i>a. americana</i>	Aug. 1950	Chadron, Nebraska
2068.6	<i>a. americana</i>	Aug. 1950	Oakdale, Nebraska
1128.10	<i>a. texana</i>	June 1941	New Orleans, Louisiana
2320.2b*	<i>a. texana</i>	July 1953	Jamestown, South Carolina
1880.6a*	<i>a. texana</i>	July 1948	Richmond, Virginia
2012.4a*	<i>a. texana</i>	June 1950	Keystone Heights, Florida
2326.1	<i>a. texana</i>	July 1953	Hollandale, Mississippi
A2.13*	<i>novamexicana</i>	Oct. 1958	Patagonia, Arizona
1720.7b*	<i>novamexicana</i>	June 1947	Cliff, New Mexico
1952.2	<i>novamexicana</i>	Aug. 1949	Antlers, Colorado
2358-6	<i>novamexicana</i>	Aug. 1953	Patagonia, Arizona

* Original culture started from isolated female.

MATERIALS AND METHODS

Flies used in this study were raised in $\frac{1}{2}$ -pint milk bottles at 24°C on standard Chicago banana medium. Stocks were obtained from the University of Texas *Drosophila* collection and, where available, University of Texas collection numbers are used to designate the strain used. A list of strains used, together with their geographic origins and collection dates, is contained in Table 1.

The following mutants of *D. virilis* were used in linkage studies. Chromosome 2: *b* (188.0), broken—posterior crossvein interrupted or absent. Chromosome 3: *t* (57.5), telescope—eye reduced; *tb* (100.0), tiny bristle—bristles narrow; *gp*² (119.0), gap-2—long veins missing in whole or part. Chromosome 4: *cd* (32.0), cardinal—eyes bright red. Chromosome 5: *pe* (203.0), peach—eyes brown. Flies that are *cd*; *pe* have yellow eyes.

Enzyme analyses were generally made with young adults about two days of age. Extracts were prepared in 0.1 M phosphate buffer, pH 6.5, containing 5% sucrose. Individual flies were ground in 0.01 ml of buffered sucrose in a glass 0.1 ml Misco microcentrifuge tube. Mass homogenates were prepared by grinding several flies in the buffered sucrose, 0.01 ml per individual. Extracts were centrifuged in a Misco microcentrifuge for 3 minutes at $21,500 \times g$.

Polyacrylamide gels were made by adding catalysts to a 5% solution of Cyanogum 41R (EC Apparatus Co.) gelling agent. When the investigations began, the method described earlier (SIMS 1965) was used for making gels. Later, 0.12% tetramethyl ethylene diamine (TMED, Aldridge) was substituted for 0.13% dimethylaminopropionitrile (DMAPN) and heat was no longer required. The gel and buffer chambers contained 0.1 M Tris-borate (1.5 mM EDTA) buffer, pH 8.9.

The electrophoresis cell was similar to that described by RAYMOND (1964) and was purchased from EC Apparatus Co. A 20-minute equilibration period preceded each run. Approximately 10 μ l of sample was applied to the gel using a microliter syringe. A sample of bovine serum albumin (BSA, Nutritional) mixed with the dye bromphenol blue was usually placed in one of the pockets to serve as a standard marker. Electrophoresis was then carried out at 500 v and about 100 ma for $1\frac{1}{2}$ to 2 hr or until the dyed BSA solution had moved 10 cm from the origin. If the current exceeded 125 ma, distortion of enzyme bands was observed; therefore, voltage was adjusted during the run when necessary.

After electrophoresis the gels were placed in 0.5 M boric acid for $\frac{1}{2}$ hr at 4°C and then in a solution containing, as substrate, 20 mg of either alpha-naphthyl acetate or beta-naphthyl acetate or 20 mg of each dissolved in about 0.3 ml acetone, and 25 mg of dye, *p*-chloro-*o*-toluidine diazonium salt (Fast Red TRN, Dajac) or 4'-amino-2', 5'-diethoxy-benzanilide, diazonium salt (Fast Blue BB, Dajac) in 100 ml 0.1 M phosphate buffer, pH 6.5. The gels were stained at room temperature under constant agitation for about 3 hours to permit maximum development of bands.

RESULTS

Esterases in D. virilis. Eight strains derived from natural populations of *Drosophila virilis* and two laboratory mutant strains, all of which are kept in large bottle cultures, were tested. After electrophoresis of mass homogenates and staining for esterase, up to 12 zones of esterase activity were seen. Identical patterns were obtained in repeated tests of the same strains except that some faint zones of activity near the anode were occasionally absent in all strains. Actual migration distance varied from gel to gel, therefore, the esterase zone in Pasadena strain which stained darkest with alpha-naphthyl acetate as substrate was arbitrarily selected as a standard for all gels. At least one sample of Pasadena was included on each gel and the relative mobility of all other sites of esterase activity was calculated by setting the distance that the standard zone had migrated equal to

1.0. (The actual distance was about 6.5 cm from the origin.) Differences in relative mobility of 0.02–0.03 are not uncommon in successive runs. To determine if sites of esterase activity from different strains had identical mobility, samples were tested side by side on the same gel.

For convenience in reference, the sites of activity were numbered from the origin, with the slowest moving component numbered 1. The phenotype, Esterase, has been abbreviated E, and it is assumed by inference, or demonstrated by genetic tests, to be determined by a gene, Esterase, abbreviated *E*. The letter *v* has been added to the symbols E and *E* to indicate that the source was the virilis group. Thus the enzyme which migrates to the site of esterase activity nearest the origin under these conditions is called Ev-1 and is here postulated to be the product of a gene, *Ev-1*. When more than one electrophoretic form of an esterase exists the relative mobility is added as a superscript.

Whereas mass homogenates had many zones of esterase activity (Figure 1), usually only the three darkest zones could be detected from single-fly homogenates. These are designated Ev-1, Ev-4 and Ev-5 in Figure 1. No variation was found in the relative mobility of Ev-1, which was .20 in all of the strains tested. The variations of Ev-4 and Ev-5 were analyzed in some detail and will be discussed in this paper.

Substrate specificity: When gels were stained for esterase activity using alpha-naphthyl acetate as substrate, Ev-5 was the darkest of the three zones (Figure 2). When beta-naphthyl acetate was used as substrate, however, Ev-4 gave the darkest zone of activity. Since the product-dye complex differs in color with

FIGURE 1.—Esterase patterns seen after staining mass homogenates of selected *D. virilis* strains with alpha-naphthyl acetate as substrate.

FIGURE 2.—Diagram of acrylamide gel staining pattern when alpha-naphthyl acetate is used as substrate (1); when beta-naphthyl acetate is used alone (2); and when alpha- and beta-naphthyl acetate are used together (3). Cross hatched zones are stained for beta-naphthyl acetate, solid zones are stained for alpha-naphthyl acetate.

these two substrates, it was possible to distinguish on the same gel esterase activity toward either or both substrates (see JOHNSON *et al.* 1966b). When both substrates were included, Ev-1 and Ev-5 had only alpha-naphthyl acetate activity and Ev-4 had only beta-naphthyl acetate activity. The esterases were clearly different in substrate preference. This property facilitated the simultaneous study of both esterases.

Variations in mobility of Ev-4: In single-fly assays individuals from eight of the ten strains of *D. virilis* had a site of beta-naphthyl acetate esterase activity with a relative mobility of .69 (Table 2). Two others, Hang Chow and one of the mutant strains, lacked any activity at .69, but had a beta-naphthyl acetate esterase site at relative mobility .78 which stained with intensity equal to that of the esterase at .69. These esterase forms have been designated Ev-4^{.69} and Ev-4^{.78}. Two strains, Argentina and Chile, had some individuals with one esterase zone at .69 or .78, and others with three, at .69, .73 and .78. It seemed likely that the Chile and Argentina strains were segregating for alleles determining esterases of

TABLE 2
Occurrence of various alleles of Ev-4 and Ev-5 in D. virilis

Strain	Ev-4		Ev-5					
	.69	.78	.74	.82	.86	.95	1.0	1.08
Argentina, 1999	x	x	x			x		x
Texmelucan, 1801.1	x			x				
Japan	x			x				
Hang Chow, 1736.6		x			x			x
Brazil	x				x	x		
Truckee	x					x		
Chile, 2375.8	x	x			x			
Pasadena	x						x	
<i>b; tb gp²; cd; pe</i>	x					x		
<i>b; t tb gp²; pe</i>		x			x			

relative mobility .69 and .78, with an additional hybrid band found at .73 in heterozygous individuals. In crosses made between individuals from strains containing only *Ev-4*^{.69} and individuals from strains containing only *Ev-4*^{.78}, an intermediate site of beta-naphthyl acetate esterase activity was indeed found in all individuals heterozygous for the two forms of *Ev-4*.

Variation in mobility of Ev-5: All strains had one or more esterase sites which stained with the same intensity as the Pasadena alpha-naphthyl acetate esterase used as standard. Seven strains had only one such zone of esterase activity, but three were clearly heteromorphous—Brazil, Argentina and Hang Chow (Table 2). Some individuals in these strains had only one intensely staining site, others, the heterozygotes, had two. Unlike the case with individuals heterozygous for *Ev-4*, no hybrid enzymes were found here. These darkly staining sites were demonstrated to be allelic forms of the Pasadena "standard" esterase in crosses and were designated *Ev-5*^{.74}; *Ev-5*^{.82}; *Ev-5*^{.95}; *Ev-5*^{1.0} and *Ev-5*^{1.08}.

Chromosome localization: Several pairs of virgin flies from each of the geographic strains which were segregating at the *Ev-4* or *Ev-5* locus, were removed from the mass culture bottles and placed in vials. If analysis of their progeny indicated that a pair was still segregating for esterase alleles, the pair mating procedure was continued until lines which bred true for *Ev-4* and *Ev-5* of a given relative mobility were obtained.

The homozygous esterase lines were crossed to the mutant *b*; *tb gp*²; *cd*; *pe* strain. When single-fly homogenates of the progeny were tested, the *F*₁ males and females of reciprocal crosses were identical in all cases indicating that the esterases being studied were not sex-linked. The *F*₁ males were backcrossed to females of the *b*; *tb gp*²; *cd*; *pe* strain. Offspring were selected for electrophoresis which were heterozygous for only one of the four marked chromosomes at a time, and homozygous for the visible mutants marking the other three. Regardless of geographic strain used, only in the *tb gp*²; *cd*; *pe* class of testcross offspring were

TABLE 3

Chromosome localization of Ev-4 and Ev-5 in D. virilis as determined by segregation in the F₁ backcross

Parental cross	Visible phenotype and esterase phenotype					
	<i>b</i> ; <i>tb gp</i> ² ; <i>cd</i>		<i>b</i> ; <i>tb gp</i> ² ; <i>pe</i>		<i>b</i> ; <i>cd</i> ; <i>pe</i>	
	<i>Ev-4</i>	<i>Ev-5</i>	<i>Ev-4</i>	<i>Ev-5</i>	<i>Ev-4</i>	<i>Ev-5</i>
<i>Ev-4</i> ^{.69} <i>Ev-5</i> ^{.95} <i>b</i> ; <i>tb gp</i> ² ; <i>cd</i> ; <i>pe</i> ♀ × <i>Ev-4</i> ^{.78} <i>Ev-5</i> ^{.74} (Argentina) ♂	.69	.95	.69	.95	.69 .95	.69 .73 .78 .74 .95
<i>Ev-4</i> ^{.78} <i>Ev-5</i> ^{.74} (Argentina) ♀ × <i>Ev-4</i> ^{.69} <i>Ev-5</i> ^{.95} <i>b</i> ; <i>tb gp</i> ² ; <i>cd</i> ; <i>pe</i> ♂	.69	.95	.69	.95	.69 .95	.69 .73 .78 .74 .95
<i>Ev-4</i> ^{.69} <i>Ev-5</i> ^{.95} <i>b</i> ; <i>tb gp</i> ² ; <i>cd</i> ; <i>pe</i> ♀ × <i>Ev-4</i> ^{.78} <i>Ev-5</i> ^{1.08} (Hang Chow) ♂	.69	.95	.69	.95	.69 .95	.69 .73 .78 .95 1.08
<i>Ev-4</i> ^{.78} <i>Ev-5</i> ^{1.08} (Hang Chow) ♀ × <i>Ev-4</i> ^{.69} <i>Ev-5</i> ^{.95} <i>b</i> ; <i>tb gp</i> ² ; <i>cd</i> ; <i>pe</i> ♂	.69	.95	.69	.95	.69 .95	.69 .73 .78 .95 1.08

two forms of *Ev-4* and *Ev-5* found (Table 3). This indicated that both *Ev-4* and *Ev-5* are on chromosome 2.

When heterozygous F_1 males and F_1 females were permitted to mate, 16% recombination was observed between *Ev-4* and *Ev-5*, indicating that there are two, separate esterase-determining loci (Table 4).

Ev-4 and *Ev-5* can be distinguished in *D. virilis* simply on the basis of substrate specificity. The esterases of two closely related members of the virilis group, *D. americana* and *D. novamexicana*, were tested to see if this kind of distinction could be made for other species.

Esterases in D. americana. Eleven strains of the subspecies *D. a. americana* and five strains of the subspecies *D. a. texana* were tested for esterase activity

TABLE 4

*Segregation and recombination of the genes determining Ev-4.⁷⁸ and Ev-5.⁹⁵ from Ev-4.⁶⁹ and Ev-5.⁸² in D. virilis**

P_1 cross: *Ev-4.⁷⁸ Ev-5.⁹⁵ Argentina* ♀ × *Ev-4.⁶⁹ Ev-5.⁸² Japan* ♂

F_1 cross: $\frac{Ev-4.78 \quad Ev-5.95}{Ev-4.69 \quad Ev-5.82}$ ♀ × $\frac{Ev-4.78 \quad Ev-5.95}{Ev-4.69 \quad Ev-5.82}$ ♂

Esterase genotype		Number observed
F_1	$\frac{Ev-4.78 \quad Ev-5.95}{Ev-4.69 \quad Ev-5.82}$	9
F_2 nonrecombinant	$\frac{Ev-4.69 \quad Ev-5.82}{Ev-4.69 \quad Ev-5.82}$	6
	$\frac{Ev-4.69 \quad Ev-5.82}{Ev-4.78 \quad Ev-5.95}$	16
	$\frac{Ev-4.78 \quad Ev-5.95}{Ev-4.78 \quad Ev-5.95}$	4
F_2 recombinant	$\frac{Ev-4.69 \quad Ev-5.95}{Ev-4.78 \quad Ev-5.95}$	1
	$\frac{Ev-4.78 \quad Ev-5.82}{Ev-4.69 \quad Ev-5.82}$	1
	$\frac{Ev-4.69 \quad Ev-5.95}{Ev-4.69 \quad Ev-5.82}$	1
	$\frac{Ev-4.78 \quad Ev-5.82}{Ev-4.78 \quad Ev-5.95}$	2
Percentage recombination		16

*Heterozygotes could always be distinguished from homozygotes and the recombination values could be calculated directly from the F_2 data.

with alpha- and with beta-naphthyl acetate as substrates. All strains had at least one zone of esterase activity which stained darkly when alpha-naphthyl acetate was used as substrate and another zone which stained darkly with beta-naphthyl acetate.

Variation in alpha-naphthyl acetate esterase: In all strains of *D. americana*, a very dark site of alpha-naphthyl acetate esterase activity appeared at relative mobility .69. This site was lightly stained when beta-naphthyl acetate was used as substrate, and thus had substrate specificity like Ev-5 of *D. virilis* and appeared to be homologous to it. Three strains, *D. a. americana* Independence and 3013.1 and *D. a. texana* 2320.2b, had more than one major alpha-naphthyl acetate esterase site. Single fly homogenates revealed that these strains were heteromorphic for electrophoretic forms of the alpha-naphthyl acetate esterase. In addition to the esterase of mobility .69 (Ev-5^{.69}), two other forms of Ev-5 were found in these strains: at .79 in all three heteromorphic *americana* strains and at .63 in *D. a. americana* 3013.1.

Variations in beta-naphthyl acetate esterase: In *D. americana* extracts there was also a zone which stained moderately when alpha-naphthyl acetate was used as substrate but darkly when beta-naphthyl acetate was used. Many strains of *D. a. americana* had more than one electrophoretic form of this esterase. Individuals from these strains had one or three bands with the same staining properties as found in *D. virilis* individuals heterozygous for Ev-4. In crosses between pure breeding *D. americana* strains which differed in form of the beta-naphthyl acetate esterase, an intermediate hybrid band of esterase activity was found in heterozygous progeny. Two forms of this enzyme which appeared to be homologous with Ev-4 in *D. virilis* were found in *D. americana* with relative mobilities .73 and .80.

The distribution of the esterase alleles in the various geographic strains of *D. americana* is shown in Figure 3.

Determination of homology of loci specifying Ev-4 and Ev-5 in D. virilis and D. americana: It is possible to obtain hybrids between *D. virilis* and *D. americana* if the cross is made using *americana* males and *virilis* females (PATTERSON and STONE 1952). Chromosomes 2 and 3 of *virilis* are linked in *americana* as a result of a chromosomal "fusion". If the beta-naphthyl acetate esterase, Ev-4 and the alpha-naphthyl acetate esterase, Ev-5, are homologous, in both, the genes determining them would be expected to be on the same chromosome and, thus, the esterase alleles of *americana* should always segregate from the alleles in *virilis*. The *americana* esterase alleles should also segregate from the *virilis* visible mutants *b*; *tb* and *gp*² in hybrid males where there is no crossing over. Therefore, *D. a. americana* Ev-4^{.80} Ev-5^{.69} (Anderson) males were mated to *D. virilis* Ev-4^{.69} Ev-5^{.95} *b*; *tb* *gp*²; *cd*; *pe* females. In the F₁, esterases were seen which had the relative mobilities .69, .75, .80 and .95 (Figure 4). F₁ flies had an esterase site at relative mobility .69 which was very dark when alpha-naphthyl acetate, as well as when beta-naphthyl acetate, was used as substrate. This zone was apparently produced by both Ev-4^{.69} of the *virilis* strain and Ev-5^{.69} of *americana*. The site at .80 was light with alpha-naphthyl acetate and moderately dark with beta-

FIGURE 3.—Distribution of esterase forms in *D. a. americana* (A), *D. a. texana* (T), and *D. novamexicana* (N) showing geographic origin of the strains.

naphthyl acetate and corresponded to Ev-4^{.80} of the *americana* strain. The site at .75 was moderately dark with alpha-naphthyl acetate and darker with beta-naphthyl acetate. This esterase was apparently a hybrid molecule formed between subunits of Ev-4^{.69} and Ev-4^{.80}. The band at .95 was very dark with alpha-naphthyl acetate and light with beta-naphthyl acetate and thus represented Ev-5^{.95} from the *virilis* parent. F₁ males were backcrossed to *b; tb gp²; cd; pe* females. The *cd; pe* class contained flies heterozygous for the esterase forms of Ev-4 and Ev-5. This indicated that Ev-4 and Ev-5 of *D. a. americana* were on the fused 2-3 chromosome.

Esterases in *D. novamexicana*. *Variations in alpha-naphthyl acetate esterase:* Four strains of *D. novamexicana* were examined. All of the strains had an esterase site which stained very darkly when alpha-naphthyl acetate was used as substrate. This site showed little activity with beta-naphthyl acetate and seemed to represent Ev-5. Esterases with properties characteristic of Ev-5 were found at relative mobilities .79 in *novamexicana* 1720.7b and at .71 in *novamexicana* 1952.2, 2358.6 and A2.13 (Figure 3).

Variations in beta-naphthyl acetate esterase: Three of the strains of *D. novamexicana* had an esterase zone at .76 with the properties characteristic of Ev-4—it stained moderately with alpha-naphthyl acetate and very darkly with beta-

FIGURE 4.—Esterase patterns of F₁ *americana/virilis* hybrid as compared to parental esterase patterns. Acrylamide gel stained with alpha-naphthyl acetate and with beta-naphthyl acetate as substrates and Fast Blue BB as dye.

naphthyl acetate as substrate. Of all the strains tested, *novamexicana* 2358.6 was unique in that it lacked an esterase which was active toward beta-naphthyl acetate used as substrate. Individuals of this strain were crossed to individuals of the other *novamexicana* strains whose extracts showed beta-naphthyl acetate esterase activity. The heterozygous progeny from this cross had beta-naphthyl acetate esterase activity. In the F₂, a ratio of three individuals with beta-naphthyl acetate esterase activity to one without was obtained. The heterozygotes often did not seem to stain as darkly for beta-naphthyl acetate as the homozygotes. The presence of Ev-4 appeared to be dominant or at least semidominant over its absence in this case.

Determination of homology of esterase loci in D. novamexicana and D. virilis. Individual males of *D. novamexicana* Ev-4.^{.76} Ev-5.^{.79} (1720.7b) were crossed to *D. virilis* Ev-4.^{.69} Ev-5.^{.95} b; tb gp²; cd; pe females. The F₁ progeny from this cross

had Ev-5 esterases of the same relative mobility as the parents. They had in addition a beta-naphthyl acetate zone which was intermediate between the beta-naphthyl acetate active sites from the two parents. This suggests that the two forms of beta-naphthyl acetate esterase are indeed homologous.

The backcross of F_1 males to the *virilis* parent was largely sterile. Only one offspring was recovered from one hundred pair matings. Its genetic constitution indicated that the esterase-determining genes were either on chromosome 2 or 4 and, thus, may be on the same chromosome element as *Ev-4* and *Ev-5* in *virilis* and *americana*.

DISCUSSION

The species, *D. virilis*, *D. americana* and *D. novamexicana*, are genetically closely related and, as has been shown, are capable of limited cross hybridization. *D. virilis* is considered to be close to the primitive parent species of the entire virilis group and has the primitive *Drosophila* karyotype of five rod chromosomes and one dot chromosome. It has a broad geographic distribution and is often associated with man. In the species, *D. americana*, there are two cytological races which are treated as subspecies: *americana* and *texana*. *D. americana* differs from *virilis* cytologically in inversions and by chromosomal fusions—2-3 in both *americana* and *texana* and X-4 in *americana* only. Populations of both races contain individuals heterozygous for chromosomal inversions, although there are fewer in *texana*. Cytologically, the eastern strains of *a. americana* and *a. texana* resemble each other more than either does the western strains of *a. americana*. These western strains are heterozygous for many inversions which are fixed in *novamexicana*. (See Figure 3 for geographic distribution of *americana*, *texana*, and *novamexicana*.) *D. novamexicana* itself is composed of small semi-isolated populations which seem to contain no chromosomal and little visible genetic variation. This species has more fixed inversion differences from *virilis* than *americana* does, but it lacks chromosomal fusions and has the primitive *Drosophila* karyotype. (Data above from ALEXANDER 1952; HSU 1952; PATTERSON and STONE 1952.)

Because these three species are so closely related, it seems reasonable to suggest that there is a good deal of genetic homology among them. Genes have classically been identified as homologous by the similarity of effects resulting from mutations combined with linkage data (STURTEVANT and NOVITSKI 1941). More recently the genetic homologies of some functionally similar proteins have been unequivocally demonstrated by evidence of the similarity of their amino acid sequences (MARGOLIASH 1963). A common function is, then, the primary clue to possible genetic homologies. Other phenotypic characteristics plus genetic information lend support to or weaken the suggestion. In the present case, two functionally different esterases were found in each of three closely related *Drosophila* species. These two esterases were the major sites of esterase activity after electrophoresis on acrylamide gel. One of them, Ev-5, stained very darkly with alpha-naphthyl acetate as substrate and the other, Ev-4 with beta-naphthyl acetate. In each species both Ev-4 and Ev-5 migrated to the central portion of the gel under

standard conditions. No hybrid enzyme of the alpha-naphthyl acetate esterase was found after electrophoresis of extracts of individuals heterozygous for alleles determining two electrophoretic forms. But, in the two species where electrophoretic variants were found, an intermediate, hybrid enzyme appeared when individuals were heterozygous at the beta-naphthyl acetate esterase-determining locus. Hybrid Ev-4 enzymes were also found in the interspecies hybrids—*virilis/americana* and *virilis/novamexicana*. This suggests that Ev-4, in all three species, is a dimer, the subunits of which are capable of recombining in heterozygotes.

In *D. virilis* and *D. americana* the two esterase-determining genes, *Ev-4* and *Ev-5*, were linked. Backcross segregation indicated that both were on chromosome 2 in *D. virilis* and possibly on chromosome 2 in *americana*, where they segregated with the fused 2-3. The segregation data for *novamexicana* was not sufficient to localize the esterase-determining genes to chromosome 2 in this species, but it did not overrule this possibility.

By both functional and genetic criteria the alpha-naphthyl acetate esterases and beta-naphthyl acetate esterases appear to be homologous in the three species. They were not, however, identical in the three species nor even identical in all strains of a given species.

No species had only one form of Ev-4 or Ev-5. In *D. virilis* and *D. americana*, some strains were segregating for two alleles of *Ev-4* and some for two or three alleles of *Ev-5*. Electrophoretic differences were found between strains of *novamexicana*, but there were no cases where a particular line was segregating at either *Ev-4* or *Ev-5*. Because of the small semi-isolated populations that make up this species, less heterozygosity might be expected than in strains derived from the larger populations of the other two species. Since all of the strains used have been kept in the laboratory for many years, these results can only serve as an approximation to the patterns of variation which actually exist in nature. They do seem to indicate that both *Ev-4* and *Ev-5* are polymorphic in populations of both *virilis* and *americana*.

The nature of the selective forces which would tend to preserve the polymorphisms of *Ev-4* and *Ev-5* are unknown at the present time. A condition which might result in a strong selective disadvantage is the absence of an enzyme's activity. A lack of activity, such as was found in *D. novamexicana* 2358.6 with Ev-4, can be the result of a structural change in the genetic locus which determines this enzyme—a change which renders it incapable of catalysing the assay reaction and perhaps also, but not necessarily, incapable of performing its true physiological function. Several *D. melanogaster* enzymes studied electrophoretically have been reported to have such "null" alleles—Esterase-C (JOHNSON 1964), Leucine aminopeptidase-A (BECKMAN and JOHNSON 1964), and alkaline phosphatase (JOHNSON 1966). In none of these instances did the absence of the enzyme seem to inflict a disadvantage on the organisms and it does not in the present case.

In this investigation only one allele was found which might be identical in two of the species—a form of Ev-5 was found in both *D. novamexicana* 1720.7b and *D. a. americana* Independence which had relative mobility .79. The two sub-

species of *americana* did share esterases of the same mobility. In general, new, different alleles were found in the different species. Speculations on evolutionary relationships cannot be useful in a multiple allelic situation when so few alleles are the same between species without a more detailed study of the enzyme structure.

Electrophoretic variants of enzymes are attracting widespread attention as useful tools for population genetics study (LEWONTIN and HUBBY 1966; JOHNSON *et al.* 1966a; JOHNSON *et al.* 1967). Because of the large amounts of variability which may be encountered—both in activity and electrophoretic mobility—parallel laboratory studies of particular enzymes are necessary in connection with field work.

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SUMMARY

Using the technique of gel electrophoresis a large number of alleles can be detected at a particular genetic locus. Variations in two esterase-determining genes in three species are described. The two esterases differed from one another in substrate specificity and the nature of the heterozygote phenotype. The esterase-determining genes are linked, but separable by recombination. The number of alleles found in each of the three species differed and the alleles found were generally not identical in the different species.

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