IMMUNOLOGICAL COMPARISON OF THE α - AND β -ESTERASES IN *DROSOPHILA* SPECIES

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In recent years biochemical methods are increasingly used in characterizing the phylogenetic relationships between various *Drosophila* species. Studies involving pteridines have been reported by Hubby and Throckmorton (1960) and Throckmorton (1962b) using paper chromatography. One of the biochemical methods of studying the protein pattern is electrophoresis, using either starch, agar, cellulose or acrylamide gel. Hubby and Throckmorton (1965) studied the evolutionary relationships between proteins within the *virilis* group of *Drosophila* by means of polyacrylamide gel electrophoresis. Based on starch gel electrophoretic studies on esterases of more than 70 *Drosophila* species, Johnson *et al.* (1966) reported that the differences in substrate specificity and alcohol inhibition-activation characteristics were correlated with taxonomic categories in the genus *Drosophila* proposed by Throckmorton (1962a). Lakovaara *et al.* (1972) discussed evolutionary relationships in eleven species of the *Drosophila obscura* group comparing electrophoretically detectable differences at 27 enzyme loci.

A second biochemical method consists in analysing the homogeneity of proteins by immunological techniques. Because of the extraordinary specificity of the antigen-antibody reactions, the immunological reaction has proved to be a useful tool in demonstrating the identity of proteins from different organisms. For 29 species from nine species groups of the genus *Drosophila*, Duke and Glassman (1968) analysed polyacrylamide gel electrophoretic patterns of xanthine dehydrogenases (XDH) and also examined from seven species the immunological reactions of XDHs to the antiserum prepared *D. melanogaster* XDH. They reported the tendency in the XDH molecule of the electrophoretic mobility being faster in those species of early evolutionary lineage over those of a later lineage. They also found a direct relationship between the degree of electrophoretic mobility and the antigenic properties of XDH in the seven species examined.

The results of previous investigations of α - and β -esterases of *D. virilis* indicated that nine allelic variants of the α -esterases were immunologically identical with each other; and the same situation was found for the five β -esterase variants controlled by the *Est- β* locus, while these two groups of esterases were completely distinct in immunological responses (Sasaki 1974b).

The present paper deals with an attempt to study the relationships between esterases of various *Drosophila* species through immunological reactions to antibodies pre-

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pared for the α - or β -esterases of *D. virilis*.

MATERIALS AND METHODS

Drosophila stocks: The majority of species used in the present study came from laboratory stocks maintained in small mass cultures for many generations at the Genetics Laboratory of Tokyo Metropolitan University. The total of thirty species of the genus *Drosophila* examined here could be classified into 13 species groups belonging to 4 subgenera (Throckmorton 1962b; Johnson *et al.* 1966; Okada 1956). In addition, one unidentified species, which was morphologically very close to *D. ezoana* and therefore tentatively designated as *ezoana*-like, could also be checked through the courtesy of Dr. A. Kaneko. The name and abbreviation of these thirty one species are listed in Table 1. For each species one or more wild strains were randomly chosen for this study, with only the exception of *virilis*, for which two strains homozygous for different esterase alleles ($\alpha^8\beta^0$ in a strain $\alpha^0\beta^B$ in another) were employed.

The esterase zymograms of the species used, were studied by means of the thin layer agar gel electrophoresis, the procedures of which were previously described elsewhere (Ohba *et al.* 1974; Sasaki 1974a). Adult flies for the immunological and electrophoretic tests were raised on either standard cornmeal-molasses or 20% sterile yeast medium, depending on the ecological characteristics of each species. Usually 4-7 day old flies were employed for the tests.

Antisera: Two kinds of rabbit antisera were prepared against partially purified esterases of α^8 or β^B strains of *virilis* and they were called anti- α and anti- β serum respectively. The antisera were the same as those employed in a preceding paper (Sasaki 1974b) and the technical details of antisera preparation have already been reported in the above paper.

Immunological tests: To examine the immunological reaction of various *Drosophila* species to two kinds of antisera described above, Ouchterlony double diffusion tests were carried out in 1.5% agarose gels adjusted to pH 8.3 with veronal-HCl buffer. Fly extracts for the immunodiffusion tests were prepared as follows: 1 g of fresh flies were homogenized in 1 ml of 0.1 M phosphate buffer (pH 6.8) and the homogenates were centrifuged at 10,000 rpm for 20 minutes. These procedures were made at 0°C and the supernatants were immediately used for the Ouchterlony tests. After diffusion was allowed to proceed for two or three days at 5°C, the gel plates were washed repeatedly with cold distilled water to remove excess proteins. Then, when anti- α serum was used, the plates were incubated at room temperature in the substrate solution consisting of a 1:5 mixture of 20% α -naphthyl acetate in acetone and distilled water. After 5 minutes, the substrate solution was replaced by a 0.1% aqueous diazo blue B solution and, uniting with the dye at room temperature, the precipitin lines involved with the α -esterase activity were colored bluish-brown within a few hours. When immunoreaction to anti- β serum was to be examined, β -naphthyl acetate was used as the substrate and the precipitin lines became red.

Table 1. Results of the Ouchterlony double diffusion tests using anti- α or anti- β serum prepared against α^8 of β^B esterase of *D. virilis*

Species group	Species	(abbreviation)	Reaction anti- α	Reaction against* anti- β
Genus: <i>Drosophila</i>				
Subgenus: <i>Scaptodrosophila</i>				
<i>bryani</i> group	<i>bryani</i>	(br)	—	—
Subgenus: <i>Sophophora</i>				
<i>obscura</i> group	<i>pseudoobscura</i>	(po)	—	—
	<i>bifasciata</i>	(bf)	—	—
<i>melanogaster</i> group				
takahashi: subgroup	<i>lutea</i>	(lu)	—	—
<i>melanogaster</i> subgroup	<i>melanogaster</i>	(mg)	—	—
<i>ananassae</i> subgroup	<i>ananassae</i>	(an)	—	—
<i>montium</i> subgroup	<i>auraria</i>	(au)	—	—
Subgenus: <i>Dorsilopha</i>	<i>busckii</i>	(bu)	—	—
Subgenus: <i>Drosophila</i>				
<i>quinaria</i> section				
<i>quinaria</i> group	<i>nigromaculata</i>	(ni)	—	—
<i>testacea</i> group	<i>testacea</i>	(te)	—	—
<i>bizonata</i> group	<i>bizonata</i>	(bz)	—	—
<i>historio</i> group	<i>historio</i>	(hi)	—	—
<i>funnebris</i> group	<i>funnebris</i>	(fu)	—	—
	<i>multispina</i>	(mu)	±	—
	<i>macrospina</i>	(ma)	—	—
	<i>subfunnebris</i>	(sf)	—	—
<i>immigrans</i> group	<i>immigrans</i>	(im)	—	—
	<i>curviceps</i>	(cu)	—	—
	<i>albomicans</i>	(al)	—	—
<i>virilis</i> section				
<i>virilis</i> group	<i>virilis</i> (α^8)	(vi- α^8)	‡	—
	<i>virilis</i> (β^B)	(vi- β^B)	—	‡
	<i>ezoana</i>	(ez)	‡	‡
	<i>ezoana</i> -like	(el)	‡	**
<i>melanica</i> group	<i>pengi</i>	(pe)	+	+
<i>robusta</i> group	<i>sordidula</i>	(so)	‡	+
	<i>lacertosa</i>	(la)	+	±
	<i>pseudosordidula</i>	(ps)	+	+
	<i>moriwakii</i>	(mo)	+	+
	<i>robusta</i>	(ro)	+	+
<i>repleta</i> group				
<i>hydei</i> subgroup	<i>hydei</i>	(hy)	±	+
<i>mercatorum</i> subgroup	<i>mercatorum</i>	(mc)	+	+
<i>melanopalpa</i> subgroup	<i>repleta</i>	(re)	+	‡

* The very intense precipitin lines were represented in the table by ‡. Precipitin lines indicated with ± are faint and not always observed. The minus (—) represents the case in which no precipitin line was observed.

** Not tested.

For some species, tests for inhibition of the enzyme activity by the anti- β serum were performed to estimate the relative amount of cross-reacting materials (CRM). For this purpose the reaction mixtures had contents of 0.0125 ml or 0.025 ml of anti- β serum and also 0.1 ml of the fly extracts under study, all serially diluted with 0.1 M phosphate buffer (pH 6.8) to 1:256. The mixtures were incubated at 30°C for 30 minutes and allowed to stand at 4°C for 48 hours. After centrifugation (3,000 rpm, 15 minutes) the supernatants were examined by the Ouchterlony tests, using the extracts of β^B strain of *virilis* in the center well.

RESULTS

Electrophoretic patterns of esterases

The electrophoretic esterase patterns of various *Drosophila* species employed in the present immunological studies have been examined. Some examples of the esterase zymograms of fly extracts used for immunodiffusion tests were shown in Fig. 1, using a mixed substrate containing α - and β -naphthyl acetate and naphthanil diazo blue B as a dye coupler. In most of the species examined, only one major band was found for either α - or β -esterase. Among the species presented in Fig. 1, the following species revealed two esterase bands, one with α -esterase activity and the other with β -esterase activity: *virilis*, *ezoana*, *pengi*, *mercatorum*, *repleta*, *hydei*, *multispina*, *lutea* and *buskii*. Among them, however, the mobilities of α - and β -bands were nearly the same in *mercatorum* and *buskii*, the α -esterase band was very faint in *lutea* and the activities of both α - and β -esterases were weak in *pengi*. These results indicate that the laboratory

Fig. 1. Photograph of esterase zymograms for fly homogenates of *Drosophila* species. The β -esterase components are marked with black dots. See vi=*virilis* ($\alpha^8\beta^0/\alpha^0\beta^B$), el=*ezoana*-like, ez=*ezoana*, ro=*robusta*, pe=*pengi*, mc=*mercatorum*, re=*repleta*, hy=*hydei*, mu=*multispina*, al=*albomicans*, fu=*funebris*, lu=*lutea*, ni=*nigromaculata*, bu=*buskii*.

strains of the above species were homozygous for the alleles at the two major esterase loci controlling α - and β -esterase bands respectively. In some species (*ezoana*-like, *robusta*, *funnebris*, *albomicans* and *nigromaculata*), on the other hand, there exist more than two major bands for α - or β -esterase. This may be explained by the fact that not all the laboratory strains were homozygous for the alleles controlling esterase bands in the zymogram and also by the fact that, when more than two strains were used for the same species, they were equally mixed for preparing a fly extract of a given species. The results of the previous immunological investigation of *virilis* esterases clearly indicate that the allelic variants of the α - or β -esterase were completely identical in reference to the immunological characteristics, while these two kinds of esterases remained distinct (Sasaki 1974b). Therefore, the immunological tests in the following sections were carried out regardless of the degree of electrophoretic homogeneity of the α - or β -esterases in the strains or species employed.

Immunodiffusion tests

Figs. 2 and 3 show some examples of Ouchterlony double diffusion tests of various species of *Drosophila* fly extracts. In the tests shown in Fig. 2 the center well was filled with the anti- α serum prepared against the α^8 esterase of *virilis*, and extracts from adult flies of the *Drosophila* species to be examined were set in peripheral wells in different combinations. In Fig. 2a in which α^8 strain of the *virilis* and *mercatorum* (subgenus *Drosophila*, *virilis* section), *funnebris* and *immigrans* (subgenus *Drosophila*, *quinaria* section), *melanogaster* and *pseudoobscura* (subgenus *Sophophora*) were tested at the same time, a precipitin line with α -esterase activity was formed only in the case of two species of the *virilis* section, viz. *virilis* and *mercatorum*. No trace of precipitin lines was detected for *pseudoobscura*, *melanogaster*, *funnebris* and *immigrans*. Similarly, in Figs. 2b-2f, anti- α serum cross-reacted only with fly extracts of species belonging to the *virilis* section; e. g., *hydei* (Fig. 2b), *moriwakii* (Figs. 2d and 2f), *robusta* (Figs. 2b, 2c and 2d), *pseudosordidula* (Figs. 2c, 2d and 2e), *repleta* (Figs. 2b and 2e) and *lacertosa* (Fig. 2f). As an exceptional case, however, *multispina* (*quinaria* section, *funnebris* group) seemed to react with the anti- α serum, although the precipitin lines were always very faint in this case (Fig. 2b). It is apparent that, when tested in adjacent peripheral wells, the precipitin lines of two species of the *virilis* group (*virilis* and *ezoana*) formed a complete converging arc, indicating immunological identity of their α -esterases (Figs. 2d and 2f). An additional species designated as *ezoana*-like is closely related to *ezoana* in morphological characteristics (Kaneko, personal communication). Although the esterase zymogram of this species differs considerably from those of *virilis* and *ezoana* (Fig. 1), the precipitin lines for anti- α serum are continuous among these three species, as shown in Fig. 2f. On the other hand, the precipitin lines are not completely identical among the above three species of the *virilis* group and those species belonging to the other species groups of the *virilis* section. In other words, the converging arc and the spur of a cross-reaction were formed when the following pairs of species were tested in adjacent wells; *virilis* and *sordidula* (Figs. 2b and 2d), *virilis* and *multispina* (Fig. 2b), *virilis* and *robusta* (Fig. 2c), *ezoana* and *moriwakii* (Figs. 2d and 2f), *ezoana* and *lacertosa* (Fig. 2f), *ezoana* and *mercatorum* (Fig. 2e)

Fig. 2. Immunodiffusion reactions of fly extract of various *Drosophila* species against anti-α serum prepared against α-esterase of *D. virilis*. The center well contained anti-α serum. The peripheral wells were filled with extracts from various *Drosophila* species abbreviated as follows: el=ezoana-like, ez=ezoana, fu=funebris, hy=hydei, im=immigrans, la=lacertosa, mc=mercatorum, mg=melanogaster, mo=moriwakii, mu=multispina, pe=pengi, po=pseudoobscura, ps=pseudosordidula, re=repleta, ro=robusta, so=sordidula, vi-α⁸=virilis (α⁸β⁰), vi-β^G=virilis (α⁰β^G).

Fig. 3. Immunodiffusion reactions of various *Drosophila* species against anti- β serum put in the center well. Abbreviations are the same in Figs. 1 and 2.

ezoana and *pengi* (Fig. 2e). Except for three species of the *virilis* group, there seems to be no differences in immunological reaction against anti- α serum among the ten species which belong to the *virilis* section, since the precipitin lines were always continuous without the spur in any pair of these species.

Results similar to those described above were obtained in the tests shown in Figs. 3a-3f in which the center well was filled with anti- β serum prepared against *virilis*

esterase β^B . In Figs. 3a and 3b in which β^B strain of *virilis*, *ezoana* (subgenus *Drosophila*, *virilis* section), *nigromaculata*, *funnebris*, *albomicans*, *multispina* (subgenus *Drosophila*, *quinaria* section), *melanogaster*, *lutea*, *bifasciata*, *ananassae* (subgenus *Sophophora*) were tested, precipitin lines with β -esterase activity were formed only in the cases of two species of the *virilis* section (*virilis* and *ezoana*). Their precipitin lines, however, were not identical, as they formed a spur of cross-reaction. This fact suggests that there is partial identity among the antigenic determinant of the β -esterase of two species of the same *virilis* group, *virilis* and *ezoana*. No precipitin line was detected for the species belonging to the *quinaria* section of the subgenus *Drosophila* or subgenus *Sophophora*.

Figs. 3c-3f show some examples of immunodiffusion tests for 11 species of the *virilis* section; *virilis* and *ezoana* (*virilis* group), *pengi* (*melanica* group), *sordidula*, *lacertosa*, *pseudosordidula*, *moriwakii*, and *robusta* (*robusta* group), *hydei*, *mercatorum* and *repleta* (*repleta* group). All species of the *virilis* section examined show the precipitin lines as usually forming the converging arc of a homologous reaction, though the spur of the cross-reaction was formed in some cases. Among 5 species of the *robusta* group (Figs. 3d and 3e) and among 3 species of the *repleta* group (Figs. 3c, 3e and 3f), the precipitin line was always respectively continuous. On the contrary, the spur was seen in the following pairs of species: *virilis* and *robusta* (Fig. 3c), *virilis* and *moriwakii* (Fig. 3c), *virilis* and *sordidula* (Fig. 3d), *virilis* and *ezoana* (Fig. 3d), *moriwakii* and *repleta* (Fig. 3c), *ezoana* and *moriwakii* (Fig. 3d), *hydei* and *pengi* (Figs. 3e and 3f) *sordidula* and *repleta* (Fig. 3e) and *pseudosordidula* and *hydei* (Fig. 3f). For *pengi* (*melanica* group), the precipitin line was weak in activity but seemed to be continuous with that of the *robusta* group. The weak esterase activity of the precipitin line of this species is probably due to the lower esterase activity of individuals and this can be seen in the zymogram in Fig. 1.

The positive results in the Ouchterlony double diffusion tests are diagrammatically illustrated in Fig. 4. Antiserum against α -esterase of *virilis* reacted with all species

Fig. 4. Diagrammatic representation of immunodiffusion tests against anti- α serum (a) and anti- β serum (b). For abbreviations of the species name see Figs. 1 and 2.

of the *virilis* section examined, regardless of the species group to which they belong, and also with a species of the *quinaria* section, *multispina* (Fig. 4a). The esterase activity of the precipitin lines, however, is strong in species of the *virilis* group, weak in species of the other species groups in the *virilis* section, and very weak, barely visible, in *multispina*. The converging arc with the spur was seen between the *virilis* group and the other species groups and also between the *quinaria* section and the *virilis* section. From these data it may be reasoned that the molecules of the α -esterase are antigenically identical within the *virilis* group, partially identical between the *virilis* group and the other species groups, and that the more distant the phylogenetic relationship, the lower the degree of the identity.

With regard to the immunological reaction of β -esterases against anti- β serum too, similar tendencies were found as shown in Fig. 4b. In this case, however, the differentiation between species or species groups seems to be greater than in the case of the α -esterases. No precipitin line was detected for *multispina* belonging to the *quinaria* section. Even between *virilis* and *ezoana* in the same *virilis* group a clear spur was always observed. Among the other three species groups in the *virilis* section (*melanica* gr., *robusta* gr., and *repleta* gr.), the *repleta* group proved to be partially different from the other two groups.

The results of a number of immunodiffusion tests against anti- α or anti- β serum, a part of which has already been illustrated in Figs. 2 and 3, are summarized in Table 1. The thirty one species examined are arranged according to the classification system of Throckmorton (1962b), Okada (1956) and Wheeler (1949). It is to be noted in this table that fly extracts from all species which belong to subgenera other than *Drosophila* (*bryani* in *Scaptodrosophila*; *busckii* in *Dorsilopha*; *pseudoobscura*, *bifasciata*, *lutea*, *melanogaster*, *ananassae* and *auraria* in *Sophophora*) reacted with neither anti- α nor anti- β serum prepared against α - or β -esterase of *virilis*. Eleven species of the *quinaria* section in the subgenus *Drosophila* (*nigromaculata*, *testacea*, *bizonata*, *histrion*, *funbris*, *macrospina*, *subfunbris*, *immigrans*, *curviceps*, *albomicans* and *multispina*) did not react with either antisera, with but one exception, the *multispina* in which very faint precipitin lines with α -esterase activity were almost always found against anti- α serum.

On the other hand, fly extracts from the twelve species which belonged to the *virilis* section of the subgenus *Drosophila* (*virilis*, *ezoana*, *ezoana*-like, *pengi*, *sordidula*, *lacertosa*, *pseudosordidula*, *moriwakii*, *robusta*, *hydei*, *mercatorum* and *repleta*) formed clear precipitin lines against both anti- α and anti- β sera without exception. It is also evident that the esterase activity of the precipitin lines, which could regard as a measure of immunological homology of esterases, were stronger for three species of the *virilis* group (*virilis*, *ezoana*, *ezoana*-like) than for the remaining species belonging to the other species groups of the *virilis* section.

Immunological homogeneity among allelic variants of esterases in D. hydei

In a previous paper, the present author demonstrated that allelic variants of the α - and β -esterases of *D. virilis* were immunologically identical (Sasaki 1974b). Since some laboratory strains of *D. hydei* had been made homozygous for different alleles at *Est- α* and *Est- β* loci, the immunological homogeneity among esterase variants with

Fig. 5. Differences in electrophoretic mobility (a) and precipitin lines in double diffusion test against anti- β serum (b). Allelic variants of *virilis* and *hydei*.

β^A =*virilis* ($\alpha^0\beta^A$), β^B =*virilis* ($\alpha^0\beta^B$), β^C =*virilis* ($\alpha^0\beta^C$),
 $\alpha^4\beta^S$ =*hydei* ($\alpha^4\beta^S$), $\alpha^3\beta^F$ =*hydei* ($\alpha^3\beta^F$), $\alpha^5\beta^S$ =*hydei* ($\alpha^5\beta^S$).

different electrophoretic mobilities was studied by using anti- β serum against *virilis*. An example is given in Fig. 5 in which the electrophoretic pattern (Fig. 5a) and the result of the immunodiffusion test (Fig. 5b) are shown for 3 strains of *virilis* ($\alpha^0\beta^A$, $\alpha^0\beta^B$ and $\alpha^0\beta^C$) and 3 homozygous strains of *hydei* ($\alpha^4\beta^S$, $\alpha^3\beta^F$ and $\alpha^5\beta^S$). For both *virilis* and *hydei*, the precipitin lines were completely continuous within the species, while the clear spurs were formed between species. This result supports the conclusion previously obtained for *virilis* that esterase isozymes controlled by different alleles at the same locus are completely identical in immunological reactions.

Relative amount of cross-reacting material

The modifications of the ordinary Ouchterlony test to demonstrate the degree of antigenic homogeneity among species of the subgenus *Drosophila* consist in treating anti- β serum with fly extracts of a serial dilution followed by subsequent immunodiffusion tests. In the samples shown in Fig. 6, the extract of *virilis* (β^B) was placed in the center well and the anti- β sera pretreated with fly extracts of a given species serially diluted, or with a control solution (0.1 M phosphate buffer pH 6.8), were filled in to eight peripheral wells. When 0.025 ml of the anti- β serum was pretreated with 0.1 ml of *virilis* extracts (β^B) of a serial dilution (1:0–1:256), a faint precipitin line first seemed to appear in the case of 1:4 dilution and this line became apparent when pretreated with the extract of 1:16 dilution (Fig. 6a). In *ezoana* of the *virilis* species group, a clear precipitin line was formed against the antiserum pretreated with the extract of 1:4 dilution but not in the case of 1:2 dilution (Fig. 6b). In *hydei* belonging to the *repleta* species group precipitin lines always developed but the line seemed to be fainter in the case of the undiluted extract than the other cases, although it is not clear in the photograph in Fig. 6c. In *funbris*, a species of the *quinaria* section, intense precipitin lines were formed even against the antiserum pretreated with undiluted extracts (Fig. 6d).

Fig. 6. Absorption of esterases by anti- β serum. Center well contains the extract of *virilis* β^B strain. Peripheral wells contain supernatants of mixtures of anti- β serum and serial dilutions (1:0-1:256) of fly extracts. a; *virilis*, b; *ezoana*, c; *hydei*, d; *funebris*, C; control.

In another series of tests in which three species of the *virilis* section were examined again for the relative amount of the cross-reacting materials in reference to the β -esterases, 0.0125 ml of the anti- β serum was pretreated with 0.1 ml of fly extracts of a more detailed serial dilution. The results are given in Fig. 7. The dilution level of fly extracts at which the precipitin lines with esterase activity first appeared was as follows; 1:16 in *virilis* (Fig. 7a), 1:8 in *ezoana* (Fig. 7b) and 1:2 in *hydei* (Fig. 7c). Summarizing the results of Figs. 6 and 7, it can be said that, in comparison with *virilis*, the amount of cross-reacting materials is about one half in *ezoana* of the same species group as *virilis*, and probably around one eighth in *hydei* belonging to a different species group of the *virilis* section. No materials which could react with the antiserum prepared against *virilis* β -esterase seemed to be involved in species not belonging to the *virilis* section, such as *funebris*. The results described above are fairly in parallel with the results illustrated in Figs. 2 and 3 and summarized in Table

Fig. 7. Immunodiffusion tests showing the relative amount of cross-reacting materials in *virilis* (a), *ezoana* (b), and *hydei* (c). Center well contains the extract of *virilis* β^B strain. Peripheral wells contain supernatants of mixtures of anti- β serum and serial dilution (1:0-1:128) of fly extracts. C; control.

1, in which no precipitin line was detected in *funbris* and the lines with very strong esterase activity were found for both *virilis* and *ezoana*.

DISCUSSION

By means of starch gel electrophoresis, Johnson *et al.* (1966) made an extensive comparative study on the esterase isozymes of various *Drosophila* species from the viewpoint of substrate specificity and alcohol inhibition-activation characteristics. They found a tendency for most *Drosophila* species to possess both α - and β -esterase forms together, and these esterases were polymorphic in some species or strains. In *D. melanogaster* there are two major esterase loci which are known to be polymorphic, *Esterase-6* (Wright 1963) and *Esterase-C* (Beckman and Johnson 1964). The use of mixed substrates revealed that the former controlled the β -esterase and the latter, the α -esterase. By Ohba and coworkers similar situations were reported for *D. virilis* in which most of the esterase activity was controlled by two esterase loci on the second chromosome, *Est- α* and *Est- β* (Ohba 1970, 1971; Ohba *et al.* 1974; Ebitani 1974). In *D. pseudoobscura* Hubby and Lewontin (1966) and Hubby and Narise (1966) found that six electrophoretic esterase variants were controlled by alleles at a single locus, *Esterase-5*, forming a hybrid enzyme in each heterozygote. Later they had been proven to be β -esterases, when a mixed substrate was employed.

The facts described above suggest that, at least in most of the *Drosophila* species, there may be generally two major esterase loci, one controlling α -esterase and another β -esterase, both of which are polymorphic in many cases. In most of the species

employed in the present study too, only one α -esterase and one β -esterase band were detected in the esterase zymograms. This may indicate that the laboratory strains of these species have already become homozygous for the alleles at the two major esterase loci controlling α - and β -esterase bands, as a result of many years of inbreeding. In some species, however, there exist more than two major bands for α - or β -esterase. This merely means that the laboratory strains of these species are still polymorphic for the alleles controlling esterase bands.

In a previous paper (Sasaki 1974b), the present author reported the results of immunological analyses of esterase variants in *D. virilis*, concluding that allelic variants of α - or β -esterases, controlled by the *Est- α* or *Est- β* locus respectively, were immunologically identical, but yet these two groups of esterases were completely distinct with reference to antigenic determinants. Further evidence was obtained in this study on β -esterase variants of *D. hydei*. In the present investigation, therefore, various *Drosophila* species could be used for immunological tests regardless of the degree of heterogeneity in α - and β -esterase loci, though most of the strains used were estimated to be homozygous as stated above.

The results of the Ouchterlony double diffusion tests against anti- α and anti- β serum, which were prepared using *virilis* α^s and β^s esterase respectively, revealed remarkable differentiation in both α - and β -esterases among 31 *Drosophila* species surveyed in the present study. Both esterases seem to be completely different among *virilis* and the eight species belonging to the subgenera *Scaptodrosophila*, *Sophophora* and *Dorsilopha*, because the fly extracts of these species never reacted with either anti- α or anti- β serum. Similar situations were also disclosed for the species in the *quinaria* section of the subgenus *Drosophila*, with the exception only of *multispina* in which a very faint precipitin line was often detected but only against the anti- α serum.

All wild strains of the species belonging to the *virilis* section of the subgenus *Drosophila* reacted immunologically with the antisera against α - and β -esterases of *virilis*. The degree of reactions, however, differs apparently between species groups. Although complete quantitative comparisons could not be made in the present study, the degree of immunological similarity of esterases was considered from three point of view: 1) the esterase activity of precipitin lines, 2) formation of the converging arc and spur in the precipitin lines, and 3) absorption of antisera by fly extracts. The following conclusions can be drawn from the data presented in this paper.

Esterases of *ezoana* which belongs to the same species group as *virilis* are undoubtedly the most similar to those of *virilis* in immunological responses among 11 species of the *virilis* section. Between *ezoana* and *virilis*, the precipitin lines formed against anti- α serum show a complete converging arc indicating homologous reactions, and the esterase activity of precipitin lines seems to be almost equal in these two species. For β -esterase, however, slight differences are noticed between *ezoana* and *virilis*. A spur of cross-reaction is always formed when extracts of these two species are put in adjacent wells in the Ouchterlony test and the esterase activity of precipitin lines is slightly weaker in *ezoana*. The results of the absorption experiment also indicate a smaller amount of cross-reacting materials in *ezoana* compared with *virilis*. Another species which is morphologically very close to *ezoana*, *ezoana*-like, also proves to be

very close to both *ezoana* and *virilis* in immunological responses of the esterases. It is therefore almost certain that this species is a close relative of the above two species.

Three species groups in the *virilis* section other than the *virilis* group (*melanica* gr., *robusta* gr., and *repleta* gr.) possess esterases partially different from the esterases of *virilis*. This is clear from the fact that, (a) when the extracts of flies belonging to these species groups and of the *virilis* group were tested side by side in the Ouchterlony tests, a spur of precipitin line was formed without exception and from the fact (b) that the esterase activity of precipitin lines was significantly lower in these species groups than in the *virilis* group. It is also noticed that, within the members of the same species group, there are no detectable differences in immunological responses to anti- α and anti- β sera prepared against *virilis* esterases. Between the species groups, however, differentiation occurs in the β -esterases, since the spur was formed between species of the *robusta* group and of the *repleta* group. On the other hand, no sign of such differentiation was found for α -esterases among the three species groups in the *virilis* section other than in the *virilis* group.

Summarizing the results discussed above, it can be generally said for *Drosophila* esterases that β -esterases are more differentiated than α -esterases. This seem to correspond to the tendency found in biochemical comparisons of α - and β -esterases of *D. virilis* (Narise 1973). In this connection it may be worth noting that in all species hitherto studied, the α -esterases are monomeric while the β -estrases are usually dimeric with exceptions such as *melanogaster* having monomeric β -esterase. In *virilis*, the molecular weight of β -esterases is estimated to be almost twice as large as that of the α -estrases (Narise 1973).

The phylogeny of the genus *Drosophila* has been studied by Patterson and Stone (1952) and Throckmorton (1962a, 1962b). According to the conclusion on morphological studies by Throckmorton (1962a), the phylogenetic relationship of 31 species used in the present study are diagrammed as shown in Fig. 8. Immunological relationships of α - and β -esterases disclosed in this investigation are in high agreement with the phylogenetic relationships proposed by Throckmorton based on both morphological characters (1962a) and biochemical characters connected with pteridine (1962b). In other terms, esterases of the subgenera *Scaptodrosophila*, *Sophophora* and *Dorsilopha* are quite different from those of *virilis*. Esterases of the *quinaria* section in the subgenus *Drosophila* are also immunologically different from *virilis*, while within the *virilis* section the esterases reacted more or less with antisera prepared against *virilis* esterases. Furthermore, the degree of reaction corresponds to the phylogenic relationships of each species group in the *virilis* section.

Duke and Glassman (1968) described an attempt to study immunologically the evolution of xanthine dehydrogenase (XDH) in the *Drosophila* species. By the use of antiserum prepared for partially purified XDH from *melanogaster*, they observed that the antiserum reacted not only with species belonging to the subgenus *Sophophora* (*melanogaster*, *simulans* and *pseudoobscura*) but also with species of the subgenus *Drosophila* (*immigrans*, *funbris*, *virilis* and *hydei*). From their results it can be said that xanthine dehydrogenases are less differentiated in the genus *Drosophila* than the esterases studied in this paper, although the antigenic relationship between XDH of the species seems

Fig. 8. Phylogenetic relationships of 31 *Drosophila* species used in this study. According to the scheme of Throckmorton (1962a).

to correspond to the scheme of evolutionary relationships described by Throckmorton (1962a, b). Duke and Glassman also reported a direct relationship between the degree of electrophoretic mobility and the antigenic properties of the enzymes of the seven species examined and also a tendency for the XDH molecule from the species of early evolutionary lineage to migrate more rapidly during electrophoresis than those from species of later lineage. In the present immunological and electrophoretic studies of esterases, however, no such tendencies were observed.

For the enzyme lactate dehydrogenase (LDH) of vertebrates, Bailey and Wilson (1968) described the homologies between the LDH isozymes of trout and those of higher vertebrates. The isozymes that they had referred to as the heart group in trout showed a definite resemblance to the H₄ isozyme of the chicken in physical, catalytic and immunological properties, while the muscle group of trout LDH resembles the chicken

M₄ enzyme. Wilson and Kaplan (1964) also found that *Rana pipiens* LDH-1 is immunologically homologous to chicken LDH-1 and *Rana* LDH-5 corresponds to chicken LDH-5.

Although slight quantitative differences are found between α - and β -esterases, the phylogenetic differentiation of *Drosophila* esterases is strikingly noted as compared with *Drosophila* xanthine dehydrogenases or vertebrate lactate dehydrogenases. Since marked differences in the degree of differentiation disclosed here might be due to the differences in physiological roles of enzymes, it would be very interesting to perform further immunological comparative studies on different kinds of enzymes and for different categories of organisms, all from the viewpoint of phylogenetic changes in protein structures.

SUMMARY

Immunological phylogenetic relationships of the α - and β -esterases of *Drosophila* have been studied for 31 species from 13 different species groups of four subgenera. The results of immunodiffusion tests against anti- α and anti- β serum, which were prepared against α - and β -esterase of *D. virilis* respectively, revealed remarkable differentiation in both these esterases among *Drosophila* species. Eight species belonging to the subgenera other than the subgenus *Drosophila* to which *virilis* belonged, reacted with neither anti- α nor anti- β serum. The same situation was found for the species in the *quinaria* section of the subgenus *Drosophila*, with the exception only of *multi-spina* (*funnebris* group) in which faint precipitin lines were found against only anti- α serum. Fly extracts from 12 species of the *virilis* section in the subgenus *Drosophila* cross-reacted with both anti- α and anti- β sera without exception. However, differentiation of esterases between species groups in the *virilis* section as well as differentiation between three species of the *virilis* group was proven to be based on (a) different esterase activity of the precipitin lines, (b) the formation of the spur and (c) on inhibition of esterase activity by antisera. The amount of cross-reacting materials in reference to β -esterase estimated in comparison with *virilis* is as follows: about one half in *ezoana* (*virilis* group), one eighth in *hydei* (*repleta* group, *virilis* section) and zero in *funnebris* (*funnebris* group, *quinaria* section). In general β -esterase seem to differentiate more than α -esterases. Differentiation of *Drosophila* esterases disclosed here by immunological methods coincides well with Throckmorton's scheme of phylogenetic relationships based upon morphological and biochemical characters.

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