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COMPARATIVE BIOCHEMICAL STUDIES ON ENZYMATIC DIFFERENCES OF TAIWAN *IMMIGRANS* GROUP OF GENUS *DROSOPHILA*[1]

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ABSTRACT

Electrophoretic variation of several enzymes in the *immigrans* group of *Drosophila* was investigated. The enzymes from several species were studied from the point of view of substrate specificity after separation by polyacrylamide gel electrophoresis. The separated enzymes were detected by histochemical techniques. Some of the enzyme patterns were found to be species specific.

INTRODUCTION

In recent years the application of the techniques of electrophoretic separation of multiple molecular forms of enzymes (isozymes) in various plant and animal species has been shown to be a useful and more direct tool for taxonomic purposes. Starch gel electrophoresis (12) combined with the histochemical techniques for enzyme assay after electrophoresis (9) has been used in investigations of inherited electrophoretic variation in enzymes in *Drosophila* species (14, 4) as well as in other organisms (11).

In insects, isozymes of fruit flies of the genus *Drosophila* have received particular attention. Beckman and Johnson (1) investigated the genetics of electrophoretic variation in esterases of laboratory strains of *D. melanogaster*. The relative constancy of esterase patterns within species and pattern differences between species have been applied in broad taxonomic comparisons among *Drosophila* species (5). The more recent technique of zone electrophoresis in polyacrylamide gel has been proved to be superior to other methods. The present investigation is a preliminary screening of various enzymatic differences of Taiwan *immigrans* group of genus *Drosophila* by using polyacrylamide gel electrophoresis. Investigation of this kind may provide useful information for further taxonomical studies among related species of this group.

MATERIALS AND METHODS

Eight species of laboratory stock of *immigrans* group maintained on banana-agar medium at 25±2°C were used in this investigation. These were collected on this island except two species, i. e. *hypocausta* and *silver orbits* from the Philippines. Species names, stock numbers and general area where original collections were made are given in Table 1. They were maintained in our laboratory, at least, for 50 generations. The

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following enzyme assay methods were mostly those described by Hubby and Lewontin (3).

Esterase: Single one-day-old adults were smashed on a glass slide and ground in about 15μl of 0.1M tris (hydroxymethyl) aminomethane borate (tris-borate) buffer at pH 8.9 containing 1.5 mM ethylenediaminetetracetic acid (EDTA) and 5% sucrose. The slurry, without centrifugation, was used for electrophoretic analysis. For the purposes of characterization and comparison, the materials obtained as described above were electrophoresed by using polyacrylamide gel as the supporting medium. Five % polyacrylamide gel was prepared according to Nerenberg(10). Zone electrophoresis was performed vertically. About 10 μl of the homogenized slurry was layered into the gel pocket and the electrophoretic run was conducted at a potential difference of 400-450 V (25V/cm, 10mA/cm^2) for 180 minutes. Unless otherwise stated, all the experiments were performed at 4°C. Before each enzyme assay, the gels were preincubated at 4°C in 0.5M boric acid for 1.5-2 hours to lower the pH of the gel to approximately 6.5. The gels were then incubated in 100 ml of 0.1M phosphate buffer pH 6.5 containing 25 mg α-naphthylacetate in acetone-water 1 : 1 (v/v), and 50 mg Fast Red TRN (both from Dajac Laboratories, Philadelphia, Pa.). When no discrimination of the α-ester and β-ester specificities of esterases on the gel was desired, both substrate α-naphthylacetate and β-naphthylacetate were included. Johnson, *et al* (6) reported that a greater apparent activity of serveral of the esterases was caused by adding n-propanol.

Alkaline Phosphatase: Gel was prepared with 0.1M Tris-borate buffer at pH 8.9 without EDTA; in addition, the gel solution contained 5mM $MgCl_2$. Third instar larvae were ground in 10μl of the buffer containing $MgCl_2$. Electrophoresis was performed at 400 volts (25 V/cm, 10 mA/cm^2) for 90 minutes. The gels were incubated in 100 ml of 0.1M Tris-HCl pH 8.5, containing 50μM $MgCl_2$, 50mg sodium α-naphthylphosphate, 2g NaCl, and 500mg polyvinylpyrrolidone. After 1-2 hours, 50mg of Fast Blue RR were added and the incubation continued for additional 30 minutes.

Leucine aminopeptidase: The gel was prepared in the same manner as for alkaline phosphatase. Late pupae (one day before emergence) were ground singly in 10μl of 0.1M Tris-borate buffer pH 8.9 containing 5mM $MgCl_2$ and 5% sucrose. For enzyme detection, the gels were preincubated at 4°C in 0.5M boric acid containing 5mM $MgCl_2$. After rinsing, the gel was incubated in 100ml of 0.2M Tris-malate buffer pH 5.2 containing 20mg L-leucyl-β-naphthylamide-HCl. After 2 hours, 50mg of Fast Black K was added to the buffer solution.

Glucose-6-phosphate dehydrogenase: One-day-old adults were extracted as above. 3mg TRN was included in the gel mixture and electrophoresis was continued for 150 minutes. The gels were incubated for 1-2 hours in 100ml of 0.5M Tris-HCl pH 8.5, containing 0.1mM KCN, 50μM $MgCl_2$, 50mg sodium glucose-6-phosphate, 10mg TRN and 50mg Nitro BT. At the end of incubation period, 2mg phenazine methosulfate (PMS) was added and the gel was incubated for additional 30 minutes.

α-Glycerophosphate dehydrogenase: The gel mixture contained, in addition to the usual ingredients, 1.5mM of α (β)-glycerophosphate; the usual extraction buffer also contained an identical concentration of the substrate. One-day-old adults were the source of the enzyme, and for enzyme detection the gels were

incubated in 100*ml* of 0.1*M* Tris HCl *p*H 8.5, containing 1*mM* α (β)-glycerophosphate, 0.1*mM* KCN, 50*mg* Nitro BT and 15*mg* NAD. After 2 hours incubation, 2*mg* of PMS was added.

Alcohol dehydrogenase: Gel and sample preparation methods were the same as for esterase determination. After electrophoretic separation, the gels were incubated in 100*ml* of 0.1*M* Tris-HCl *p*H 8.5 containing 40*mg* NAD, 20*mg* Nitro BT and 0.75*ml* isopropanol. After incubation for 1 hour, 2*mg* of PMS was added.

RESULTS

Esterase: A number of esterases can be detected in the flies with the present method. The species which have been examined are listed in Table 1. From three to nine sites of activity have regularly been observed in the zymograms. These included α-naphthylacetate and β-naphthylacetate hydrolyzing esterases. Under the experimental condition described, however, all the enzyme bands could hydrolyze both α- and β-esters, indicating that the fly esterases might not be able to distinguish the two types of esters. When only one of the esters was included in the histochemical staining mixture, the zone formed by the hydrolysis of α-naphthylacetate were brownish in color and those of β-naphthylacetate appeared reddish.

TABLE 1

Species, Stock Numbers, and Collection Localities of Specimens Used in the Investigation

Species	Stock No.	Locality
Immigrans group		
immigrans	0008.1	Ahli Shan
formosana	0020.1	Wulai
ruberrima	0017.1b	Tai Ru Kou
grey nasuta	0005.1	I-lan
komaii	0008.3	Ken Ting
silver nasuta (?)	0002.1	Chi-tou
silver orbits		Philippines
hypocausta		Philippines

The electrophoretic patterns of the esterases of the individuals of different species are illustrated in Figure 1. Some lightly stained bands do not show up in the photograph. Fig. 2 is the diagramatic expression of Fig. 1.

In Fig. 2, it was revealed that each species has its own specific esterase pattern. Comparisons of the patterns between males and females of the same species had also been made. It showed only slight differences which were considered to be tissue specific (8). The present survey demonstrated that the patterns of esterases were species-specific.

In a strain analysis, we found some variations in the esterase group-IV (Est-IV) among individuals of the same

species (Table 2). The numbers of individuals analyzed were small and thus the results were presented merely to indicate the need of strain purification before performing a genetic cross experiment.

TABLE 2

Variation of Phenotypes of Est-IV Among Individuals of Each Species.

Species Name / Type of Est-IV	*silver orlits*	*ruber-rima*	*immi-grans*	*grey nasuta*	*hypo-causta*	*komaii*	*formo-sana*	*silver nasuta* (?)
Fast	4	7	0	0	0	8	0	8
Intm.	11	0	0	0	0	0	0	4
Slow	0	0	2	0	0	0	0	0
Null	1	10	14	8	15	0	13	0
3-Bands (F. I & S)	0	0	0	0	0	8	0	0
Total No. Examined	16	17	16	8	15	16	13	12

Alkaline Phosphatase: From two to five sites of alkaline phosphates (AP) activity were demonstrated in late third-instar larvae from different species. The patterns are shown in Fig. 3. This enzyme, at least in a certain band, was extremely sensitive to the presence of Mg^{++} ions in the gel (3). Gels made without including the ion resulted in fewer bands.

Leucine aminopeptidase: As many as six sites of activity were detected. In all the species there was a clear band which remained at the origin. The second band from the origin was most consistently observed. As leucine aminopeptidase is a proteolytic enzyme, it is considered to be an enzyme related to metamorphosis. The different enzyme patterns are shown in Fig. 4.

Glucose-6-phosphate dehydrogenase: Under the conditions employed, only two substrate specific sites of activity were detected. The major bands displayed the fastest migration rate. No electrophoretic variants of this enzyme were formed among the species analyzed, except in *immigrans* where only one major band was present (Fig. 5). A very conspicuous difference of migration rate in *silver orbits* and *komaii* was observed.

α-Glycerophosphate dehydrogenase: No electrophoretic variants of this enzyme were observed.

Alcohol dehydrogenase: This enzyme existed in several forms separable by electrophoresis. The multiple forms of the enzyme suggested that it may exist as a dimer of two polypeptide subunits (2). Fig. 6 showed different isozyme patterns among species analyzed. There was no difference in isozyme patterns between males and females of the same species. Under the conditions tested, two sites of activity migrating toward the cathode were observed in *hypocausta*, one site each in *silver nasuta* and *ruberrima* and none in *silver orbits* or *grey nasuta*.

DISCUSSION

Recently the introduction of electrophoretic techniques into taxonomy has promised to be very useful at the levels from a whole population to a single species (13). This paper is intended to demonstrate that comparative biochemistry can serve to confirm and even extend findings of morphological taxonomy. Morphological variation is the external manifestation of underlying biochemical differences between organisms. The number of electrophoretic

fractions and their rates of migration and concentration form different enzyme patterns which illustrate basic similarities and differences between taxonomic groups. One must realize, however, that mutations resulting in changes in electrophoretic properties of proteins could become established within populations of organisms without necessarily altering their status as species. Probably, the greater the genetic affinity between two kinds of animals the greater will be the number of electrophoretically identical proteins found in the two forms.

In this investigation, the electrophoretic patterns of esterases, alkaline phosphatases, leucine aminopeptidases and alcohol dehydrogenases of *D. immigrans* showed species-specific variations, but the patterns of glucose-6-phosphate dehydrogenase and α-glycerophosphate dehydrogenase did not show variations significant enough for permitting species differentiation. The patterns between males and females of the same species showed only slight differences. A preliminary attempt of genetic crosses has been made; it showed that some species could be inter-crossed and hybrid esterase patterns were obtained. This indicated that an extended study of the strain composition of Est-IV and a genetic cross experiment using purified strains may eventually give clues to comprehending the degree of interactions of the *Drosophila* species in the natural habitat.

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LEGEND OF FIGURES

Fig. 1. Photograph showing the esterase patterns of whole individuals from the stocks of various species used in this investigation.
A: *silver orbits*; B: *ruberrima*; C: *immigrans*; D: *grey nasuta*
E: *hypocausta*; F: *komaii*; G: *formosana*; H: *silver nasuta*(?)

Fig. 2. Diagram of esterase patterns including the lightly stained bands not visible in Fig. 1. For comparative purposes the esterases were classified into six groups according to their electrophoretic mobilities.

Fig. 3. Diagram of alkaline phosphatase patterns from different species. Species names were given according to Fig. 1.

Fig. 4. Diagram of leucine aminopeptidase patterns from different species. Species names were given according to Fig. 1.

Fig. 5. Diagram of glucose-6-phosphate dehydrogenase patterns from different species. Species names were given according to Fig. 1.

Fig. 6. Diagram of alcohol dehydrogenase patterns from different species.
A: *silver orbits*; B: *ruberrima*; D: *grey nasuta*; E: *hypocausta*; F: *komaii*

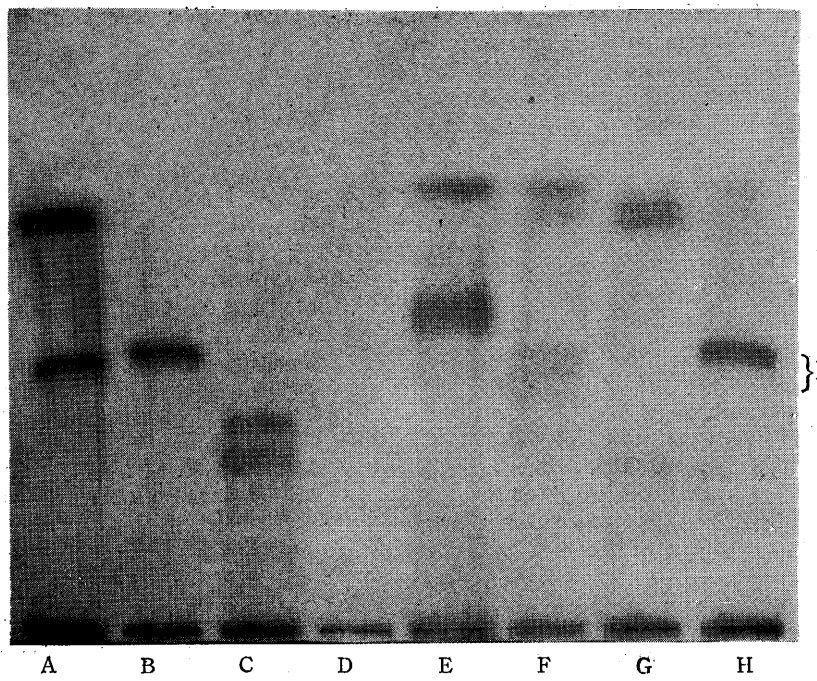

FIGURE 1

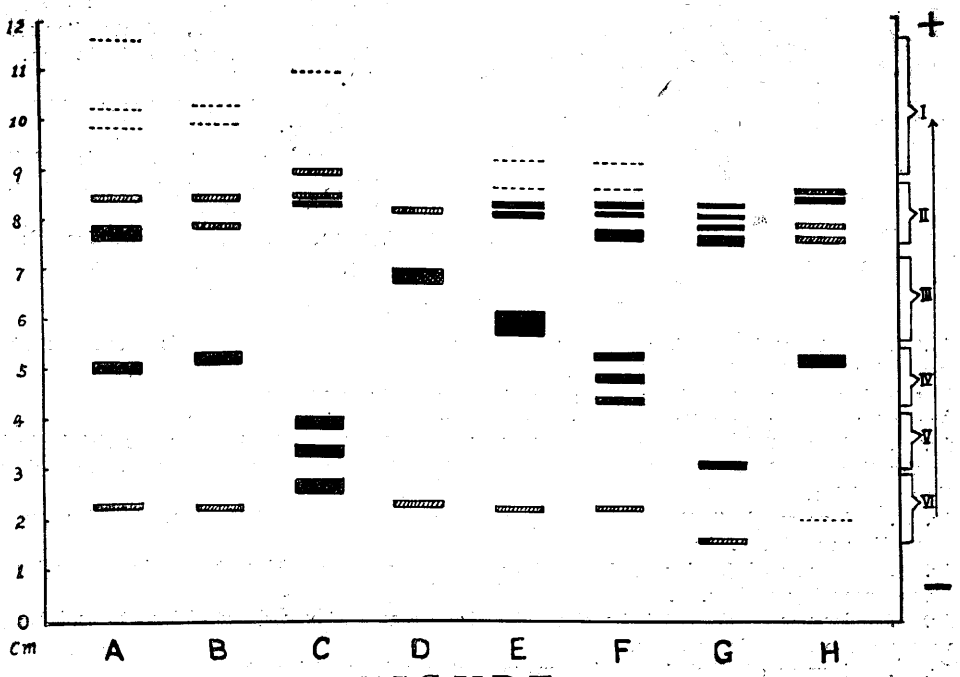

FIGURE 2

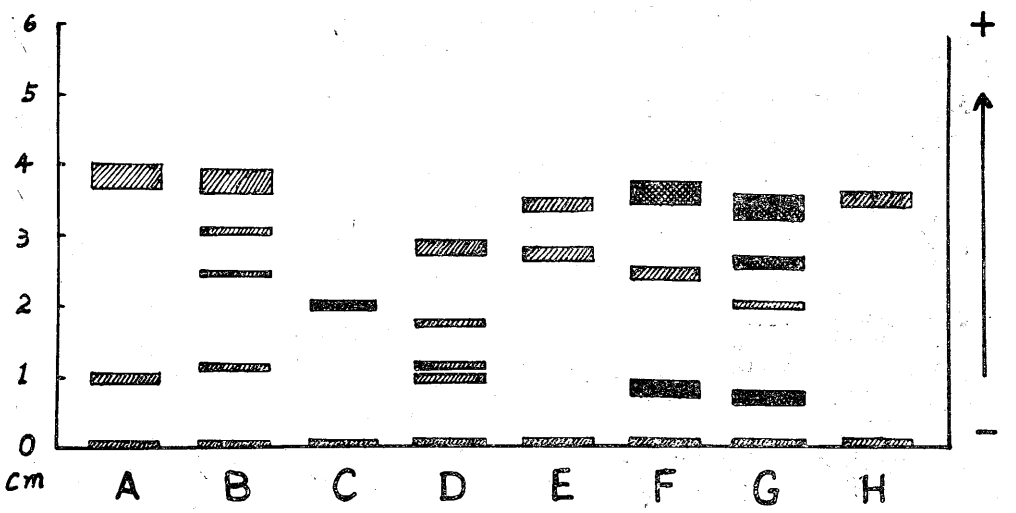

FIGURE 3

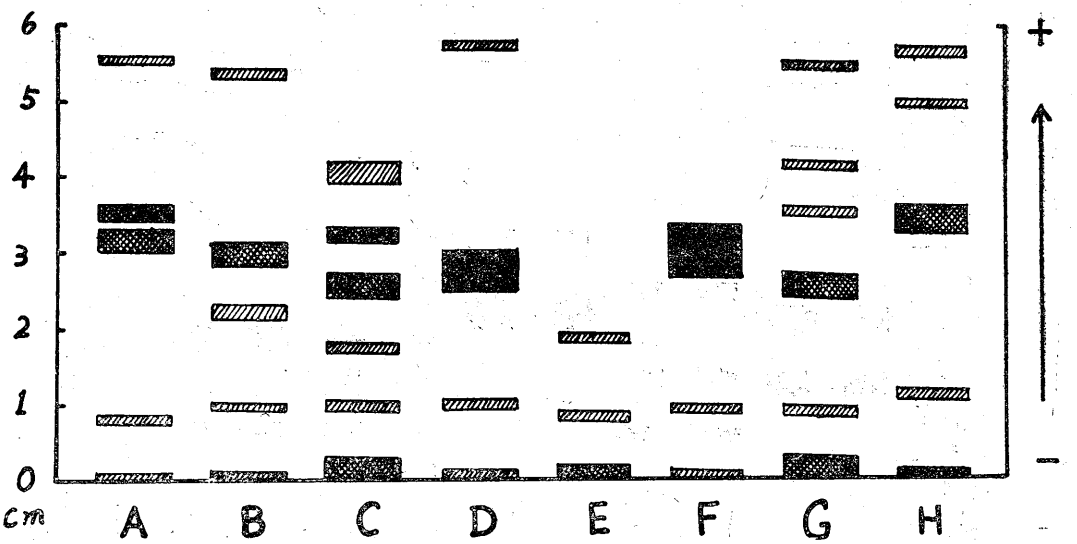

FIGURE 4

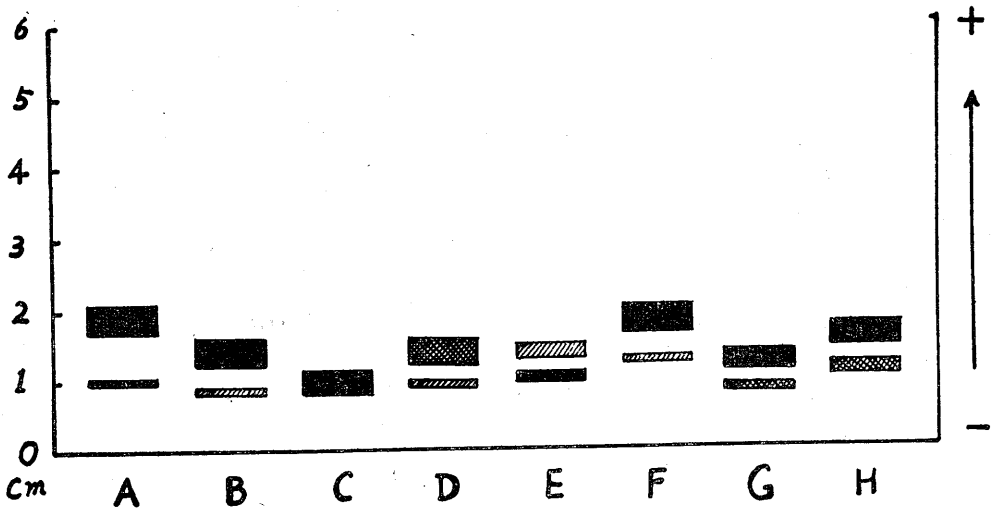

FIGURE 5

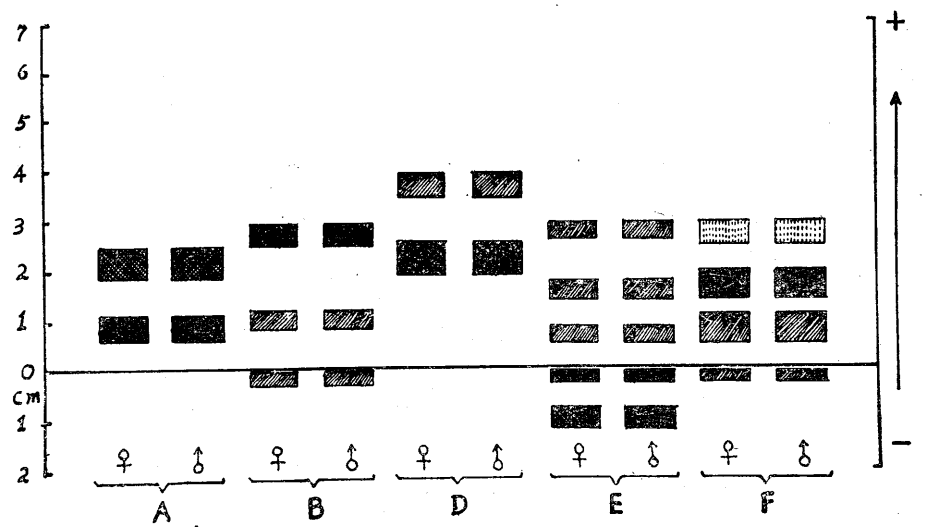

FIGURE 6