A quantitative analysis of modifier mutations which occur in mutation accumulation lines in *Drosophila melanogaster*

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Seven enzyme activities were measured in *Drosophila melanogaster* lines in which spontaneous mutations had accumulated over about 300 generations under the minimum pressure of natural selection. These enzymes included alcohol dehydrogenase (ADH), α-glycerol-3-phosphate dehydrogenase (αGPDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) and α-amylase (AMY). A significant genetic variance was observed for some enzyme activities. The mutations which alter the enzyme activities are called modifier mutations. The magnitudes of the genetic variance in modifier mutations differed greatly among enzymes but were often similar between two series of mutation accumulation lines (AW and JH). This may therefore indicate that the number of modifiers is specific for each enzyme system. The modifier mutation rate is suggested to be one of the clues for assessing the maintenance mechanism of protein polymorphism in natural populations.

Keywords: *Drosophila melanogaster*, enzyme activities, genetic variance, modifier mutations, mutation rates, protein polymorphism.

Introduction

In the past two decades a natural variation of enzyme activities has been reported in various organisms such as in *Zea mays* (Scandalios & Baum, 1982), *Mus musculus* (Paigen, 1979) and especially in *Drosophila* (Ward & Hebert, 1972; Choudhary & Laurie, 1991; Laurie *et al.*, 1991). These studies have shown that large variation in enzyme activities exists in natural populations. Mutations that affect enzyme activities are generally called modifiers and are considered to be quantitative characters (Laurie-Ahlberg *et al.*, 1982). The candidates for such mutations may be the ones that occur in regulatory elements on DNA sequences or the genes encoding regulatory proteins such as transcription factors. The idea of co-ordinate expression of unlinked genes by common regulatory elements in eukaryotes, as first envisaged by the model of Britten & Davidson (1969) is well established today (Ingham, 1988; Morimoto *et al.*, 1990).

The potential importance of polymorphisms in such regulatory genes in natural populations as the source of adaptive evolution has been discussed (Wallace, 1963; King & Wilson, 1975; Mukai &

Cockerham, 1977; Hedrick & McDonald, 1980). Therefore, in order to evaluate the evolutionary significance of regulatory genes it is important to determine the extent of the polymorphism in natural populations and to estimate how such genetic variation could accumulate in populations. The effect of regulatory mutations may be revealed in the genetic variation in the enzyme activities. In order to evaluate the accumulation rate of such mutations we have estimated the genetic variances in several enzyme activities using lines of *Drosophila melanogaster* in which spontaneous mutations have been accumulated for about 300 generations. It was shown that significant genetic variance arose in the enzyme activities of alcohol dehydrogenase (ADH: EC 1.1.1.1; map position, 2–50.1) (Mukai *et al.*, 1984) α-glycerol-3-phosphate dehydrogenase (αGPDH: EC 1.1.1.8, map position, 2–17.8) (Koga *et al.*, 1992) and α-amylase (AMY: EC 3.2.1.1; map position, 2–77.7) (Tachida *et al.*, 1989). In this study I extend the screening to seven enzymes and analyse the relation among the enzyme activities. The additional enzymes are malate dehydrogenase (MDH: EC 1.1.1.37; map position, 2–37.2), isocitrate dehydrogenase (IDH: EC 1.1.1.42; map position, 3–27.1),

glucose-6-phosphate dehydrogenase (G6PD: EC 1.1.1.49; map position, 1–63) and 6-phosphogluconate dehydrogenase (6PGD: EC 1.1.1.44; map position, 1–0.6). The results of the previous experiments mentioned above are also reanalysed here.

Materials and methods

Establishment of lines

One thousand second chromosome lines were established using four ancestral chromosomes: two balancer chromosomes, *In(2LR)SM1* marked by the dominant gene *Curly* (abbreviated as *Cy*) and two lethal-carrying chromosomes *l*(AW) and *l*(JH), which were derived in 1967 from a cage population, W-1. The lines were maintained by single-pair matings and accumulated spontaneous mutations until 1975. Subsequently the lines were maintained in a mass culture. The detailed method of mutation accumulation has been described elsewhere (Yamaguchi & Mukai, 1974; Mukai & Cockerham, 1977), and was developed at North Carolina State University by Mukai and his collaborators. In early 1980, about 100 lines, 50 from the AW and 50 from the JH lines, were randomly chosen and transferred to Kyushu University. The genetic backgrounds (X, third and fourth chromosomes) of these lines were then substituted by repeated back-crosses to two kinds of balanced lethal stocks of C-160, that is *In(2LR)SM1/In(2LR)bw^{V1}*, abbreviated as *Cy/Pm*. One was C-160(G37), which had the isogenic background of W-1, and the other was C-160(K), with the isogenic background of the Kaduna population (Clark *et al.*, 1979). The former substitution lines were called AW(G) and JH(G), and the latter AW(K) and JH(K). The activity assays were performed using AW(G) and JH(G) lines for ADH, αGPDH, MDH, G6PD, 6PGD and AMY. The activity assay of IDH was carried out using AW(K) and JH(K) lines. The original lines and the substituted lines (experimental lines) were maintained at 18°C in a mass culture. About 300 generations of accumulation of mutations had occurred in these lines before the present experiments were conducted.

Experimental crosses

The experimental crosses for enzyme activity assay were performed as follows: In each of the experiments, four vials of 5 *Cy/l_i* × 5 *Cy/l_i* crosses were made for each line, where *i* indicates the line number. They were divided into two groups with two

vials for each line. The first group and the second group of each experiment were called sets 1 and 2, respectively. The newly emerged F_1 offspring from each vial were collected and aged for 4–6 days in a fresh vial. Then a sample of five male flies was collected in a plastic centrifuge tube and used for the activity measurement. Otherwise it was frozen at −20°C or at −80°C until used for the measurement. The activity measurements of the AW and JH lines were carried out independently. The measurements of different sets were undertaken on different days. The control lines were derived from a single male from one of the randomly chosen AW or JH experimental lines for each enzyme activity assay. Each was crossed with a female from a marked inversion of the C-160 stock which had the same genetic background as the experimental lines. A single pair of *Cy* phenotype male and female was established and was expanded to about the same number of experimental lines. The control crosses were performed at the same time as the experimental lines. Samples were then randomized within the sets for the activity assay.

Measurement of enzyme activities

A sample of five male flies was sonicated with 0.2 mL (for ADH, αGPDH, MDH and IDH) or 0.4 mL (for G6PD, 6PGD and AMY) of distilled water and centrifuged at 2054 *g* for 10 min at 4°C. The supernatant was used for the activity assay. For the NAD$^+$ and NADP$^+$ dependent reactions, activities were measured by observing the absorbance change resulting from the reduction of NAD$^+$ to NADH (ADH and αGPDH) or NADP$^+$ to NADPH (IDH, G6PD and 6PGD), or resulting from the oxidation of NADH to NAD$^+$ (MDH), at 340 nm by a spectrophotometer. The reaction was observed at an interval of 30 s for 2 min at 25°C. The α-amylase activity was measured by observing the chromatic change of DNSA (3,5-dinitrosalycilic acid reagent) resulting from the production of reducing sugars. This reaction was carried out at 25°C. The assay mixtures were made as follows. ADH: 20 μL of the sample solution was added to 2.6 mL of the reaction mixture of 2.3 mM NAD$^+$ and 0.14 M isopropanol in 0.05 M Tris–HCl buffer at pH 8.5. αGPDH: 25 μL of the sample solution was added to 2.5 mL of the reaction mixture of 4.2 mM NAD$^+$ and 16 mM glycerol-3-phosphate in glycine–NaOH buffer at pH 8.5. MDH: 25 μL of the sample solution was added to 0.65 mL of the reaction mixture of 0.2 mM NADH and 0.33 mM oxaloacetate in 0.1 M Tris–HCl buffer at pH 7.5. IDH: 40 μL of the sample solution

was added to 0.8 mL of the reaction mixture of 0.8 mM $NADP^+$, 20 mM $MgCl_2$ and 5.8 mM trisodium citrate in 0.1 M Tris–HCl buffer at pH 8.5. G6PD: 50 μL of the sample solution was added to 0.65 mL of the reaction mixture of 0.525 mM $NADP^+$, 19 mM $MgCl_2$ and 4.2 mM glucose-6-phosphate in 0.2 M Tris–HCl buffer at pH 8.5. 6PGD: 100 μL of the sample solution was added to 0.6 mL of the reaction mixture of 1.4 mM $NADP^+$, 24.5 mM $MgCl_2$ and 3.5 mM 6-phosphogluconate in 0.2 M Tris–HCl buffer at pH 8.5. For α-amylase, the substrate solution was prepared with 1 per cent soluble starch (Merck–Zulkovsky) and 0.05 per cent glucose in 0.1 M Tris–HCl buffer at pH 7.4. It was boiled for a short time, then cooled down. Fifty μL of sample solution was added to 1.05 mL of the substrate solution. Two reactions were initiated at a 10 min interval, and 10 min after the second reaction they were stopped by adding 3 mL of DNSA solution. The optical density (OD) at 550 nm was measured after 10 min of boiling. The difference in the OD of the two reactions was taken to be the activity. The total amount of soluble protein was measured by the Lowry method (Lowry *et al.*, 1951). The unit enzyme activity is defined as the amount of the enzyme which consumes 1 μmol of the substrate per minute. The 'specific activity' is defined as the unit activity per mg soluble protein.

Electrophoresis

The electrophoretic mobilities of the enzymes were determined in all of the experimental lines. Starch gel was used for the electrophoresis of NAD^+ and $NADP^+$ dependent enzymes. The electrophoresis of α-amylase was carried out using acrylamide gel.

Results

Electrophoresis

Electrophoresis was performed using the corre-

sponding lines of the activity assay. The results are shown in Table 1. No altered electromorphs were found in any of the lines except one null mutant found in αGPDH in AW(G) lines. This line was not used for the αGPDH activity assay.

Enzyme activity assays

Two-way ANOVA was performed on the enzyme activity data. Two factors, sets and lines, are involved. Therefore, the sources of variation comprise the set effect, the line effect, the interaction between the sets and the lines and the sampling error. The genetic effect of mutation accumulation is represented in the differences between the lines. We therefore called the variance between the lines the 'genetic variance'. The results of the analysis for each enzyme activity assay are summarized in Table 2 and are as follows.

ADH Four-day-old male flies were used for the activity assay. Control lines were derived from one of the JH(G) lines. A highly significant line effect occurred in both the AW(G) and JH(G) lines, but not in the control lines. The set effect was significant in both the experimental lines. The interaction was not significant in any of the experiments. This means that the experimental conditions well controlled regardless of any large differences between the sets.

αGPDH An activity assay was carried out using 5-day-old males for set 1 and using 6-day-old male flies for set 2. The control lines were derived from one of the JH(G) lines. The line effect was significant at the 5 per cent level in both the AW(G) and JH(G) lines but was not significant in the control lines. The set effect was highly significant in all the experiments. No significant interaction occurred in any of the experiments.

MDH Four-day-old male flies were used for the

Table 1 Electrophoretic mobilities of the AW and JH experimental lines of *Drosophila melanogaster*

Lines	ADH	αGPDH	MDH	IDH	G6PD	6PGD	AMY
AW	FF	FS	SS	SS	SS	FF	1,6
JH	FF	FS	SS	SS	SS	FF	1

Electrophoretic mobilities were examined in AW(G) and JH(G) lines for ADH, αGPHD, MDH, G6PD, 6PGD and AMY.
Electrophoretic mobilities of IDH were examined in AW(K) and JH(K) lines.
FF, FS and SS indicate the homozygotes with the *F* allele, the heterozygotes with *F* and *S* alleles and the homozygote with the *S* allele, respectively.

activity assay. The control lines were derived from both the AW(G) and JH(G) lines. The line effect was not significant in any of the experimental or control lines. The set effect was significant in both the AW(G) and AW-control lines, but was not significant in the JH(G) and JH-control lines. None of the interactions was significant.

IDH An activity assay was carried out using 4-day-old male flies for set 1 and 5-day-old male flies for set 2. The control lines were derived from both the AW(K) and JH(K) lines. The line effect was not significant in any of the experimental or control lines. The set effect was highly significant in the JH(K), JH-control and AW-control lines but not significant in the AW(K) line. No significant interaction was observed in any of the experiments.

G6PD and 6PGD Activity assays were performed using 4-day-old male flies. The control lines were derived from both the AW(G) and JH(G) lines. The same samples were used for the G6PD and 6PGD activity assays. For G6PD, the line effect was significant at the 5 per cent level in both the AW(G) and JH(G) lines but was not significant in the control lines. The set effect was highly significant in all of the lines. The interaction was not significant in any of the experiments. For 6PGD, the line effect was significant at the 5 per cent level in both the AW(G) and JH(G) lines but was not significant in the control lines. The set effect was highly significant in the AW(G) lines but not significant in the JH(G) and both the AW- and JH-control lines. No significant interaction was observed in any of the experiments.

α-amylase An activity assay was undertaken using 4-day-old male flies. Control lines were derived from both the AW(G) and JH(G) lines. Two sets of crosses were performed in two different media, starch (set 1) and normal corn meal (set 2). The line effect was significant at the 1 per cent level in the AW(G) lines and at the 5 per cent level in the JH(G) lines. None of the control lines showed any significant line effect. Set effect was highly significant in all the experiments and showed that the mean activity of the sets was significantly higher in the starch medium. No significant interaction was observed between the sets and the lines except in the JH-control lines. The ratio of α-amylase activity in an inducible medium (starch) compared to that in a noninducible medium (corn meal) is defined as 'inducibility' (Yamazaki & Matsuo, 1984). One-way ANOVA was applied for the analysis of inducibility. No significant line effect was observed concerning the inducibility in both the AW(G) ($F_{29,30} = 1.20$) and JH(G) ($F_{25,26} = 0.75$) lines. This shows that no appreciable genetic variation occurred in the inducibility in the mutation accumulation lines.

Variance components analysis

Genetic variance was estimated from the ANOVA tables as follows:

$$\hat{\sigma}_G^2 = \tfrac{1}{4}\{MS(Line) - MS(Set \times Line)\}, \tag{1}$$

where MS(Line) and MS(Set × Line) are the mean squares of the lines and the interaction between the sets and the lines, respectively, and $\hat{\sigma}_G^2$ is the estimate of the variance component for the lines. The standard errors of the genetic variances were calculated after Mukai (1978). These values were calculated for all the enzyme activities and are summarized with activity means in Tables 3 and 4. In order to measure the relative magnitude of the genetic variance, the genetic variance to the error variance ratio, R, was calculated as follows:

Table 2 Summary of the significance tests in the two-way ANOVAs of the enzyme activities of *Drosophila melanogaster*

Enzyme	AW-experimental			AW-control			JH-experimental			JH-control		
	Set	Line	Set × Line	Set	Line	Set × Line	Set	Line	Set × Line	Set	Line	Set × Line
ADH	**	***	NS	—	—	—	***	***	NS	***	NS	NS
αGPDH	***	*	NS	—	—	—	***	*	NS	***	NS	NS
MDH	***	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS
IDH	NS	NS	NS	***	NS	NS	***	NS	NS	***	NS	NS
G6PD	***	*	NS	***	NS	NS	***	*	NS	***	NS	NS
6PGD	***	*	NS	NS	NS	NS	NS	*	NS	NS	NS	NS
AMY	***	**	NS	***	NS	NS	***	*	NS	***	NS	*

F-test: *$P<0.05$, **$P<0.01$, ***$P<0.005$.
NS, not significant. — Not available.

Table 3 The enzyme activity means and genetic variance components in AW lines of *Drosophila melanogaster*

Enzyme	No. of lines	Mean (U/mg)	Genetic variance $(U/mg)^2 \times 10^{-5}$	R
Experimental				
ADH	37	0.242 ± 0.023	21.7 ± 7.1	0.780
αGPDH	31	0.305 ± 0.031	7.3 ± 4.3	0.242
MDH	29	0.986 ± 0.137	34.6 ± 73.3	0.033
IDH	31	0.0488 ± 0.0057	0.222 ± 0.334	0.083
G6PD	30	0.0215 ± 0.0030	0.189 ± 0.098	0.263
6PGD	30	0.00888 ± 0.00184	0.0725 ± 0.0393	0.302
AMY	30	0.260 ± 0.066	69.5 ± 33.6	0.304
Control				
ADH	—	—	—	—
αGPDH	—	—	—	—
MDH	28	0.971 ± 0.165	-137.4 ± 167.6	0.000
IDH	25	0.0504 ± 0.0064	-0.287 ± 0.361	0.000
G6PD	28	0.0255 ± 0.0036	0.066 ± 0.130	0.064
6PGD	28	0.00990 ± 0.00173	-0.0267 ± 0.0353	0.000
AMY	16	0.171 ± 0.054	-13.2 ± 13.3	0.000

R is considered to be zero when the genetic variance shows a negative value.

Table 4 The enzyme activity means and genetic variance components in JH lines of *Drosophila melanogaster*

Enzyme	No. of lines	Mean (U/mg)	Genetic variance $(U/mg)^2 \times 10^{-5}$	R
Experimental				
ADH	32	0.317 ± 0.035	40.9 ± 16.4	0.708
αGPDH	39	0.286 ± 0.027	14.3 ± 7.2	0.258
MDH	25	1.090 ± 0.261	424 ± 636	0.059
IDH	25	0.0526 ± 0.0079	-0.039 ± 0.550	0.000
G6PD	27	0.0191 ± 0.0032	0.139 ± 0.080	0.183
6PGD	27	0.00869 ± 0.00209	0.0981 ± 0.0550	0.263
AMY	26	0.213 ± 0.089	154.9 ± 88.1	0.411
Control				
ADH	20	0.302 ± 0.037	1.6 ± 10.2	0.012
αGPDH	21	0.203 ± 0.160	0.5 ± 2.0	0.021
MDH	27	1.007 ± 0.2245	34.9 ± 399.5	0.013
IDH	25	0.0504 ± 0.0058	-0.112 ± 0.247	0.000
G6PD	28	0.0159 ± 0.0037	0.054 ± 0.104	0.071
6PGD	28	0.00898 ± 0.00209	-0.0029 ± 0.0294	0.000
AMY	17	0.108 ± 0.030	-7.0 ± 11.6	0.000

R is considered to be zero when the genetic variance shows a negative value.

$$R = \frac{\hat{\sigma}_G^2}{\hat{\sigma}_E^2}, \qquad (2)$$

where $\hat{\sigma}_G^2$ and $\hat{\sigma}_E^2$ are the estimates of the variance components of the lines and the errors, respectively.

R was used for evaluating the magnitude of the genetic effect of modifier mutations between the different enzyme activities as well as between the AW and JH experimental lines. The value of R for each experiment is also listed in Tables 3 and 4. Here, genetic variances which have negative values are

considered to be zero. The values are ranged from 0.033 (MDH) to 0.780 (ADH) in the AW experimental lines, and from zero (IDH) to 0.708 (ADH) in the JH experimental lines. The maximum increasing rate of the relative magnitude of the genetic variance was thus obtained in ADH activity and was 0.00260 and 0.00236 per second chromosome per generation for the AW and JH lines, respectively.

Correlation in enzyme activities

Many examples of the co-ordinate control of related genes by common regulatory factors have been shown by recent molecular studies. In order to test such co-ordinate expression of enzyme genes, correlations of enzyme activities were calculated for all of the pairwise comparisons. Phenotypic as well as genetic correlations were calculated. The genetic correlation (r_g) is calculated as follows:

$$r_g = \frac{Cov(G,G')}{\sqrt{V(G)V(G')}}, \qquad (3)$$

where $Cov(G,G')$ is the genetic covariance of two enzyme activities and $V(G)$ and $V(G')$ are the genetic variances of the respective enzyme activities. Standard errors of r_g were obtained by the method of Tallis (1959). The results are summarized in Tables 5 and 6. In the AW lines, significant phenotypic correlations were obtained for the activities of MDH and 6PGD ($P < 0.01$) and G6PD and 6PGD ($P < 0.01$). The genetic correlation of the G6PD and 6PGD activities was apparently large, although not significant. In the JH lines, significant phenotypic correlations were obtained for the activities of ADH and MDH, αGPDH and IDH, αGPDH and G6PD, and MDH and α-amylase. All were significant at the 5 per cent level; however, none of the corresponding genetic correlations appeared to be significant. Among these phenotypically significant correlations three were negative and three positive.

The results obtained in the AW and JH lines were not consistent. A meaningful correlation was observed only for the activities of G6PD and 6PGD in the AW experimental lines. Using recently substituted lines, a highly significant positive correlation was observed in G6PD and 6PGD activities in both the AW and JH lines (K. Harada, unpublished results). A significant positive correlation in these enzyme activities has also been noted in natural populations (Bijlsma, 1980; Hori & Tanda, 1981; Laurie-Ahlberg *et al.*, 1981; Miyashita & Laurie-Ahlberg, 1984). These two enzymes are closely related in the metabolic pathway of pentose phosphate

Table 5 The correlation of enzyme activities in AW experimental lines of *Drosophila melanogaster*

	ADH	αGPDH	MDH	IDH	G6PD	6PGD
αGPDH	−0.00 (n = 31) 0.03±0.34					
MDH	0.06 (n = 24) 0.53±3.30	−0.22 (n = 21) −0.23±1.09				
IDH	−0.08 (n = 16) −0.31±0.51	−0.00 (n = 14) 0.14±0.82	−0.61 (n = 12) —			
G6PD	0.17 (n = 23) 0.29±0.31	0.03 (n = 20) −0.27±0.54	−0.33 (n = 30) −0.91±1.04	0.30 (n = 12) 1.29±7.42		
6PGD	0.12 (n = 23) 0.29±0.42	0.08 (n = 20) 0.77±1.01	−0.51 (n = 30)** −1.90±2.03	0.50 (n = 12) 1.98±11.24	0.52 (n = 30)** 0.87±0.28	
AMY	−0.24 (n = 26) −0.34±0.27	−0.18 (n = 21) 0.03±0.45	0.17 (n = 28) 0.25±1.19	0.55 (n = 12) 5.27±36.16	−0.27 (n = 27) −0.30±0.34	−0.09 (n = 27) −0.10±0.45

In each combination of the enzymes, the top figure is the phenotypic correlation and the bottom figure is the genotypic correlation with standard deviation.
n, number of lines compared.
**P<0.01. —, Not available.

Table 6 The correlation of enzyme activities in JH experimental lines of *Drosophila melanogaster*

	ADH	αGPDH	MDH	IDH	G6PD	6PGD
αGPDH	-0.14 (n = 29) -0.03±0.49					
MDH	-0.45 (n = 20)* -0.75±0.40	0.32 (n = 22) 0.98±0.96				
IDH	0.17 (n = 10) 0.14±0.64	0.59 (n = 12)* 1.07±0.78	-0.07 (n = 11) -0.46±1.20			
G6PD	-0.04 (n = 18) 0.17±0.39	0.46 (n = 20)* 2.27±6.64	-0.05 (n = 26) 0.26±0.54	0.15 (n = 10) -0.50±2.21		
6PGD	0.07 (n = 18) —	0.19 (n = 20) 0.38±1.64	0.24 (n = 26) 0.40±0.55	-0.20(n = 10) -0.16±1.00	-0.04 (n = 27) -0.07±0.39	
AMY	0.29 (n = 20) 0.42±0.36	0.05 (n = 22) 0.01±0.44	-0.41 (n = 23)* -1.10±0.62	0.03 (n = 10) -0.46±1.20	-0.11 (n = 22) 0.26±0.54	-0.40 (n = 22) 0.40±0.55

In each combination of the enzymes, the top figure is the phenotypic correlation and the bottom figure is the genotypic correlation with standard deviation. *n*, number of lines compared.
*$P<0.05$. —, Not available.

shunt. It is probable that some common factors are operating in the co-ordinate control of the expression of G6PD and 6PGD genes.

Discussion

A significant genetic variance occurred in some enzyme activities after the accumulation of mutations over about 300 generations. However, the magnitude is very different among enzymes. Significant variances apparently result from the mutations accumulated on the second chromosomes. Although mutations have been accumulated independently in the AW and JH lines, the results are in good agreement with each other. Accordance in the magnitudes of the genetic variance between the AW and JH lines was tested by Kendall's rank correlation coefficient, τ, for R in the AW and JH lines. $\tau = 0.81$ ($n = 7$); the probability of 0.81 ($n = 7$); the probability of obtaining a higher value than this is less than 0.5 per cent indicating a highly significant association in the order between the AW and JH lines. The differential magnitude of the genetic variance may indicate that either the number of modifiers involved in each enzyme system is different or the modifier mutation rate is different among enzyme systems.

The probability that the structural genes of the enzymes acquired mutations during the accumulation of modifier mutations is calculated as follows. Band-morph and null mutation rates for enzyme loci were estimated in the AW and JH lines (Mukai & Cockerham, 1977). They are 1.81×10^{-6} and 10.3×10^{-6}/locus/generation, respectively. Considering the detection rate for band-morph to be 1/3 (Nei & Chakraborty, 1973), the expected number of lines carrying at least one new mutation in a total of 60 lines is

$$60 \times (1 - e^{-0.00000181 \times 3 \times 300 \times 2}) = 0.195.$$

Here, the null mutation rate is not considered because no nulls were used in this experiment. Because this is much less than one, the possibility that mutations of the structural gene affect the enzyme activity is ruled out. Aquadro *et al.* (1990) examined the restriction pattern of 15 kb regions including the ADH structural genes using 45 mutation accumulation lines of the AW and JH lines and found no major change in these regions. This observation suggests that the structural change of the ADH locus and flanking regions was not the source of activity variation. The same conclusion was derived from the survey of a 26 kb region surrounding the entire *Gpdh* gene (Koga *et al.*, 1992).

Restriction site variation in the 14 kb region that includes the structural genes for α-amylase was also investigated (Tachida et al., 1989). Contrary to the two enzymes mentioned above, it was found that most of the activity variation results from the substitution of the SM1(Cy) chromosomal region by the corresponding regions of the lethal chromosomes. Gene conversion or double recombination was suggested to be a mechanism for this replacement. It is not known whether the occurrence of such events is specifically high in the α-amylase coding region. The duplicated structure of the Amy gene (Gemmill et al., 1986) may contribute to this occurrence. The structural genes for G6PD, 6PGD and IDH are clearly not involved in the activity variation because the structural genes of these enzymes are located on the isogenic X (G6PD and 6PGD) or the third (IDH) chromosomes. Thus, the main sources of the activity variation are suggested to exist in regions other than the enzyme coding regions.

In order to find a relationship between the modifiers and the parameters relating to population dynamics in natural populations, the correlation coefficient of R with the average heterozygosity of the corresponding loci detected by electrophoresis was calculated. Significant positive correlations ($r = 0.960$, d.f. = 4 for AW, and $r = 0.932$, d.f. = 4 for JH) were obtained using a data set collected over 18 species of Drosophila with a sample size of more than 60 (Gojobori, 1979). The α-amylase locus was excluded from this analysis because the assay method is basically different from the other methods. When I used the heterozygosity data of only D. melanogaster alone (Singh & Rhornberg, 1987), r was 0.741 (d.f. = 4) for the AW lines and 0.723 (d.f = 4) for the JH lines. These values are not significant, but close to the 5 per cent level. In the past two decades many authors have attempted to establish a relationship between an enzyme function and its genetic variation in natural populations (Gillespie & Kojima, 1968; Kojima et al., 1970; Gillespie & Langley, 1974; Yamazaki & Maruyama, 1974). It is generally considered that those enzymes showing a smaller amount of heterozygosity have stronger functional constraint than the enzymes showing a larger amount of heterozygosity. The former group of enzymes is often involved in the main single metabolic pathways and thus require a single physiological substrate. On the other hand, the latter group consists of the enzymes that are involved in multiple pathways or substrate-nonspecific enzymes (Gojobori, 1979). Our observations suggest that the latter group has a higher modifier mutation rate than the former group. The substantial entity of

modifier mutations is totally unknown, but the mutations in the regions of regulatory function are the most probable candidates. Therefore, it is suggested that larger DNA regions are involved in the regulation of the gene expression in the latter group of enzymes. A consideration of the quantitative variations of the modifiers can thus be introduced as a new clue in the discussion of the maintenance mechanism of protein polymorphism in the context of the long-lasting debate of the neutralist–selectionist controversy. Because our data are restricted to modifiers on the second chromosomes and only a limited number of loci were examined, more extensive studies are required to obtain a general conclusion.

Finally, I must mention the role of transposable elements as the major agent of mutations. It has been suggested that many of the spontaneous visible mutations are caused by mobile genetic elements (Green, 1980; Spradling & Rubin, 1981). Many examples of regulatory mutations caused by mobile elements have been reported in various organisms including yeast (Errede et al., 1980; Williamson et al., 1981), plants (Burr & Burr, 1982; Döring et al., 1984), vertebrates (Hayward et al., 1981; Jenkins et al., 1981; Neel et al., 1981) and especially in Drosophila (Snyder et al., 1982; Bender et al., 1983; McGinnis et al., 1983; Scott & Weiner, 1984; Tsubota et al., 1985; Zacher et al., 1985; Cote et al., 1986). Furthermore, some of the suppresser mutations which can modify gene expressions have been reported (Modolell et al., 1983; Mount et al., 1988; Geyer et al., 1991). Hence, it is possible that some of the activity mutations may be caused by mobile genetic elements. It was noticed that both the AW and JH lines carried active hobo and I elements that are known to induce hybrid dysgenesis in certain matings (Harada et al., 1990). The number of these elements was very different among lines. It has been suggested that transposon mutagenesis is more efficient for the generation of regulatory mutations than EMS (Tsubota et al., 1985) because the cis-acting regulatory elements are relatively insensitive to single base pair changes (Eissenberg & Elgin, 1987). Although there is no evidence for trans-acting regulatory factors, the above findings support the view that modifying mutations detected in this experiment largely result from the insertion of transposable elements.

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