GENETIC AND BIOCHEMICAL BASIS OF ENZYME ACTIVITY VARIATION IN NATURAL POPULATIONS. I. ALCOHOL DEHYDROGENASE IN *DROSOPHILA MELANOGASTER*.

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ABSTRACT

Recent studies by various authors suggest that variation in gene regulation may be common in nature, and might be of great evolutionary consequence; but the ascertainment of variation in gene regulation has proven to be a difficult problem. In this study, we explore this problem by measuring alcohol dehydrogenase (ADH) activity in *Drosophila melanogaster* strains homozygous for various combinations of given second and third chromosomes sampled from a natural population. The structural locus (*Adh*) coding for ADH is on the second chromosome. The results show that: (1) there are genes, other than *Adh*, that affect the levels of ADH activity; (2) at least some of these "regulatory" genes are located on the third chromosome, and thus are not adjacent to the *Adh* locus; (3) variation exists in natural populations for such regulatory genes; (4) the effect of these regulatory genes varies as they interact with different second chromosomes; (5) third chromosomes with high-activity genes are either partially or completely dominant over chromosomes with low-activity genes; (6) the effects of the regulatory genes are pervasive throughout development; and (7) the third chromosome genes regulate the levels of ADH activity by affecting the number of ADH molecules in the flies. The results are consistent with the view that the evolution of regulatory genes may play an important role in adaptation.

ELECTROPHORETIC techniques and other methods have shown that an enormous wealth of genetic variation exists in natural populations of most organisms. The adaptive significance of this variation remains, however, largely undetermined.

The variation uncovered by electrophoresis (and by other techniques such as immunological and serological methods, protein sequencing, etc.) consists of differences in the amino acid sequence of proteins. Yet, recent studies suggest that variation in gene regulation (modifying, for example, the amount or timing of protein synthesis) may be common in nature, and might be of great evolutionary consequence. But the ascertainment of variation in gene regulation has proven to be a very difficult problem. One of the most important challenges facing contemporary evolutionary genetics is to ascertain the levels and adaptive significance of variation in genetic regulation.

Our approach to this problem has been to focus our attention on the variation in levels of enzyme activity, that exists in natural populations of Drosophila. Enzyme activity is a functionally important and quantifiable property of enzyme systems and can be under structural or regulatory gene control. This paper presents a preliminary genetic and molecular analysis of variation in alcohol dehydrogenase (ADH:E.C.1.1.1.1) activity found in a natural population of *Drosophila melanogaster*.

ADH in *D. melanogaster* is coded by a single gene locus (*Adh*) mapped at position 50.1 on the second chromosome (GRELL, JACOBSON and MURPHY 1965). Several studies have shown that the level of ADH activity can, however, be affected by other gene loci (WARD and HEBERT 1972; MCDONALD *et al.* 1977), some of which are not adjacent to the *Adh* structural gene locus (PIPKIN and HEWITT 1972; WARD 1975).

Although the adaptive and physiological functions of the enzyme are far from completely known, levels of ADH activity have been shown to correlate with the ability of *D. melanogaster* flies to cope with environmental alcohol (MCDONALD and AVISE 1976)—a substrate often encountered by this species in natural habitats (BROWN 1934; MCKENZIE and PARSONS 1972, 1974; BRISCOE, ROBERTSON and MALPICA 1975).

This study is the beginning of an investigation to determine whether variation in gene regulation exists in natural populations and what the adaptive significance of such variation is. With respect to the existence of regulatory variation, we raise the following questions: (1) how much, if any, of the variation in ADH activity existing in natural populations can be attributed to regulatory loci, (2) whether regulatory elements are variable within a population, and (3) where in the genome the regulatory elements are located.

A nomenclatural note: According to the recognized meaning among geneticists, structural genes are those whose RNA products are translated into proteins (polypeptides). There is, however, no universally accepted definition of regulatory genes. We define regulatory genes as those that control or modify the activity of other genes (MACINTYRE and O'BRIEN, 1976; DOBZHANSKY *et al.* 1977, pp. 24–25). Thus defined, regulatory and structural genes are not two mutually exclusive classes (nor do they include all kinds of genes, *e.g.*, those coding for ribosomal RNA and transfer RNA); some genes are both structural and regulatory, such as those coding for "effector" polypeptide molecules that regulate the transcription of other genes.

MATERIALS AND METHODS

The chromosomes studies in the present work are derived from *D. melanogaster* flies collected in Napa County, California, in September 1974. Flies were made homozygous for a wild second and a wild third chromosome, using the balanced-lethal-inversion systems *SM5* (*Cy/Bl* L^s) and *TM3* (*Sb Sr/*e^{11}) (LINDSLEY and GRELL 1968), as shown in Figure 1. Wild males are individually crossed with females from the balanced stock (generation P); since male offspring from these crosses are again mated with the balanced stock in the next generation (F_1), the *X* chromosomes of the double homozygous strains come in all cases exclusively from the balanced stock. Chromosomes II and III jointly represent about 80 percent of the genome of *D. melanogaster*.

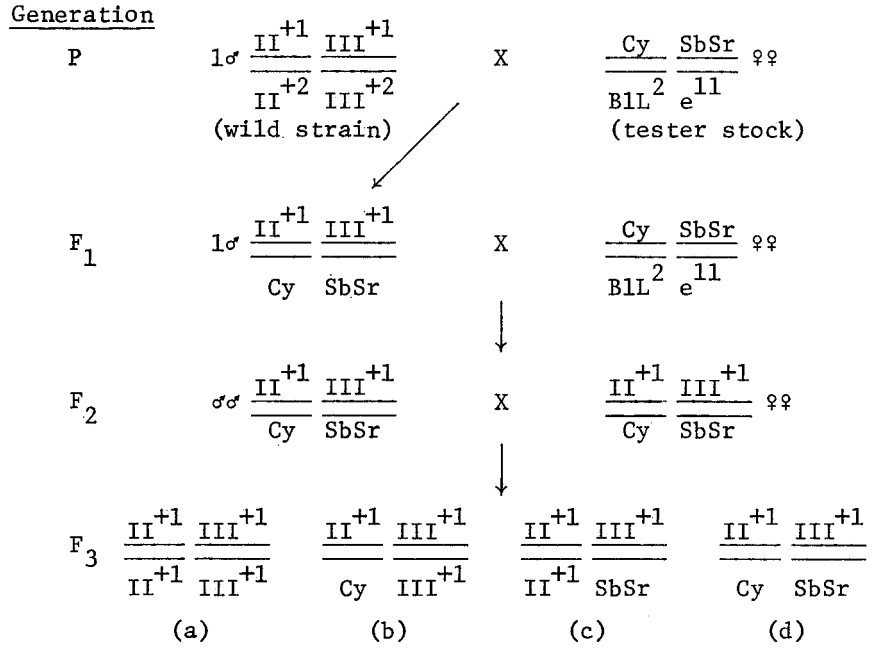

FIGURE 1.—Mating scheme used to obtain strains homozygous for both a second and a third wild chromosome. The superscripts "+1" and "+2" represent any two wild chromosomes. Flies homozygous for the *Cy* or the *Sb Sr* chromosomes die before completing metamorphosis, and thus are not shown in the F_3 generation.

Second and third chromosomes originating from different wild flies were combined in double homozygous condition, using the mating scheme outlined in Figure 2A. Flies homozygous for a wild second chromosome, but heterozygous for two different wild third chromosomes were obtained following the procedure outlined in Figure 2B. The superscripts "+1" and "+3" in Figure 2 represent any two chromosomes derived from different wild flies.

In all, nine second and nine wild third chromosomes were used in the experiment. Of the nine second chromosomes, three coded for the fast (F) form and the other six for the slow (S) form of ADH.

ADH activity was measured by observing the reduction of NAD+ to NADH at 340 mμ, following the techniques of MCDONALD and AVISE (1976). Activities were measured on samples within 30–40 minutes after homogenization. We have, however, monitored ADH activity levels of individual samples for periods of up to four hrs. No significant decrease in ADH activity was observed in any samples over that period of time. We conclude that ADH is reasonably stable under our conditions and apparently not subject to significant temperature or protease degradation. One unit of activity is defined as 8×10^{-4} moles of NAD+ reduced per ml reaction mixture per minute per mg live weight. All assays were carried out on a Gilford model 250 spectrophotometer at 22°. Electrophoretic techniques were as described in AYALA *et al.* (1972).

The comparative studies were made using flies six to ten days posteclosion; we found that activities do not vary significantly over this developmental time span.

In order to separate the variation due to genotype, environmental effects and experimental error, we used the experimental design outlined in Figure 3, which lends itself readily to analysis of variance. Three samples of males and three samples of females were taken from three separate cultures (maintained under identical laboratory conditions) for each strain tested. Three independent assays of enzyme activity were carried out on each sample. Variation among

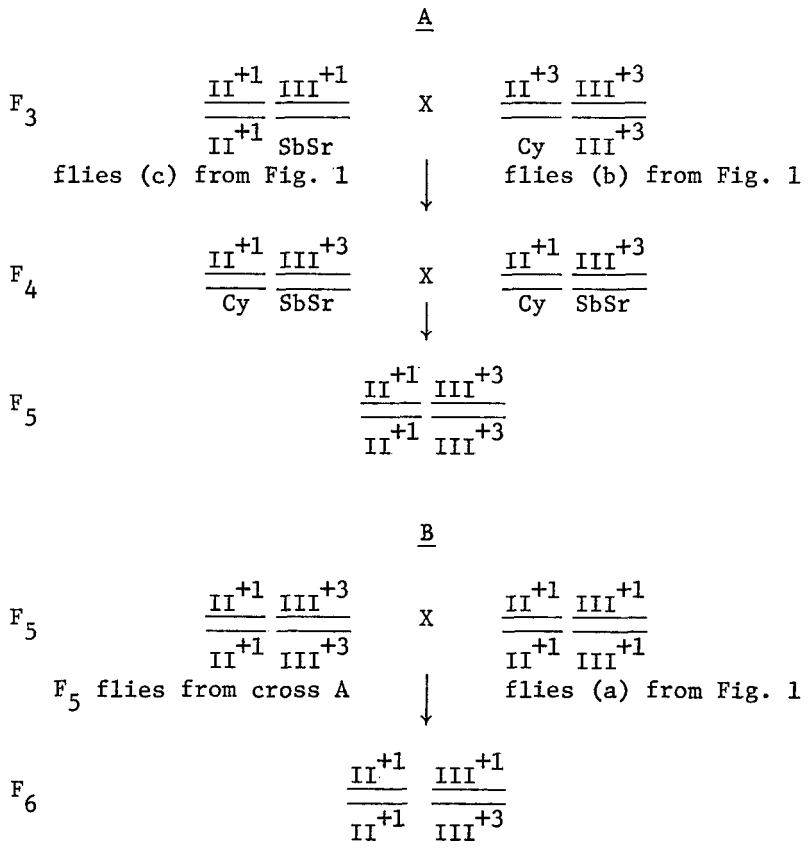

FIGURE 2.—(A) Mating scheme used to obtain flies homozygous for a second and a third wild chromosome derived from different original strains. The superscripts "+1" and "+3" represent any two chromosomes extracted from different wild-collected flies. (B) Mating scheme used to obtain flies homozygous for a second chromosome and heterozygous for two third chromosomes derived from different wild-collected flies.

the assays of the same sample may be attributed to error; variation among samples is due to environmentally induced differences between cultures (and to error in weighing the flies). All additional variation will be due to strain and electrophoretic genotype. The significance of sex was tested in each strain by means of a two-way analysis of variance (sex × samples).

Quantitative immunoelectrophoresis (LAURELL 1966) was performed following the techniques of DAY, HILLIER and CLARKE (1974) as modified by MCDONALD *et al.* (1977).

The techniques of MCDONALD and AVISE (1976) were used to carry a developmental study of ADH activity at three life stages—late third instar larvae, late pupae (eyes and wing pads visible) and six- to ten-day old adults.

RESULTS

The main results of our experiment are summarized in Table 1. The following are given for each of the 39 strains studied: the electrophoretic genotype, the source of chromosomes *II* and *III*, and the mean ADH activity and standard

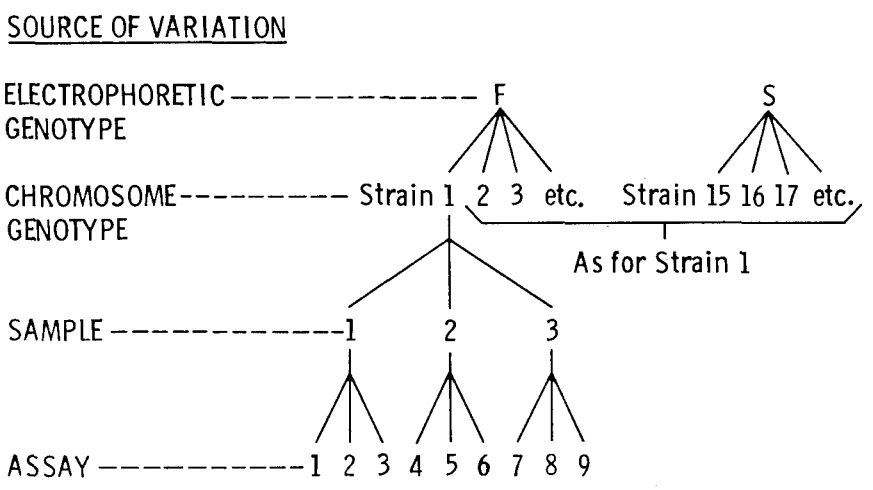

FIGURE 3.—Experimental design to determine the contribution of various factors to variation in ADH activity. Details are given in text.

error for the nine assays performed with male samples, for the nine assays performed with female samples, and for all 18 assays carried out for each strain. As for the source of chromosomes *II* and *III*, the symbols 1F, 2F, 1S, etc., represent, respectively, a chromosome from the first strain that had the F electrophoretic phenotype, a chromosome from the second strain that had the F phenotype, a chromsome from the first strain that had the S phenotype, and so on. When a strain is heterozygous for two given chromosomes, this is indicated in Table 1 (*e.g.*, strain five carries two different third chromosomes, 1F and 6S); otherwise the strains are homozygous for the chromosomes given. As pointed out in the MATERIALS AND METHODS, all *X* chromosomes are derived from the balanced laboratory stock.

Original homozygous strains: Using the mating scheme outlined in Figure 1, nine strains were obtained [flies labeled (a) in the figure] homozygous for a second and a third chromosome both derived from the same original wild fly. Three of the nine strains had the *F* allele at the *Adh* structural locus (1F, 2F, 3F), the other six had the *S* allele (1S, 2S, ... 6S).

The mean levels of ADH activity for these nine strains are given in Table 1 (strains 1, 6, 12, 15, 22, 26, 31, 32, and 33), and are shown graphically in Figure 4. Clearly the strains carrying the *F* allele at the *Adh* locus have substantially higher ADH activity than the strains carrying the *S* allele. For this particular set of strains, the mean activity value is 22.46 ± 0.26 units for the *F* strains and 9.11 ± 0.21 for the *S* strains; the difference is highly significant. This is consistent with results obtained by other investigators (see below).

An analysis of variance was carried out to separate the effects of electrophoretic genotype, strain, samples and individual assays on the total variation. A separate analysis was carried out for each sex, and the results are presented in Table 2.

TABLE 1

Levels of activity of ADH in 6 to 10-day-old adult Drosophila melanogaster *from 39 different strains*

Strain	Electrophoretic genotype	Chromosome genotype		Mean activity ± standard error		
		II	*III*	Males	Females	Overall
1.	F/F	1F	1F	19.44 ± 0.44	17.33 ± 0.55	18.39 ± 0.32
2.	F/F	1F	2F	26.11 ± 0.75	25.33 ± 0.41	25.72 ± 0.72
3.	F/F	1F	3S	22.56 ± 0.93	17.28 ± 0.80	19.90 ± 0.45
4.	F/F	1F	6S	22.22 ± 0.32	16.00 ± 0.17	19.11 ± 0.42
5.	F/F	1F	1F/6S	22.89 ± 0.51	16.78 ± 0.28	19.80 ± 0.43
6.	F/F	2F	2F	27.55 ± 0.29	23.44 ± 0.24	25.50 ± 0.35
7.	F/F	2F	3F	46.22 ± 0.52	43.33 ± 0.78	44.39 ± 0.46
8.	F/F	2F	2F/3F	43.56 ± 0.60	32.67 ± 0.67	38.11 ± 0.57
9.	F/F	2F	1S	22.00 ± 0.47	23.33 ± 0.55	22.50 ± 0.30
10.	F/F	2F	3S	26.56 ± 0.65	22.61 ± 0.74	24.47 ± 0.40
11.	F/F	2F	5S	21.33 ± 0.58	21.67 ± 0.99	21.50 ± 0.36
12.	F/F	3F	3F	25.66 ± 0.80	21.33 ± 0.40	23.50 ± 0.40
13.	F/F	3F	1S	16.00 ± 0.33	14.78 ± 0.28	15.39 ± 0.25
14.	F/F	3F	5S	16.67 ± 0.60	16.22 ± 0.36	16.44 ± 0.28
15.	S/S	1S	1S	9.67 ± 0.24	8.17 ± 0.20	8.92 ± 0.24
16.	S/S	1S	2S	16.89 ± 0.48	12.78 ± 0.17	14.83 ± 0.40
17.	S/S	1S	1S/2S	15.33 ± 0.33	11.89 ± 0.31	13.61 ± 0.33
18.	S/S	1S	6S	11.11 ± 0.26	9.66 ± 0.17	10.39 ± 0.23
19.	S/S	1S	1S/6S	10.55 ± 0.55	12.11 ± 0.35	11.33 ± 0.25
20.	S/S	1S	3F	18.56 ± 0.41	13.25 ± 0.39	16.00 ± 0.40
21.	S/S	1S	3F/1S	17.22 ± 0.22	11.44 ± 0.29	14.39 ± 0.41
22.	S/S	2S	2S	9.50 ± 0.35	8.33 ± 0.26	8.92 ± 0.24
23.	S/S	2S	2F	11.78 ± 0.33	11.61 ± 0.17	10.92 ± 0.38
24.	S/S	2S	3F	32.67 ± 0.53	28.11 ± 0.54	28.89 ± 0.45
25.	S/S	2S	3F/2S	31.77 ± 0.64	26.11 ± 0.26	28.94 ± 0.45
26.	S/S	3S	3S	6.11 ± 0.39	5.44 ± 0.18	5.78 ± 0.23
27.	S/S	3S	1F	11.11 ± 0.51	10.94 ± 0.70	11.00 ± 0.31
28.	S/S	3S	1F/3S	10.66 ± 0.23	10.22 ± 0.22	10.44 ± 0.20
29.	S/S	3S	2F	7.78 ± 0.15	6.22 ± 0.22	7.00 ± 0.23
30.	S/S	3S	6S	5.67 ± 0.24	5.17 ± 0.14	5.42 ± 0.19
31.	S/S	4S	4S	11.67 ± 0.29	11.89 ± 0.35	11.78 ± 0.22
32.	S/S	5S	5S	7.89 ± 0.26	5.22 ± 0.28	6.55 ± 0.30
33.	S/S	6S	6S	11.56 ± 0.44	9.83 ± 0.12	10.64 ± 0.24
34.	S/S	6S	2F	20.33 ± 0.33	15.22 ± 0.40	17.78 ± 0.40
35.	S/S	6S	3F	29.00 ± 0.53	20.44 ± 0.75	24.72 ± 0.51
36.	S/S	6S	3F/6S	30.55 ± 0.24	21.33 ± 0.84	25.94 ± 0.53
37.	S/S	6S	1S	8.39 ± 0.31	7.33 ± 0.37	7.86 ± 0.25
38.	S/S	6S	5S	23.89 ± 0.54	20.89 ± 0.51	22.44 ± 0.35
39.	S/S	6S	5S/6S	21.11 ± 0.39	14.56 ± 0.50	17.83 ± 0.45

The means and standard errors are based on nine measurements for each sex. One unit of activity is defined as 8×10^{-4} moles of NAD^+ reduced per ml reaction mixture per minute per mg live weight.

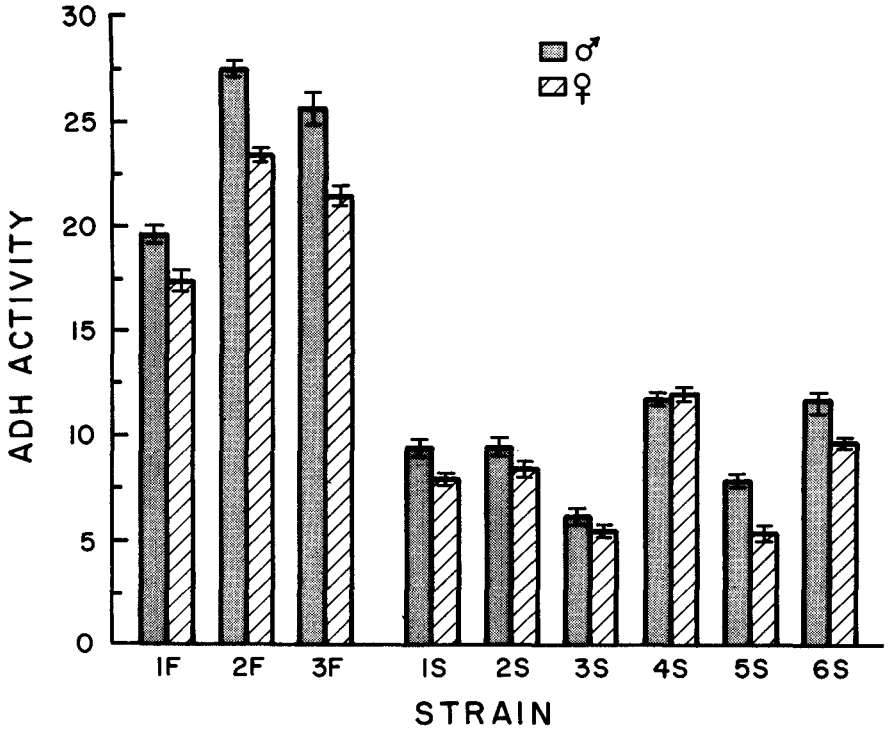

FIGURE 4.—Mean ADH activity of each sex in the original double homozygous strains (*i.e.*, the strain represented as 1F is homozygous for the second 1F chromosomes as well as for the third 1F chromosome, and so on). The range of one standard error on each side of the mean is indicated. Data are from Table 1.

TABLE 2

Analysis of variance of ADH activity in homozygous strains of D. melanogaster *with the same electrophoretic genotype*

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F	P
Males:					
Genotype	1	3965.44	3965.44	52.70	$p < 0.001$
Strain	7	526.70	75.24	81.53	$p < 0.001$
Sample	18	16.61	0.92	0.51	N.S.
Assay	54	96.83	1.72	——	——
Total	80	4605.58			
Females:					
Genotype	1	2837.19	2837.19	42.40	$p < 0.001$
Strain	7	468.42	66.92	94.60	$p < 0.001$
Sample	18	12.72	0.71	0.75	N.S.
Assay	54	51.17	0.95	——	——
Total	80	3369.50			

The effects of electrophoretic genotype and strain are highly significant, while variation between samples, *i.e.*, that due to experimental error and environmental effects, is not significant.

It can be seen in Figure 4 (and in Table 1) that males, as a rule, exhibit higher ADH activity than females. A two-way analysis of variance shows sex to be a significant factor ($p < 0.001$) in all but one of the strains (strain 4S).

A number of authors have reported differences in ADH activity between *D. melanogaster* strains with different electrophoretic phenotypes, and also between strains with a given electrophoretic phenotype (WARD and HEBERT 1972; WARD 1975; BIRLEY and BARNES 1973; HEWITT *et al.* 1974). However, no attempt was made in these studies to obtain flies homozygous for a major fraction of the genome. WARD and HEBERT (1972) made flies homozygous for the structural *Adh* locus, but not for the rest of the genome. In spite of the various techniques used, the studies quoted, as well as our present results, all agree in the following two points: (1) mean ADH activity is higher in F strains than in S strains, and (2) significant variation in ADH activity exists between strains exhibiting the same electrophoretic phenotype, whether this be F or S.

Combined homozygous strains: The differences in ADH activity between the F and the S strains may be due to structural differences in the enzyme itself affecting the "turnover rate" (DAY, HILLIER and CLARKE 1974). Variation among F strains, or among S strains, might possibly be due to differences in the primary structure of the enzyme as well. However, WARD and HEBERT (1972), WARD (1975), PIPKIN and HEWITT (1972), and MCDONALD *et al.* (1977) have all presented evidence indicating that genes other than the one coding for the ADH polypeptide may also play a significant role in determining ADH activity levels.

WARD and HEBERT (1972) obtained a strain homozygous for a given *S* allele and subjected it for selection for increased and decreased activity levels; substantial differences were detected after only two generations of selection. WARD (1975) combined the *X*, second, and third chromosomes of high and low activity strains, and concluded that significant effects on activity levels were attributable to genes in the third and in the *X* chromosome, although most of the activity difference was due to genes in the second chromosome. PIPKIN and HEWITT (1972) also found that genes located on the *X* chromosome influenced ADH activity levels.

In order to determine whether any of the variation in ADH activity could be due to genes on the third chromosome (the large autosome not carrying the *Adh* structural locus), we constructed strains with various combinations of second and third chromosomes following the mating scheme outlined in Figure 2A. A total of 21 homozygous combination strains were obtained; for each strain, ADH activity was measured in 18 assays, nine for males and nine for females, according to the scheme in Figure 3. The mean values are shown in Table 1. The differential effects on ADH activity of the second-third chromosome combinations examined are summarized in Table 3. Three main results are observed:

TABLE 3

ADH activity of double homozygous strains with second and third chromosomes from different original strains

Second chromosome	Third chromosome								Activity effects of third chromosomes*
	1F	2F	3F	1S	2S	3S	5S	6S	
1F	18.4 (0)	25.7 (+7.3)	—	—	—	19.9 (+1.5)	—	19.1 (+0.7)	2F >> 3S = 6S = 1F
2F	—	25.5 (0)	44.4 (+18.9)	22.5 (−3.0)	—	24.5 (−1.0)	21.5 (−4.0)	—	3F >> 2F = 3S > 1S = 5S
3F	—	—	23.5 (0)	15.4 (−8.1)	—	—	16.4 (−7.1)	—	3F >> 5S = 1S
1S	—	—	16.0 (+7.1)	8.9 (0)	14.8 (+5.9)	—	—	10.4 (+1.5)	3F > 2S >> 6S > 1S
2S	—	10.9 (+2.0)	28.9 (+20.0)	—	8.9 (0)	—	—	—	3F >> 2F = 3S
3S	11.0 (+5.2)	7.0 (+1.2)	—	—	—	5.8 (0)	—	5.4 (−0.4)	1F >> 2F = 3S = 6S
6S	—	17.8 (+7.2)	24.7 (+14.1)	7.9 (−2.7)	—	—	22.4 (+11.8)	10.6 (0)	3F >> 5S >> 2F >> 6S >> 1S

Differences in activity between each given strain and the original double homozygous strain with the same second chromosome are given in parentheses.

* Results of Student-Newman-Keul's multiple comparison test, where the influence of each third chromosome on ADH activity is ranked and the statistical significance between each pair of two adjacent chromosomes is tested (STEEL and TORRIE 1960). The meanings of the symbols are: =, not significantly different; >, greater than with $P < 0.05$, >>, greater than with $P < 0.01$.

(1) There is at least one genetic locus (but there could be more) in the third chromosome of *D. melanogaster* that can significantly affect the levels of ADH activity in the flies—although the structural locus coding for ADH is located in the second chromosome. This evidence is apparent in Tables 1 and 3. For example, the mean activity of a strain homozygous for the 2F second chromosome is 25.50 ± 0.35 units when it is homozygous for the third chromosome 2F, but is 44.35 ± 0.46 units when it is homozygous for the third chromosome 3F. This increase of 74 percent in mean activity is statistically highly significant.

(2) There is variability segregating in the population for the gene or genes in the third chromosome that modify ADH levels of activity. Different third chromosomes have different effects on ADH activity when combined with any given second chromosome. For example, take the strains homozygous for the second chromosome 6S; their mean ADH activities when homozygous for various third chromosomes are as follows: 7.86 with 1S, 10.64 with 6S (135 percent of the activity with 1S), 17.78 with 2F (226 percent), 22.44 with 5S (285 percent), and 24.72 with 3F (315 percent). All the activity differences between any two of these values are significant.

(3) There is variability segregating in the population for a gene (or genes) located in the second chromosome that interact with the third chromosome gene(s) in modifying the levels of ADH activity. As an example, consider the effects of the third chromosomes 2F and 3S. Both result in similar ADH activities when combined with either the 2F or the 3S second chromosomes; however, when combined with the 1F second chromosome, the 2F third chromosome results in significantly higher activity than the 3S third chromosome (see Table 3). Whether the variable interaction effects of the second chromosome are due to variation at the *Adh* locus or at some other (regulatory) loci, remains to be seen. However, if the variation is due to the *Adh* locus, it must be that variation exists within a given electrophoretic class: as just noted the effects of the 2F and 3S third chromosomes are different when combined with the 1F and 2F second chromosomes, although both of these have the *F* allele at the *Adh* locus.

Combined heterozygous strains: A question that arises is what is the dominance relationship between the allelic variants at the third chromosome gene(s) that modify *Adh* activity. We explored this question by obtaining strains heterozygous for different third chromosome combinations while keeping them homozygous for the second chromosome. Nine such strains were obtained following the mating scheme outlined in Figure 2B. The results are given in Table 1 (strains 5, 8, 17, 19, 21, 25, 28, 36, and 39); the comparisons between such heterozygous strains and the corresponding homozygous strains are shown in Figure 5.

The results indicate that "higher-activity" third chromosomes are either partially or fully dominant over "lower-activity" third chromosomes. In every case, the activity of the strain heterozygous for the third chromosome is higher than the average of the activity of the strains homozygous for one and for the other of the two third chromosomes. The difference between the heterozygous strain

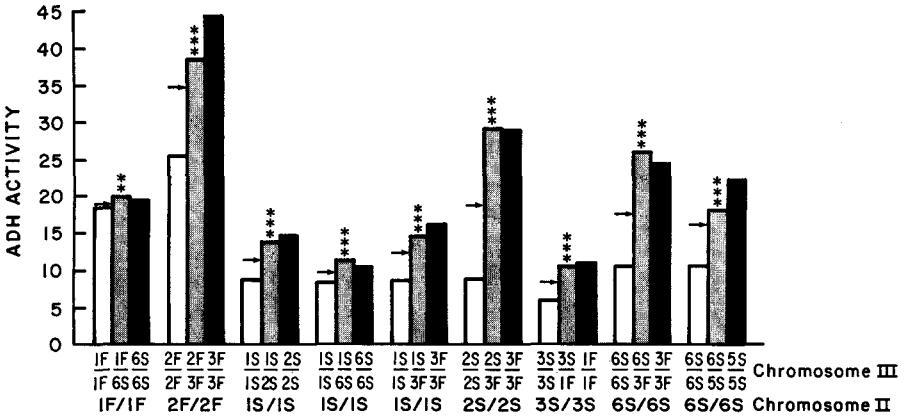

FIGURE 5.—Mean ADH activity (averaged between the two sexes) compared between strains heterozygous for two wild third chromosomes and strains homozygous for each one of the two third chromosomes. The arrows indicate the expected co-dominance values. The mean activity of the heterozygous strain is in every case significantly higher (using Standard *t*-tests) than the expected co-dominance value, ** = $P<0.01$, *** = $P<0.001$.

and the average of the two homozygous strains is highly significant in all nine cases tested (see Figure 4). The dominance effects of higher-activity over lower-activity third chromosomes are not always complete: in five of the nine cases (*i.e.*, those involving heterozygous strains 8, 17, 21, 28, and 39) the homozygous higher-activity strain exhibits significantly higher activity than the corresponding heterozygous strain.

Molecular basis of ADH activity differences: The precise nature of genetic regulation in higher organisms is far from adequately known, although theoretical models and some experimental evidence suggest that interchromosomal control may not be uncommon (MCCLINTOCK 1965; BRITTEN and DAVIDSON 1969; WARD 1975; STEIN and STEIN 1976; NELSON, LEHMAN and METZENBERG 1976). The postulated mechanisms of such nonadjacent gene regulation are generally believed to involve the synthesis of macromolecules. The action of these regulatory macromolecules may involve either direct modification of the target gene product or regulation of structural gene synthesis *via* specific binding at adjacent control sites (BRITTEN and DAVIDSON 1969; STEIN and STEIN 1976; NELSON, LEHMAN and METZENBERG 1976).

A preliminary exploration of the molecular basis of the activity differences caused by the third chromosome was carried out using two homozygous combination strains: 2F(*II*)–2F(*III*) and 2F(*II*)–3F(*III*), where the roman numerals in parentheses refer respectively to the second and the third chromosome. These two strains (6 and 7 in Table 1) differ by 75 percent in adult ADH activity (25.00 ± 0.35 versus 44.39 ± 0.46 units).

The rationale is as follows. If the 3F third chromosome increases ADH activity relative to the 2F third chromosome exclusively by some form of post-translational modification of the ADH molecule, then the amount of protein present in

the 2F(*II*)–2F(*III*) and 2F(*II*)–3F(*III*) strains should not be significantly different. The amount of ADH protein present in the flies was measured using the Laurell-rocket immunoelectrophoretic method referred to in the MATERIALS AND METHODS. With this method, the length of the "rockets" in the gels is directly proportional to the number of protein molecules in the sample.

The results are given in Figure 6. A crude extract homogenate plus two serial dilutions (one-half and one-quarter) of the higher activity strain (2F–3F) were tested against the crude homogenate of the lower-activity strain (2F–2F). The results indicate that indeed there are more ADH molecules in the high-activity strain than in the low-activity strain. The differences in the amount of ADH protein can be due to modification of the rate of synthesis of the polypeptide or

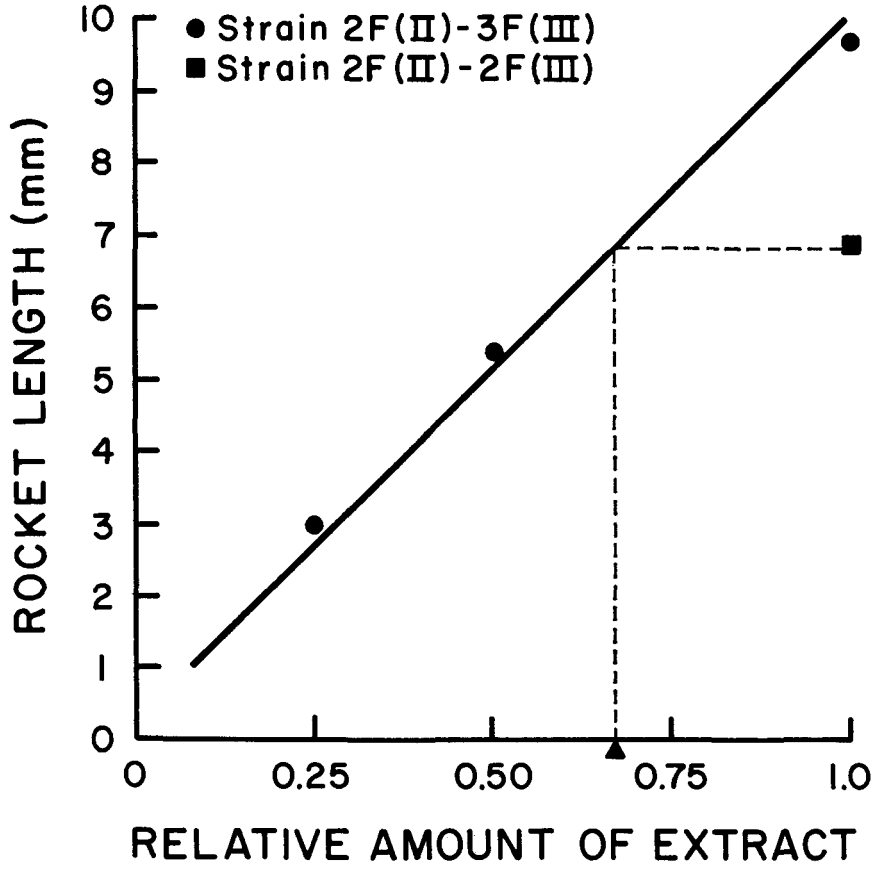

FIGURE 6.—Relative number of ADH molecules as estimated by the length of Laurell "rockets." The two serial dilutions of strain 2F(*II*)–3F(*III*) demonstrate the linear relationship between amount of ADH and rocket length. The number of ADH molecules in strain 2F(*II*)–2F(*III*) is estimated at about 68 percent that of strain 2F(*II*)–3F(*III*) as indicated by the triangle on the abscissa. ADH activity measured in the samples used for the immunoelectrophoresis "rocket" test was 38.90 ± 0.58 and 24.20 ± 0.42 (or 62 percent of 38.90) for strains 2F–2F, respectively.

of the rate of degradation, or both. Changes in the rate of synthesis would be consistent with the hypothesis that the regulation of ADH activity takes place *via* the specific binding of macromolecules at control sites adjacent to the *Adh* locus.

ADH activity differences through development: The measurements of ADH activity so far reported were carried out on adult flies, six to ten days after eclosion. An experiment was conducted using the strains 2F(*II*)–2F(*III*) and 2F(*II*)–3F(*III*) to test whether the changes in ADH activity caused by the third chromosome are specific to the adult stage or extend throughout development. ADH activity was measured for each of the two strains in late third-instar larvae, late pupae, and six to eight-day-old adults. The data, given in Figure 7, show that ADH activity is significantly higher in strain 2F(*II*)–3F(*III*) throughout the three developmental stages tested. These results indicate that at least some of the control elements of the ADH system located in the third chromo-

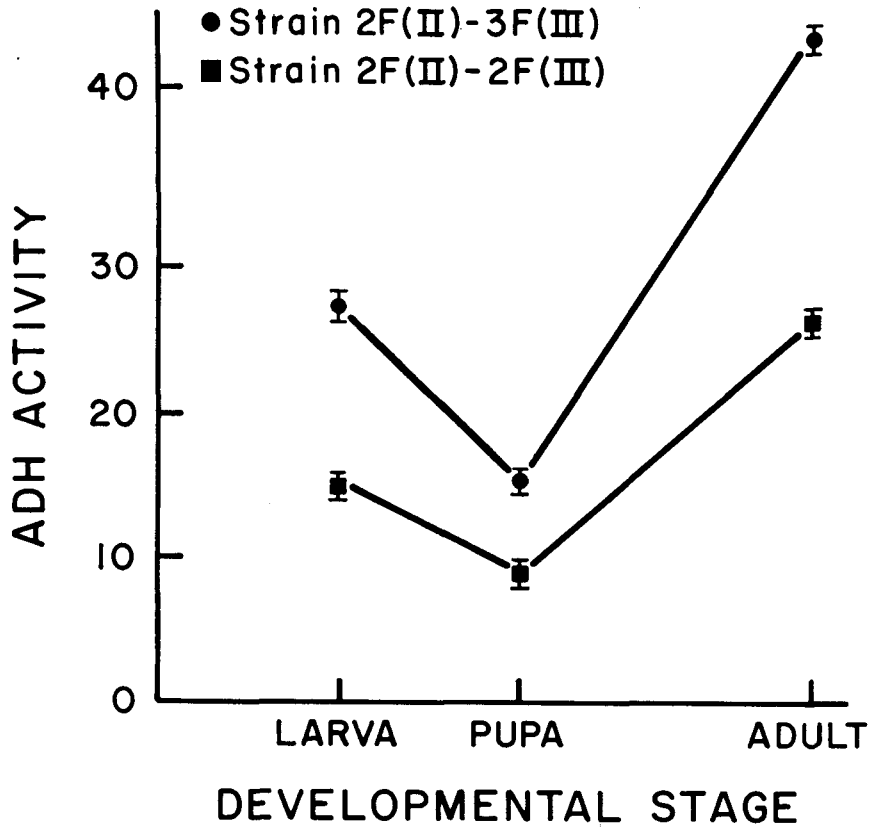

FIGURE 7.—Developmental pattern of ADH activity in two double homozygous strains. Third-instar larvae, late pupae, and six to ten day-old adults were assayed. The range of one standard error on each side of the mean is indicated. The points along the abcissa are approximately proportional to the length of development since the egg stage.

some are active throughout ontogeny. This in turn is consistent with the findings of WARD (1975) and MCDONALD *et al.* (1977), who observed in *D. melanogaster* that selection for increased ADH activity at a given life stage results in a regulatory-mediated increase in activity at other stages of development.

DISCUSSION

Two fundamental questions in population genetics are: (1) how much genetic variation exists in natural populations, and (2) what is the adaptive significance of such variation.

The amount of genetic variation in a population determines the evolutionary potential of the population. Considerable progress has taken place during the last decade towards obtaining estimates of genetic variation in natural populations through application of the concepts and techniques of molecular biology. Gel electrophoresis in particular has provided estimates of the levels of variation in gene loci coding for enzymes and other soluble proteins. The amount of genetic variation uncovered is very high; we now know that vertebrate individuals, including humans, are heterozygous, at least, at about six percent of their gene loci, while a typical invertebrate may be heterozygous at twice as large a proportion of loci as vertebrates (see summaries in SELANDER 1976, and DOBZHANSKY *et al.* 1977).

Two major unresolved problems plague the estimates of genetic variation obtained by electrophoretic techniques. One is that electrophoresis detects only a fraction of all amino acid substitutions in proteins; we do not yet know how much additional variation exists, although this is a question currently being actively investigated. The other unresolved problem emanates from the fact that only genes that qualitatively affect the structure of soluble proteins are examined by electrophonetic methods. We do not know how much variation exists in other classes of gene loci, notably those whose function is to regulate the amount or timing of protein synthesis. This ignorance may be critically important with respect to the question of adaptive significance.

The second fundamental question of population genetics, posed above, concerns the adaptive significance of the genetic variation that exists in natural populations. Evolution occurs through genetic changes in the constitution of populations; population genetics is concerned with the processes responsible for such genetic changes. The adaptive significance of genetic variation determines the processes by which genetic changes will occur, whether by sampling drift or by natural selection, and by what mode of selection whenever this is involved.

In recent years considerable experimental and theoretical work has been addressed to investigating of the adaptive significance of the wealth of variation uncovered by electrophoretic methods. This important work should, and doubtless will, continue. Recently attention has been directed at the intriguing possibility that, on the whole, the most significant evolutionary changes may not involve changes in the structural gene loci themselves, but rather changes in gene regulation (*e.g.*, BRTTEN and DAVIDSON 1969; KING and WILSON 1975; DOBZHAN-

SKY *et al.* 1977). Therefore, investigation of genetic regulation might perhaps be the most challenging question facing population geneticists. We need to ascertain how much variation exists at regulatory gene loci, and we need to know the adaptive significance of such variation. The investigation of genetic regulation in higher organisms has, however, proved to be an elusive task.

The study presented here is a preliminary attempt to investigate the questions raised, *i.e.*, whether variation in genetic regulation exists in natural populations and whether such variation is adaptively significant. The approach followed is one frequently used by developmental geneticists and involves detailed analysis of the factors controlling the activity of specific enzyme systems (RAWLS and LUCCHESI 1974a,b; DICKINSON and SULLIVAN 1974; CHOVNICK *et al.* 1976).

With respect to these fundamental questions, our study of the ADH enzyme system in *D. melanogaster* leads to the following conclusions:

(a) There are genes, other than the structural locus coding for ADH, that affect the levels of ADH activity. These may be called regulatory genes because they modify the activity of other genes, namely the structural *Adh* locus.

(b) At least some of these regulatory genes are located in the third chromosome, while the *Adh* structural locus is in the second chromosome. That is, regulation is due (at least in part) to genes not adjacent to the structural gene locus.

(c) Variation exists in natural populations at regulatory gene loci modifying the activity of the *Adh* locus. We have examined nine different third chromosomes extracted from a natural population, and have found among them considerable variation in modifying ADH activity levels.

(d) The effects of the regulatory genes located on the third chromosome depend on gene(s) located on the second chromosome. That is, the relative effects of two given third chromosomes may change as they are combined with different second chromosomes, even when these carry the same electrophoretic allele at the *Adh* locus.

(e) Third chromosomes with "higher-activity" regulatory genes are either partially or totally dominant over third chromosomes with "lower-activity" genes.

(f) The regulatory effects of the third chromosome genes are not necessarily specific to any given stage of development, some, at least, are pervasive throughout the life cycle.

(g) At least some third chromosome genes regulate the levels of ADH activity by affecting the number of ADH molecules. This could be due to either an increase in the rate of synthesis of ADH or a decrease in the rate of degradation of the enzyme, or both.

(h) Finally, the question of the adaptive significance of the observed variation in gene regulation arises. Alcohol is common in environments where *D. melanogaster* live. In particular, it has been shown that flies with higher ADH activity levels survive better in the presence of high levels of environmental alcohol (*e.g.*, MCDONALD and AVISE 1976), and also the converse, namely that flies more tolerant of environmental alcohol have a higher ADH activity (and, in fact, greater numbers of ADH molecules; see MCDONALD *et al.* 1977).

Some of the conclusions reached in this study were also established by other investigators; *e.g.*, (a) and (c) above (see references in the Introduction and the RESULTS section). We consider the results reported as only preliminary—many questions remain unsolved, such as the number of regulatory elements, and the precise fashion in which they regulate the amount of ADH protein in the organisms. Nevertheless, if the finding presented here are typical of enzyme systems in Drosophila (and other organisms), the implications for evolutionary genetics are important. Adaptive evolution may take place by changes not only in the structural gene loci involved in a particular adaptation, but also by changes in gene regulation modifying the activity of such loci.

Others have recently suggested that changes in gene regulation may be the most decisive process in the evolution of higher organisms (BRITTEN and DAVIDSON 1969; VALENTINE and CAMPBELL 1975; KING and WILSON 1975; WILSON 1976; DOBZHANSKY *et al.* 1977). The provocative hypotheses advanced are supported by some indirect evidence. Nevertheless, these hypothese possess little empirical validity until the term "genetic regulation" is translated into precise genetic mechanisms in higher organisms and is investigated experimentally in natural populations. We suggest that the approach taken in this study will help explore this relatively unknown dimension of evolutionary change.

Our results indicate that the adaptive significance of any structural gene needs to be evaluated not only within the the context of other metabolically cooperating structural genes, but also in reference to relevant regulatory loci as well. If the variability of regulatory genes is as extensive as our results suggest there are considerable implications concerning the much debated question regarding the adaptive significance of the widespread allozyme variation observed within and between species.

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