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Isozyme Variability in Species of the Genus *Drosophila*. VI. Frequency–Property–Environment Relationships of Allelic Alcohol Dehydrogenases in *D. melanogaster*[1]

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Adult Drosophila melanogaster *from naturally occurring populations in the Eastern United States were examined by gel electrophoresis for their alcohol dehydrogenase (ADH) phenotype. The ADH enzymes were partially purified and characterized. Frequencies of the controlling alleles,* Adh^4 *and* Adh^6*, were discovered to vary in a clinal pattern.* Adh^6 *reaches a maximum frequency of about 0.90 in the South and minimum of about 0.50 in the North. Partially purified enzymes from the three* Adh *genotypes varied according to specific activity, substrate specificity, and heat stability. A differential influence of* pH *was indicated. There was little variation in* K_m *values for ethanol and* DPN^+ *among the enzymes.*

INTRODUCTION

After several years of investigation of isozyme polymorphisms in natural populations, there is still considerable controversy about their maintenance. The argument is principally between those supporting "balancing" selective forces (*cf.*, e.g., Prakash *et al.*, 1969; Selander *et al.*, 1969; Richmond, 1972) and those believing that the variation is selectively neutral (*cf.*, e.g., Kimura

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and Ohta, 1971; Mukai *et al.*, 1971; Yamazaki, 1971). Several approaches to the problem have been made, but usually conflicting interpretation has been possible from the results. Perhaps more promising will be the multidimensional methods in current development. These involve investigation on a locus-by-locus basis with regard to genetic, environmental, biochemical, and physiological considerations in some combination. Thus selective evidence might be partially based on patterns of association between certain genotypes and natural environmental conditions (e.g., Koehn and Rasmussen, 1967; Johnson *et al.*, 1969), with supporting evidence provided by relating functional differences in enzymatic properties to the genotype–environment associations (Koehn, 1969; Merritt, 1972). Notwithstanding the possibility that some correlation could exist by way of unrecognized nonselective mechanisms, the approach at least begins to provide plausible selective mechanisms which then can be tested experimentally.

The present paper records preliminary results with regard to genetic, environmental, and biochemical aspects of the alcohol dehydrogenase (ADH) polymorphism in *Drosophila melanogaster*. Possible means of selective involvement are suggested which should be amenable to future experimental testing and verification.

MATERIALS AND METHODS

Flies were collected from localities in 11 of the Eastern United States ranging from Maine to Florida (Table I). In the table, the localities are identified by cities near the actual sites, which were generally in outlying wooded areas. Collections were made from pails that contained a yeasted banana-mash bait and that had been placed in the field a few days prior to collection.

Alcohol dehydrogenase (ADH) phenotypes and gene frequencies were determined from starch gel electrophoretic analyses performed on single-fly homogenates of wild-caught individuals. Methods have been published, e.g., Stone *et al.* (1968). The two predominant alleles, now identified as Adh^4 and Adh^6, found in the population samples are apparently identical with those previously identified as "fast" and "slow", respectively (Johnson and Denniston, 1964). Multiple forms of ADH result from flies of all three genotypes: Adh^4/Adh^4, Adh^4/Adh^6, and Adh^6/Adh^6. The combinations of forms within a genotype are referred to as the ADH^4, ADH^{4-6}, and ADH^6 enzymes, respectively.

ADH activity in crude and purified extracts was routinely measured by a modification of a technique used by Jacobson *et al.* (1970). A 3 ml reaction mixture consisting of enzyme extract, 1.9 mM NAD^+, and 0.13 M substrate in 0.03 M tris–HCl, *p*H 9.0, was used. Activity was determined by monitoring the increasing absorbancy at 340 nm with a Beckman DB-GT grating spectro-

Table I. Localities, Dates of Collections, *Adh* Allelic Frequencies, and Numbers of Flies in the Various ADH Phenotypic Categories for Collections of *D. melanogaster* from the Eastern United States

Collection locality	Date	*Adh* alleles		*N* alleles	ADH phenotypes			*N* individuals
		4	*6*		4	4–6	6	
Swainsboro, Ga.	5/70	0.299	0.701	354	15	76	86	177
Sylvester, Ga.	5/70	0.277	0.723	350	12	73	90	175
Boston, Mass. (P.C.)	8/70	0.299	0.701	756	31	164	183	378
Boston, Mass. (R)	8/70	0.255	0.745	106	4	19	30	53
Auburn, Ala.	8/70	0.256	0.744	994	39	177	281	497
Knoxville, Tenn.	8/70	0.341	0.659	290	13	73	59	145
Miami, Fla. (S_1)	4/71	0.116	0.884	172	4	12	70	86[a]
Miami, Fla. (S_3)	4/71	0.133	0.867	128	2	13	49	64
Miami, Fla. (S_4)	4/71	0.044	0.956	136	0	6	62	68
Miami, Fla. (S_9)	4/71	0.118	0.882	220	1	24	85	110
Miami, Fla. (S_{10})	4/71	0.108	0.892	130	1	12	52	65
Miami, Fla. (S_{11})	4/71	0.698	0.902	184	0	18	74	92
Miami, Fla. (several)	4/71	0.142	0.858	232	3	27	86	116
Miami, Fla. (S_3)	6/71	0.109	0.891	256	1	26	101	128
Miami, Fla. (S_5)	6/71	0.145	0.855	256	6	25	97	128[b]
Miami, Fla. (several)	6/71	0.106	0.894	188	2	16	76	94
Columbia, S.C. (S_1)	7/71	0.243	0.757	288	9	52	83	144
Columbia, S.C. (S_2)	7/71	0.258	0.742	236	10	41	67	118
Columbia, S.C. (S_3)	7/71	0.266	0.734	158	6	30	43	79
Columbia, S.C. (S_4)	7/71	0.282	0.718	294	12	59	76	147
Portland, Maine	8/71	0.430	0.570	614	45	174	88	307[a]
Niagara Falls, N.Y. (S_9)	8/71	0.556	0.444	288	43	74	27	144
Niagara Falls, N.Y. (S_{11})	8/71	0.458	0.542	358	39	86	54	179
Winchester, Va. (S_1)	8/71	0.487	0.513	156	17	42	19	78
Winchester, Va. (S_2)	8/71	0.359	0.641	504	29	123	100	252
Winchester, Va. (S_3)	8/71	0.350	0.650	340	21	77	72	170
Erie, Pa. (S_6)	8/71	0.520	0.480	244	37	53	32	122
Erie, Pa. (S_7)	8/71	0.383	0.617	282	17	74	50	141
Erie, Pa. (S_8)	8/71	0.376	0.624	298	24	64	61	149
Jacksonville, Fla. (S_1)	11/71	0.352	0.648	128	8	29	27	64
Jacksonville, Fla. (several)	11/71	0.247	0.753	170	4	34	47	85
Orlando, Fla. (S_{13})	12/71	0.147	0.853	170	2	21	62	85
Orlando, Fla. (S_{14})	12/71	0.171	0.829	240	3	35	82	120
L. Placid, Fla. (S_{11})	12/71	0.117	0.883	196	2	19	77	98
L. Placid, Fla. (S_{12})	12/71	0.139	0.861	158	0	22	57	79
Raleigh, N.C. (S_1)	7/70	0.334	0.666	644	44	127	151	322[b]
Raleigh, N.C. (S_2)	7/70	0.321	0.679	458	27	93	109	229
Raleigh, N.C. (S_3)	8/70	0.317	0.683	1132	67	225	274	566
Raleigh, N.C. (S_4)	8/70	0.285	0.715	130	3	31	31	65
Raleigh, N.C. (S_3)	9/70	0.320	0.680	384	24	75	93	192
Raleigh, N.C. (S_4)	9/70	0.289	0.711	142	5	31	35	71
Raleigh, N.C. (S_2)	9/70	0.292	0.708	298	12	63	74	149

[a] $P<0.01$.
[b] $P<0.05$.

photometer and a Heath log/linear recorder. One unit of activity was defined as a 1.00 increase in optical density per minute. Enzyme concentrations were adjusted to be within the range of linear response. Where temperature control was necessary, it was regulated by a Haake water bath connected to the sample compartment of the spectrophotometer. Temperature readings were obtained from thermometers placed in the cuvettes. Protein concentration was determined by the method of Lowry *et al.* (1951).

ADH enzymes were obtained from each of the two homozygous genotypes maintained separately in mass cultures. These stocks were each originally derived from single wild-caught females captured near Raleigh, N.C. The enzymes of heterozygotes were obtained from a balanced laboratory strain, *Cy/Pm*, courtesy of Professor Terumi Mukai. The flies were cultured on standard banana medium at 23 C and preserved by freezing at −20 C.

ADH was partially purified from adult flies of homozygous and heterozygous genotypes. The technique used employs aspects of the methods used by Jacobson *et al.* (1970) and Sofer and Ursprung (1968). Two and one-half grams of frozen flies was placed in 7.5 ml of 0.03 M tris–HCl, *p*H 9.0, and homogenized for 1 min with a Sorvall Omni-Mixer run at maximum speed. The crude homogenate was centrifuged at 27,000 × *g* for 20 min and the pellet discarded. After the supernatant fluid had been passed through glass wool to remove lipid material, freshly prepared 2% protamine sulfate was added (0.06 ml per 1.0 ml of supernatant fluid). The mixture was allowed to stand for 15 min in ice and then centrifuged at 8000 × *g* for 20 minutes. The supernatant fluid was removed, adjusted to *p*H 9.0 with 0.5 M NaOH, and applied to a 35 by 45 mm column (fitted with a coarse-fritted glass filter) containing 10 g of Whatman DE-52 DEAE-cellulose equilibrated with 0.03 M tris–HCl buffer, *p*H 9.0. Elution with approximately 15 ml 0.05 M tris–HCl, *p*H 9.0, resulted in an effluent containing essentially all recoverable activity. With some lots of DEAE-cellulose, irreversible binding of the enzyme occurred under the above conditions. This was found to be preventable by reducing the *p*H of the enzyme preparation, the column, and the influent to *p*H 8.0. The DEAE-cellulose eluate was subsequently applied to another 35 by 45 mm column containing 2.0 g of Bio-Rad Laboratories' powdered hydroxylapatite equilibrated with 0.03 M tris–HCl, *p*H 9.0. Elution with 10 ml 0.05 M tris–HCl, *p*H 9.0, followed by 10 ml of the same buffer of 0.07 M, produced an eluate containing all recoverable activity. This served as the working enzyme preparation. The enzymes from all three genotypes were purified according to the same procedure (Table II).

Following purification, the ADH enzymes from the three genotypes were compared on the basis of several characterization parameters as follows:

1. Relative reaction rates were determined at ambient temperature using

Table II. Purification of Alcohol Dehydrogenase from the Adh^6/Adh^6 Genotype of *D. melanogaster*

Fraction	Volume (ml)	Total protein (mg)	Specific activity (units/mg protein)	Overall purification (-fold)	Total activity	Overall yield (%)
Homogenate	4.5	118.00	0.23	—	27.1	100
Protamine sulfate	3.8	70.00	0.37	1.6	25.9	96
DEAE-cellulose	9.8	6.80	1.44	6.3	9.8	36
Hydroxylapatite	5.5	0.13	42.30	183.9	5.5	20

different substrates (ethanol, 2-butanol, 1-butanol, 2-propanol, and 1-propanol). A constant amount of enzyme was used in each reaction mixture as described in the above procedure for determining ADH activity.

2. The influence of *p*H on the reaction rates was investigated by substituting 0.01 M glycyl glycine buffer at various hydrogen ion concentrations (*p*H 7.0–9.5) for the tris in the reaction mixture. A similar test was done using a borate buffer (0.1 M boric acid, 0.1 M KCl adjusted to various *p*H levels in the range between *p*H 7.8 and *p*H 9.9 with 0.1 N NaOH). Both were done at room temperature with ethanol as substrate.

3. Michaelis constants (K_m) were determined for DPN^+ and ethanol at *p*H 9.0, room temperature. The K_m for DPN^+ was established using concentrations between 0.01 mM and 1.00 mM with the ethanol concentration held constant at 60 mM. For ethanol, the K_m was determined from concentrations between 1.3 mM and 50 mM with a constant concentration of DPN^+, 0.5 mM.

4. Temperature-dependent activity profiles were measured using 1-butanol as substrate (with checks for consistency performed with ethanol). The data were obtained as initial reaction rates when enzyme was added to reaction mixture held constant at various specific temperatures.

5. Temperature inactivation was further investigated by warming the reaction mixture to particular set temperatures, adding enzyme, and then monitoring activity over time, as temperature was held constant. Reaction rates were calculated at several different times for each temperature. In control runs at low temperature (e.g., 25 C), no change in rate occurred even though the reactions were allowed to continue for prolonged periods of time (until twice the optical density range of the actual experimental determinations had been reached). Therefore, neither reactants nor products were likely to be major rate-limiting factors in activity changes associated with higher temperatures. Both 1-butanol and ethanol were used as substrates.

All experimental data were obtained in at least duplicate from independent enzyme preparations.

RESULTS

The usual ADH electrophoretic phenotype of single adult individuals of the most common genotype (Adh^6/Adh^6) is shown in Fig. 1. There are two predominant zones (bands 3 and 5) and three that are very faint and sometimes not visible (bands 1, 2, and 4). The other homozygote (Adh^4/Adh^4) displays essentially the same pattern, but the entire set of bands is shifted toward the anode. Heterozygotes show all the bands of both homozygotes plus additional bands midway between the prominent corresponding parental bands. Sensitivity of the staining mixture and time of development determine phenotype to a degree. Bands 3 and 5 develop readily; bands 1, 2, and 4 very slowly if at all. The numbering system is that of Grell *et al.* (1968).

After purification, the same set of bands is present on electrophoresis of the preparation as that found after electrophoresis of single adult flies. Relative intensities of the bands, however, are somewhat different, and additional faster-migrating bands appear. Bands 3, 4, and 5 stain quite deeply, while bands 1 and 2 and the new faster-moving bands are much fainter. Comparative tests were done on the enzyme preparations without regard to possible functional differences of the various forms occurring within one genotype. The results, therefore, reflect an average of the properties for the enzymes within each ADH phenotype.

The *Adh* genotypes and gene frequencies found in the population samples are shown in Table I. Generally, there is close agreement between observed numbers in the genotypic classes and those expected on the basis of Hardy–Weinberg equilibrium. Exceptions are three cases of excess in the homozygous

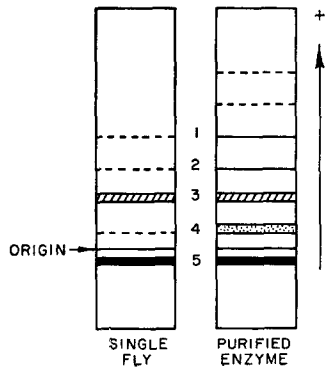

Fig. 1. Diagram of the electrophoretic pattern of ADH from a single fly and a purified enzyme preparation. Genotype in both is Adh^6/Adh^6 (homozygous "slow").

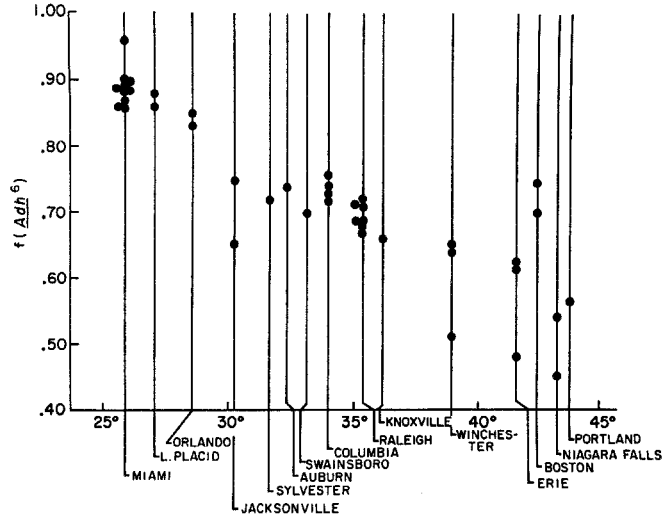

Fig. 2. Frequency of the *Adh*^{6} gene in localities arranged according to latitude.

classes (two in Florida and one in North Carolina) and one case of excess in the heterozygous class (Maine). Thus out of 42 total cases there are only four statistically significant discrepancies. When the entire sampling range is considered, there is wide variation in allelic frequencies, however, and the pattern of geographic change is clinal in nature. Figure 2 is a plot of *Adh*^{6} frequencies according to latitude of the collection localities. The frequency drops from approximately 0.90 in the South to about 0.50 in the North. Perhaps there are many environmental factors varying similarly that might be correlated with the frequency change, but the most obvious one is tempera-

Table III. Representative Activities of ADH in Crude Supernatants of Flies of Different *Adh* Genotypes Using 1-Butanol as Substrate

Genotype	Specific activity (units/mg protein)					Average relative activity
Adh^4/*Adh*^{4}	0.39	0.37	0.33	0.23	0.45	100
Adh^4/*Adh*^{6}	0.22	0.21	0.22	0.28	0.22	66
Adh^6/*Adh*^{6}	0.24	0.10	0.12	0.21	0.23	51

ture. During most times of the year, average temperature shows a pattern of decrease similar to the frequency of *Adh*^{6} (*cf.* U.S. Weather Bureau records).

Differences in biochemical properties among genotypes first became apparent during purification. Specific activity varied in the following decreasing order: ADH^4 > ADH$^{4-6}$ > ADH^6 (Table III). This was most consistently apparent in crude supernatants, because technical variations in procedure obscured differences in later stages of purification. Similar differences in specific activity among genotypes have been reported by Gibson (1970) for crude larval homogenates and by Rasmuson *et al.* (1966) for crude adult homogenates.

Comparisons of activity with various substrates are presented in Table IV. The three ADH enzymes are generally similar in order of reactivity: 2-butanol > 2-propanol > 1-butanol ≅ 1-propanol > ethanol. ADH^6 is apparently less specific for 2-butanol than ADH$^{4-6}$ and ADH^4, because ADH^6 shows the highest values with respect to the other alcohols. Since the activities are all relative to 2-butanol within each phenotypic classification, interpretation requires considerable caution. Preference of the ADH enzymes for secondary alcohols was suggested by Sofer and Ursprung (1968) and Jacobsen *et al.* (1970).

Figure 3 shows the influence of *p*H on activity in borate buffer. The enzymes from all three genotypes respond almost identically with a sharp activity peak at *p*H 8.4. Comparable data for glycine buffer are presented in Fig. 4. Peak activity is observed at approximately *p*H 9.0 for all forms, but at lower *p*H values the ADH^6 and ADH$^{4-6}$ enzymes retain a greater level of activity than do the ADH^4 enzymes.

Table V gives the results of the K_m determinations for DPN$^+$ and ethanol. Between DPN$^+$ and ethanol there is approximately a hundredfold difference for all forms, but among forms there is little variation.

Table IV. Relative Activities of the ADH Enzymes with Different Substrates[a]

Substrate	Percent maximum activity								
	ADH^4			ADH$^{4-6}$			ADH^6		
ethanol	12.8	13.0	(12.9)	15.3	23.8	(19.6)	26.8	19.4	(23.1)
2-butanol	100.0	100.0	(100.0)	100.0	100.0	(100.0)	100.0	100.0	(100.0)
1-butanol	26.0	29.0	(27.5)	29.2	40.0	(34.6)	47.6	47.0	(47.3)
2-propanol	72.0	78.5	(75.3)	88.8	84.6	(86.7)	91.0	86.9	(88.9)
1-propanol	21.2	21.4	(21.3)	29.7	30.2	(30.0)	41.9	36.7	(39.3)

[a] Numbers in parentheses are averages of two separate determinations.

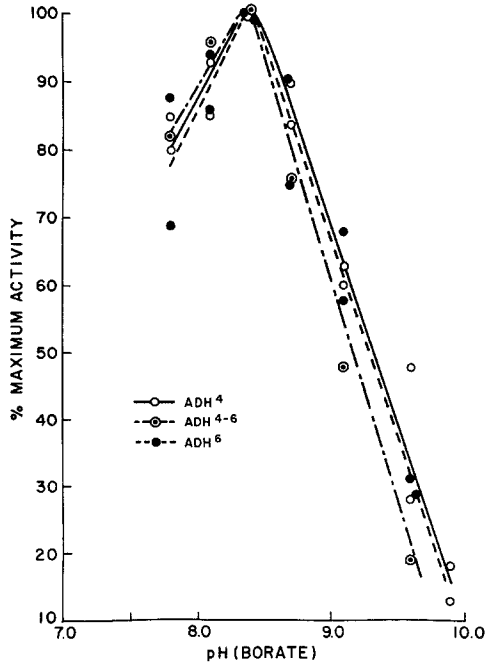

Fig. 3. Activity of the ADH enzymes as a function of *p*H in borate buffer.

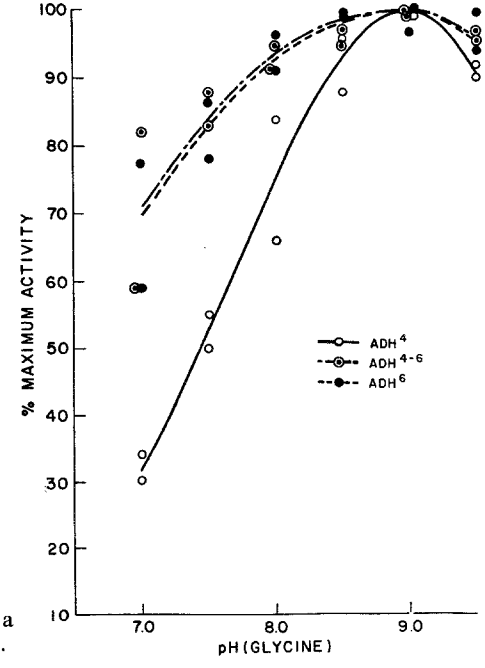

Fig. 4. Activity of the ADH enzymes as a function of *p*H in glycyl glycine buffer.

Table V. Results of K_m Determinations for DPN$^+$ and Ethanol for the Three ADH Enzymes[a]

Enzyme	K_m (mM DPN$^+$)			K_m (mM EtOH)		
ADH^4	0.076	0.100	(0.088)	7.1	8.3	(7.7)
ADH$^{4-6}$	0.045	0.053	(0.049)	5.0	5.0	(5.0)
ADH^6	0.140	0.073	(0.106)	6.9	4.5	(5.7)

[a] Values in parentheses are averages.

Temperature-dependent activity profiles with 1-butanol as substrate are shown in Fig. 5. The ADHs of all three genotypes produce curves of similar shape, but they are not completely overlapping. ADH^4 and ADH$^{4-6}$ peak at about 38 C, while ADH^6 peaks at a distinctly higher temperature, 43 C. At 40 C and below, ADH^4 and ADH$^{4-6}$ express more of their maximum activity than does ADH^6. Above 40 C, the order is reversed. When ethanol was substituted for 1-butanol, several spot checks yielded results comparable to the values obtained with 1-butanol. When the curves are adjusted according to the specific activity observed in the crude supernatants, additional distinction among the ADH enzymes is noted (Fig. 6). Although this is a somewhat artificial comparison, it suggests a possible biological relationship. That is, at temperatures below a certain point, flies may be expected to contain decreasing

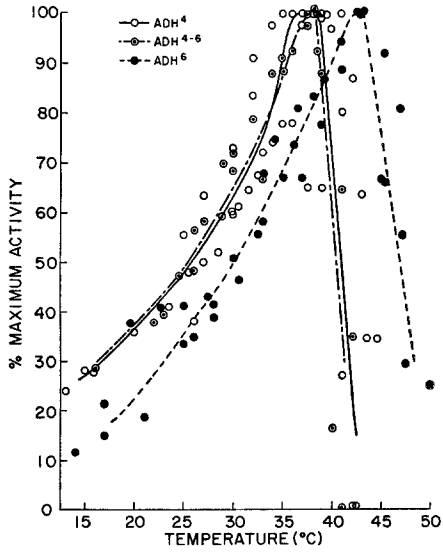

Fig. 5. Percent of maximum initial reaction velocity according to temperature for the ADH^4, ADH$^{4-6}$, and ADH^6 enzymes.

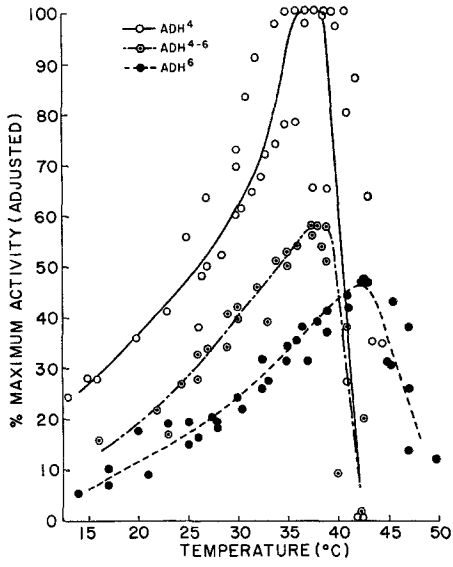

Fig. 6. Percent of maximum initial reaction velocity according to temperature for the ADH^4, ADH$^{4-6}$, and ADH^6 enzymes with adjustment for differences in specific activity in crude supernatants.

amounts of ADH activity according to genotype, $Adh^4/Adh^4 > Adh^4/Adh^6 > Adh^6/Adh^6$, while at higher temperatures the order may be reversed. The data suggest that 40 C is the critical point, but the value is not necessarily applicable to the enzymes in intact organisms.

Temperature inactivation is partially reflected in the activity profiles,

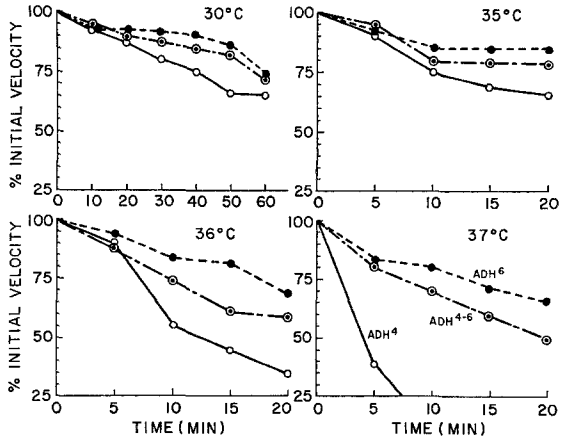

Fig. 7. Comparison of ADH^4, ADH$^{4-6}$, and ADH^6 in relation to decreases in reaction velocities with time and temperature using 1-butanol as substrate.

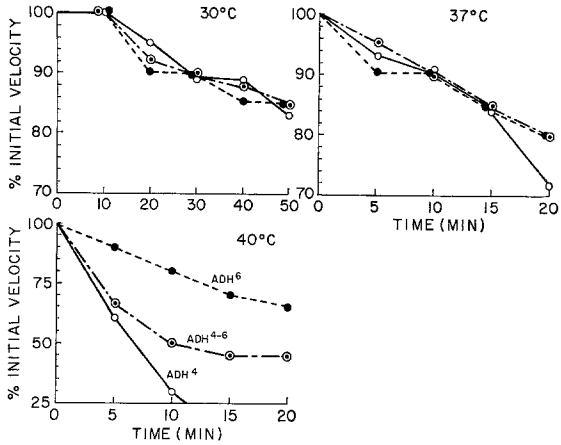

Fig. 8. Comparison of ADH^4, ADH^{4-6}, and ADH^6 in relation to decreases in reaction velocities with time and temperature using ethanol as substrate.

but it was further investigated in other experiments. Figure 7 shows the patterns of decay in activity according to time, temperature, and genotype using 1-butanol as substrate. At the lower temperatures (30–35 C), the enzymes are inactivated slowly, and differences among the enzymes from different genotypes are slight. The order of stability is $ADH^6 > ADH^{4-6} > ADH^4$. At high temperatures (37 C), ADH^4 is distinctly more labile than the other forms. Comparable data using ethanol as substrate are shown in Fig. 8. The degree of inactivation is appreciably less with ethanol for all forms, and in the 30–37 C range there is apparently no significant difference among the ADH enzymes. At 40 C, however, variation in heat stability becomes clearly evident, and the order of stability is the same as when 1-butanol is used. Possibly ethanol has some stabilizing effect or butanol some denaturing effect on the enzymes. Gibson (1970) reported differences in heat stability of the ADHs from crude larval extracts which are generally consistent with the present results.

DISCUSSION

The hypothesis that isozyme polymorphisms are directly maintained by selection requires that functional differences exist among the alternate genetic forms. If differences cannot be found, only the postulation of undiscovered or undiscoverable differences can prevent rejection of the hypothesis. For the ADH enzymes, there is now little doubt that differences exist. The ADH enzymes are not the same with regard to specific activity, substrate

specificity, influence of *p*H, and heat stability. Since various concentrations and kinds of alcohols, different *p*H levels, and variations in temperature are a part of the natural environment, it can be concluded that conditions exist which could, *in concept*, bring advantage to one or another of *Adh* genotypes. Certainly, then, there is no reason to reject selection on the basis of the results.

Whether or not the environmental variation is *actually and directly* concerned with maintenance of the ADH polymorphism is another question. Part of the problem lies in identifying the characteristics of the environment that might confer advantage to one genotype over another. There is also difficulty in experimental duplication of the environmental characteristics, and there is no precise way of knowing how the environment of the organism is reflected at the microenvironmental level of the enzymes themselves. Thus environmental variables cannot be accurately quantitated, and artificial compromises must be made in characterizing the allelic types. As a result, it is almost certain that some important differences in properties will go unnoticed and/or that some spurious distinctions will arise. Given the inherent limitations of the experiments, possible selective relationships are suggested by the data. Selection could favor high specific activity in the North and/or low in the South to most efficiently tolerate and/or utilize quantitative and qualitative differences in the alcoholic phase of the environment. Differences in substrate specificity and the influence of *p*H on activity could also reflect selective importance either related to or independent of the variation in specific activity. ADH is present in numerous tissues including parts of the digestive system (Dunn *et al.*, 1969; Ursprung *et al.*, 1970). Thus localization is such that ADH may directly act on and be influenced by dietary alcohols and other compounds in the diet. Since many different food sources are utilized by *D. melanogaster*, e.g., citrus fruits, apples, grapes, and strawberries, the geographico-nutritional relationships are extremely complex. However, the average net pattern for individual nutritional components, including *p*H, could well be clinal as are the *Adh*-gene frequencies.

The thermal properties of the enzymes show suggestive consistency with the genetic variation. The ADH^6 enzymes, those of greatest frequency of occurrence in the South, are the most reactive at higher temperatures, and the ADH^4 and ADH^{4-6} enzymes, found in greater frequency in the North, are more reactive at lower temperatures. It could be that there is no causal connection, but there is logical appeal in the relationship. Fallen fruit exposed to the direct rays of the sun and warm to the touch will often contain living *Drosophila*, and the situation is certainly more prevalent in the Southern climates. Thus, even though changes in relative enzyme activities apparently take place only at fairly high temperatures, such extremes would seem to be a part of the natural environment.

The various forms of ADH occurring within a homozygous genotype might bring further complication to the selective mechanism. Relative activities of these forms change during development (Dunn *et al.*, 1969), and *in vitro* interconversion has been demonstrated (Jacobson, 1968). The *in vitro* interconversion has been shown to be controlled by one or more of several factors (NAD^+ concentration, acetone concentration, and *p*H), and the conformational changes accompanying conversion confer heat-stabilizing properties on the molecule (Jacobson *et al.*, 1970; Jacobson *et al.*, 1972; Knopp and Jacobson, 1972). Other loci in the genetic background as well as outside environmental factors could, therefore, be envisaged to have possible regulatory control over total ADH activity.

Future work must be concerned with relating the biological utility of the activity differences with the fitness of the organism. Experiments under various controlled environmental conditions should be helpful. If environmental factors can be experimentally demonstrated to influence the relative fitness values associated with different enzyme genotypes (in accordance with the properties of the enzymes and in accordance with the genotypic distribution in the natural environment), there will be few contingencies left whereby neutrality can remain a tenable hypothesis. The ADH polymorphism is an attractive system for continued pursuit in this direction.

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