ASSESSMENT OF VARIABILITY WITHIN ELECTROMORPHS OF ALCOHOL DEHYDROGENASE IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Ninety-six isochromosomal lines of *Drosophila melanogaster* from a natural population were screened electrophoretically for unusual mobility variants at the alcohol dehydrogenase locus, using a total of eight conditions of acrylamide electrophoresis. No additional mobility variation was found among the 50 "slow" and 46 "fast" mobility lines beyond that detected by standard methods of electrophoresis. However, two thermostability variants recovered by R. MILKMAN from a natural population, whose electrophoretic mobilities were previously thought to be indistinguishable from those of "standard" alleles, are distinguishable from the standard electromorphs by these procedures. These results suggest that the *Adh* locus, although polymorphic, does not harbor substantial amounts of "hidden" allelic variability. This study also reports the appearance of substantial mobility variation among isogenic lines that can be induced under specific conditions of sample preparation involving the pretreatment of samples with NAD and acetone. However, genetic analysis demonstrates that this variability cannot be attributed to allelic differences at the structural locus, but instead appears to be dependent upon the concentration of the enzyme in a sample. These results are discussed in relation to the distribution of allelic variation at other enzyme loci.

THE extent to which standard gel electrophoresis underestimates the actual genic variation at structural loci remains a central issue in population genetics. However, a variety of techniques have now been employed in the detection of this so called "hidden" variation, and several patterns of its distribution among enzyme-coding loci have emerged. In particular, results of studies utilizing a sequential method of gel electrophoresis to detect protein variation have led to the suggestion that the relative increase in the number of electromorphs resolved by this methodology will be proportional to the number that have previously been detected by standard techniques (COYNE and FELTON 1977; COYNE, FELTON and LEWONTIN 1978). This prediction is based on the observation that already highly polymorphic loci become more variable upon closer electrophoretic inspection, while relatively monomorphic loci remain essentially invariant (SINGH, LEWONTIN and FELTON 1976; COYNE 1976; BECKENBACH and PRAKASH 1977). This apparent dichotomy between highly polymorphic *versus* monomorphic loci implies the existence of two distinct classes of loci, and it has been proposed that such a

pattern can only be the result of different selective mechanisms operating within these two classes of loci (COYNE, FELTON and LEWONTIN 1978).

However, the extent to which such observations can be generalized is, at present, uncertain. Perhaps the most serious concern is that the methodologies employed in the detection of protein variation may not be equally sensitive to similar types of structural variation in different proteins. There is no *a priori* reason to expect that the rigid application of a single methodology will yield an equivalent resolution of allelic variation for all proteins. Yet, a recent study by RAMSHAW, COYNE and LEWONTIN (1979) on allozymic variation in fully sequenced human hemoglobins has demonstrated that the sequential method *is* capable of detecting a large proportion of nominal charge-change substitutions in this molecule, and similar investigations using other proteins are presently underway in this laboratory that should provide information on this important problem. A second major objection is that there are no established criteria or procedures to assess the adequacy or degree of bias in the sample of enzymes that have been examined thus far. If, for example, this is not a representative sample, the actual distribution of alleles at structural loci could be considerably different from the one suggested by these few loci. At present, this possibility can be investigated only by the examination of a broader range of proteins.

In this study, a number of conditions of acrylamide electrophoresis were used to investigate the possibility of additional allelic variation at the alcohol dehydrogenase locus in a natural population of *Drosophila melanogaster*. The sensitivity of this method to the detection of "hidden" variation at this locus was also investigated by the electrophoretic analysis of a number of thermostability mutants that have previously been reported to have the standard "fast" or "slow" electrophoretic mobilities. In addition, this study reports on the appearance of a surprising and substantial amount of mobility variation between isogenic strains when subjected to certain running conditions and sample treatment. However, this variation is shown to be nongenetic in origin.

Two structural forms of alcohol dehydrogenase can be distinguished on the basis of their differential electrophoretic mobilities, and both forms occur throughout the geographic range of *D. melanogaster* (DAVID 1977). This two-allele polymorphism appears to be stable at intermediate frequencies of the two electromorphs in both natural (JOHNSON and SCHAFFER 1973; LANGLEY, TOBARI and KOJIMA 1974; LANGLEY, ITO and VOELKER 1977) and laboratory (CAVENER and CLEGG 1978) populations, although latitudinal and altitudinal clines have been described (VIGUE and JOHNSON 1973; PIPKIN *et al.* 1976).

Several features of this polymorphism have contributed to its choice for this study. First, the two-allele condition at this locus is somewhat intermediate and in marked contrast to the highly polymorphic esterase-5 and xanthine dehydrogenase loci at one extreme, for which up to a dozen electromorphs can be identified under standard conditions of electrophoresis, and the nearly monomorphic octanol dehydrogenase and malic dehydrogenase loci at the other in *D. pseudoobscura*. Together, these results form the basis for the previously described pattern. Second, *Adh* is a small molecule with a subunit molecular weight of 24,000

(THATCHER 1977). This is also in direct contrast to the high subunit molecular weights of the polymorphic xanthine dehydrogenase and esterase-5 proteins, which are 150,000 and 54,000, respectively (SEYBOLD 1974; COBBS 1976). Third, heat denaturation studies have been used in an attempt to detect additional variability at this locus in natural populations; several variants have been discovered by this method (SAMPSELL 1977; MILKMAN 1976; THORIG, SCHOONE and SCHARLOO 1975) suggesting that other forms of enzyme exist. However, this technique has failed to uncover substantial amounts of additional variation such as that uncovered in the cases of XDH and EST-5. Finally, this locus is of interest because it has been intensively studied in *D. melanogaster* at both the populational level in natural and laboratory populations and at the biochemical level (see DAVID 1977 for a recent review). In this regard, the considerable interest in understanding the maintenance of the *Adh* polymorphism in natural populations of the species will be critically dependent upon the actual number of alleles segregating at this locus.

METHODS

Ninety-six independently derived lines of *D. melanogaster*, isogenic for the second chromosome, were kindly supplied by C. LANGLEY. All extracted chromosomes were maintained in the laboratory over the *SM1* balancer chromosome. Detailed information regarding the collection site and extraction procedure is reported in VOELKER *et al.* 1980. Of the 96 lines, 50 were known to carry the slow *Adh* electromorph and 46 the fast electromorph (C. LANGLEY, personal communication).

Vertical slab-gel electrophoresis using the discontinuous tris-HCl-glycine buffer of DAVIS (1964) produces excellent resolution of the 2 common electromorphs at the *Adh* locus, with both homozygotes exhibiting the typical 3-banded pattern and heterozygotes a 5-banded pattern. The staining procedure is reported in AYALA *et al.* (1972). All lines were subjected to 8 gel conditions representing combinations of 3 gel concentrations, 5%, 7.5% and 10%, and three pH's, 7.5, 8.5 and 10.1. A concentration of 5% bis was used at all gel concentrations. One combination, 10% pH 7.5, was excluded from this analysis due to the excessive running time that would be required. Running times varied from 4 hr for the 5% pH 10.1 gel to 16 hr for the 10% pH 7.5 gel to ensure adequate migration. All gels were run at 15.5 volts/centimeter.

Two methods of sample preparation were utilized in the study. Samples were first run through the sequence of gel conditions after having been treated for 3 hr at 10° with 0.1 M tris-HCl pH 8.5 containing 1.0 mM acetone and 30 mM NAD. This procedure results in the complete conversion of the "slow" and "intermediate" migrating isozymes (the numbers 5 and 3 bands, respectively) into the "fast" form (the number 1 band), thereby facilitating visual comparisons between samples (JACOBSON *et al.* 1972). Samples were then run a second time without NAD or acetone treatment in order to compare the isozymic forms of the enzyme.

RESULTS AND DISCUSSION

Electrophoretic analysis consisted of separate comparisons of 50 "slow" and 46 "fast" lines under eight conditions. This analysis failed to reveal a single additional mobility variant among any of the 96 lines tested. Such a result, quite naturally, raises the possibility that additional allelic variation might exist among the lines tested, but that the methodology employed is insensitive to the types of amino acid substitutions coded for by this additional allelic variation. In order to evaluate such a possibility, five lines of *D. melanogaster* were obtained carrying

variant forms of the enzyme isolated on the basis of their unusual heat sensitivity or kinetic properties. Two "fast" and one "slow" variants were originally isolated on the basis of their heat-sensitivity (one heat-restant fast allele, F^r, one heat-sensitive slow allele, S^s, supplied by R. MILKMAN and one heat-sensitive fast allele, *71-k*, supplied by G. THÖRIG, and two slow variants were synthesized from null alleles by MARONI ($Adh^{B7}rd^s$ and $Adh^{B1}rd^s$) (MILKMAN 1976; THÖRIG, SCHOONE and SCHARLOO 1975; MARONI 1978). These lines were tested against standard "fast" and "slow" lines under the same eight gel conditions. The important characteristic of this set of variants is that they are indistinguishable from the standard "fast" and "slow" forms of the enzyme on the basis of their mobility under "normal" conditions of gel electrophoresis. The purpose of this test, then, was to determine whether the methodology employed in this study could resolve by means of mobility variation any of these five putative allelic variants, which by previous electrophoretic determinations could not be distinguished from their respective standard alleles. The two methods of sample preparation were also used in order to evaluate the contention that co-factor binding to the enzyme conceals electrophoretic variation in this protein (JOHNSON 1978).

In the untreated samples, two of the three thermosensitive alleles, the slow-sensitive and fast-resistant alleles of MILKMAN, were distinguishable from their respective standard alleles on the basis of electrophoretic mobility (Figure 1). The slow-migrating thermosenstive allele produces a slightly faster migrating number 5 and 3 band on pH 8.5 and 7.5 gels, while the fast-resistant allele produces a

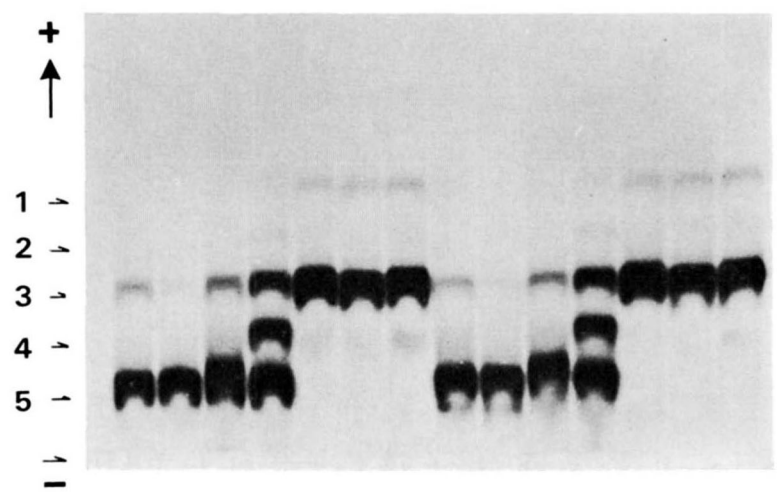

FIGURE 1.—Comparison of thermostability and null-synthesized variants with standard alleles (7.5%, pH 8.5). Numbers 1 through 5 on left indicate position of major isozymes of the standard alleles. From left to right: Pockets 1 and 2 contain Adh^{B7} and Adh^{A1} (MARONI) that are indistinguishable from the standard slow allele; Pocket 3: S^s (MILKMAN) showing a faster-migrating number 5 isozyme compared to a standard slow allele; Pocket 4: heterozygote between standard fast and slow alleles; Pocket 5: 71^k (THORIG) which is indistinguishable from a standard fast allele (pocket 7); Pocket 6: F^r (MILKMAN) number 3 isozyme migrates slightly slower than a standard fast allele; Pockets 8 through 14: repeat.

slightly slower number 5 band at pH 7.5 and 8.5. However, it is interesting to note that in neither case could the two mobility variants be distinguished on the basis of the mobility of the fast-migrating number 1 form of the enzymes, nor could they be distinguished after NAD and acetone treatment (which converts the two slow-migrating isozymic forms into the fast-migrating form). This, then, confirms the observation of JOHNSON (1978) that co-factor binding (such as NAD) to proteins may indeed obscure electrophoretic mobility variation that might otherwise be detectable and should serve as a caveat for future electrophororetic work of this sort. The third thermosensitive allele, the fast-migrating variant of THÖRIG, could not be distinguished from standard fast alleles, nor could the two slow-migrating synthetic alleles of MARONI be distinguished from a standard slow allele. However, since it has not yet been unequivocally demonstrated that these forms of the enzyme represent structural variants, which can be demonstrated only by sequencing, the ability of this procedure to distinguish the two as being electrophoretically different suggests that this technique is sensitive to at least certain types of amino acid substitutions that have previously gone undetected. Furthermore, the ability to distinguish the two variant forms of the enzyme that were isolated from natural populations lends support to the previous evidence that substantial amount of additional variability do not exist at the *Adh* locus.

Although no mobility of variation was uncovered among either treated or untreated samples, a surprising amount of mobility variation was obtained for "partially" treated samples—either samples that were treated with NAD in the grinding buffer but were immediately subjected to electrophoresis or samples that were untreated but were run on gels containing 20 to 30 mg NAD (Figure 2). By these methods, for example, three variant patterns could be distinguished at pH 7.5 and 8.5 among five randomly chosen lines. Furthermore, the positions of the variant isozyme bands did not conform to the positions of any of the isozyme bands of untreated samples, suggesting that the partial treatment procedure might have exposed structural differences among the proteins that affect their inter-

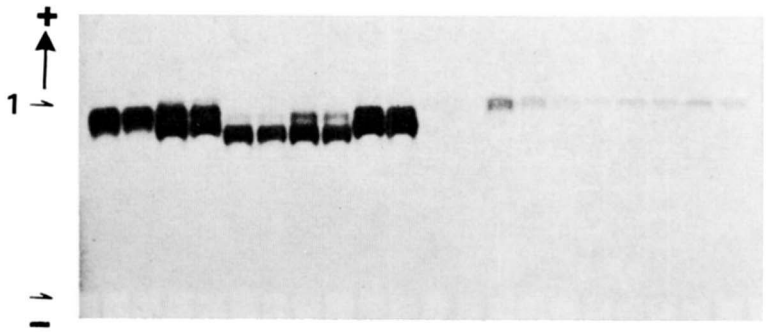

FIGURE 2.—Induced mobility variation among five isogenic lines homozygous for the fast allele (5%, pH 7.5). All lines are replicated and are run in adjacent pockets. Each sample was divided into two equal aliquots. Pockets 1 through 10: no NAD in grinding buffer; 11 through 20: identical samples in same order, but with NAD and acetone added to grinding buffer 30 min prior to electrophoresis.

action with the co-factor NAD. However, it is also possible that the observed mobility differences between isogenic lines after partial treatment may be caused by differences in the amount of enzyme being produced among lines and therefore would not necessarily be due to differences at the structural locus. According to this alternative explanation, variation in the concentration of enzyme present in the sample during electrophoresis would lead to the observed mobility variation by affecting the stochiometry of the isozyme conversion process during electrophoresis (see ORNSTEIN 1964).

In order to evaluate the two hypotheses, reciprocal crosses were set up among homozygous "fast" lines that exhibited unusual migration patterns and between these fast lines and a standard slow line. Randomly selected F_1 and F_2 offspring from 10 of the crosses were then analyzed electrophoretically to determine the segregational properties of the mobility differences. Two lines of evidence clearly indicate a nongenetic basis for the mobility variation between lines. First, there was no evidence of Mendelian segregational patterns in either the F_1 or F_2 of the Fast × Fast crosses, although some within-vial variability was observed between F_2 individuals. However, similar variability was also observed in F_2 homozygous "fast" individuals of the Fast × Slow crosses that could not be due to structural differences, since all "fast" alleles in these crosses had a common ancestry. Further experiments in which aliquots were prepared from single lines in grinding solutions at 2×, 1×, 1/2×, 1/4× and 1/8× dilutions revealed that the electrophoretic patterns could be modified to resemble the variant patterns by varying the concentration of the sample. Therefore, it is clear from this, as well as from the genetic analysis, that the variability observed in partially treated samples cannot be attributed to the structural locus, but is most likely an artifact of the sample concentration and local gel conditions around the migrating protein front during electrophoresis. It is important to note, however, that similar types of variability were never observed during the examination of the 96 isogenic lines in the present study, nor has it been observed in previous work in this laboratory on the detection of genic variability at other enzyme loci.

These results do point out, however, the importance of establishing genetic bases for the sometimes subtle mobility variation exposed by these electrophoretic methods. It is also possible that this provides an explanation that reconciles the results presented here with the results of JOHNSON (1978) on *Adh* variability in *D. melanogaster*. In his study, he reported the presence of considerable *ADH* mobility variation in 43 isogenic lines of *D. melanogaster*, but found little evidence for conformational variability in 20 individuals of a highly inbred line, which suggests a genetic basis for the mobility variation among lines. In addition, this was localized by genetic analysis to the second chromosome. An alternative explanation is that the mobility variation he described may merely be a consequence of between-line differences in *Adh* activity. CLARKE *et al.* (1979) have shown that by varying the amount of yeast in a cornmeal-molasses medium the quantity of *ADH* in individual flies can be changed by a factor of four. Furthermore, between-line differences in *Adh* activity have also been described that appear to have a genetic basis, but that do not map to the structural locus (MCDONALD and AYALA

1978). Such variability might contribute to slight mobility differences between lines, but this variability would not be expressed among individuals within a single inbred line.

The inability to uncover even a single electrophoretic variant among 96 lines of *D. melanogaster* indicates that natural populations of this species do not harbor large amounts of "hidden" genetic variability at the *Adh* locus, although some certainly exists (MILKMAN 1976; SAMPSELL 1977). Identical findings have also been reported by BECKENBACH and PRAKASH (1977) for several hexokinase loci in *D. pseudoobscura* and *D. persimilis*. They were unable to detect any additional allelic variation using several techniques, including sequential gel electrophoresis. Likewise, little additional variation was uncovered by COYNE and FELTON (1977) at the nearly monomorphic malic dehydrogenase and octanol dehydrogenase loci in *D. pseudoobscura* and in other species of Drosophila for alpha-glycerophosphate dehydrogenase (COYNE *et al.* 1979). This study confirms the conclusions of BECKENBACH and PRAKASH that the large amounts of additional allelic variation uncovered at the *Xdh* and *Est-5* loci in *D. pseudoobscura* will not extend to all loci. Indeed, the high levels of polymorphism at these two loci may turn out to be exceptional. However, such assessments will first require a better knowledge of the relative sensitivity of gel electrophoresis to detect amino acid substitutions in proteins of different sizes. This study does demonstrate, at least, that polymorphism *per se* is not a sufficient indicator of the extent to which sequential acrylamide electrophoresis will detect additional allelic variation at a locus since, on the basis of heterozygosity estimates, the *Adh* locus is certainly polymorphic. Rather, it would appear that the number of previously detected electromorphs provides the better indication of the extent of variation at a locus.

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