Copyright © 1987 by the Genetics Society of America

Reexamination of Alcohol Dehydrogenase Structural Mutants in Drosophila Using Protein Blotting

Hope Hollocher[1] and Allen R. Place[2]

Department of Biology, Leidy Laboratories, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Manuscript received October 16, 1986
Revised copy accepted March 10, 1987

ABSTRACT

Using protein blotting and an immuno-overlay procedure, we have reexamined the cross-reacting material produced by ADH null-activity mutants generated with ethyl methanesulfonate (EMS). Of the 13 mutants, 11 have an immunodetectable polypeptide of wild-type size. The native and urea denatured isoelectric points (pI) establish that 7 of 13 of the mutations have no effect on protein charge. The electrophoretic mobilities of each variant on increasing percent acrylamide gels (Ferguson analysis), reveal that 9 of the 11 immunodetectable mutants have retained the ability to form dimers under native conditions. None of the inactive mutant proteins has the ability to form the "adduct-bound" isozyme. We have found no correlation between protein pI and *in vivo* stability. The observed frequencies of specific charge class alterations do not dispute the propensity of G:A transitions previously found for EMS mutagenesis.

ETHYL methanesulfonate (EMS) mutagenesis has been applied extensively to the alcohol dehydrogenase (*Adh*) locus of *Drosophila melanogaster* (GRELL, JACOBSON and MURPHY 1968; SCHWARTZ and SOFER 1976a). Based on the assumption that any null-activity mutant which synthesized a protein contained a structural gene defect, better than 75% of mutations were classified as being within the structural gene. Our approach has been to reexamine the ADH-negative mutants using a series of "mini-gel" electrophoresis techniques coupled with a protein blotting procedure and a sensitive immuno-overlay protein detection assay on crude extracts. Because of the lower *in vivo* stabilities of the mutant proteins (PELLICCIA and SOFER 1982), it was important to analyze them with as little prior fractionation as possible in order to reduce artifacts produced by proteolytic degradation.

The methods used and the parameters estimated by each method are as follows: (1) native and urea-denaturing polyacrylamide gel isoelectric focusing (PAGIEF) for determining isoelectric points (pI); (2) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for determining subunit molecular weights (M_r); and (3) native polyacrylamide gel electrophoresis (PAGE) on increasing percent acrylamide (Ferguson analysis) for determining the charge to surface area ratio (Y_o) and effective volume (K_r) of the proteins. We will show that all of these methods can be successfully performed using mutants which in some instances have ADH present in levels only 1% of that found in the wild type.

By reexamining the protein produced by the EMS-generated mutants, we set out to relate their physical properties to their observed *in vivo* stabilities (PELLICCIA and SOFER 1982), and to infer whether the G:C to A:T transitions previously found in three *Adh* mutants (RETZIOS and THATCHER 1979; MARTIN *et al.* 1985) adequately described the mutagenic event.

MATERIALS AND METHODS

Drosophila strains: Most of these second chromosome mutants have been described more completely by LINDSLEY and GRELL (1967).

Adh^S carries the electrophoretic "slow" variant of ADH in the Schenk Forest Strain.

w; Adh^F (WEP) carries the *X*-linked gene white eyes and the naturally occurring electrophoretic "fast" protein variant of ADH. The ADH of Adh^F differs from that of Adh^S by a threonine amino acid change at residue 192 (THATCHER 1980).

Adh^D *pr cn* carries an electrophoretic variant of ADH that migrates slightly faster than ADH^F. It is an active variant derived from the Samarkand stock of Adh^F by EMS mutagenesis (GRELL, JACOBSON and MURPHY 1968). Adh^D differs from Adh^F by a glycine to glutamate amino change at residue 232 (SCHWARTZ and JORNVALL (1976).

Adh^{n1} through Adh^{n3} are three inactive ADH strains generated by GRELL, JACOBSON and MURPHY (1968) from the Canton S Adh^S strain.

Adh^{n4} and Adh^{n5} are two strains derived from Adh^D (GRELL, JACOBSON and MURPHY 1968). Adh^{n4} does not produce an active ADH (SCHWARTZ and SOFER 1976b). Adh^{n5} is a temperature sensitive mutant which exhibits ADH activity under the conditions used in these experiments (VIGUE and SOFER 1974).

Adh^{n6} through Adh^{n13} are inactive ADH strains generated in Adh^F *b cn vg* and selected using the pentenol procedure (GERACE and SOFER 1972).

Flies were cultured at 25° on Carolina Drosophila Me-

[1] Present address: Department of Biology, Washington University, St. Louis, Missouri 63130.
[2] To whom all correspondence should be addressed.

dium, Blue 4-24. Flies were aged 4–10 days prior to their use in all assays.

Antibody production: Goat anti-ADH antibody was produced by immunizing a young female goat according to the method of SPIELMAN, ERICKSON and EPSTEIN (1974). Purification of the goat antisera by ammonium sulfate fractionation and antigen affinity chromatography was performed as described by PELLICCIA and SOFER (1982). The anti-ADH antibody constituted approximately 1% of the IgG fraction.

Protein extraction: Soluble protein was extracted by homogenizing flies (1 fly per 10 μl extraction buffer) with a motorized glass pestle designed to fit microcentrifuge tubes (1.5 ml). The crude homogenate was filtered by centrifugation through a glass-fiber filter. Native proteins were extracted on ice. Typically, 0.1 to 0.5 fly equivalents were analyzed.

Gel methods: Horizontal native polyacrylamide gel isoelectric focusing (PAGIEF) was performed using the Pharmacia Fine Chemical Co. Flatbed apparatus FBE 3000. The gels (113 mm × 230 mm) consisted of 5.0% acrylamide, 0.3% bis-acrylamide, 13.3% glycerol, 1% ampholytes (a 2:1 mixture of pH range 3–9.5 and pH range 5–8), 0.0152% ammonium persulfate and 0.05% TEMED. Samples were prepared at 4° in Na-phosphate buffer (pH 7.5) with 1 mM EDTA, 5 mM β-mercaptoethanol (BME), and 15% glycerol. Prefocusing was carried out at 15 watts (constant wattage) for 1 hr. Samples were then applied to the surface of the gel and focused for 90 min at 15 watts (constant wattage). The gel temperature was regulated by using a circulating water bath set to 4°. The isoelectric focusing points (pIs) were estimated by measuring the distance migrated from the cathode boundary relative to the mobility of the three major electrophoretic variants, ADH^F, ADH^S and ADH^D, as well as to four visual markers, methyl red (pI 3.75), hemoglobin A (pI 7.0), hemoglobin S (pI 7.3), and horse heart cytochrome *c* (pI 10.75). The pIs for ADH^F, ADH^S and ADH^D were determined for the above set of conditions by using the BDH visual isoelectric focusing standards with pIs ranging from 5.65 to 8.30, and by staining the gels directly for activity. ADH activity was detected by staining in 0.02 M Na-phosphate buffer (pH 7.5) containing 0.18% NAD, 0.1% nitro blue tetrazolium, 0.004% phenazine methosulfate, and 3.6% 2-butanol at 25° for 50 min.

Isoelectric focusing in the presence of 9 M urea was performed with the same apparatus described for native PAGIEF. The gels were made with the constituents described by O'FARRELL (1975), modified to include only 1% ampholytes (a 2:1 mixture of pH range 3–9.5 and pH range 5–8). The gel solution was deionized by lightly shaking for 1 hr with 0.25 g of Amberlite MB-1 ion exchange resin/30 ml of solution and was filtered prior to pouring. Protein samples were made using fresh extraction buffer consisting of a filtered solution of 9.5 M urea, 2% NP-40, 5% BME, and 10% Amberlite MB-1 ion exchange resin. The flies were ground at room temperature and the samples were heated in an 80° water bath for 3 min before centrifugation. The gels were not prefocused prior to loading the samples. Focusing was done for 4 hr at 15 watts (constant wattage) at 20°.

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) was performed using 12.5% acrylamide and 0.8% bis-acrylamide running gels (65 mm × 70 mm) consisting of 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS; stacking gels (10 mm) were made using 4.5% acrylamide containing 0.8% bis-acrylamide in 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS. The electrophoresis buffer contained 0.025 M Tris, 0.19 M glycine and 0.1% SDS. The flies were homogenized in 0.12 M Tris-HCl (pH 6.8), 0.4% SDS, 10% BME, 0.02% bromophenol blue, and 20% glycerol. The samples were then heated for 3 min in a boiling water bath before centrifugation. The gels were run at room temperature at 90 V for the first 15 min, then 120 V for the next 0.5 hr and finally 150 V for the remaining 1.5 hr. The low molecular weight range visual markers from Bethesda Research Laboratories (BRL) were used to estimate the subunit molecular weight of the mutants. The standards are transferable onto nitrocellulose and remain highly visible even after several washings.

Native polyacrylamide gel electrophoresis using a modified version of the Jovin Output System 2860 (JOVIN, DANTE and CHRAMBACH 1970) was performed using concentrations of acrylamide ranging from 4% to 9% with 2.5% bis-acrylamide cross-linker for the Ferguson analysis of the ADH null-activity mutants. Gel slabs (65 mm × 70 mm × 8 mm) were run with and without stacking gels. Those run without stacking gels still contained the same relative combination of buffers as used in the gels with stacking gels. In the case of those without stacking gels, the composition of the sample buffer was 0.0374 M Tris and 0.0283 M H_3PO_4 (pH 6.25) and included 1 mM EDTA, 5 mM BME, 15% glycerol, and 1 mg/ml of α-macroglobulin. The six different percent acrylamide gels were run simultaneously at 110 V at 4° with electrophoresis being terminated when the tracking dye (bromophenol blue) reached a premeasured distance of 7.0 cm. To get a representation of the electrophoretic moving boundary within the gel, hemetin, which co-migrates with bromophenol blue, was used as a transferable, visible marker of the ion front. Hemetin does not wash out of the nitrocellulose during incubations, and the color intensifies under the staining conditions used to detect the ADH mutants because of heme-mediated oxidation of 3,3'-diaminobenzidine tetrahydrochloride (see *Immunodetection*). Samples run on gels containing 1 cm stacking gels were prepared (see *Protein extraction*) using 0.02 M Na-P buffer (pH 7.5) with 1 mM EDTA, 5 mM BME, 15% glycerol, and 1 mg/ml α-macroglobulin.

Protein blotting: The GD4 Destainer apparatus and the Destainer power supply by Pharmacia were used to perform protein blotting as described by TOWBIN, STAEHELIN and GORDON (1979) and BITTNER, KUPFERER and MORRIS (1980) under the conditions as described by BARINGA *et al.* (1981) except that native gels were transferred using 0.375 M Tris-HCl (pH 8.8). The proteins were transferred using a relatively high current (0.5–2.5 A) and a low voltage (12–24 V) onto nitrocellulose paper of 0.45 μm pore size from Schliecher and Schuell. Whatman 3 MM chromatography paper was used to ensure complete contact between the gel and the nitrocellulose.

Immunodetection: Protein blots were probed using the procedure described by TOWBIN, STAEHELIN and GORDON (1979). After transfer, the filters were incubated in 3% (w/v) bovine serum albumin (BSA) in a Tris-saline buffer consisting of 10 mM Tris-HCl (pH 7.6), 0.9% (w/v) NaCl and 0.01% (w/v) NaN_3 for a minimum of 1 hr. The filters were washed with Tris-saline for 30 min and then incubated in 10 ml of 1.5% (w/v) BSA containing 10% (v/v) horse serum and 1.4 μg/ml of goat anti-ADH antibody solution for 3 hr. After rinsing three times, the sheets were then incubated in 10 ml of 2 μg/ml of rabbit anti-goat horseradish peroxidase conjugate for 3 hr. Peroxidase activity was detected by incubating the nitrocellulose sheets in 0.15 M phosphate-citrate buffer (pH 5.0), containing 0.04% (v/v) H_2O_2 and 0.2% (w/v) 3,3'-diaminobenzidine tetrahydrochloride. To intensify the color of the precipitate, 3 mg of nickel sulfate and 3 mg of cobalt chloride were added to each 25 ml of incubation solution (DEBLAS and CHERWINSKI 1983). The

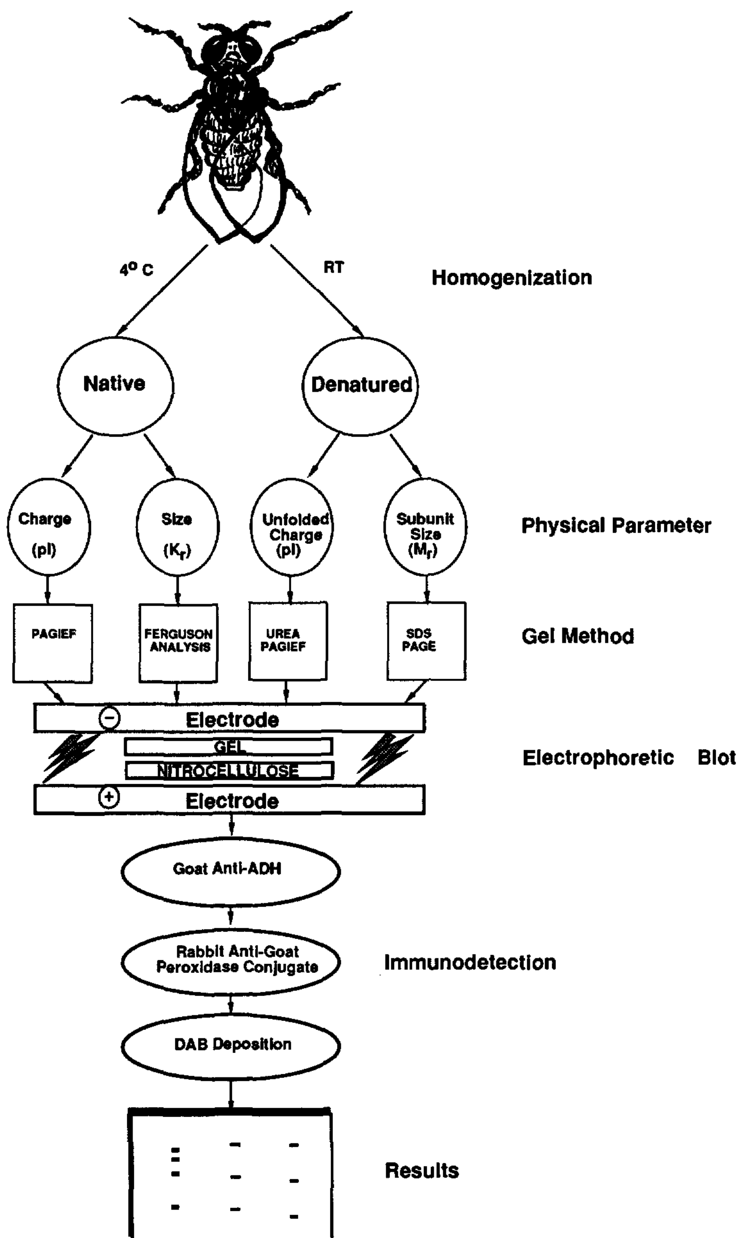

FIGURE 1.—Schematic of a protein blotting strategy for analysis of mutant proteins.

reaction was stopped by rinsing thoroughly in water and air drying away from direct light.

Mobility measurements and data analysis: Distances were measured to the nearest 0.1 mm by using a GTCO bitpad. The program package PAGEPACK was used for data analysis (RODBARD and CHRAMBACH 1974).

Materials: The Trizma base, glycine, bis-acrylamide, rabbit anti-goat peroxidase conjugated IgG, β-mercaptoethanol, TEMED, 3,3′-diaminobenzidine tetrahydrochloride, 30% hydrogen peroxide, nitro blue tetrazolium, phenazine methosulfate, NAD^+, Amberlite MB-1, and acrylamide for the SDS-PAGE were purchased from the Sigma Chemical Co. Acrylamide of 99.9% purity used for gels other than SDS was purchased from Bio-Rad. Carrier ampholytes for PAGIEF were the Ampholine brand from LKB. Ultrapure urea was obtained from Schwartz/Mann, Inc. SDS low molecular weight standards were bought from Bethesda Research Laboratories, and the PAGIEF standards were bought from BDH. Nitrocellulose and Whatman 3mm paper were from Schleicher and Schuell. Drosophila stocks were supplied by WILLIAM SOFER.

RESULTS

A schematic representation of the techniques used to characterize the EMS-generated ADH nulls is shown in Figure 1. Using four "mini-gel" techniques, PAGIEF, urea-PAGIEF, SDS-PAGE, and native PAGE of various percents of acrylamide coupled with immunodetection, we can list five parameters to describe each of the EMS-generated mutant proteins in this study. Three parameters, native pI, urea-denatured pI, and Y_o, provide information on protein charge, and the other two, M_r and K_r, estimate size.

Protein charge alterations: Figure 2 is an electrophoretic transfer of a typical native isoelectric focusing gel. Of the 13 mutants Adh^{n2}, Adh^{n4}, Adh^{n10} and Adh^{n13} do not produce immunodetectable protein under these conditions. SCHWARTZ and SOFER (1976b) and PELLICCIA and SOFER (1982) have shown that

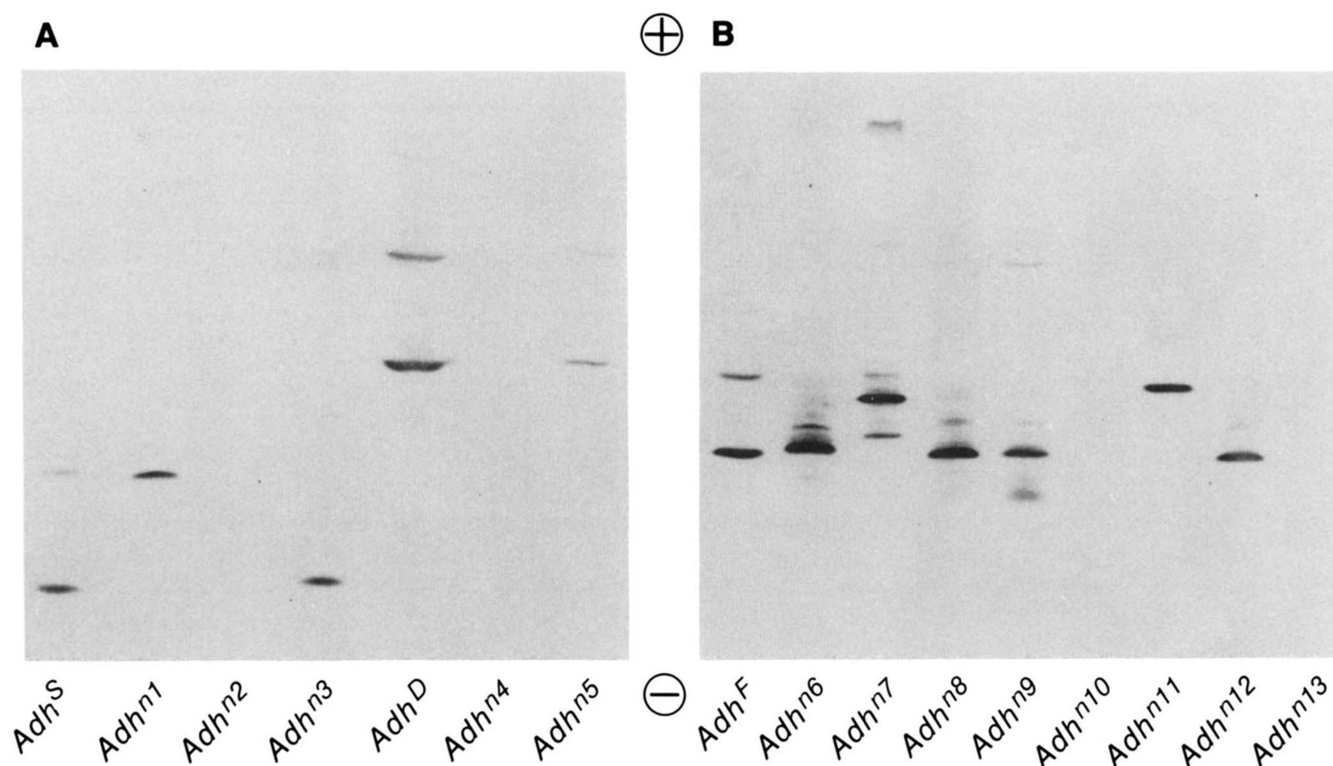

FIGURE 2.—Nitrocellulose transfers of the mutant ADH proteins after isoelectric focusing in polyacrylamide. Extracts were made in 0.02 M sodium phosphate (pH 7.5) containing 5 mM β-mercaptoethanol, 1 mM EDTA, and 15% (v/v) glycerol. Gels (115 × 230 mm) were prefocused at 15 watts for 1 hr, then focused at the same wattage for 1 hr after samples were applied. Proteins were transferred onto nitrocellulose as described by TOWBIN, STAEHELIN and GORDON (1979) and BITTNER, KUPFERER and MORRIS (1980), at 12 V for 25 min in 0.375 M Tris-HCl (pH 8.8). Protein blots were probed for ADH CRM using the procedure described by TOWBIN, STAEHELIN and GORDON (1979) modified as described in MATERIALS AND METHODS.

Adh^{n4} and Adh^{n10} do not produce a polypeptide capable of cross-reacting with the goat anti-ADH used in these studies. ADH^{n2} and ADH^{n13} do cross-react with the anti-ADH antibody (SCHWARTZ and SOFER 1976b). The *in vivo* steady state levels of ADH^{n2} and ADH^{n13} are less than 5% of wild-type levels (PELLICCIA and SOFER 1982). We have determined that as little as 1–5 ng of purified wild-type enzyme is detectable under these conditions, yet no ADH^{n2} or ADH^{n13} protein was detectable even when eight times the concentration of extract was analyzed.

All the immunodetectable mutant proteins focus within the pI boundary set by the most basic ADH protein variant, ADH^S (pI 7.7), and the most acidic variant, ADH^D (pI 6.2), used for comparison in this study. Nitrocellulose was placed on both sides of the gel during transfer to ensure that the mutants were not being lost during electroblotting. Under native conditions, immunodetectable protein transfers anodally and was observed only on the nitrocellulose side facing the gel.

Each immunodetectable mutant protein (except ADH^{n5}) is detected as one major band. The characteristic "adduct-bound" isozymes of Drosophila ADH (JOHNSON and DENNISTON 1964) are not found for the inactive variants. The minor bands associated with the null variants do not correspond to the single charge differences which characterize "adduct-bound" isozymes of Drosophila ADH (JOHNSON and DENNISTON 1964; URSPRUNG and LEONE 1965; JACOBSON, MURPHY and HARTMANN 1970; JACOBSON *et al.* 1972; KNOPP and JACOBSON 1972; SCHWARTZ *et al.* 1976; SCHWARTZ and SOFER 1976a; SCHWARTZ, O'DONNELL and SOFER 1979; PAPEL *et al.* 1979; WINBERG, THATCHER and MCKINLEY-MCKEE 1983) and are seen for ADH^S, ADH^D, ADH^F and ADH^{n5} in Figure 2. Moreover, feeding flies a 3% (w/v) sucrose solution containing 0.5% (v/v) acetone for 17 hr (PAPEL *et al.* 1979) converted the major bands of ADH^F and ADH^{n5} to their adduct forms. No change occurred in ADH^{n11}. We suspect the minor bands seen in Figure 2 are degradation products or deamidated forms of the enzyme (WINBERG, THATCHER and MCKINLEY-MCKEE 1983).

Table 1 summarizes the results obtained through all the gel methods used to analyze the mutants, and includes the estimated pIs for the various proteins as calculated by linear regression. Each gel was analyzed separately and the individual values averaged.

Isoelectric focusing was also performing under denaturing conditions to determine whether there were any hidden charge differences. The mutant proteins

TABLE 1

Characterization of Drosophila ADH null mutants from immunoblots

Genotype	Ferguson analysis: K_r (95% confidence)	Ferguson analysis: Y_o (95% confidence)	Isoelectric focusing: Native pI (SD)	Isoelectric focusing: Urea pI (SD)	SDS-PAGE: Mol wt × 10^3 (95% confidence)
Adh^F					
major	0.0593 ± 0.0045	0.401 ± 0.026	6.91 ± 0.060	6.91 ± 0.060	24.62 ± 0.65
adduct1	0.0575 ± 0.0028	0.547 ± 0.022	6.38 ± 0.056	6.38 ± 0.056	
adduct2			5.68 ± 0.020		
Adh^D					
major	0.0563 ± 0.0019	0.554 ± 0.016	6.24 ± 0.059	6.24 ± 0.059	24.82 ± 0.78
adduct1	0.0569 ± 0.0018	0.740 ± 0.019	5.65 ± 0.062	5.65 ± 0.062	
adduct2	0.0575 ± 0.0016	0.912 ± 0.020	5.11 ± 0.085		
Adh^S					
major	0.0546 ± 0.0076	0.199 ± 0.022	7.74 ± 0.073	7.74 ± 0.073	24.82 ± 0.78
adduct1	0.0574 ± 0.0052	0.361 ± 0.027	6.88 ± 0.068	6.88 ± 0.068	
Adh^n1	0.0624 ± 0.0050	0.328 ± 0.024	6.95 ± 0.082	6.95[a] (6.24)[a]	25.45 ± 0.58
Adh^n2 pr cn	Could not be stabilized under native conditions			7.68 ± 0.021 (6.24)[a]	24.79 ± 0.58
Adh^n3 pr cn	0.0660 ± 0.0063	0.320 ± 0.027	7.57 ± 0.061	7.68 ± 0.021 (6.95)[a]	24.79 ± 0.58
Adh^n4 pr cn	Does not produce a detectable protein				
Adh^n5					
major	0.0602 ± 0.0026	0.591 ± 0.022	6.25 ± 0.046	6.29[a]	24.80 ± 0.58
adduct1	0.0608 ± 0.0029	0.789 ± 0.033	5.66 ± 0.107	5.45[a]	
Adh^n6 cn vg	0.0562 ± 0.0046	0.430 ± 0.027	6.92 ± 0.067 (6.30 ± 0.049)	7.02 ± 0.035	24.80 ± 0.58
Adh^n7 cn vg	0.0572 ± 0.0020	0.543 ± 0.016	6.57 ± 0.129	6.30 ± 0.049 (5.49)[a]	24.69 ± 0.69
Adh^n8 cn vg	0.0563 ± 0.0055	0.391 ± 0.032	6.97 ± 0.084 (6.28 ± 0.021)	7.02 ± 0.035	24.79 ± 0.74
Adh^n9 cn vg	0.0604 ± 0.0054	0.410 ± 0.033	6.94 ± 0.101	7.02 ± 0.035 (6.28 ± 0.021)	24.84 ± 0.73
Adh^n10 cn vg	Does not produce a detectable protein				
Adh^n11 cn vg	0.0558 ± 0.0023	0.563 ± 0.018	6.41 ± 0.099	6.24 ± 0.071 (5.53)[a]	24.84 ± 0.73
Adh^n12 cn vg	0.0564 ± 0.0055	0.390 ± 0.031	6.94 ± 0.091	7.00 ± 0.064 (6.25 ± 0.014)	24.84 ± 0.73
Adh^n13 cn vg	Could not be stabilized under native conditions			7.67 ± 0.084 (7.04 ± 0.130)	24.84 ± 0.73

The 95% confidence limits of the linear regression reverse predictions used for determining the pIs, never exceed 4.5% of the estimate. Estimates for the pIs of the urea-denatured proteins were obtained assuming that the relative pIs of the wild-type proteins remain unchanged when denatured. The second number for pIs of the urea-denatured proteins (in parentheses) represents a second major band visible on the protein blots.

[a] Number of determinations too small for estimate of error.

focused to the same relative pIs obtained under the native conditions except ADH^{n7}, which appears to be more acidic. *Adh*^{n4} and *Adh*^{n10} produced no immunodetectable protein, as was expected. The ADH^{n2} and ADH^{n13} proteins, which could not be stabilized under native conditions, were detected in the presence of urea. The relative pI estimates for the denatured polypeptides are found in Table 1.

In addition to the major band, a secondary band was detected on the nitrocellulose after urea PAGIEF for each variant in the positions where "adduct-bound" isozymes (JOHNSON and DENNISTON 1964; URSPRUNG and LEONE 1965; JACOBSON *et al.* 1972; KNOPP and JACOBSON 1972; SCHWARTZ *et al.* 1976; SCHWARTZ and SOFER 1976a; SCHWARTZ, O'DONNELL and SOFER 1979; PAPEL *et al.* 1979; WINBERG, THATCHER and MCKINLEY-MCKEE 1983) would be expected to focus under native conditions. Purified ADH^{F}, which showed a single band upon focusing under native conditions, exhibited two bands when focused in 9 M urea. These bands may represent two conformational forms of the same polypeptide (CREIGHTON 1979; GOLDENBERG and CREIGHTON 1984). No "stepladder" effect is seen as would be expected if multiple carbamylations were responsible for the new bands (HICKMAN *et al.* 1979; CHRAMBACH and RODBARD 1981); however, the more acidic band may result from single carbamylation of a highly reactive lysine residue (HICKMAN *et al.* 1979; CHRAMBACH and RODBARD 1981).

Subunit molecular weights: Wild-type Drosophila ADH exists as a dimer with a monomer molecular weight of 27,600 (THATCHER 1980). Our measurements of M_r (Table 1) indicate that the immunodetectable mutants express a polypeptide of equal length to wild-type polypeptide, confirming the results of

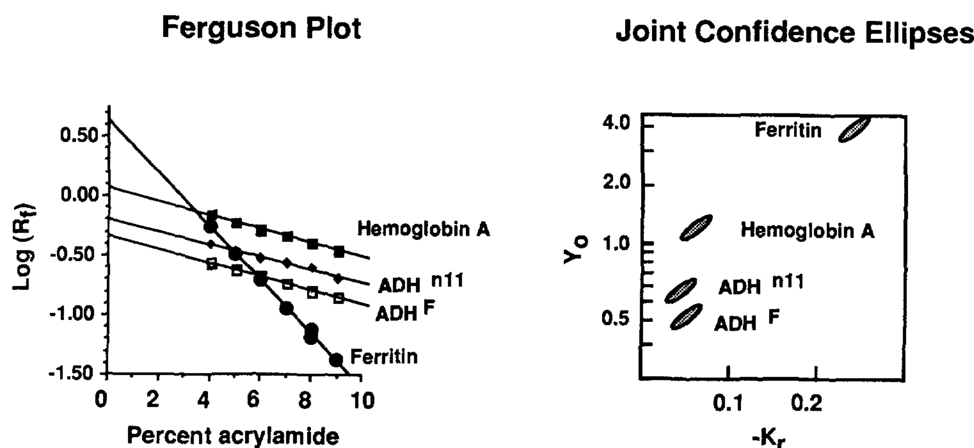

FIGURE 3.—Ferguson analysis of purified proteins. By measuring the relative distances proteins migrate for different acrylamide concentrations with constant crosslinking, the retardation coefficient (K_r—a measure of molecular size) and the free mobility (Y_o—a measure of charge to mass) can be determined by plotting the logarithm of the relative mobility (R_f) *vs.* the percent of acrylamide (%T). The slope of the resultant line gives estimates of K_r, while the extrapolated value of R_f at 0% acrylamide gives estimates for Y_o. These "Ferguson plots" shown in the figure are for purified enzymes electrophoresed at 25° using buffer system 2860 (JOVIN, DANTE and CHRAMBACH, 1970). The data clearly show the utility of this analysis. Ferritin and *Drosophila* ADH have nearly identical electrophoretic mobilities at 6% acrylamide, yet differ widely in physicochemical properties. These differences are only manifested when the acrylamide concentrations are varied.

SCHWARTZ and SOFER (1976b). ADH^{n4} and ADH^{n10} again are not detectable by this method. ADH^{n2} and ADH^{n13} were stabilized sufficiently in SDS to show that their subunit molecular weights are the same as wild type.

Charge and size analysis: Charge and size analyses of the native proteins were performed using the method developed by FERGUSON (1964) and refined by RODBARD and CHRAMBACH (1974), CHRAMBACH *et al.* (1976), and CHRAMBACH and RODBARD (1981). Figure 3 represents the log R_f dependency on %T for purified ADH^F, ADH^{n11}, hemoglobin A, and ferritin. Table 2 provides the parameters K_r and Y_o calculated by linear regression (PAGEPACK). The resulting 95% confidence ellipses for the estimates of K_r and Y_o for purified ADH^F, ADH^{n11}, hemoglobin A, and ferritin are shown in Figure 3. Based on this data, ADH^F, ADH^{n11} and hemoglobin A are similar in effective volumes, while ferritin is much larger. The charge to mass ratio for each protein is significantly different.

Representative electroblots of 4% and 9% acrylamide gels of crude ADH extracts are shown in Figure 4. The same immunodetectable proteins as seen with the native PAGIEF were visualized with these gels. The estimates for K_r and Y_o are shown in Table 1. The proteins differ most in their net charge (Y_o), indicative of charge isomers. The variation observed in molecular size (K_r) does not indicate differences in the number of subunits contained in each molecule. Each is capable of dimerization, and none showed any signs of forming aggregates larger than two subunits.

The K_r estimates obtained for purified ADH^F and ADH^{n11} on gels stained directly for protein correspond to the values obtained by immunodetection on protein blots. The higher mobilities found for the purified proteins result from the electrophoresis being performed at 25° rather than 4°, the temperature used for screening the crude extracts.

TABLE 2

Charge and size analysis of purified proteins

Protein	Retardation coefficient (K_r)	Free mobility (Y_o)	Correlation coefficient (R)
ADH^F	0.0574 ± 0.0014	0.4642 ± 0.0090	0.9997
ADH^{n11}	0.0533 ± 0.0018	0.6462 ± 0.0169	0.9994
Hemoglobin A	0.0581 ± 0.0010	1.1870 ± 0.0180	0.9998
Ferritin	0.2265 ± 0.0028	4.4660 ± 0.1402	0.9999

The Y_o values obtained for the mutant proteins confirm the charge differences observed in native PAGIEF, with the exception of ADH^{n1} and ADH^{n3}. The 95% confidence ellipses for ADH^{n1} and ADH^{n3} are identical, which indicates the proteins produced by these two mutants are indistinguishable (Figure 5). However, ADH^{n1} has an apparent pI identical to ADH^F, whereas ADH^{n3} has an apparent pI similar to ADH^S. The 95% confidence ellipse for these two null variants shows no significant overlap with those of the wild types. The native gels confirm that none of the immunodetectable mutants exhibit the "adduct-bound" isozymes with the exception of ADH^{n5} which is known to have residual activity at the temperatures used to rear the flies (VIGUE and SOFER 1974; and H. HOLLOCHER and A. R. PLACE, unpublished data).

DISCUSSION

The protein blot technique is a crucial step in this assay procedure. The nitrocellulose provides a permanent "hard copy" of the information in the gel and

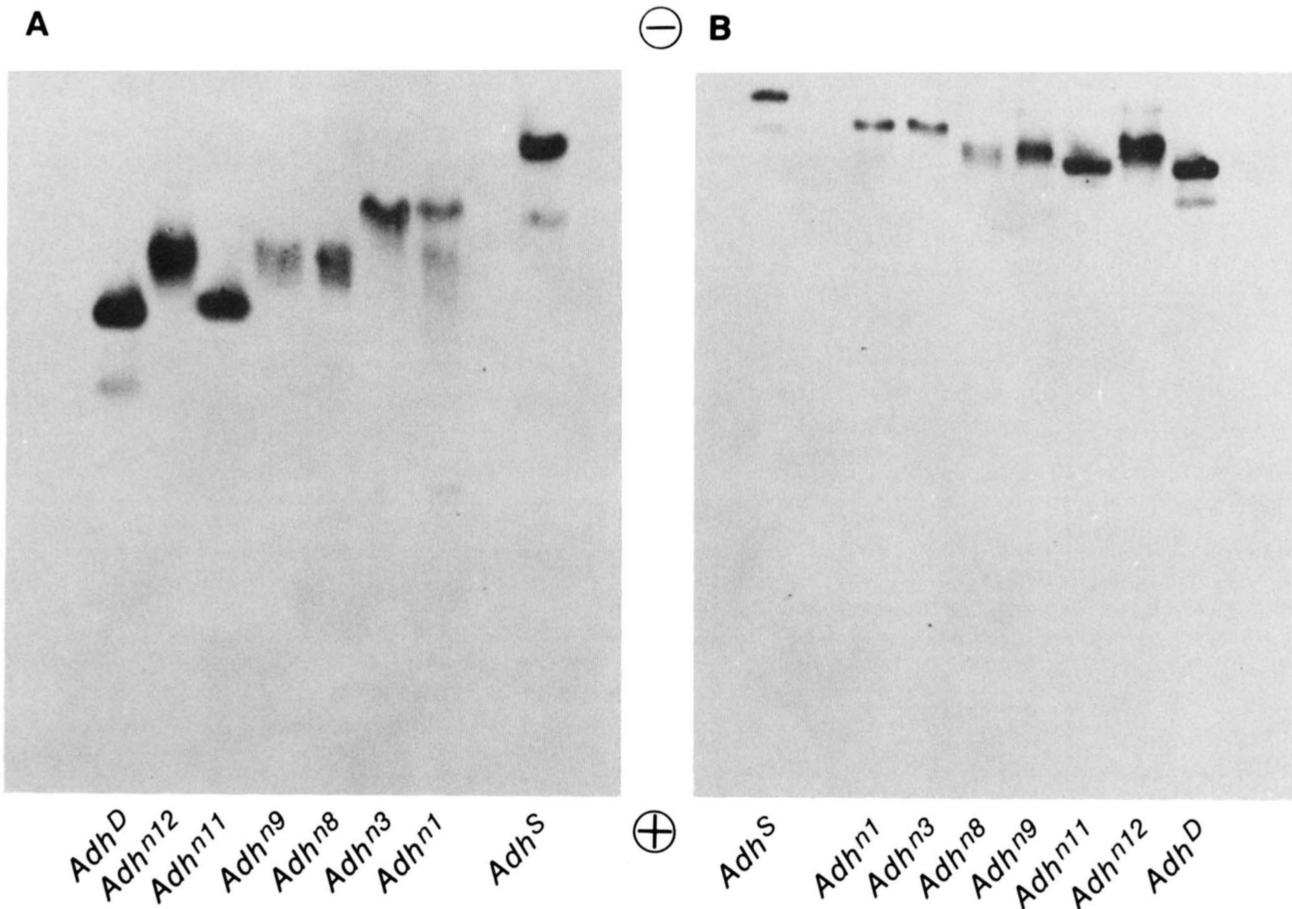

FIGURE 4.—Nitrocellulose transfers on the mutant ADH proteins after native polyacrylamide gel electrophoresis. Samples were made in Tris-H_3PO_4, (pH 6.9), containing 5 mM β-mercaptoethanol, 1 mM EDTA, 1 mg/ml α-macroglobulin, and 15% (v/v) glycerol. Gels (65 mm × 75 mm) containing various percents of acrylamide from 4% (A) to 9% (B) and constant 2.5% crosslinking were electrophoresed at 4° at 110 V until the tracking dye reached 7.0 cm. Hematin was used as a transferable tracking dye marker. Transfer and immunodetection methods are the same as those described for PAGIEF.

can be analyzed using multiple probes (RENART, REISER and STARK 1979; LEGOCKI and VERMA 1981). The electrophoretic mobility measured on the blot is an accurate representation of the original distances migrated in the gel, unlike measurements made directly on a gel which has been subject to the shrinkage and swelling associated with normal staining procedures. The immuno-overlay procedure (TOWBIN, STAEHELIN and GORDON 1979) in our hands has a lower detection limit of 1–5 ng of ADH. Since ADH comprises approximately 1–2 μg per adult fly (PELLICCIA and SOFER 1982), mutants with steady state levels as low as 5% of wild type (PELLICCIA and SOFER 1982) are within the detection range of this method.

An important advantage in using protein blotting and immunodetection is the ability to analyze crude homogenates, even when the protein of interest has no easily detectable enzymatic activity. Prefractionation, which could introduce artifacts and which might prove technically difficult because of the known decreased stability of the mutants (PELLICCIA and SOFER 1982), is unnecessary. Another advantage over other conventional staining methods is the increased sensitivity of detection with the peroxidase mediated deposition of diaminobenzidine (TOWBIN, STAEHELIN and GORDON 1979). The ease with which null variants can be screened for in natural populations is a further advantage over the conventional methods. There is also a disadvantage: some mutants will have lost their cross-reactivity with the antibody through the mutagenic process, making them difficult to detect by this means. Polyclonal antibodies are best for screening a large number of unknown protein variants.

Of all the mutants analyzed by the methods outlined above, only Adh^{n4} and Adh^{n10} proved to have no detectable ADH. These same two mutants were classified as cross-reactivity material (CMR) (−) by PELLICCIA and SOFER (1982). Solution hybridization and northern blot analysis has detected low levels of messenger RNA wild-type in length (A. R. PLACE, unpublished data). Since the major antigenic determinants are thought to lie in the first third of the molecule (A. R. PLACE, unpublished data), the defect may involve early translation termination because of a base substi-

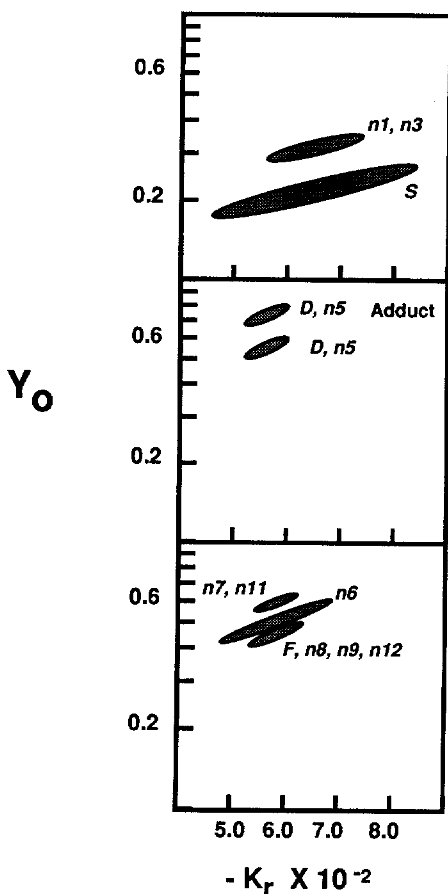

FIGURE 5.—Confidence ellipses (95%) for the K_r and Y_o estimates of ADH^F, ADH^S, ADH^D and the mutant ADH proteins derived from each one. Those ellipses having identity with one another are drawn as a single ellipse derived from a composite of the data for the individual mutants.

tution, or it may involve a frameshift mutation caused by a small deletion. Both events could cause the loss of the antigenic determinants.

The remaining mutants are detectable under SDS-PAGE and exhibit subunit polypeptide lengths equal to the wild-type length. This confirms the results of SCHWARTZ and SOFER (1976b) indicating that these mutants are most probably point mutations.

Estimated values of K_r reveal that ADH^{n1}, ADH^{n3}, ADH^{n5} through ADH^{n9}, ADH^{n11}, and ADH^{n12} are capable of dimerization. ADH^{n4} and ADH^{n10} are not detectable, as mentioned earlier. ADH^{n2} and ADH^{n13} are unstable under native conditions and could not be detected by Ferguson analysis.

The importance of using more than one technique to characterize mutant proteins is clearly evident from the results obtained for ADH^{n1} and ADH^{n3}. Estimated values of Y_o confirm the relative charge differences among all mutants determined by PAGIEF, except for these two variants. These proteins are electrophoretically identical on native PAGE with free mobilities intermediate to ADH^S and ADH^F, yet were clearly distinguishable on native PAGIEF: ADH^{n1} has a pI identical to ADH^F, and ADH^{n3} has a pI similar to ADH^S. Both mutants were derived from ADH^S (GRELL, JACOBSON and MURPHY 1968). Since electrophoretic mobility is dependent on both the charge to surface area ratio and the effective volume of a protein, the discrepancy between Y_o and pI can be attributed to differences in conformation. For example, ADH^{n1} might have a looser conformation and hence, a larger effective surface area. This would be manifested by a slower electrophoretic mobility.

PELLICCIA and SOFER (1982) have estimated the *in vivo* steady state levels of ADH^{n2} and ADH^{n13} CRM to be only 5% of wild-type levels. Since the rates of synthesis are equivalent, PELLICCIA and SOFER (1982) attribute the lower *in vivo* steady state levels to a greater degradation rate. It has been suggested that molecules of smaller molecular weights are more susceptible to degradation (BUKARI and ZIPSER 1973; GARFINKLE and TERSHAK 1972; KUEHL and SCHARFF 1974). The lack of detectable material under native conditions can be explained if these two mutants exist primarily as monomers, and are thus more easily degraded. Therefore, we believe that the two mutant proteins, ADH^{n2} and ADH^{n13}, do not form stable heterodimers.

Two popular generalizations about protein turnover can be addressed using the data obtained for the nulls. By examining the pIs for the nulls in conjunction with the percent CRM (PELLICCIA and SOFER 1982), it can be seen that there is no direct correlation between increased turnover and decreasing pI as first proposed by DICE and GOLDBERG (1975). ADH^{n5}, ADH^{n7}, and ADH^{n11} have the lowest pIs and have 4%, 54%, and 21% CRM, respectively. ADH^{n2}, ADH^{n3}, and ADH^{n13} have the highest pIs, and have 5%, 15%, and 5% CRM, respectively. This finding is also supported by recent studies (ANDERSON and MCDONALD 1981b) which showed that ADH^F is found in higher steady state levels than ADH^S at different developmental stages, even though ADH^F has a more acidic pI than ADH^S.

The "error-catastrophe" hypothesis of ORGEL (1963, 1973) postulates the selective removal of abnormal proteins from the total protein pool. Since all the mutants except ADH^{n5} are inactive, we defined "abnormality" as the deviation from the charge of the parental strain used in the mutagenic process. Amino acid substitutions that resulted in no charge change from the parental type have *in vivo* steady state levels ranging from 4% CRM for ADH^{n5} to 73% CRM for ADH^{n12}. ADH^{n1}, ADH^{n7}, and ADH^{n11} deviate from their respective parental strains by a charge of approximately -1, and have CRM of 20%, 54%, and 21%, in that order (PELLICCIA and SOFER 1982). From these two observations, the selective recognition and removal of aberrant proteins is not obvious. It may be

TABLE 3

Expected percentage of charge classes resulting from amino acid substitutions in the *Adh* gene of Drosophila

Result of substitution	All codons	*Adh* gene		
		All possible changes	Only G ⇒ A and C ⇒ T	Only A ⇒ G and T ⇒ C
No change	24.96	24.96	50.00	16.05
No charge change	42.36 (55.71)	47.87 (63.79)	33.63 (67.26)	60.19 (71.69)
Single acidic	12.15 (15.98)	10.07 (13.42)	6.28 (12.56)	8.95 (10.66)
Double acidic	0.69 (0.91)	0.95 (1.27)	0	5.56 (6.62)
Single basic	12.15 (15.98)	11.89 (15.84)	3.81 (7.62)	8.95 (10.66)
Double basic	0.69 (0.91)	0.95 (1.27)	2.24 (4.48)	0
Terminator	3.99 (5.25)	2.99 (3.99)	3.81 (7.62)	0
Antiterminator	3.99 (5.25)	0.30 (0.40)	0.22 (0.45)	0.31 (0.37)
Total changes	576	2304	446	324

The first column represents the percentages based on the codons available in the genetic code. The last three columns contain the percentages based on the coding strand codons contained in the *Adh* gene. The values in parentheses are the percentages obtained after subtracting the codon substitutions that result in redundant coding. The start codon ATG was not subject to a substitution event. G to A and C to T changes (column 3) represent all possible G to A changes for the *Adh* gene occurring on either strand. A to G and T to C changes (column 4) represent all possible A to G changes for the *Adh* gene occurring on either strand.

that slight conformational changes independent of charge deviations are involved in the recognition process.

A common attribute of the immunodetectable mutants which may help explain their lower *in vivo* stabilities is their inability to form the "adduct-bound" isozyme characteristic of the *D. melanogaster* ADH (JOHNSON and DENNISTON 1964; URSPRUNG and LEONE 1965; JACOBSON, MURPHY and HARTMANN 1970; JACOBSON *et al.* 1972; KNOPP and JACOBSON 1972; SCHWARTZ *et al.* 1976; SCHWARTZ and SOFER 1976b; SCHWARTZ, O'DONNELL and SOFER 1979; PAPEL *et al.* 1979; WINBERG, THATCHER and MCKINLEY-MCKEE 1983). These "adduct-bound" isozymes are more stable than the apoenzyme both *in vivo* (ANDERSON and MCDONALD 1981a) and *in vitro* (JACOBSON 1968). Hence, the lower steady state levels may result from the mutant proteins not being able to shift between the apoenzyme and the more stable "adduct-bound" isozyme, rather than representing an intrinsic instability of the polypeptides themselves. The one exception is ADH^{n5} which has been characterized as a temperature sensitive mutant protein (VIGUE and SOFER 1974) and which does form the "adduct-bound" isozymes (Figure 2). In this case, it has been determined that the polypeptide made by ADH^{n5} is intrinsically less stable than that of the wild-type (THATCHER and SHEIKH 1981). It appears that ADH activity is coupled with the ability to form the "adduct-bound" isozyme in *D. melanogaster,* since all inactive protein variants that have been isolated do not form the "adduct-bound" isozymes.

The last of our objectives was to determine whether G:C to A:T transitions, common in EMS mutagenesis (PRAKASH and SHERMAN 1973; COULONDRE and MILLER 1977; MILLER 1983), are consistent with the frequency of observed charge alterations in the ADH null-activity variants. The first column in Table 3 displays the expected frequency of the different charge classes resulting from amino acid substitution for all possible changes in each codon. These frequencies are similar to those obtained by MARSHALL and BROWN (1975) with the "no change" and "no charge change" classes being the most abundant, followed by "acidic" and "basic" classes having approximately equally expected frequencies. Two other classes of amino acid substitutions, the "terminators" and "antiterminators," are the least expected.

Since the mutants were selected on basis of having no ADH activity (GRELL, JACOBSON and MURPHY 1968; GERACE and SOFER 1972), those single base substitutions which result in "no change," hence retaining activity, are not relevant. By eliminating the "no change" class from the evaluation, the expected percentages increase to approximately 56% for "no charge change," 16% each for "basic" and "acidic," and 5% each for "terminators" and "antiterminators."

If we now take into account the codon usage for the *Adh* gene, the expected percentages differ slightly from those obtained with the entire DNA code (column 2, Table 3), with the number of "no charge changes" increasing at the expense of "acidics" and "basics." In addition the expected frequency of "antiterminators" is greatly reduced.

For the ADH null-activity mutant proteins, the percent "no charge changes" is 54%, the percent "single acidics" is 23%, and the percent "single basics" is 8%. These percentages are based on the comparison between the pI values of the variants and the pIs of the strains from which they were derived (*i.e.*, ADH^S, ADH^F and ADH^D). If the two mutants not immunodetectable are the result of early translation termination, the percent "terminators" observed is 15%. There were no "antiterminators" detected, though

not an unexpected finding if translation anti-terminators are most likely to produce slightly longer, yet still functional proteins. The selection scheme used would have eliminated them. If we compare these percentages with the percentages expected for all possible single base changes (column 2 of Table 3) there is an excess of observed acidic changes. Moreover only 3–4% of single base changes would give early termination as compared to the nearly 15% observed. The percentages expected for all possible A:G transitions also predict equal acidic and basic changes and no early terminator mutants. Only with G:A transitions are acidic mutants more probable than basic mutations. The percent termination mutants is also higher. Hence the data for the ADH nulls is consistent with the observed trend for EMS mutagenesis to cause G:A transitions.

Work by SCHWARTZ (1981) for ADH in maize showed equal numbers of acidic and basic amino acid substitutions. However, SCHWARTZ found a difference in the step charge change between the acidic and basic type substitutions. Fourteen out of the 16 basic substitutions resulted in +2 charge changes while all of the acidic substitutions were −1 charge changes. This observation could not be explained based on the codon frequency of the maize ADH gene, since it was determined that single G-A changes in only two codons could give the observed two-step basic change and that these 14 basic mutants did not represent identical substitutions (SCHWARTZ 1981). It should be noted that we did not observe any two-step charge alterations in the Drosophila ADH mutants. It is difficult at this stage to describe the consequences of EMS mutagenesis in either Drosophila or maize without DNA sequencing. Our current efforts are directed toward obtaining these data.

The utility of the approach we have adopted in this study for characterizing the ADH null-activity mutants is clearly evident. Based on the various physical parameters determined, the 13 mutants can be classified into nine distinct groups, six of which (Adh^{n1}, Adh^{n3}, Adh^{n5}, Adh^{n6}, Adh^{n7} and Adh^{n11}) represent unique mutations. The remaining three groups (Adh^{n2} and Adh^{n13}; Adh^{n4} and Adh^{n10}; Adh^{n8}, Adh^{n9} and Adh^{n12}) contain members which are indistinguishable from each other. It is possible to perform these studies with single fly extracts and with mutants whose steady state levels are only 5% that of wild-type levels. The ability to form competent oligomers is easily determined as is the charge alteration. Although slightly more complex than typical large scale screening procedures, these methods could be applied to analyzing nulls found in natural populations. In our case, these procedures have helped describe the effect of each mutation on the ADH enzyme. Once the amino replacement is determined for each mutant, we can begin to catalog residues important in catalysis.

We thank W. SOFER for the mutant *Adh* Drosophila strains. We also thank A. CHRAMBACH for generously supplying the PAGE-PACK programs and advice on selecting an appropriate gel system for Ferguson analysis. This research was supported by the National Science Foundation (PCM81-10819).

LITERATURE CITED

ANDERSON, S. M. and J. F. MCDONALD, 1981a Effect of environmental alcohol on *in vivo* properties of *Drosophila* alcohol dehydrogenase. Biochem. Genet. **19:** 421–430.

ANDERSON, S. M. and J. F. MCDONALD, 1981b Changes in the levels of alcohol dehydrogenase during the development of *Drosophila melanogaster.* Can. J. Genet. Cytol. **23:** 305–313.

BARINGA, M., R. FRANCO, J. MEINKOTH, E. ONG and G. M. WAHL, 1981 Methods for the transfer of DNA, RNA, and protein to nitrocellulose and diazotized paper solid supports. In: *Schleicher and Schuell Instruction Manual.* Schleicher and Schuell, Keene, New Hampshire.

BITTNER, M., P. KUPFERER and C. F. MORRIS 1980 Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. Anal. Biochem. **102:** 459–471.

BUKARI, A. I. and D. ZIPSER, 1973 Mutant of *Escherichia coli* with a defect in the degradation of nonsense fragments. Nature **243:** 238–240.

CHRAMBACH, A. and D. RODBARD, 1981 "Quantitative" and preparative polyacrylamide gel electrophoresis. pp. 93–143. In: *Gel Electrophoresis of Proteins,* Edited by B. D. HAMES and D. RICKWOOD. IRL Press, Oxford, England.

CHRAMBACH, A., T. M. JOVIN, P. J. SVENDSEN and D. RODBARD, 1976 Analytical and preparative polyacrylamide gel electrophoresis: an objectively defined fractionation route, apparatus, and procedures. In: *Method of Protein Separation,* Vol. 2, Edited by N. CATSIMPOOLAS. Plenum, New York.

COULONDRE, C. and J. H. MILLER, 1977 Genetic studies of the Lac repressor. IV. Mutagenic specificity in the Lac I gene of *Escherichia coli.* J. Mol. Biol. **117:** 577–606.

CREIGHTON, T. E., 1979 Electrophoretic analysis of the unfolding of proteins by urea. J. Mol. Biol. **129:** 235–264.

DEBLAS, A. L. and H. M. CHERWINSKI, 1983 Detection of antigens on nitrocellulose paper immunoblots with monoclonal antibodies. Anal. Biochem. **133:** 214–219.

DICE, J. F. and A. L. GOLDBERG, 1975 Relationship between *in vivo* degradative rates and isoelectric points of proteins. Proc. Natl. Acad. Sci. USA **72:** 3893–3897.

FERGUSON, K. A., 1964 Starch-gel electrophoresis-applications to the classification of pituitary proteins and polypeptides. Metabolism **13:** 985–1002.

GARFINKLE, B. D. and D. R. TERSHAK, 1972 Degradation of poliovirus polypeptide *in vivo.* Nature **243:** 238–239.

GERACE, L. and W. SOFER, 1972 Adh^{n}: alcohol dehydrogenase negative mutants. Drosophila Inform. Serv. **49:** 39.

GOLDENBERG, D. P. and T. E. CREIGHTON, 1984 Gel electrophoresis in studies of protein conformation and folding. Anal. Biochem. **138:** 441–445.

GRELL, E. H., K. B. JACOBSON and J. B. MURPHY, 1968 Alterations of genetic material for analysis of alcohol dehydrogenase isozymes of *Drosophila melanogaster.* Ann. NY Acad. Sci. **151:** 441–445.

HICKMAN, B. J., N. L. ANDERSON, K. E. WILLARD and N. G. ANDERSON, 1979 Internal charge standardization for two dimensional electrophoresis. pp. 341–350. In: *Electrophoresis '79,* Edited by B. J. RADOLA. Walter de Gruyter Press, New York.

JACOBSON, K. B., 1968 Alcohol dehydrogenase of *Drosophila*: interconversion of isozymes. Science **159:** 324–325.

JACOBSON, K. B., J. B. MURPHY and F. C. HARTMANN, 1970 Isoenzymes of *Drosophila* alcohol dehydrogenase. I.

Isolation and interconversion of different forms. J. Biol. Chem. **245:** 1075–1083.

JACOBSON, K. B., J. B. MURPHY, J. A. KNOPP and J. R. ORTIZ, 1972 Multiple forms of *Drosophila* alcohol dehydrogenase. III. Conversion of one form to another by nicotinamide adenine dinucleotide or acetone. Arch. Biochem. Biophys. **149:** 22–35.

JOHNSON, F. M. and C. DENNISTON, 1964 Genetic variation of alcohol dehydrogenase in *Drosophila melanogaster.* Nature **204:** 906–907.

JOVIN, T. M., M. L. DANTE and A. CHRAMBACH, 1970 Multiphasic buffer systems catalogue. National Technical Information Service, Springfield, Va.

KNOPP, J. A. and K. B. JACOBSON, 1972 Multiple forms of Drosophila alcohol dehydrogenase. IV. Protein fluorescence studies. Arch. Biochem. Biophys. **148:** 36–41.

Kuehl, W. M. and M. D. SCHARFF, 1974 Synthesis of a carboxyl-terminal (constant region) fragment of the immunoglobin light chain by a mouse myeloma cell line. J. Mol. Biol. **89:** 409–421.

LEGOCKI, R. P. and D. P. S. VERMA, 1981 Multiple immunoreplica technique: screening for specific proteins with a series of different antibodies using one polyacrylamide gel. Anal. Biochem. **111:** 385–392.

LINDSLEY, D. L. and E. H. GRELL, 1967 Genetic variation of *Drosophila melanogaster.* Carnegie Inst. Wash. Publ. 627.

MARSHALL, D. R. and A. H. D. BROWN, 1975 The charge-state model of protein polymorphism in natural populations. J. Mol. Evol. **6:** 149–163.

MARTIN, P., A. R. PLACE, E. PENTZ and W. SOFER, 1985 UGA nonsense mutation in the alcohol dehydrogenase gene of *Drosophila melanogaster.* J. Mol. Biol. **184:** 221–229.

MILLER, J. H., 1983 Mutational specificity in bacteria. Annu. Rev. Genet. **17:** 215–238.

O'FARRELL, P. H., 1975 High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **10:** 4007–4021.

ORGEL, L. E., 1963 The maintenance of the accuracy of protein synthesis and its relevance to aging. Proc. Natl. Acad. Sci. USA **49:** 517–521.

ORGEL, L. E., 1973 Aging of clones of mammalian cells. Nature **243:** 441.

PAPEL, I., M. HENDERSON, J. VANHERREWEGE, J. DAVID and W. SOFER, 1979 *Drosophila* alcohol dehydrogenase activity *in vitro* and *in vivo*: effects of acetone feeding. Biochem. Genet. **17:** 553–563.

PELLICCIA, J. and W. SOFER, 1982 Synthesis and degradation of alcohol dehydrogenase in wild type and ADH-null activity mutants of *D. melanogaster.* Biochem. Genet. **20:** 297–313.

PRAKASH, L. and F. SHERMAN, 1973 Mutagenic specificity: reversion of iso-1-cytochrome *c* mutants of yeast. J. Mol. Biol. **79:** 65–82.

RENART, J., J. REISER and G. R. STARK, 1979 Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. Proc. Natl. Acad. Sci. USA **76:** 3116–3120.

RETZIOS, A. D. and D. R. THATCHER, 1979 Chemical basis of the electrophoretic variation observed at the alcohol dehydrogenase locus of *Drosophila melanogaster.* Biochimie **61:** 701–704.

RODBARD, D. and A. CHRAMBACH, A. 1974 Quantitative polyacrylamide gel electrophoresis: mathematical and statistical analysis of data. pp. 28–62. In: *Electrophoresis and Isoelectric Focusing in Polyacrylamide*, Edited by R. C. Allen and H. R. MAURER. Walter de Gruyter, New York.

SCHWARTZ, D., 1981 ADH locus in maize for detection of mutagens in the environment. *Environ. Health Prospect.* **37:** 75–77.

SCHWARTZ, M. F. and H. JORNVALL, 1976 Structural analyses of mutant and wild-type alcohol dehydrogenases from *Drosophila melanogaster.* Eur. J. Biochem. **68:** 159–168.

SCHWARTZ, M. and W. SOFER, 1976a Diet-induced alteration in distribution of multiple forms of alcohol dehydrogenase in *Drosophila.* Nature **263:** 129–131.

SCHWARTZ, M. and W. SOFER, 1976b Alcohol dehydrogenase-negative mutants in *Drosophila*: defects at the structural locus? Genetics **83:** 125–136.

SCHWARTZ, M., J. O'DONNELL and W. SOFER, 1979 The origin of multiple forms of alcohol dehydrogenase from *Drosophila melanogaster.* Arch. Biochem. Biophys. **194:** 365–378.

SCHWARTZ, M., L. GERACE, J. O'DONNELL and W. SOFER, 1976 Drosophila alcohol dehydrogenase: origin of the multiple forms. pp. 725–751. In: *Vol. I, Isozymes: Molecular Structure* (Third International Conference on Isozymes). Academic Press, New York.

SPIELMAN, H., R. P. ERICKSON and C. J. EPSTEIN, 1974 The production of antibodies against mammalian LDH-1. Anal. Biochem. **59:** 462–467.

THATCHER, D. R., 1980 The complete amino acid sequence of three alcohol dehydrogenase alleloenzymes (Adh^{n11}, Adh^{S} and Adh^{UF}) from the fruitfly *Drosophila.* Biochem. J. **187:** 875–886.

THATCHER, D. R. and R. SHEIKH, 1981 The relative stability of the alcohol dehydrogenase alleloenzymes of the fruitfly *Drosophila melanogaster.* Biochem. J. **197:** 111–117.

TOWBIN, H., T. STAEHELIN and J. GORDON, 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA **76:** 4350–4354.

URSPRUNG, H. and J. LEONE, 1965 Alcohol dehydrogenases: a polymorphism in *Drosophila melanogaster.* J. Exp. Zool. **160:** 147–154.

VIGUE, C. and W. SOFER, 1974 Adh^{n5}: a temperature-sensitive mutant at the ADH locus in *Drosophila.* Biochem. Genet. **11:** 387–396.

WINBERG, J.-O., D. R. THATCHER and J. S. MCKINLEY-MCKEE, 1983 *Drosophila melanogaster* alcohol dehydrogenase: an electrophoretic study of Adh^{S}, Adh^{F}, and Adh^{UF} allelozymes. Biochem. Genet. **21:** 63–80.

Communicating editor: W. J. EWENS