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Conditional Overdominance at an Alcohol Dehydrogenase Locus in Yeast

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ABSTRACT

Documented examples of heterosis attributable to overdominance at specific protein-encoding gene loci have rarely been reported, the association of sickle cell hemoglobin with malarial resistance being the best documented example of this phenomenon. Here we report an example of overdominance that is temperature- and allyl alcohol-dependent and due to heterozygosity at the *ADH1* locus, involving two ADHI functional mutants. Overdominance appears to be due in part to an intermediate level of ADHI activity in the heterozygote. Unlike previous work with this system using haploid strains, the NAD^+/NADH ratios show no negative correlation with allyl alcohol resistance. This system is formally equivalent to that of sickle cell hemoglobin and shows promise as a tool for investigating the physiological basis for overdominance.

THE reasons for the widespread occurrence of allelic enzyme polymorphisms in natural populations have been debated for decades and alternatively attributed either to some kind of overdominance of alleles in heterozygous condition (LEWONTIN 1974; WALLACE 1986) or to the random fixation of selectively neutral mutations (KIMURA 1983). Single gene overdominance, in which an individual heterozygous for a structural gene (such as an enzyme locus) possesses an unequivocally higher fitness than either homozygote under a particular set of environmental conditions, has rarely been documented. Hemoglobin S is the most-cited example of this phenomenon. Although other systems are likely candidates, particularly in man (WILLS 1981), their molecular basis is not as well established or understood. This paucity of known, well-documented examples has been a key argument against its playing a significant role in population adaptation. Heterozygotes for virtually all of the well-characterized allelic enzyme polymorphisms exhibit the expected functional or kinetic intermediacy rather than overdominance. As a consequence this explanation for the maintenance of enzyme polymorphisms has become less frequently invoked, and population geneticists with a selectionist bent have resorted to mechanisms involving marginal overdominance to explain protein polymorphisms. In recent years increasing reports of significant positive correlations between heterozygosity at enzyme loci and aspects of the phenotype related to fitness (MITTON and GRANT 1984) have renewed interest in the role protein polymorphisms *per se* play in the adaptation of populations. Unfortunately, there has been no experimentally manipulable system available to investigate overdominance and its metabolic consequences.

Several laboratories have developed model systems using microorganisms for investigating the consequences of "experimental evolution" at the molecular level. These systems have been useful for examining how the metabolic machinery of cells may be altered by selection pressure for the utilization of novel carbon sources. The mutational responses to selection in such experiments are quite diverse. Constitutive synthesis of a normally repressed enzyme with a poor specificity for a novel substrate can enhance the utilization of that substrate, as in the ribitol dehydrogenase exploitation of xylitol in *Klebsiella aerogenes* (*e.g.*, LIN, HACKING and AGUILAR 1976) or the utilization of novel amides by *Pseudomonas aeruginosa* (CLARKE 1974, 1984). Other responses include increasing the permease activity for the transport of poorly utilized substrates into the cell to increase its utilization (ribitol dehydrogenase) (LIN, HACKING and AGUILAR 1976; WU, LIN and TANAKA 1968), gene duplication and amplification to increase enzyme activity (ribitol dehydrogenase) (RIGBY, BURLEIGH and HARTLEY 1974; HARTLEY 1984); *Escherichia coli* β-galactosidase (HORIUCHI, HORIUCHI and NOVICK 1963), "mobilization" of enzymes from preexisting pathways (propanediol metabolism by *E. coli*) (LIN and WU 1984), and the expression of previously unexpressed or "silent" genes, coding for enzymes already showing partial activity for the new substrate ("evolved" β-galactosidase in *E. coli*) (HALL 1983). Mutations in the structural genes that enhance the specificity of such enzymes toward the novel substrates almost always arise once the enzymes have become expressed.

Selection for allyl alcohol resistance in respiratory-deficient *Saccharomyces cerevisiae* strains also yields

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functional enzyme mutants (WILLS 1984), but unlike the other experimental systems mentioned above the target of selection is remarkably specific for *ADH1*, the gene coding for the alcohol dehydrogenase isozyme, ADHI (CIRIACY 1975). A tetrameric enzyme and one of two isozymes located in the cytoplasm, ADHI is largely constitutively expressed and functions primarily in glucose fermentation (BRANDEN *et al.* 1975). The other isozyme, ADHII, is repressed or noninduced in the presence of glucose and in anaerobically-grown or petite cells and is thought to function primarily in the aerobic oxidation of ethanol (WILLS 1976). Yeast strains having no cytoplasmic ADH activity (either ADHI or ADHII) cannot survive anaerobically or as petite phenotypes, where respiration is genetically (ρ^- strains) or pharmacologically (*e.g.*, with Antimycin A) blocked (WILLS and PHELPS 1975), since this enzyme is largely responsible for regenerating NAD^+ in glycolysis (SOLS, GANCEDO and DELAFUENTE 1969). This fact was exploited for the selection of functional mutants at the *ADH1* locus (WILLS and PHELPS 1975; WILLS 1976). Yeast ADH oxidizes allyl alcohol (2-propen-1-ol) to acrolein (2-propenal), a toxic aldehyde known to stop growth and to kill the cell (RANDO 1974). The only petite strains capable of growth in media containing allyl alcohol are those that reduce the rate of acrolein production or minimize its toxic effects without the concomitant loss of cytoplasmic ADH activity. Allyl alcohol resistant mutants typically arise at frequencies between 10^{-7} and 10^{-9} in such selection experiments depending on specific conditions. A large proportion (usually >40%) of such mutants are structural changes in ADHI that retain catalytic function (WILLS 1976; WILLS and JORNVALL 1979).

Functional ADHI mutants have recently been isolated in which allyl alcohol resistance is conditional upon the temperature of growth (HALL and WILLS 1987). Several mutants exhibit allyl alcohol resistance that is significantly reduced at either high (37°) or low (19°) temperatures. These mutants are not merely thermostability mutants, since the temperature-conditional mutants grow well at both temperatures in the absence of allyl alcohol. Here we report a case of conditional overdominance when two of these temperature-conditional *ADH1* functional mutants occur together in heterozygous condition.

MATERIALS AND METHODS

Strains and growth conditions: The parental strains and experimental conditions were as described in HALL and WILLS (1987). Complete YEPD medium (1% yeast extract, 2% bactopeptone, and 2% dextrose, all w/v from Difco) to which 1 ppm Antimycin A and either 2% ethanol or 0.04% acetaldehyde had been added following autoclaving were used in all selection and growth experiments. These media were also supplemented with 0–10 mM allyl alcohol. Haploid *ADH1* (ρ^-) mutant strains were backcrossed twice to the parental grande strain of S288C from which the ρ^- strains and the *ADH1* mutants were originally derived, and the various diploids were constructed from wild-type and mutant haploids. To test the effects of gene dosage on allyl alcohol resistance, diploid strains were constructed by crossing mutants and S288C wild-type to a cytoplasmic ADH-negative strain (*adh1*, *adh2*), which also had been backcrossed twice to S288C. Clonal growth of individually isolated cells on agar slabs was monitored as previously described (HALL and WILLS 1987). The presence of 1 ppm Antimycin A in the media for these experiments converted all strains to phenotypic petites (*i.e.*, respiratory incompetent, repressed ADHII). Consequently, all energy for growth and other cellular processes was derived from the fermentation of glucose under these conditions.

NAD^+/NADH ratios: Levels of NAD^+ and NADH in yeast cells were determined by a modification of the method of LILIUS, MULTANEN and TOIVONEN (1979). Cultures were inoculated into 5 ml of YEPD medium to which filter-sterilized 2% v/v ethanol and 1 ppm Antimycin A had been added following autoclaving. Cultures were grown 24 hr at 30°, reinoculated into the same medium at 37°, grown overnight at this temperature, diluted to $\sim 5 \times 10^6$ cells/ml in fresh medium in 1-ml aliquots, and shaken at 37° for 2 hr. NAD^+ and NADH were immediately extracted at 70° in 0.1 N HCl or KOH, respectively, for 10 min. Standard curves for the oxidized and reduced pyridine nucleotides, performed identically and simultaneously with the experimental samples, were used to determine the respective concentrations of the latter. NAD^+ and NADH concentrations were determined simultaneously for each sample. The NAD^+/NADH ratios were calculated separately for each sample.

Enzyme activities: Yeast cells, grown for 48 hr at 37° in 100 ml liquid medium, containing 2% ethanol and 1 ppm Antimycin A, were broken open and homogenized, as previously described (HALL and WILLS 1987). Cytosolic supernatants were obtained by centrifugation of extracts at $10{,}000 \times g$ for 2 min and then appropriately diluted in 0.1 M potassium phosphate buffer, pH 7.0 at 20°. Enzyme activities were determined in 7.5 mM NAD^+, 300 mM ethanol, and 100 mM potassium phosphate, pH 7.0, and are expressed per mg cytosolic protein. ADHII activities were repressed with the growth conditions employed in this study, and the mitochondrial enzyme (ADHM) activity was extremely low in cell extracts. Consequently, ADH specific activities represented primarily those of the ADHI isozymes. Protein concentrations were determined by the method of LOWRY *et al.* (1951).

RESULTS AND DISCUSSION

Two of the temperature-conditional mutants described by HALL and WILLS (1987), EAA5-19II-S6 and EAA5-37II-S7, exhibit ADHI electrophoretic mobilities on starch gels that are 96% and 80% that of the parental wild-type enzyme. As haploids these two *ADH1* mutants are resistant to 10 mM allyl alcohol at 19° but not at 37°. The meiotic products of crosses of these mutants to S288C co-segregate 2:2 with respect to allyl alcohol resistance and altered electrophoretic mobility in 31 and 32 tetrads, respectively, when the segregants are grown on agar plates of complete medium, supplemented with 2% ethanol, 1

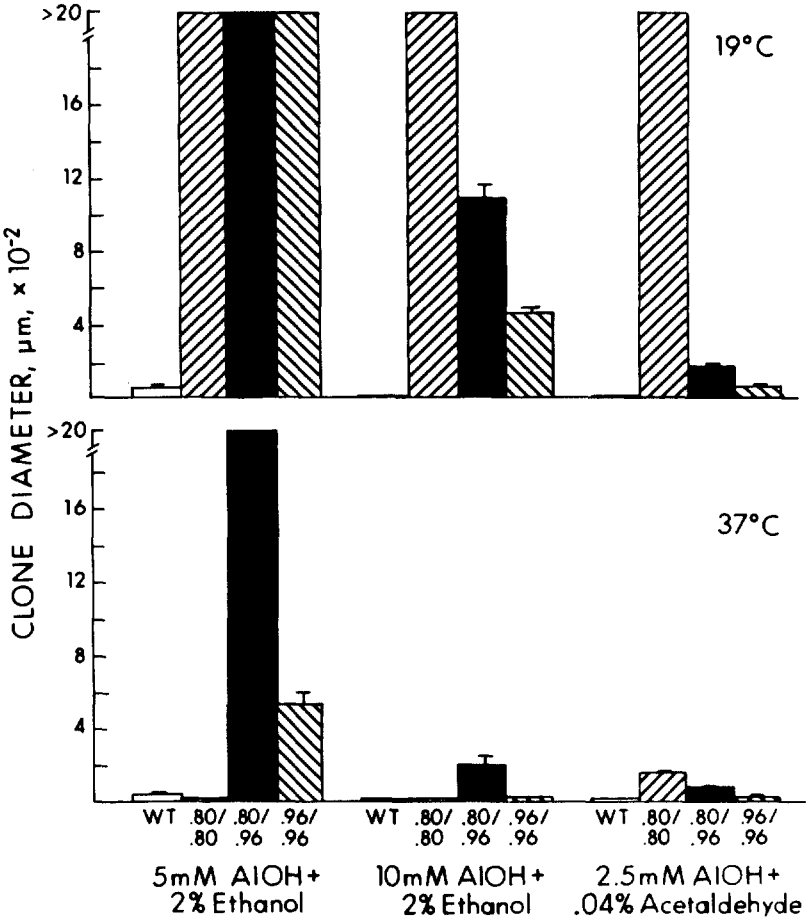

FIGURE 1.—Asymptotic clone diameters (μm ± SEM) for yeast strains homozygous and heterozygous for *ADH1* mutants 19II-S6 (0.96) and 37II-S7 (0.80). Clones of all strains grow to final diameters >2000 μm in the absence of added allyl alcohol.

ppm Antimycin A, and 5 or 10 mM allyl alcohol. These data indicate that the allyl alcohol resistance is a single gene trait and that the mutations are within 1.5 cM of the ADHI structural gene and probably coincident with it. The meiotic segregants of homozygous and heterozygous diploids constructed from these ADHI mutants are all allyl alcohol resistant (>40 tetrads, respectively).

The diploid heterozygotes for the two *ADH1* mutants—hereafter referred to as 0.96 and 0.80, based on their relative electrophoretic mobilities—are overdominant in terms of their resistance to 5 or 10 mM allyl alcohol (Figure 1). The clonal growth kinetics are somewhat complex; however the overall resistance of both haploid and diploid strains may be represented accurately by the asymptotic diameter at which growth ceases for the clone (HALL and WILLS 1987). Overdominance is conditional on the presence of 2% ethanol in the medium and is apparent only at 37° (Figure 1). These two mutants are co-dominant in resistance at 19° and when 0.04% acetaldehyde is substituted for ethanol in the growth medium, 0.96 is dominant to 0.80 at 19° and is co-dominant at 37° (2.5 mM allyl alcohol).

Earlier experiments with haploid strains have shown that allyl alcohol resistance mutants have lower $NAD^+/NADH$ ratios than wild-type strains and that both resistance to allyl alcohol and this ratio may be coordinately manipulated through the addition of ADH substrates (either ethanol or acetaldehyde) to the growth media (WILLS and PHELPS 1978; WILLS and MARTIN 1980). ADH-mediated lowering of this ratio has been implicated as a possible mechanism for conferring resistance by means of reducing the equilibrium concentration of acrolein (WILLS 1984). No such relationship is apparent with these diploid strains; in fact, the more resistant heterozygote exhibits an apparent *higher* ratio (Table 1). The apparent inconsistency in $NAD^+/NADH$ ratio patterns between earlier and the present work might be attributable to the use of diploid strains in the present work or to the higher growth temperature employed in the present study to determine ratios under environmental conditions in which the strains exhibit overdominance. The variation in the ratios for these diploid strains is much less than that reported for haploid strains (WILLS and MARTIN 1980) and may possibly be a consequence of diploidy. An alternative explanation is that the favored amino acid substitutions may be those that increase the specificity of ADHI for ethanol relative to allyl alcohol, thereby decreasing the accumulation of acrolein while incidentally lowering the

TABLE 1

NAD$^+$ and NADH concentrations and NAD$^+$/NADH ratios for strains homozygous and heterozygous for *ADH1* mutants, 19II-S6 (0.96) and 37II-S7 (0.80)

Diploid Strain	[NAD$^+$]	[NADH]	NAD$^+$/NADH*
0.80/0.80	9.00 ± 0.39	11.48 ± 0.62	0.79 ± 0.040
0.96/0.96	8.24 ± 0.12	10.35 ± 0.70	0.81 ± 0.040
0.80/0.96	8.46 ± 0.25	9.09 ± 0.27	0.93 ± 0.016
0.96/null	10.89 ± 1.18	11.68 ± 1.19	0.93 ± 0.028
0.80/null	9.11 ± 0.13	12.30 ± 0.97	0.76 ± 0.057
WT/null	9.07 ± 0.19	11.74 ± 1.15	0.82 ± 0.112
WT/WT	8.25 ± 0.13	12.01 ± 1.12	0.73 ± 0.212

* Kruskal-Wallis test on NAD$^+$/NADH ratios for all seven genotypes indicates significant heterogeneity among genotypes ($P < 0.05$). NAD$^+$ and NADH concentrations (nmol/10^6 cells ± SEM) were determined by a modification of the method of LILIUS, MULTANEN and TOIVONEN (1979). For concentrations and ratios, $N = 6$.

NAD$^+$/NADH ratio. Both the NAD$^+$/NADH ratios and the sensitivity to allyl alcohol increase for petites with either wild-type or mutant ADHIs, when low concentrations of acetaldehyde are added to the medium. This might be attributed to acetaldehyde driving the ADH reaction towards ethanol and NAD$^+$. Higher levels of NAD$^+$ would imply higher steady state levels of the ADH-NAD$^+$ binary complex, which would lead to higher allyl alcohol oxidation rates. The fact that the addition of 0.04% acetaldehyde to growth medium in the absence of allyl alcohol stimulates the growth rates of mutant ADHI strains to wild-type levels (HALL and WILLS 1987) implies that NAD$^+$ availability limits growth in the mutant strains.

Overdominance due to ADHI heterozygosity could be due either to a functional interaction between dissimilar subunits in the heterotetramer or, alternatively, to a requirement for an intermediate level of enzyme activity because of competing functional requirements (*i.e.*, retaining ADH activity *vs.* minimizing acrolein toxicity). To examine the latter possibility wild-type and mutant strains heterozygous for a null mutant of ADHI (*adh1*) were constructed. As expected these strains exhibit approximately half the specific activities of the corresponding homozygotes. The 0.96 allele is also codominant with the 0.80 allele in this respect in the heterozygote (Table 2). Since the other cytoplasmic isozyme, ADHII, is repressed in petite phenotypes and since the activity of the mitochondrial isozyme is very low in cytoplasmic cell extracts—the specific enzyme activities reported in Table 2 almost entirely represent those of ADHI. Growth experiments with these strains indicate that the dosage of ADH-I specific activity may be responsible in part for overdominance in this system (Figure 2). The 0.96/null and WT/null heterozygotes are more resistant than the corresponding homozygotes at either the high or low temperature, the null/null

TABLE 2

Specific activities of alcohol dehydrogenase from cytoplasmic extracts of diploid mutant and wild-type *ADH1* strains

Adh1 Genotype	Specific Activity (μmol/min/mg)
WT/WT	5.34 ± 0.33
WT/null	2.63 ± 0.037
0.80/0.80	0.40 ± 0.0045
0.80/null	0.24 ± 0.0048
0.96/0.96	1.97 ± 0.055
0.96/null	0.80 ± 0.13
0.80/0.96	1.07 ± 0.052

Activities reflect primarily those of ADH-I. See text for growth and assay conditions.

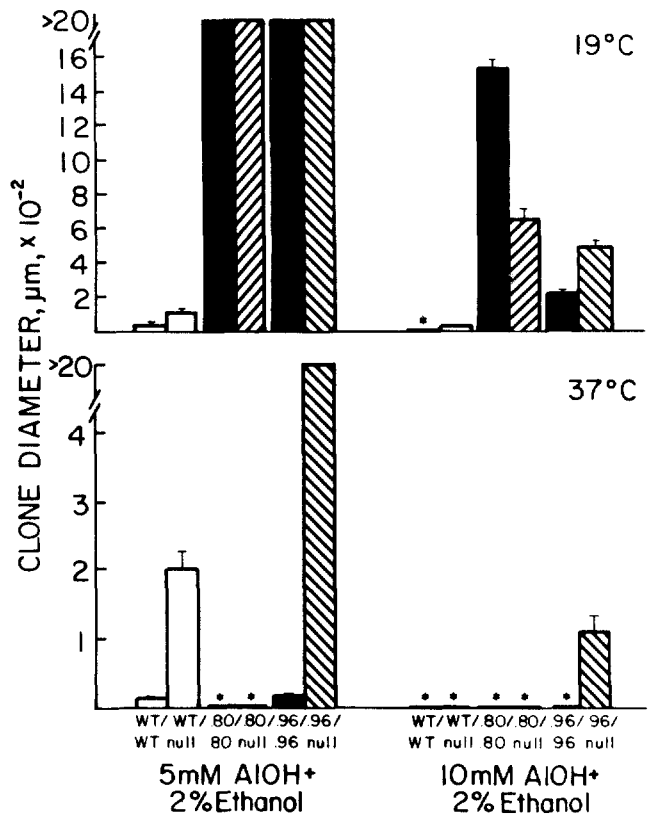

FIGURE 2.—Asymptotic clone diameters (μm ± SEM) for diploid yeast strains homozygous and heterozygous for *ADH1* mutants 19II-S6 (0.96) and 37II-S7 (0.80), and *adh1* (null); null/null homozygotes are lethal as petite phenotypes. Clones of all strains grow to final diameters >2000 μm in the absence of added allyl alcohol with the exception of 0.80/null, which shows no growth at 37° under these conditions. Asterisks indicate no growth of isolated cells.

homozygote being lethal as a petite phenotype. While 0.80/null shows a lower resistance than the 0.80/0.80 homozygote, its specific activity is very low. A single dose of the 0.80 allele may not be sufficient to meet cellular glycolytic requirements as a diploid. Indeed, the 0.80/null does not grow at all at 37° in the presence of Antimycin A, even in the absence of allyl alcohol.

Consideration of other data, however, suggests that the effects of ADHI dosage are not entirely sufficient

to explain the overdominance in resistance. First, unlike the 0.96/0.80 heterozygote, an increased allyl alcohol resistance is apparent at both 19° and 37° for both the 0.96/null and WT/null heterozygotes, compared to the allyl alcohol resistant homozygotes (Figure 2). In addition, all three null heterozygotes show slightly higher clonal growth rates than the respective homozygotes in the absence of allyl alcohol at 19° but not at 37°; this is not observed for the 0.96/0.80 heterozygote (data not shown). Third, there is no resistance overdominance when heterozygous strains are constructed from these two mutant strains and other *functional ADH1* mutants. EAA5-37-S7 (R_f = 1.00) (HALL and WILLS 1987) exhibits dominance in allyl alcohol resistance with respect to both 0.96 and 0.80 mutants at 37°. A fourth mutant, EAA5-37-S4 (R_f = 0.96) is co-dominant with 0.80 *ADH1* under these same conditions. These observations and the fact that resistance mutations frequently involve amino acid substitutions in ADHI suggest that some other functional change in the *ADH1* allelic products of EAA5-19II-S6 and EAA5-37II-S7 besides the reduction in specific activity also contributes to the overdominant phenotype.

The results obtained with this experimental system suggest that selection for intermediate enzyme kinetic phenotypes can produce overdominance with respect to aspects of the phenotype related to fitness. A corollary of this conclusion is that the specific activities of enzymes in cell extracts are not necessarily correlated directly with fitness parameters.

There are several other instances in which biochemical intermediacy has been shown to produce phenotypic overdominance. Studies with *Neurospora* heterokaryons indicate that a single dose of a gene may provide an enhanced viability over a double dose through the production of an intermediate enzyme level or indirectly through the production of an optimal level of a required metabolite, which may be toxic at higher concentrations (*e.g.*, EMERSON 1948; REISSIG 1960). However, the best-documented case for overdominance—the association of HbS with malarial resistance—is a clear case of a conditional selection for an intermediate hemoglobin functional capacity in regions infested with *Plasmodium falciparum* (*cf.* WILLS 1981; INGRAM 1986). While HbS possesses an unimpaired oxygen affinity, it readily forms polymeric chains in homozygotes at only slightly lower oxygen tensions. This leads to the clinical symptoms of erythrocyte sickling, severe anemia, and liver and spleen damage. Individuals homozygous for the wild-type (HbA) chain have no sickling capacity but are very susceptible to malarial infection. Erythrocytes of heterozygotes sickle at oxygen tensions significantly lower than those that induce sickling in HbS homozygotes, and this occurs only under unusual environmental circumstances, such as at high altitudes. However, when a malarial parasite infects an erythrocyte of a heterozygote, the parasite induces the pH to drop sufficiently in infected cells to induce polymerization. The selective sickling that occurs leads to a precipitous decrease in K^+ ion levels in the cell, killing the parasite. In the environmental context of a high incidence of malaria, the intermediate polymerization capacity of HbS heterozygotes constitutes an important biological function for this molecule. This intermediate sensitivity to erythrocyte sickling poises the individual for malarial resistance while eliminating most of the toxic effects of polymerization that occur in mutant homozygotes.

The environmental conditions necessary for overdominance in allyl alcohol resistance in yeast are directly analogous to the HbS case in that a balance is struck between conflicting selection pressures: an excessive ADHI functional capacity (*i.e.*, enzyme activity) leads to acrolein toxicity, while too little provides insufficient NAD^+ to prime glycolysis, which effectively shuts down energy production in respiratory incompetent yeast. We emphasize again that intermediacy in enzyme specific activity is apparently not an entirely sufficient explanation for our observations. Within the limits of these two extreme constraints single amino acid substitutions are capable of producing subtle functional differences, such as temperature-conditional allyl alcohol resistance in haploids (HALL and WILLS 1987) as well as the temperature- and substrate-conditional nature of the overdominance reported here.

Selection for an intermediate kinetic or biochemical phenotype is one of two potential mechanisms for producing overdominance. The second—and most frequently invoked—mechanism requires the interaction of allelic products in the heterozygote. Consideration of the evidence for these two mechanisms suggests reasons (aside from problems in detection and other technical problems) why documented examples of this phenomenon are rare. The kinetic interaction mechanism requires quite restrictive conditions. First, it must be limited to oligomeric proteins for any functional interaction between allelic polypeptides to be possible. Second, such interactions in the allelic heterooligomer must be sufficiently large to overcome the averaging effects of the presence of both homooligomers in the binomial proportions usually found in the enzyme population. For a dimeric enzyme a 20% change in the function of the enzyme population in the heterozygote requires a 40% change in the interactive function of the heterodimeric isozyme. While there are numerous documented reports of interallelic complementation for oligomeric enzymes (FINCHAM 1966; ZABIN and VILLAJERO 1975; HOLLACHER and PLACE 1987), all of these notably

involve the interaction of mutant polypeptides that are inactive as homooligomers (*e.g.*, SCHLESINGER and LEVINTHAL 1963; SCHWARTZ and LAUGHNER 1969). We know of no sufficiently characterized examples of kinetic interactions between mutually *functional* allelic subunits—the kinds of interactions that are usually envisioned to play roles in adaptation (*cf.* FINCHAM 1972; MITTON and GRANT 1984). While there are a few reports of apparent kinetic overdominance involving measurements of specific activities and/or various kinetic parameters for both crude extracts and purified enzyme preparations (*e.g.*, WATT 1983; ZERA 1987), the heterooligomer to our knowledge has not been isolated from the "contaminating" homooligomers and characterized for any allelic isozyme system. Such information and appropriate analysis of these data are required in order to demonstrate any kinetic interactions. Inappropriate fitting of kinetic data, obtained from a heterogeneous population of isozymes or active sites, to the Michaelis-Menten model for a single enzyme form in some cases may yield kinetic artefacts resembling biochemical overdominance [C. ZIMMERLE, personal communication; see CLELAND (1970), pp. 56–57, for a brief discussion].

Considering these arguments and the paucity of sufficiently documented examples of the "kinetic interaction" mechanism, the cases and the data we have discussed in this article suggest that when overdominance does occur in nature, it is most commonly the consequence of the other possible mechanism—selection for an intermediate kinetic or biochemical function. Substitution of a low activity variant obviously may have detrimental effects upon organismal fitness by impeding flux through a metabolic pathway and through the accumulation of metabolites in steps antecedent to the block. On the other hand, substitution of an alternative variant with a higher functional efficiency would generally be expected to have either positive or negligible effects on fitness, as no metabolic cost is incurred for the substitution of the same amount of a more efficient enzyme (*cf.* KACSER and BURNS 1973, 1981; HARTL, DYKHUIZEN and DEAN 1985; DEAN, DYKHUIZEN and HARTL 1986). The only conceivable environmental circumstance where a more effective protein function might be at a selective disadvantage would be one in which the product of the protein's function is in some way detrimental, as in our examples of high ADH activity induction of acrolein poisoning in petite yeast when allyl alcohol is present, and the excess "sickling" capacity of HbS homozygotes. Such conditions are restrictive *a priori*, implying that conditions in which biochemical intermediacy produces simple overdominance are also uncommon. Given these specific conditions required for the manifestation of overdominance by either mechanism, it is perhaps not surprising that this phenomenon has rarely been observed in nature or in the laboratory.

The relative potential for biochemical intermediacy to yield overdominant phenotypes does not appear to be generally appreciated, as is apparent in the suggestion by several workers of a paradox between the universal observation (and expectation) of heterozygote biochemical intermediacy and the evidence for some kind of functional relationship between heterozygosity at enzyme loci *per se* and measures of fitness (MITTON and GRANT 1984). Such statements imply that overdominance at the phenotypic level requires overdominance at the level of enzyme function. The evidence presented in this report and in the examples cited above suggest that such a speculation is not expected to be generally valid. While some form of "marginal overdominance" in heterogeneous physical, physiological, or genetic environments, may remain the most attractive adaptive mechanism for the correlations between enzyme heterozygosity and measures of fitness, the mechanism of biochemical intermediacy should also be considered in these kinds of investigations. The model system reported here will be useful for investigating the metabolic basis for this phenomenon.

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