

Natural and Synthetic Alleles Provide Complementary Insights Into the Nature of Selection Acting on the *Men* Polymorphism of *Drosophila melanogaster*

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ABSTRACT

Two malic enzyme alleles, *Men*^{113A} and *Men*^{113G}, occur at approximately equal frequency in North American populations of *Drosophila melanogaster*, while only *Men*^{113A} occurs in African populations. We investigated the population genetics, biochemical characteristics, and selective potential of these alleles. Comparable levels of nucleotide polymorphism in both alleles suggest that the *Men*^{113G} allele is not recently derived, but we find no evidence in the DNA sequence data for selection maintaining the polymorphism. Interestingly, the alleles differ in both V_{\max} and K_m for the substrate malate. Triglyceride concentration and isocitrate dehydrogenase (IDH) and glucose-6-phosphate dehydrogenase (G6PD) activities are negatively correlated with the *in vivo* activities of the *Men* alleles. We examined the causality of the observed correlations using *P*-element excision-derived knockout alleles of the *Men* gene and found significant changes in the maximum activities of both IDH and G6PD, but not in triglyceride concentration, suggesting compensatory interactions between MEN, IDH, and G6PD. Additionally, we found significantly higher than expected levels of MEN activity in knockout heterozygotes, which we attribute to transvection effects. The distinct differences in biochemistry and physiology between the naturally occurring alleles and between the engineered alleles suggest the potential for selection on the *Men* locus.

SIGNIFICANT progress has been made in characterizing population variability at the level of DNA sequences, but the central challenge of elucidating rules that connect genotype and phenotype remains. In many cases, genotype may give rise to phenotype through interactions among multiple genes and metabolite intermediates. Presumably, these interactions are at least partly the product of natural selection. The challenge, however, is not to simply infer the presence of selection, but to elucidate its action and distribution across such multiple gene networks. In this study we begin an analysis of the variation and functional interactions among three oxidative enzymes known to be the primary source of the reduced form of the metabolic cofactor NADP.

A single amino acid polymorphism was identified in cytosolic malic enzyme (*Men*) as part of a study of geographic variation of single-nucleotide polymorphisms associated with the genes of central metabolic enzymes in *Drosophila melanogaster* (SEZGIN *et al.* 2004). The polymorphism is an alanine-to-glycine substitution at amino acid 113 and the two alleles defined by the

amino acid substitution occur in approximately equal frequency in all 10 North American populations examined. Allele frequency clines were detected at a number of other metabolic genes, but these *Men* alleles and associated haplotypes showed no clinal changes with latitude. While the presence of clinal change in allele frequency can be interpreted as evidence for selection on a locus, the lack of such a pattern does not indicate that a polymorphism is necessarily neutral. Here we examine other aspects of the population genetics, and the biochemistry, of the two malic enzyme alleles for evidence of selection or the potential for selection between the two alleles. In the second part of this article, we compare these findings with those from synthetic *Men* alleles produced in the laboratory by *P*-element excision, again with the goal of determining the potential for selection between alleles of the *Men* gene.

Malic enzyme oxidizes malate to pyruvate with the concurrent reduction of NADP to NADPH, a major reductant in lipid synthesis (WISE and BALL 1964). It has been estimated that MEN produces ~30% of the available NADPH in larval *D. melanogaster*, with the remainder coming from cytosolic isocitrate dehydrogenase (IDH) activity (~20%) and the oxidative enzymes of the pentose shunt (glucose-6-phosphate dehydrogenase, G6PD, and 6-phosphogluconate, 6PGD, combined ~40%) (GEER *et al.* 1979a,b). Induction studies have shown significant interactions between these loci, presumably

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to maintain a constant NADPH/NADP ratio and supply of reduced cofactor for lipogenesis (GEER *et al.* 1976, 1978, 1981; WILTON *et al.* 1982). This explanation is, however, likely an oversimplification. Apparently compensatory changes in enzyme activity have been documented between the oxidative pentose shunt enzymes and MEN; extreme reduction in MEN activity is associated with an increase in G6PD and 6PGD activity (WILTON *et al.* 1982; GEER and LAURIE-AHLBERG 1984). However, no such compensation has been found between IDH and MEN; reduction in MEN is associated with reduction, not increase, in IDH activity (WILTON *et al.* 1982; GEER and LAURIE-AHLBERG 1984). Similarly, an increase in dietary carbohydrate increases G6PD, 6PGD, and MEN activity, but decreases IDH activity (GEER *et al.* 1978; GEER and LAURIE-AHLBERG 1984). Finally, reduction in IDH activity was associated with an increase in G6PD and 6PGD, but a decrease in MEN activity (BENTLEY *et al.* 1983).

This project first examines biochemical characteristics of the *Men* alleles to determine the possible phenotypic effects of the observed naturally occurring polymorphism. *In vitro* biochemical characteristics do not, however, necessarily correspond to *in vivo* differences in physiology and fitness (CAVENER and CLEGG 1981; DYKHUIZEN and DEAN 1990). Furthermore, large differences in enzyme activity unrelated to the observed polymorphism (*i.e.*, chromosome effects) may mask or inflate true allelic differences (LAURIE-AHLBERG *et al.* 1981, 1982). Studying polymorphisms in different genetic backgrounds will to some extent randomize chromosome differences between alleles, but this may not suffice to characterize allelic differences if they are small. Thus, to complement our examination of the naturally occurring alleles, we examined an independent set of laboratory-derived alleles in which phenotypic variation was exclusively attributable to changes in enzyme concentration and for which background genetic variation was minimized.

MATERIALS AND METHODS

Flies: basic lines: Isothird chromosome lines were a subsample of a set of nonlethal, third chromosomes extracted from isofemale lines established in 1997 (see DUVERNELL and EANES 2000; VERRELLI and EANES 2001). Inbred lines used as common genetic backgrounds were either obtained from the Bloomington Drosophila Stock Center at Indiana University (6326, line no. 6326) or developed in the Eanes lab by 20 generations of full-sib mating (*VT83i*, *HFL8i*). Inbred lines developed in the Eanes lab were cytologically and enzymatically screened for genetic homozygosity. Line 6326 is isogenic for the second and third chromosomes (HOSKINS *et al.* 2001). A line containing a deficiency that covers the entire malic enzyme gene, *Df(3R)kar³¹*, was obtained from the Bloomington Drosophila Stock Center at Indiana University (line no. 6160). Samples of flies from east Africa, Z(H) and Z(S), were kindly donated by Chung-I Wu (University of Chicago). Thirteen *D. simulans* lines, full-sib inbred for 14 generations (see DUVERNELL

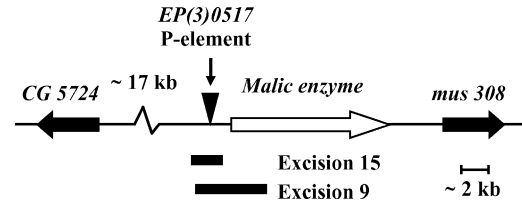


FIGURE 1.—The genomic region surrounding the *Men* gene including the insertion site of the *EP(3)0517* P element and the pair of P-element excision-mediated deletions used in this study. The *Men* gene is shown by the open arrow. The two flanking genes are shown by the solid arrows. The regions covered by the deletions are indicated by the solid bars. Excision 15 removed 2418 bases, including 160 bp of the 5' end of the cDNA UTR. Excision 9 removed 4080 bp, including 1925 bp of the cDNA, which covers the entire first exon.

and EANES 2000), were also included in our sequence analysis. All flies were maintained on standard cornmeal media. All assays for enzyme activity, protein, and triglyceride content were conducted on male flies aged 5 days after emergence at 25°.

Naturally occurring *Men* alleles for activity, protein, and triglyceride analysis: Genetic background effects were minimized by replacing the X and second chromosomes of *Men*^{113A} and *Men*^{113G} (hereafter simply *Men*^A and *Men*^G) third chromosome lines with those from inbred genetic backgrounds. Four lines of each genotype were placed in the *VT83i* background and one of each genotype was placed in the *6326* background.

Pelement excision series for *Men*: P-element-mediated deletion (TSUBOTA and SCHEDL 1986; SALZ *et al.* 1987) was used to generate activity variant alleles for the *Men* gene. The *EP(3)0517* element (RORTH 1996) is inserted 473 bases 5' to the *Men* start codon (Figure 1). Crosses were carried out to create dysgenic males, using the *EP(3)0517* element line and the Bloomington Drosophila Stock no. 2030 as a source of transposase. Recovered excision lines were screened for MEN activity. Four chromosome lines, two with wild-type levels of MEN, designated *MenEx3⁺* and *MenEx5⁺*, and two with no apparent MEN activity (knockout lines), designated *MenEx9⁻* and *MenEx15⁻*, were placed in a common genetic background (*HFL8i*). The excision site for each of these four lines was amplified and then sequenced using oligonucleotide primers flanking the initial insertion site.

Sequence analysis: The primary sequence of the *Men* gene was initially recovered, prior to the whole-genome sequence (ADAMS *et al.* 2000), using the enriched cDNA method described by VERRELLI and EANES (2000). Oligonucleotide primers were designed from the cDNA sequence to amplify the complete coding region of the *Men* gene from genomic DNA. Amplifications were conducted under the following conditions: 10 ng of genomic DNA was amplified in 10 mM Tris (pH 8.3), 50 mM KCL, 0.01% gelatin, 1.5 mM MgCl₂, 2 units of platinum *Taq* polymerase (Life Technologies, Rockville, MD), and 100 nM of each primer. The amplification products were purified (Prep-A-gene DNA purification kit; Bio-Rad, Hercules, CA) and double-stranded templates (~500 ng per reaction) were sequenced manually (Sequenase, v.2.0; United States Biochemical, Cleveland), using primers spaced at ~300-bp intervals. The primary sequence was determined for both strands and all ambiguous sites were verified on both strands. The excision sites of the alleles created through P-element excision were sequenced by the DBS sequencing facility, University of California (Davis, CA), using Big Dye Terminator version 3.1 (ABI) and then sequenced on a 3730 DNA Analyzer

(ABI). Base calls from the chromatograms were checked, and the 2-kb fragments assembled, using Sequencher (Gene Codes, Ann Arbor, MI). All sequences have been deposited in GenBank (accession nos. DQ148977–DQ149022).

DnaSP 4.0 (ROZAS *et al.* 2003) was also used to calculate θ and π , to test for differences in θ using a coalescence simulation, to carry out the TAJIMA (1989) and FU and LI (1993) tests, and to generate standardized estimates of linkage disequilibrium (R^2).

Fly homogenization: Flies were homogenized in grinding buffer (100 mM Tris-HCl, 0.15 mM NADP, pH 7.4) at a “concentration” of one fly per 200 μ l of buffer and spun at 13,000 rpm for 5 min at 4° to pellet all solids. In general, all assays were conducted using samples of five flies (five flies in 1 ml grinding buffer). In a few cases, insufficient flies were available and fewer flies were assayed.

Soluble protein content: Soluble protein was measured using a commercially available kit (Bio-Rad kit no. 500-0006) following the manufacturer’s protocol. The assays contained either 25 μ l homogenate and 1 ml reagent (Beckman spectrophotometer) or 10 μ l homogenate and 100 μ l reagent (Molecular Devices spectrophotometer) and were incubated at room temperature for 10 min. Reactions were measured at OD₅₉₅ and total soluble protein concentrations (micrograms per milliliter) were determined by comparison with bovine serum albumen standards. Each sample was assayed twice for protein concentration and the mean was used in analysis. Enzyme activities and triglyceride concentrations (below) were standardized by soluble protein content to account for differences in mass or body size between individual flies and for possible differences in the degree of homogenization between samples.

Soluble triglyceride content: Soluble triglyceride was measured using a commercially available kit (Infinity triglyceride assay; Thermo Electron, Arlington, TX; catalog no. 2780), following the manufacturer’s protocol. The assays contained either 25 μ l homogenate and 1 ml reagent (for samples assayed on the Beckman spectrophotometer) or 10 μ l homogenate and 100 μ l reagent (the Molecular Devices spectrophotometer) and were incubated at 37° for 10 min. Reactions were measured at OD₅₀₀ and total soluble triglyceride concentrations (micrograms per milliliter) were determined by comparison with a commercially available standard (Sigma-Aldrich, St Louis; catalog no. T2522). Each sample was assayed twice and the mean was used in analysis. Results are reported as micrograms triglyceride per microgram soluble protein.

Enzyme activity measurements: Enzyme activity assays were carried out using either a Beckman DU 640 or a Molecular Designs SpectraMax 384 Plus 96-well plate spectrophotometer. Assays on the Beckman spectrophotometer used 25 μ l of fly homogenate and 400 μ l assay buffer. The initial reaction rate was calculated by sampling absorbance (OD₃₄₀) every 12 sec over 3 min. Assays on the Molecular Designs spectrophotometer used 10 μ l of fly extract and 100 μ l of assay buffer and absorbance was measured every 9 sec over 3 min. All activity assays were conducted at 25°. In all experiments, samples were assayed twice and the average was used in analysis. The estimates of thermal stabilities, substrate K_m , and cofactor K_m and the initial examination of the MEN deletion series activities were determined using the Beckman spectrophotometer. Enzyme activity is expressed as micromoles NADP⁺ reduced per minute per microgram soluble protein times 10,000.

The assay buffers for the three enzymes assayed in this study were as follows: MEN, 100 mM Tris-HCl, 0.34 mM NADP, 50 mM MnCl₂, 50 mM malate, pH 7.4; G6PD, 100 mM Tris-HCl, 0.32 mM NADP, 3.5 mM D-glucose-6-phosphate, pH 7.4; and IDH, 100 mM Tris-HCl, 0.10 mM NADP, 0.84 mM MgSO₄, 1.37 mM DL-isocitrate, pH 8.6. Initial values for appropriate pH, substrate,

and cofactor concentrations for the reactions were taken from the literature and modified to give maximum enzyme activity.

Thermal stability: Allele-specific enzyme thermostabilities were estimated by following MEN activity decline over time at 50° (VERRELLI and EANES 2001). Two replicate copies from three isochromosomal lines of each haplotype were assayed for MEN activity on a Beckman spectrophotometer. A single aliquot of fly homogenate was immediately placed on ice for use as a reference. Ten aliquots were placed in a 50° heat block, and at 1-min intervals a single aliquot was removed and placed on ice. All aliquots were subsequently kept on ice until their maximum activities were measured. The activity of each sample was compared to that of the reference sample to determine the proportion of activity remaining at each time point. The decline in enzyme activity with time was treated as a first-order exponential decay process and denaturing constants (K_d) were determined using the relationship $(E/E^0)_t = e^{-K_d t}$, where $(E/E^0)_t$ is the proportion of initial enzyme activity remaining at time t and K_d is the denaturation rate (HALL 1985). The slope of the line from the linear regression of $\ln(E/E^0)_t$ on time is an estimate of K_d . A mean K_d was calculated for each line from the two replicates and a mean K_d for each allele was calculated from the three lines.

Estimation of Michaelis-Menton constants (K_m): Allele-specific Michaelis-Menton constants for both malate and NADP were determined using the two-substrate concentration method (DUGGLEBY 1979; CORNISH-BOWDEN 1995). Reaction rates were determined in parallel for four isochromosomal lines of each allele using the Beckman spectrophotometer. Individual cuvette use was randomized with respect to line. Initial estimates of the apparent K_m values for both NADP and malate were obtained at 25° using nine substrate concentrations from 0.5 K_m to 20 K_m (initial guesses were from GEER *et al.* 1980). Fits of initial rate *vs.* concentration did not appear to deviate from Michaelis-Menton kinetics over this range of substrate concentrations. From these initial estimates, the K_m of each line was determined from 12 replicate initial velocity estimates made at each of two substrate concentrations approximating 0.5 K_m and V_{max} conditions (DUGGLEBY 1979; CORNISH-BOWDEN 1995). Homogenate concentrations were adjusted so that all lines showed effectively identical V_{max} values during K_m estimation. K_m estimation under this design is robust if preliminary estimates of K_m are close to the true values (CORNISH-BOWDEN 1995). K_m values, and their associated standard errors, were estimated from initial rate data by nonlinear least squares using the computer program Leonora (CORNISH-BOWDEN 1995). For estimation of the K_m of malate, cuvette homogenate concentrations were held such that <5% of the substrate was consumed during a 3-min reaction. For estimation of the K_m of NADP, the low concentration of NADP required for estimation of the initial rate at $\sim 0.5 K_m$ precluded this control, and instead homogenate concentration was held such that a plot of absorbance against time was linear for the duration of a 4-min assay.

Data analysis: In all cases, crosses were replicated in multiple vials (two vials in examination of the wild *Men* alleles, four vials in the later experiments using the excision *Men* alleles) and each vial was sampled twice. Initial ANOVAs were conducted to determine the presence or absence of a genotype effect and Tukey’s honestly significant difference (HSD) multiple-comparison tests were conducted to determine whether or not individual crosses could be grouped within genotype classes. Grouped data were then analyzed using ANOVA. Planned pairwise comparisons were conducted using the method of SOKAL and ROLF (1994, p. 230), which calculates a sum-of-squares for the difference between two means and the group mean (from the ANOVA) and tests this value over the within-group mean square from the ANOVA.

A

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111 111111
1345689012 233455
4367672223 527249
1850067683 431889
CGACCGGGGT CCGATT
DPF 3.0
DPF 94.1
SC 96 12.3 .....A. T....C
SC 96 19.4 .....C.....C
HFL 97 12 .....C.... T....C
HFL 97 13 .....C..A. T....C
HFL 97 17 .....C.... T....C
HFL 97 20 .....C.....C
HFL 97 28 .....C..A. T....C
HFL 97 74 .....C.....C
DPF 90.2 .....TT.TCC
DPF 4.2 .C.....TT.TCC
DPF 23.1 .C.....TT.TCC
DPF 38.3 .C.....TT.TCC
DPF 48.1 .C.....TT.TCC
DPF 74.2 .C.....TT.TCC
DPF 84.3 .C.....TT.TCC
HFL 97 8 .C.....TT.TCC
HFL 97 15 .C.....TT.TCC
HFL 97 34 .C.....T..TCC
HFL 97 16 .C.G...A.. TT.TCC
HFL 97 18 .C.....TTTCC
HFL 97 46 .C.....TTTCC
HFL 97 93 .C.....TTTCC
Z(S) 15 .C...A... TT..CC
Z(S) 24 .C...C... TT.TCC
Z(S) 35 .CT..C... T..T.C
Z(S) 37 .C...A... TT..CC
Z(S) 48 .C...C... TT.TCC
Z(S) 49 .CT..C... T..T.C
Z(S) 38.4 AC...C... T..T.C
Z(S) 39 AC...C... T..T.C
Z(S) 44 .CG.T.... T...CC

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B

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11 1111111
344567811 1123466
3925385713 5756945
9663416647 5612749
HFL97 1.1s ACCCTCTACG TTCGCCC
HFL97 4.1s
GA96 2.1s
GA96 4.1s G.....
VA96 5.1s G.....
VA96 8.1s G.....
CT96 6.1s ....A....
HFL97 2.1s GT..A...A CC...T
VA96 7.1s GT..A...A CC...T
CT96 5.1s ...T..CC.A CCTT...
CT96 2.1s G.T..T..A CC...T
GA96 5.1s G.T..T..TA ...TTT
DPF96 3.1s G.T..T..TA ....TTT

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TABLE 1

Levels of silent polymorphism in the *Men* gene across sampled populations of *Drosophila melanogaster* and *D. simulans*

	<i>n</i>	No. sites	<i>S</i>	π	θ
<i>D. melanogaster</i>	33	433.81	16	0.0082	0.0091
African	9	433.76	10	0.0097	0.0085
North American	24	433.83	10	0.0073	0.0062
<i>Men^G</i>	11	433.83	7	0.0055	0.0055
<i>Men^A</i>	13	433.83	4	0.0020	0.0030
<i>D. simulans</i>	13	433.23	17	0.0130	0.0127

FIGURE 2.—Nucleotide polymorphism in the amino acid coding region of the *Men* gene of (A) *Drosophila melanogaster* and (B) *D. simulans*. The variable site (338) responsible for the amino acid polymorphism in *D. melanogaster* is in boldface type. The first 11 sequences have the *Men^G* (glycine-coding) allele, while the remaining sequences all have the *Men^C* (alanine) allele. *D. simulans* sequences are indicated by a final “s.”

RESULTS

Naturally occurring *Men* alleles: *Sequence variation:* We amplified and sequenced all 1737 bases of the *Men* coding region from 33 *D. melanogaster* isochromosomal and 13 *D. simulans* inbred lines. Polymorphic sites are shown in Figure 2. We observed 16 polymorphic sites in *D. melanogaster*, only one of which was a nonsynonymous change, the previously reported (SEZGIN *et al.* 2004) G/C polymorphism at position 338 that results in the alanine/glycine amino acid polymorphism (Figure 2, boldface type). The sequence and three-dimensional (3D) structure of the pigeon cytosolic malic enzyme and the human mitochondrial malic enzyme are known (YANG *et al.* 2000, 2002; YANG and TONG 2000). Alignment of the *D. melanogaster* sequence with the human and pigeon sequences and comparison with the known pigeon (cytosolic MEN) structure indicates that the *D. melanogaster* polymorphic site is in a α -helical segment of the molecule, in the region of the active site. The *D. melanogaster* MEN sequence is 55% identical to the pigeon sequence. Given that the human and pigeon MEN sequences are 59% identical, and all major struc-

tural features are conserved between them (YANG *et al.* 2002), we expect that the pigeon MEN structure is a reasonable predictor of the structure of the *D. melanogaster* molecule.

In our sample, the *Men* gene shows normal levels of synonymous polymorphism for *D. melanogaster* and *D. simulans* genes (Table 1) (MORIYAMA and POWELL 1996). African samples showed somewhat higher levels of polymorphism than North American samples (Table 1) although the difference was not significant at the $P < 0.05$ level using a coalescence simulation (ROZAS *et al.* 2003). African lines carried the *Men^A* allele, as did all the *D. simulans* lines, suggesting that *Men^A* is the ancestral allele. Approximately half, 13 of 24, of the North American *D. melanogaster* lines sequenced were *Men^A*, reflecting the approximately equal frequencies reported for these alleles across North American populations sampled by SEZGIN *et al.* (2004). There are no fixed synonymous differences between the *Men^A* and *Men^G* alleles, although three positions, 1323, 1428, and 1548, are fixed in almost all *Men^A* lines; only a single *Men^G* line, DPF 90.2, shares the “*Men^A*” sequence (Figure 2). The linkage disequilibrium between the amino acid-altering polymorphism and these three sites is statistically significant at the $P < 0.05$ level by a Bonferroni corrected chi-square test. The level of polymorphism within the *Men^G* alleles is not significantly different from that within the North American *D. melanogaster Men^A* alleles (Table 1). All of the TAJIMA (1989) and FU and LI (1993) tests conducted on the *D. melanogaster* (combined or exclusively on the North American sequences) or *D. simulans* sequences were not statistically significant.

MEN thermal stability and Michaelis-Menton constants (K_m): Table 2 summarizes thermal stability and *K_m* measures for the two alleles. Thermal stability values from three *Men^G* and three *Men^A* isochromosomal lines did not differ between the two alleles or between lines of either allele ($F_{1,4} = 2.90, P < 0.16, F_{4,6} = 0.93, P < 0.50$, respectively). Similarly, the *K_m* for the NADP cofactor from four isochromosomal lines of each allele did not differ between alleles or lines ($F_{1,6} = 0.01, P < 0.92, F_{4,6} = 0.87, P < 0.53$). The *K_m* for malate from four isochromosomal lines of each allele did, however, differ

TABLE 2

Men genotype values (\pm SE) for thermal stability and K_m for both cofactor and substrate

	Thermostability ^a	K_m NADP ^b	K_m malate ^b
<i>Men</i> ^G	0.13 \pm 0.005	0.003 \pm 0.0000	0.12 \pm 0.018*
<i>Men</i> ^A	0.11 \pm 0.011	0.003 \pm 0.0002	0.07 \pm 0.005*

* $P < 0.05$.

^a Absolute value of K_d .

^b In micromoles per liter.

between alleles and lines ($F_{1,6} = 8.00$, $P < 0.03$, $F_{6,8} = 77.6$, $P < 0.0001$).

MEN V_{max} : We assumed that the allele-specific activity under saturating substrate levels is a relative V_{max} estimate. Our interests were to determine whether the two *Men* allelic proteins differed in enzymatic activity under saturating substrate conditions (V_{max}) and whether the V_{max} observed in the *Men*^{A/G} heterozygous genotype equaled the average of the mean activity of the *Men*^A and *Men*^G homozygous genotypes (this is our expectation if any difference in activity between the two alleles is simply additive, but see KACSER and PORTEOUS 1987 for a cautionary note about this simplification). The four *VT83i* background-replaced *Men*^A and *Men*^G lines were crossed to the single *6326* background-replaced *Men*^A and *Men*^G lines to produce sets of *Men*^A and *Men*^G homozygous and heterozygous flies (e.g., *w*; *VT83i/6326*; *Men*^{A1}/*Men*^{A5} or *w*; *VT83i/6326*; *Men*^{A1}/*Men*^{G5}). This cross scheme produces *Men*^A and *Men*^G homozygotes in four different third chromosome combinations and heterozygotes in eight, all in a pseudo-outcrossed second chromosome background (*VT83i/6326*), effectively randomizing any background effects on V_{max} . This scheme eliminates "line" effects (individual chromosomes are never homozygous) and replaces them with possible "cross" effects (the combination of any pair of third chromosomes).

The ANOVA of the V_{max} estimates with four genotypes (G/G, A/A, G/A, and A/G) indicated a significant genotype effect ($F_{3,31} = 36.93$, $P < 0.001$). A Tukey's HSD test found no significant V_{max} difference between the reciprocal heterozygotes and these were combined for further analysis. The subsequent two-way ANOVA of polymorphism and cross effects on the combined data again indicated a significant amino acid polymorphism effect ($F_{2,32} = 10.32$, $P < 0.002$). There were no significant differences between crosses within a genotype ($F_{13,32} = 1.40$, $P < 0.26$), but there were differences between vials within a cross ($F_{16,32} = 5.37$, $P < 0.001$). The V_{max} estimates were 0.38 ± 0.01 (mean \pm SE across the four crosses) for *Men*^G flies, 0.26 ± 0.01 for *Men*^A flies, and $0.33 \pm .01$ for the heterozygotes (combined, eight crosses). We found a significant difference in V_{max} between the *Men*^A and *Men*^G activities ($F_{1,32} = 116.21$, $P < 0.001$), but no significant difference between the

heterozygote V_{max} and the mean of the homozygote V_{max} -values ($F_{1,32} = 2.25$, $P > 0.10$). The lack of significant difference between the heterozygotes and the average of the homozygotes suggests that the V_{max} of the two alleles is additive in the heterozygous genotypes.

Intergenic and physiological effects: Substrate concentrations *in vivo* are generally believed to be low, on the order of the K_m (FERSHT 1977; HOCHACHKA and SOMERO 2004), and under such conditions V_{max} , the reaction rate at saturating substrate concentration, does not reflect the true reaction rate. Instead, the ratio V_{max}/K_m provides a more accurate indicator of the relative *in vivo* reaction velocities of alleles differing in both V_{max} and K_m (HALL and KOEHN 1983; WATT 1985; WATT and DEAN 2000). Given our estimates of V_{max} and malate K_m , $V_{max}/K_m = 3.26 \pm 0.158$ (mean \pm SE across the four crosses using the mean K_m -value calculated above) for *Men*^G flies, 3.87 ± 0.186 for *Men*^A flies, and 3.59 ± 0.165 for the (combined) heterozygotes. The two *Men* alleles differ in V_{max}/K_m by $\sim 19\%$ ($t_{14} = -2.50$, $P < 0.013$). Note that because of the inverse relationship in V_{max} and K_m observed between the two alleles, the genotype with the higher V_{max} , *Men*^G, has the lower predicted *in vivo* activity.

We examined whether the predicted genotypic difference in MEN activity was associated with detectable changes in adult physiology. As an assessment, we measured total triglycerides, a major lipid component highly correlated with total lipid content (CLARK and KEITH 1988). We also examined the induction by MEN activity of two other NADPH-generating enzymes, cytosolic IDH and G6PD. Figure 3 shows plots of triglyceride concentration and IDH and G6PD activities against the predicted *in vivo* activities (V_{max}/K_m) for each *Men* genotype. Using the same pairwise comparison of means that we used for the V_{max} data, we found that the triglyceride concentration and IDH and G6PD activity all differ significantly between the *Men*^G and *Men*^A homozygotes ($F_{1,32} = 7.19$, $P < 0.025$; $F_{1,32} = 13.86$, $P < 0.025$; and $F_{1,32} = 5.90$, $P < 0.05$; respectively), whereas the *Men*^{A/G} heterozygotes do not differ from the mean of the homozygotes in triglyceride concentration or IDH or G6PD activity ($F_{1,32} = 1.28$, $P > 0.05$; $F_{1,32} = 3.27$, $P > 0.05$; and $F_{1,32} = 0.40$, $P > 0.05$; respectively). Triglyceride concentration and IDH and G6PD activity all increase as MEN genotype activity decreases (Figure 3). The negative relationship between MEN, IDH, and G6PD might be expected as a physiological response to maintain a pool of NADPH at a constant concentration. The decrease in triglyceride concentration was unexpected.

P-element excision-derived Men alleles: *Sequence characterization:* Using the *EP(3)0517* element, we recovered and characterized a chromosome set of 28 excision lines derived from *P*-element transposition 5' to the *Men* gene. We identified two lines with essentially no MEN activity (knockout lines) and selected two lines with relatively normal levels of activity (full-activity

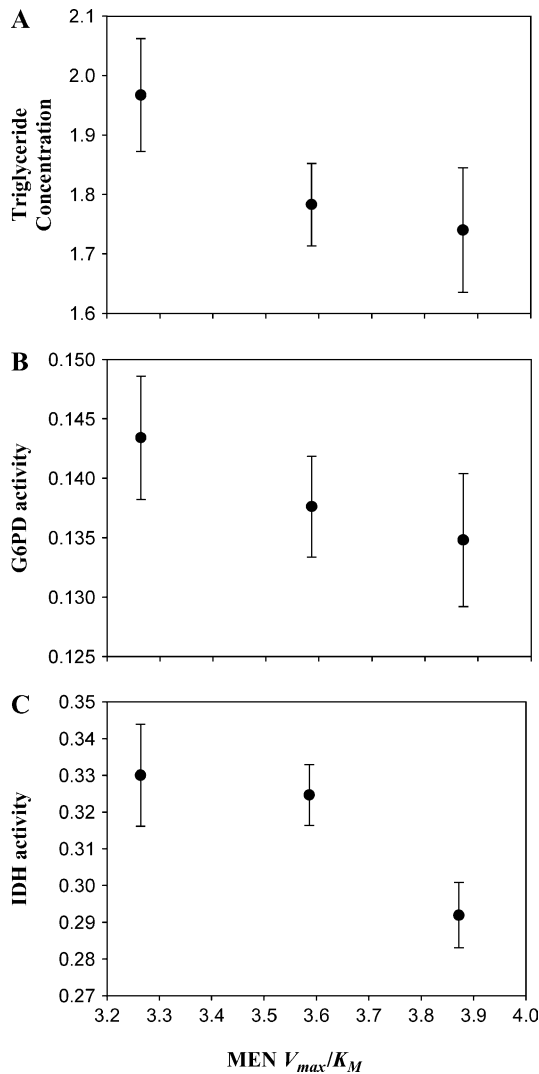


FIGURE 3.—Relationship between triglyceride concentration (micrograms per microgram protein), IDH activity or G6PD activity ($10,000 \times \mu\text{M NADPH}/\text{min}/\mu\text{g protein}$), and predicted *in vivo* MEN activity (V_{max}/K_m) of the *Men* genotypes. The genotypes are, left to right, $Men^{G/G}$, $Men^{G/A}$, and $Men^{A/A}$. Error bars indicate one standard error.

lines). Approximately 1 kb of DNA, centered on the original *P*-element insertion site, was sequenced from each line. The sequences of the excision chromosomes with normal activity ($MenEx3^+$ and $MenEx5^+$) were identical to the standard *D. melanogaster* genome sequence (both have the Men^G allele). The knockout lines ($MenEx9^-$ and $MenEx15^-$) possessed deletions covering portions of the *Men* gene (Figure 1). $MenEx15^-$ had a 2418-bp deletion, roughly centered on the *P*-element insertion site, removing 160 bp of the 5'-UTR of the *Men* gene. $MenEx9^-$ had a 4080-bp deletion that included the entire first exon (44 aa) of the *Men* gene. Annotation is based on the *D. melanogaster* genome sequence (ADAMS *et al.* 2000) (<http://www.fruitfly.org>). Both deletion lines were homozygous viable, but clearly possessed poor viability and low fertility. An initial screening of MEN

activity of these lines found that $MenEx9^-$ and $MenEx15^-$ had $\leq 10\%$ the activity of either $MenEx3^+$ or $MenEx5^+$. Closer examination of the activity of these two alleles (below) indicated that they show $<2\%$ normal MEN activity, indicating that they are, as expected from the sequence data, clean knockout alleles.

MEN V_{max} : The four excision lines (in a common *VT83i* genetic background) were intercrossed to create flies with varying levels of MEN activity. Our intention was to use these crosses to create individuals with 100% ($MenEx5^+/MenEx5^+$, $MenEx5^+/MenEx3^+$, $MenEx3^+/MenEx3^+$), 50% ($MenEx5^+/MenEx15^-$, $MenEx5^+/MenEx9^-$, $MenEx3^+/MenEx15^-$, $MenEx3^+/MenEx9^-$), and 0% ($MenEx15^-/MenEx15^-$, $MenEx15^-/MenEx9^-$, $MenEx9^-/MenEx9^-$) relative MEN activities. We detected a significant genotype effect in MEN activity among the 10 groups ($F_{9,39} = 219.13$, $P < 0.0001$). When the crosses were grouped in the three activity classes (0, 50, and 100%), we again detected a significant difference in MEN activity ($F_{2,39} = 975.50$, $P < 0.0001$, Figure 4A). The Men^+/Men^+ flies had average MEN activities of 0.45 ± 0.01 (mean \pm SE across all crosses); the Men^-/Men^+ flies, of 0.41 ± 0.01 ; and the Men^-/Men^- flies, of 0.01 ± 0.0001 . The “0” and 100% groups differed significantly in activity ($F_{1,32} = 3282.48$, $P < 0.001$) with the Men^-/Men^- flies showing $<2\%$ the activity of the normal-activity flies. The 50% group, however, showed $\sim 90\%$ the activity of the 100% group, significantly higher activity than the average of the 0 and 100% groups ($F_{1,32} = 861.10$, $P < 0.001$). This higher than expected amount of MEN activity is presumably from an upregulation of the functional copy of the gene because the Men^-/Men^- flies show essentially no activity. The upregulation could be the result of intrinsic autoregulation of MEN activity *per se* or of interallelic transvection, an interchromosomal interaction between a chromosome with a non-functional gene and the paired chromosome with an intact, functional, copy of the gene (HENIKOFF and COMAI 1998; WU and MORRIS 1999).

Intergenic and physiological effects: Figure 4, B–D, shows triglyceride concentration, and IDH and G6PD activities, plotted against the estimated MEN V_{max} of the excision genotypes. Recall that the third chromosomes of all genotypes in this experiment are identical, derived from the single *P*-element-containing parent chromosome, *EP(3)0517*, and that the remainder of the genome has been replaced with a common background (*HFL8i*). No differences in K_m between the genotypes are expected, and V_{max} should accurately reflect the *in vivo* activities. Both triglyceride and G6PD significantly differed across the three MEN-activity groups ($F_{2,39} = 18.45$, $P < 0.0001$ and $F_{2,39} = 4.79$, $P < 0.02$, respectively). The triglyceride levels of the knockout and “100%” flies were significantly different, while there was no significant difference between levels in the two high-activity groups (Tukey’s HSD test, $P < 0.05$, Figure 4B). The homozygous *Men* knockouts had

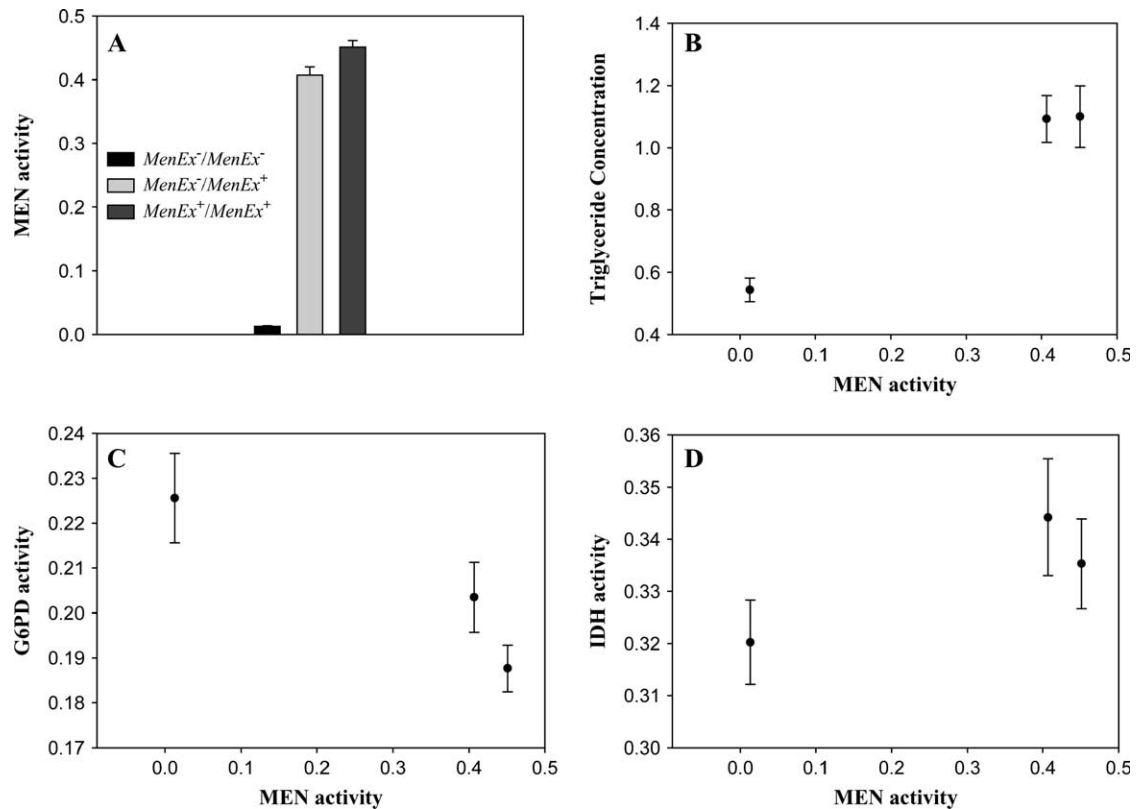


FIGURE 4.—(A) MEN V_{max} from crosses of the *EP(3)0517* excision series. Crosses were expected to generate three different activity classes: “0%,” “50%,” and “100%.” The 0% and 100% groups are significantly different. The 50% flies show significantly greater 50% activity, but are significantly lower in activity than the 100% flies. Error bars indicate one standard error. (B–D) Graphs of triglyceride concentration (micrograms per microgram protein), G6PD activity, and IDH activity, respectively, against Men activity ($10,000 \times \mu\text{M NADPH}/\text{min}/\mu\text{g protein}$).

approximately half the triglyceride levels of the 50 and 100% MEN-activity groups. The steep decline in triglyceride concentration in parallel with decline in MEN activity is consistent with the expected central role of MEN (along with IDH and G6PD) in producing reduced NADP for fatty acid synthesis. Similarly, a Tukey’s HSD test indicates that G6PD levels are significantly higher in the *Men* knockouts than in the two high-activity classes ($P < 0.05$). IDH activity did not vary significantly with MEN activity ($F_{2,39} = 1.26$, $P < 0.31$), although there was an apparent trend toward reduction in IDH activity in the MEN knockouts, consistent with earlier reports in the literature (WILTON *et al.* 1982; GEER and LAURIE-AHLBERG 1984).

To examine whether the much higher than expected levels of MEN activity in the knockout/full-activity heterozygotes were the result of transvection or upward autoregulation the *Men* gene, we crossed the four *Men* excision lines to a third chromosome line containing a large-scale deficiency [*Df(3R)kar^{3l}*] that covers the *Men* gene (VOELKER *et al.* 1981) and a second line with a wild-type, *Men^C*, copy of the *Men* gene (*CT68*). These crosses yielded four groups of genotypes: normal activity (expected 100% relative activity, *MenEx⁺/Men⁺: MenEx³⁺/CT68* and *MenEx⁵⁺/CT68*), deletion heterozygote (ex-

pected 50% relative activity, *MenEx⁻/Men⁺: MenEx⁹⁻/MenCT68*, *MenEx15/MenCT68*), deficiency heterozygote [expected 50% relative activity, *MenEx⁺/MenDf: MenEx3/Df(3R)kar^{3l}*, *MenΔ5/Df(3R)kar^{3l}*], and knockout (expected 0% relative activity, *MenEx⁻/MenDf: MenEx9-/Df(3R)kar^{3l}*, *MenEx15-/Df(3R)kar^{3l}*).

Transvection appears to result from homology-dependent chromosome pairing (HENIKOFF and COMAI 1998). It is generally thought that chromosomes with large deletions or rearrangements might not pair and therefore would not show transvection (HENIKOFF and COMAI 1998). If the higher than expected levels of MEN activity observed in the knockout/full-activity heterozygotes were the result of transvection, then the excision-deletion group should show higher levels of MEN activity (elevated by transvection) than the deficiency group (incapable of transvection by the reasoning above). We detected a significant effect of genotype on MEN activity ($F_{7,63} = 224.89$, $P < 0.0001$). A Tukey’s HSD pairwise comparison showed no difference within a genotypic group [*i.e.*, *MenEx³⁺/Df(3R)kar^{3l}* and *MenEx⁵⁺/Df(3R)kar^{3l}* did not significantly differ in activity] and the data were therefore combined by class. The combined data showed a significant genotype effect ($F_{3,63} = 1090.46$, $P < 0.0001$) and a Tukey’s

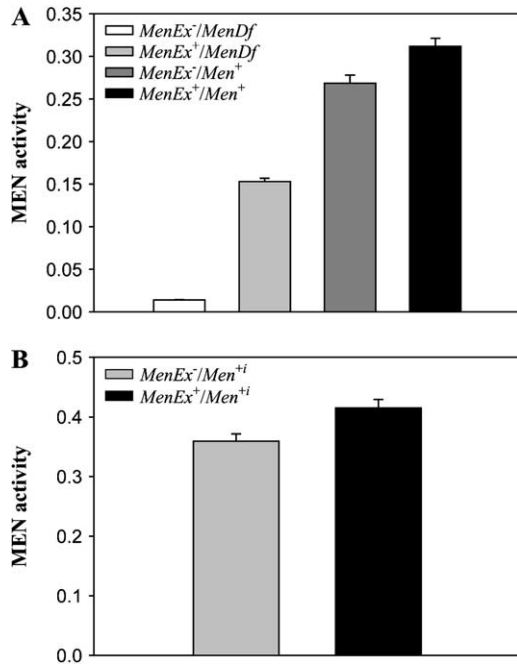


FIGURE 5.—(A) Mean MEN activity estimates from crosses of *EP(3)0517* excision series with a line carrying a large chromosomal deficiency [*Df(3R)kar^{3l}*] that covers the *Men* gene and a line carrying a wild-type copy of the gene. The four groups are *MenEx⁻/MenDf*, *MenEx⁺/MenDf*, *MenEx⁻/Men⁺*, and *MenEx⁺/Men⁺*, reading from left to right. (B) Mean MEN activity from crosses of *MenEx⁻* (left) and *MenEx⁺* (right) to 10 different *Men⁺* lines. Error bars indicate one standard error.

HSD pairwise comparison of MEN V_{\max} between the four classes found that knockout < deficiency heterozygote < deletion heterozygote < 100% (Figure 5A). The significantly higher levels of MEN activity in the deletion heterozygote group than in the deficiency heterozygote group indicate that having only a single functional copy of the *Men* gene does not necessarily result in upregulation of that locus. Additionally, pairwise comparisons indicated that the deletion heterozygote flies (*MenEx⁻/MenEx⁺*), but not the deficiency heterozygote flies [*Df(3R)kar^{3l}/Men⁺*], had significantly greater than half the activity of the normal-activity flies (*MenEx⁺/Men⁺*; $F_{1,40} = 248.00$, $P < 0.001$). Together, these two results suggest that the increased activity in deletion flies is the result of transvection. This is not, however, a perfect test in that the comparison between the *Df(3R)kar^{3l}/Men⁺* group and the *MenEx⁻/Men⁺* group is confounded by potential *trans*-acting differences between the *Df(3R)kar^{3l}* chromosome and the *CT68* (*Men⁺*) chromosome (*i.e.*, line effects) in addition to any effects from the *Men* deficiency. However, comparison of the activity in flies that combine *Df(3R)kar^{3l}* and other third chromosomes consistently found that the *Df(3R)kar^{3l}* lines had half the activity of their counterparts (data not shown), supporting our conclusion that the higher levels of MEN activity in the *MenEx⁻/Men⁺* flies are the result of transvection.

To test for possible differences in the level of transvection across different chromosomes, we crossed the *MenEx⁹* knockout line and the *MenEx³⁺* normal-activity line to 10 different isothird chromosome lines (*Men⁺*, $i = 1-10$) that shared a common genetic background (X and second chromosomes from line 6326). All lines carried the *Men^G* allele (the same as the normal-activity excision lines) to avoid differences in K_m between alleles from possibly preventing us from accurately predicting the amounts of *in vivo* MEN activity. The *MenEx⁻/Men⁺* and *MenEx⁺/Men⁺* flies differed in activity ($F_{9,175} = 23.86$, $P < 0.001$). Across the 10 isothird lines, the activities of the *MenEx⁻/Men⁺* genotypes averaged 87% (± 0.02) of those of the *MenEx⁺/Men⁺* genotypes, with a range from 75 to 93% (Figure 5B). Individual third chromosome lines varied in MEN activity (a significant line effect was detected, $F_{9,175} = 4.67$, $P < 0.0001$), but there was no significant line-by-excision interaction ($F_{9,175} = 0.97$, $P < 0.47$), indicating that the amount of transvection did not significantly differ across the different third chromosome lines. The significant line effect allows us to estimate the amount of variation in MEN activity contributed by the different third chromosomes. Across all 10 lines the mean MEN activity of the *MenEx⁺/Men⁺* flies was 0.041, with a line-specific standard deviation of 0.00346 or 11% of the mean activity.

The *MenEx⁻/Men⁺* and *MenEx⁺/Men⁺* samples were assayed for triglyceride concentration and IDH and G6PD activity (Figure 6, A–C). The data were analyzed for *Men* genotype and line effects following the same methods as the wild *Men* allele data. We found no consistent variation in triglyceride concentration with changes in MEN activity ($F_{1,175} = 0.004$, $P < 0.95$). A significant line effect on triglyceride concentration was observed ($F_{1,175} = 2.13$, $P < 0.04$), but there was no significant interaction between line and excision chromosome ($F_{9,175} = 1.67$, $P < 0.11$). For G6PD and IDH, we found highly significant increases in activity as MEN activity decreased across this small range ($F_{1,175} = 8.41$, $P < 0.005$ and $F_{1,175} = 11.42$, $P < 0.001$, respectively), similar to the results from the comparison between *Men^A* and *Men^G* alleles and the initial characterization of the *Men* excision set. The results also show a significant difference in IDH, but not in G6PD, activity among third chromosome lines ($F_{9,175} = 5.86$, $P < 0.0001$ and $F_{9,175} = 1.39$, $P < 0.21$, respectively). There were no significant line-by-excision chromosome interactions for IDH or G6PD activity ($F_{9,175} = 0.64$, $P < 0.76$ and $F_{9,175} = 0.42$, $P < 0.92$, respectively).

DISCUSSION

This study examines the potential of an amino acid polymorphism in *D. melanogaster* malic enzyme to be under selection, examining the population genetics and biochemistry of the two alleles as well as the

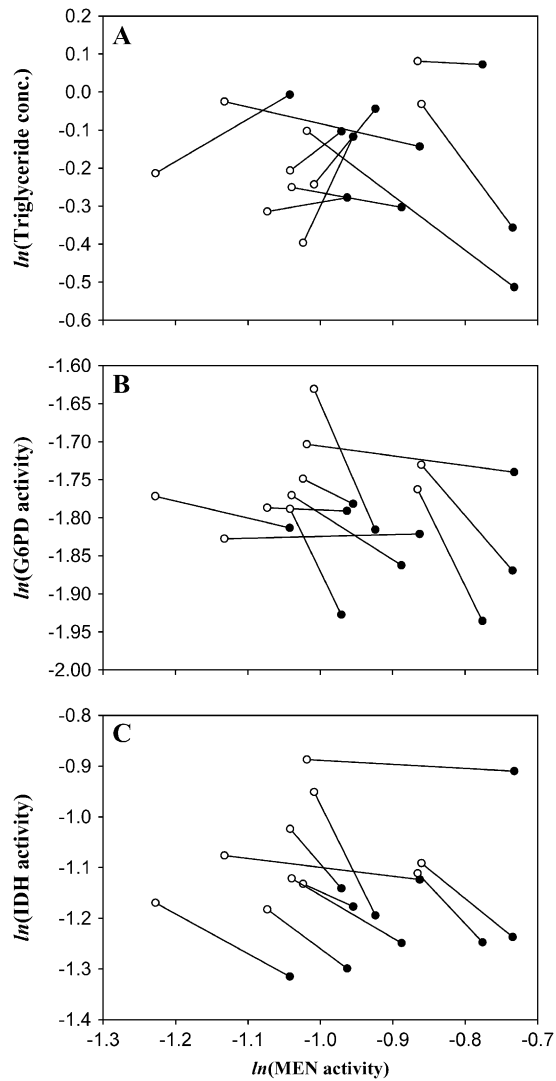


FIGURE 6.—Comparison in response to changes in MEN activity across 10 different different *Men^G* third chromosome lines. *MenEx⁺* and *MenEx⁻* flies were crossed to 10 different *Men^G* (*Men⁺*) lines to produce *MenEx⁻/Men⁺* and *MenEx⁺/Men⁺* heterozygotes. Each pair of points shows the change in (A) triglyceride concentration (micrograms per microgram protein), (B) G6PD activity, or (C) IDH activity with change in MEN activity ($10,000 \times \mu\text{M NADPH}/\text{min}/\mu\text{g protein}$) in a different third chromosome background (*Men⁺*). Values from *MenEx⁻/Men⁺* flies are shown in open circles, and those from *MenEx⁺/Men⁺* flies are in closed circles. Pairs of values for each *Men⁺* are connected by a line. Log values are plotted so that the slope of each line is an estimate of the control (A) or elasticity (B and C) coefficient in each line. Note the large difference in MEN values (x-axis) reflecting line-specific differences in MEN activity. Note also the relative constancy in the sign of slope of the lines (direction of interaction) in B and C, but not in A.

interaction between the two alleles and IDH and G6PD activity and triglyceride concentration. To complement our description of the two natural alleles, we used *P*-element excision-derived synthetic alleles to examine the effects of differences in MEN activity on IDH and

G6PD activity and on triglyceride concentration. The strength of this approach is our ability to contrast the results from our examination of naturally occurring alleles with those from these laboratory-derived alleles.

The presence of both *Men^A* and *Men^G* alleles across all North American populations (SEZGIN *et al.* 2004) suggests the possibility that the polymorphism may be under selection. There is, however, no DNA sequence-based evidence for selection maintaining this polymorphism; all Tajima's and Fu and Li's tests were not significant and SEZGIN *et al.* (2004) found no evidence for geographic clinal variation in the frequency of the two alleles (which could have indicated selection between them). Tajima's and Fu and Li's tests are, however, relatively weak tests for selection and not all polymorphisms under selection will necessarily show clinal patterns of allele frequency. Further, similar amounts of synonymous polymorphism within samples of the two alleles (Table 1) suggest that the *Men^G* allele is not a recent mutation. There is also apparent partitioning of silent polymorphisms between the two allele classes (Figure 2). This partitioning and the apparent age and widespread distribution of the amino acid polymorphism are intriguing, but do not implicate any one factor in the maintenance, or possible selective significance, of the amino acid polymorphism.

Under standard *in vitro* characterizations, the *Men* glycine/alanine amino acid polymorphism appears to result in differences in both V_{max} and K_m for malate. Comparison with the structure of the cytosolic malic enzyme from pigeon (from YANG *et al.* 2002) suggests that the amino acid polymorphism is structurally close to the active site of the molecule, particularly close to a tyrosine residue that is thought to interact with the substrate in the active site and is conserved across MEN proteins (YANG and TONG 2000). While assigning functional consequences to amino acid substitutions in a 3D structure of any enzyme is complex, we propose that the close proximity of the amino acid polymorphism to the enzyme active site may be responsible for the observed difference in both V_{max} and K_m for malate. Also, the polymorphism is in a helical region of the protein and glycine/alanine substitutions are known to affect helical stability (GANTER and PLÜCKTHUN 1990; CHAKRABARTY *et al.* 1991), suggesting that the differences between the two alleles could be a product of differing protein stability. We found no evidence of any difference in thermal tolerance between the two alleles, however, suggesting that stability, at least as reflected in thermal stability, is not the mechanism conferring the observed differences.

V_{max} is a function of enzyme concentration and the catalytic rate constant, k_{cat} . In addition to possible modification of k_{cat} by the amino acid substitution, the observed difference in V_{max} could also result from, or be modified by, a linked regulatory polymorphism resulting in a greater amount of MEN^G (*i.e.*, upregulation of transcription of the *Men^G* allele). The structure of the

regulatory region of the *Men* gene is not known but the linkage disequilibrium associated with the amino acid polymorphism and sites 1.2 kb away suggests that association between that site and a regulatory polymorphism is conceivable. Further, the amino acid change itself could result in differences in protein turnover (half-life) altering protein concentration and resulting in differences in V_{\max} .

The differences in V_{\max} and malate K_m predict a 19% difference in *in vivo* activity (V_{\max}/K_m) between alleles. This difference in V_{\max}/K_m is correlated with significant differences in triglyceride concentration and IDH and G6PD activity; all three increase with decreasing MEN activity across this range (Figure 3). Additionally, in all three measures from the *Men*^{G/A} heterozygotes are intermediate to those from the homozygous flies, suggesting that overdominance for activity between the two alleles does not occur. The IDH and G6PD results are consistent with a model of compensation to maintain a constant pool of reduced cofactor, NADPH, for lipogenesis (GEER *et al.* 1976). The observed increase in triglyceride concentration with decrease in MEN activity was unexpected given the role of MEN in lipogenesis. It is possible that this increase results from the observed increase in G6PD and IDH activities in effect overcompensating, leading to a significant increase in available NADPH and the standing concentration of triglyceride.

To complement our analysis of the naturally occurring *Men* alleles, we used *P*-element-mediated excision to create a set of *Men* alleles in isogenic backgrounds. This allele set included two lines containing deletion knockout *Men* alleles and two lines containing full-, or normal-, activity *Men* alleles. Because the alleles are derived from a single parent chromosome and result from small deletions spanning the promoter and/or beginning of the coding region they must differ only in the amount of transcript produced (not K_m) and therefore V_{\max} accurately reflects the amount of *in vivo* activity of each allele. Additionally, because the lines are entirely isogenic they are free of differences in *trans*-acting effects and any observed differences in triglyceride concentration or IDH or G6PD activity can be directly attributed to the allele-specific differences in MEN activity.

Interactions between MEN, triglyceride, IDH, and G6PD are apparent in comparison of the excision alleles. Triglyceride concentration is halved with the complete loss of MEN activity, consistent with the proposal that MEN is a major source of NADPH for lipogenesis (WISE and BALL 1964; GEER *et al.* 1979a). There is no apparent difference in triglyceride between the two high-MEN-activity groups, in contrast to the negative correlation between MEN and triglyceride seen between the naturally occurring alleles. G6PD activity increases as MEN activity decreases, which is consistent with activating the pentose shunt to supply reduced NADPH for lipogenesis and also consistent with our results from the *Men*

polymorphism. There is an apparent trend toward an initial increase in IDH with a small decrease in MEN followed by a larger decrease in IDH activity with decreasing MEN activity, although this was not statistically significant (Figure 4D). The initial increase in IDH activity is consistent with the results from the wild *Men* alleles. The final decrease in IDH activity with the *Men* complete knockout does not fit a model of activity compensation, but is consistent with some earlier observations of the interaction across large-scale differences in activity between these loci in larvae (WILTON *et al.* 1982; BENTLEY *et al.* 1983; GEER and LAURIE-AHLBERG 1984). GROMNICKI and BENTLEY (1991), however, found compensatory changes in activity between these enzymes. The interaction between IDH and MEN is apparently complex, at least over large-scale changes in activity.

It appears that we have identified a clear case of transvection at an enzyme locus; the *MenEx*⁻/*Men*⁺ heterozygotes had significantly higher than expected MEN activity irrespective of whether the *Men* deletions are placed in *trans* with the precisely excised allele (Figures 4 and 5A) or with the 10 wild chromosome alleles (Figure 5B). Our finding that *Df(3R)kar*³¹/*Men*⁺ (large deficiency) heterozygotes have only half the activity of wild-type flies and significantly less than levels of activity in the *MenEx*⁻/*Men*⁺ (small deletion) heterozygotes (Figure 5A) suggests that upregulation of the functional gene in response to some physiological cue is not the source of the observed higher than expected activity. The consistency in levels of upregulation in crosses to different third chromosomes (Figure 5B) indicates that the apparent transvection effect is robust to genetic background. The 5' upstream region of *Men* has no recognizable coding regions for >17 kb, yet possesses several small highly conserved noncoding regions (in comparison with other *Drosophila* species, data not shown) that could be enhancer sites. Both *P*-element excision-derived deletions remove >2 kb upstream of the *Men* transcription start site and can be assumed to have removed the *Men* promoter, likely a requisite for transvection (MORRIS *et al.* 1999, 2004).

The *MenEx*⁻/*Men*⁺ and *MenEx*⁺/*Men*⁺ flies were assayed for triglyceride concentration and IDH and G6PD activity to examine the consequences of the excision-associated difference in MEN activity in conjunction with a variety of background chromosomes (Figure 6, A–C). The difference in activity between the *MenEx*⁻/*Men*⁺ and *MenEx*⁺/*Men*⁺ flies was similar in magnitude to that between the naturally occurring *Men*^A and *Men*^G alleles, meaning that comparison of the synthetic allele genotypes is both biologically meaningful and directly comparable to that of the naturally occurring alleles. The results for G6PD and IDH activity are similar to those seen in our examination of the wild *Men*^A and *Men*^G alleles; both G6PD and IDH activity increase as MEN activity decreases (Figure 6, B and C), again

suggesting compensation. In contrast, no consistent pattern of change in the average triglyceride concentration was detected across this upper range of MEN activity and in these 10 genetic backgrounds (Figure 6A). While the lack of consistent change in triglyceride concentration was unexpected, the concordance in results between the synthetic and natural *Men* allele experiments for IDH and G6PD activity supports our use of V_{\max}/K_m , and not simply V_{\max} (which would have implied an opposite interaction), to estimate relative *in vivo* activity between the two naturally occurring *Men* alleles.

We can use this comparison of *MenEx⁻/Men⁺* with *MenEx⁺/Men⁺* flies to quantify the interactions between MEN activity and triglyceride concentration, IDH activity, and G6PD activity. A change in metabolite concentration in response to a change in enzyme activity can be quantified as a concentration control coefficient (KACSER and BURNS 1981; FELL 1997), the ratio of change in concentration, S , to change in enzyme activity, E , scaled by S and E : $C_E^S = (\delta S/S)/(\delta E/E)$. Across small changes in enzyme, $C_E^S = (\delta \ln S)/(\delta \ln E)$. Control coefficients can therefore be calculated as the slope of a plot of $\ln S$ vs. $\ln E$ across small changes in enzyme concentration (KACSER and PORTEOUS 1987; FELL 1997). Control coefficients generally range from 0 to 1, but values >1 , or <0 , are possible in special circumstances. An enzyme with a coefficient near 1 has a large immediate influence over a given metabolite concentration. Figure 6A shows the changes in triglyceride concentration for each of the third chromosome crosses. The slope of the line connecting a pair of MEN values is an estimate the control of triglyceride concentration by malic enzyme, $C_{\text{MEN}}^{\text{TRIG}}$, in that comparison (*Men⁺* background). The mean slope, and associated error, across all 10 lines gives an overall estimate of the control coefficient. The triglyceride results show no uniform pattern across lines; as MEN activity increased triglyceride concentration went up in 5 lines, but down in 5 lines (Figure 6A) and $C_{\text{MEN}}^{\text{TRIG}} = 0.23 \pm 0.400$ (mean \pm SE). Overall, malic enzyme does not show significant control on triglyceride concentration across these 10 genetic backgrounds.

Elasticity coefficients (ϵ) quantify change in enzyme activity in response to change in some modifier (temperature, metabolite concentration, etc.) in a manner similar to that of a control coefficient. Strictly speaking, while the elasticity coefficient of an enzyme on itself, e.g., $\epsilon_{\text{MEN}}^{\text{MEN}}$, is expected to be one (enzyme activity in exact proportion to enzyme concentration), the elasticity of one enzyme on another is expected to be zero; enzymes are not generally expected to directly interact (WESTERHOFF and VAN DAM 1987). The changes we observe in G6PD and IDH activity are most likely not direct, but mediated by the NADP/NADPH cofactor pool. Because our interest is in quantifying the possible responses between these loci, not necessarily the exact molecular mechanism, we can take the simplifying step of calculating the elasticity of IDH and G6PD with

reference to MEN activity levels. We make this simplification with the understanding that we are quantifying the change in NADPH levels through changes in MEN activity and the associated responses of IDH and G6PD activity. In 9 of the 10 lines examined (Figure 6, B and C), G6PD activity increased as MEN activity decreased, with a mean elasticity coefficient (slope) of $\epsilon_{\text{MEN}}^{\text{G6PD}} = -0.76 \pm 0.236$. Similarly, in all 10 lines examined IDH activity increased as MEN activity decreased, with a mean elasticity coefficient of $\epsilon_{\text{MEN}}^{\text{IDH}} = -0.88 \pm 0.208$. The activities of MEN, IDH, and G6PD appear to be tightly interconnected, consistent with a need to maintain a stable pool of reduced NADPH. This interconnection is consistent in examination of both the naturally occurring and the excision series *Men* alleles. The relationship between MEN activity and triglyceride concentration is likely complicated by the interaction between MEN and these other sources of NADPH.

The glycine/alanine polymorphism in *D. melanogaster* malic enzyme is widespread in North America, but we find no population genetic evidence of selection maintaining its existence. However, to repeat a truism, an absence of evidence is not evidence of absence and sequence-based tests of selection are weak. In the absence of convincing sequence-based evidence, examination of the ability of genotypic differences to modify the physiology of the organism is a reasonable approach in examining selection potential. In sum, our results from the wild and engineered *Men* alleles indicate that MEN activity can affect the physiology of the fly and suggest that levels of MEN, IDH, and G6PD activity are coregulated to maintain the homeostasis of NADPH. Given the predicted difference in activity between the two naturally occurring *Men* alleles it seems unlikely that they are selectively neutral under all conditions. Further, the results presented here are from whole adult males fed *ad lib* on a complete diet and are likely minimum measurements of the effects of MEN activity. Future research, on both adult and larval flies, incorporating variation and stress in diet and environment, as well as direct modification of IDH and G6PD activities, will further clarify the interactions in this system and shed light on the extent of selection on this polymorphism.

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