The Relationship Between Dipeptidase Activity Variation and Larval Viability in *Drosophila melanogaster*

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ABSTRACT

The enzyme dipeptidase-A (DIP-A) in *Drosophila melanogaster* is coded by a second chromosome locus that is polymorphic for three allozymes in natural populations. DIP-A appears to be the only enzyme in *D. melanogaster* capable of hydrolyzing the dipeptide glycyl-L-isoleucine, since flies homozygous for null alleles at this locus have no detectable glycyl-L-isoleucine-ase activity. DIP-A activity occurs in many tissues and throughout development, but is particularly high in the larval midgut, suggesting an important role in protein digestion. These observations suggested an experimental design for investigating the adaptive significance of genetic variation in DIP-A activity. Fitness components of DIP-A variants could be estimated and compared under two environmental conditions (defined diets under axenic conditions). In the restrictive environment, the essential amino acid L-isoleucine is provided only in the form of glycyl-L-isoleucine, whereas in the permissive environment, L-isoleucine is provided in free form. We predicted that DIP-A activity would be essential in the restrictive, but not in the permissive environment. The results reported here clearly contradict this prediction. Two stocks homozygous for DIP-A null alleles from different geographic locations are each viable on the restrictive diet. Furthermore, relative viability experiments in which null allele larvae compete with larvae having DIP-A activity provide no evidence for even a partial reduction in egg to adult survival on the restrictive diet. Apparently, the null allele larvae have some alternative mechanism for obtaining L-isoleucine from the dipeptide, even though no glycyl-L-isoleucine-ase activity can be detected in vitro. These results, along with the viability of null alleles for many other enzymes, support the idea that eukaryotes have an intricate network of alternative biochemical pathways through which the same necessary function may be achieved. Such "buffering capacity" makes it very difficult to analyze the effects of enzyme variants on fitness components.

The debate concerning the forces responsible for maintaining allozyme polymorphisms in natural populations has not reached a satisfactory resolution. The direct assessment of fitness component differences among allozymic genotypes has been frustrated by a number of very serious problems (LEWONTIN 1974). Many eukaryotes do not have life histories suitable for fitness component estimation in the field or even in the laboratory, and even when it is possible to measure fitness, extremely large sample sizes are required. There is also the problem of whether observed differences among genotypes actually apply to the locus under observation, because of the potential for linkage disequilibrium. Another serious problem is whether important environmental factors or other selection pressures are actually operational during the fitness estimation. In spite of these problems, some important information about the effects of allozyme variation has been obtained from carefully designed laboratory experiments. One approach to detecting selection operating on a particular allozyme polymorphism is to manipulate the environment of the organism with respect to a specific function of the enzyme and predict fitness component differences based on biochemical differences among the allozymes (CLARKE 1975; KOEHN 1978). The problem of sensitivity in detection of fitness differences is alleviated by creating large selection pressures that directly impinge on the enzyme under observation, such as exposure to environmental alcohol in the investigation of alcohol dehydrogenase polymorphism in *Drosophila* (CLARKE 1975). The problem of linkage disequilibrium is alleviated by comparing fitness component differences in environments in which the putative selection pressure is present and absent and by making predictions about the direction of fitness differences based on biochemical parameters of the allozymes. This approach has been implemented in a most elegant and sensitive manner in the analysis of allozyme polymorphisms in chemostat populations of *Escherichia coli* (HARTL and DYKHUIZEN 1984 and references therein).
The studies reported here were undertaken to determine whether the dipeptidase enzymes in *Drosophila melanogaster* are suitable for selection studies of the type described above. This species has at least three peptidases, designated Dip-A, Dip-B and Dip-C, that partially overlap in their substrate specificities (Laurie-Ahlberg 1982). Each enzyme hydrolyzes a variety of dipeptides, but locus-specific substrates have been identified. Their genetic map positions are 55.2 on the second chromosome for the Dip-A structural locus (Voelker and Langley 1978), 53.6 on the third chromosome for Dip-B (Laurie-Ahlberg 1982), and the cytogenetic region 87B5-6 to 87B8-10 on the third chromosome for Dip-C (Ohnishi and Voelker 1981). Genetic localization studies of Dip-A and Dip-B by Hall (1986) agree with these. Three Dip-A allozymes have been found segregating in natural populations of *D. melanogaster*, while Dip-B and Dip-C appear to be essentially monomorphic (Voelker and Langley 1978). Screens of lines that vary only in the second or third chromosome have revealed genetic bases for a continuous distribution of dipeptidase activities (Laurie-Ahlberg 1982).

The Dip-A and Dip-B enzymes are found in nearly every tissue studied throughout development, but are particularly high in the larval midgut and in the adult female reproductive tissues (Laurie-Ahlberg 1982). The physiological functions of these enzymes are unknown, but their ubiquitous distribution suggests a general metabolic function. Collett (1976a) suggested they play a vital role in regulation of the amino acid and oligopeptide pools in the hemolymph and other tissues. Free amino acids and their derivatives occur in high concentrations in insects (Chen 1966, 1971), and Drosophila is of no exception (Collett 1976a, b, c). The occurrence of particularly high Dip-A activities in the larval midgut suggests an important role in the final stages of protein digestion. If this is the case, then selection pressures might be applied specifically to the Dip-A locus through dietary manipulation of its substrates. This manipulation could be accomplished through rearing larvae axenically on precisely defined media (Geer 1966b; Hunt 1970).

The experiments described here were performed to determine whether the Dip-A enzyme has a vital role in releasing free amino acids from dietary peptides for use in growth and development. Egg to adult viability for individuals with and without Dip-A activity was analyzed under two environmental conditions. The restrictive environment was designed to make Dip-A activity essential. Dip-A appears to be the only locus through dietary manipulation of its substrates. The restrictive environment provides the essential amino acid L-isoleucine only in the form of glycyll-L-isoleucine. In the permissive environment, L-isoleucine is provided in free form. The prediction was that Dip-A null genotypes would be lethal under the restrictive, but not the permissive environment. The results clearly contradict this prediction.

### MATERIALS AND METHODS

**Stocks**: Two Dip-A null activity lines, Dip-A<sup>ene</sup> and Dip-A<sup>ence</sup> (from a fly collection in North Carolina) and Dip-A<sup>ck</sup> and Dip-A<sup>ck</sup> (from Great Britain), were generous gifts from R. A. Voelker. Two cytogenetic deficiencies for Dip-A, Df(2R)bwVDe2'/G1a and Df(2R)M-S2/Gla, were obtained from the Mid-America Drosophila Stock Center at Bowling Green State University. Their genetic descriptions are given by Lindesly and Grell (1968). The origin and construction of RIOJJI, a high Dip-A activity outlier line, is given by Laurie-Ahlberg et al. (1980). Hochi-R, a wild-type inbred line that has been maintained under standard laboratory conditions, was also used in these experiments.

**Axenic culture**: Lines were maintained under axenic conditions on a dead yeast-sucrose medium (12.5% brewers' yeast; 10% sucrose; 2% agar) at 23°C on a 12-hr light cycle for at least 1 yr before the experiments. All transfers and crosses were performed under sterile conditions in a laminar flow hood, and anesthesia was performed using filter-sterilized carbon dioxide.

To monitor microbial contamination, food from randomly selected vials was plated onto slants of mycological agar, Sabouraud dextrose agar, and tryptic soy agar. These were chosen on the basis of their suitability to cultivate molds, yeasts, fungi, and microorganisms in general. Slants were incubated at 37°C for 7 days. Because of the nutritional richness of the Drosophila synthetic media, contamination could often be detected by color changes in the food itself.

**Media preparation**: The three synthetic media in this study were driven from Hunt's (1970) recipe with the following modifications: (1) *Control medium*: l-isoleucine was omitted entirely from the recipe; (2) *Amino acid medium*: to Hunt's medium, glycine was added at the same concentration specified for l-isoleucine; and (3) *Dipeptide medium*: an equimolar amount of glycyll-l-isoleucine was substituted in the recipe for l-isoleucine and glycine.

Batches of glycyll-l-isoleucine, l-isoleucine, and glycine were checked for purity by reversed-phase high-performance liquid chromatography (HPLC) using the o-phthaldehyde pre-column derivatization technique with Waters RESOLVE C<sub>18</sub> column. Amino acid-dipeptide profiles were obtained for both the amino acid and the dipeptide media before and after autoclaving of the food.

**Enzyme assays**: Dip-A activity was assayed according to the procedures of Laurie-Ahlberg (1982) with the following modifications. Instead of boiling to stop the hydrolysis of the dipeptide, 50 µl of 0.5 N HCl were added to the reaction mixture. Before the addition of l-amino acid oxidase reagent (LAOR), each sample tube was treated with 50 µl of 0.5 N NaOH. The LAOR step was allowed to proceed for 25 min, then stopped by the addition of 100 µl of 0.5 N HCl. Absorbance was read at 410 nm using a spectrophotometer.

1-Leucine aminopeptidase (LAP) activity was assayed using a modification of the method of Pyleider (1970). For each line, 20 third instar larvae were homogenized in 300 µl of 0.05 M Tris-HCl buffer (pH 7.2). The homogenate was divided into two equal fractions, then centrifuged in 1.5-ml polypropylene tubes at 6390 X g for 10 min. For one
fraction, the pellet was dissolved in 0.4 ml of 0.05 M Tris-HCl buffer (pH 7.2), and the supernatant was mixed with 0.05 ml of the same buffer. For the other fraction, the pellet was dissolved in 0.4 ml of 0.05 M Tris-HCl buffer (pH 7.2) that also contained 1% Triton X-100, and the supernatant was mixed with 0.05 ml of the same buffer. All four preparations were assayed spectrophotometrically for both DIP-A and LAP activities. LAP activity was quantitated by the spectrophotometric monitoring of the hydrolysis of 0.2 M leucine-β-nitroanilide in 0.05 M Tris-HCl, pH 7.2. Absorbance changes were recorded continuously at 410 nm after 50 μl of a sample was added to 1 ml of the substrate solution.

Absolute larval viability on axenic media: Larval survival in D. melanogaster depends upon the availability of dietary essential amino acids of which L-isoleucine is one (Geer 1966a). Since extracts of DIP-A null activity lines show no hydrolysis of glycyl-L-isoleucine under optimal DIP-A assay conditions, one would predict no survival of null lines when the sole dietary source for L-isoleucine is in the form of the dipeptide. The survival of the stocks under axenic conditions on completely defined synthetic media was tested by placing ten males and ten females of a given stock on a given medium, and monitoring larval development. Adults were cleared from vials after four days to insure the detection of emerging adults.

Larvae from Hochi-R, RIO3, II, Dip-A^{ANC1}, and Dip-A^{GB1} that were grown on each medium were assayed for DIP-A activity. To understand the functional and physiological relationship between LAP and dipeptidases, Hochi-R, Dip-A^{ANC1}, and Dip-A^{GB1} were assayed for both DIP-A and LAP under optimal conditions for LAP activity.

Relative larval viability on axenic media: Relative larval viability was estimated by measuring the proportions of DIP-A null vs. active progeny from a cross. The two DIP-A null alleles (Dip-A^{+}) and the two DIP-A cytogenetic deficiencies (Df(2R)) described were used. In each cross, DIP-A/DIP-A females were crossed to Df(2R)/DIP-A^{+} males. Four such crosses were made, each corresponding to a different combination of null allele and deficiency. In the progeny, DIP-A activity cosegregates with the dominant Glazed eye phenotype, so the numbers of DIP-A null and active phenotypes were easily scored. Each cross was made on each of two defined media under axenic conditions, the amino acid and dipeptide media. Viability differences due to DIP-A activity variation were expected to appear as a smaller proportion of null progeny on the restrictive dipeptide diet than on the amino acid diet.

Amino acid and dipeptide media were prepared as described above. A block refers to one batch of medium that was prepared at a particular time. Vials, each containing 5 ml of medium, were kept refrigerated until the time of use. Crosses were performed by mating five virgin females to five males. After 3 days, the parents were transferred to a new set of vials. The two sets of vials are referred to as broods. Within each block, crosses were made on each of 2 days, repeated to as sets. A total of five replicate five-pair matings were made within each set of each cross. Under these rearing conditions, F1 progeny could be collected until the 28th day after the first day of mating. A total of 33,716 flies were scored for eye phenotype from the 320 vials used in this experiment. Three of the vials contained microbial contamination. Results of analyses of the data with and without these vials were essentially identical.

Relative larval viability is reported in terms of the proportion of null activity flies in the total progeny. Since the wild type eye phenotype cosegregates with null activity, the segregation parameter is expressed as the number of wild type eyed flies divided by the total count of progeny flies. In an experiment of this sort, such effects as meiotic drive, cytoplasmic and extranuclear parental components are confounded with viability in the segregation ratio, and thus the classical Mendelian 1:1 ratio cannot be assumed. However, since the fundamental question addresses the relative larval viability differences associated with the two diets, it is sufficient to simply use the segregation parameter as the dependent variable for statistical analysis.

Relative larval viability on nonaxenic medium: One of the objectives of this experiment was to detect any reciprocal effects that may have been present in the previous experiment. The same lines were used to perform a relative larval viability test with the following additions. Hochi-R was included in the crosses, and reciprocal crosses were performed. Five pairs of females and males were mated in each vial containing standard cornmeal-molasses medium, and adults were cleared from vials after three days. A block refers to a week when crosses were made, and sets refer to different days when the flies were mated. Replicates (vials) are independent matings of the same cross. Adults were scored for eye phenotype through the 18th day after the initial day of mating. A total of 18,235 progeny flies from 240 vials were scored in this experiment. The segregation parameter, as defined in the previous experiment, was used as the dependent variable for statistical analysis.

RESULTS

Absolute viability on axenic media: Since L-isoleucine is an essential amino acid and since the two homozygous null lines have no detectable glycyl-L-isoleucine-ase activity, we predicted that these lines could not complete egg to adult development on a medium in which the only source of L-isoleucine is in the form of the dipeptide, glycyl-L-isoleucine. The results of three independent experiments, summarized in Table 1, clearly contradict the prediction. Both DIP-A null and activity lines survive on the dipeptide medium as well as on the positive control (amino acid) medium. None of the lines survived on the negative control (total omission of L-isoleucine in any form), which confirms Geer's (1966a) finding that this amino acid is absolutely essential for egg to adult development.

Different batches of glycyl-L-isoleucine that were used for these experiments were analyzed for purity.

### Table 1

<table>
<thead>
<tr>
<th>Line Activity</th>
<th>Medium Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIP-A activity</td>
<td>No L-ile</td>
</tr>
<tr>
<td>Hochi-R</td>
<td>+</td>
</tr>
<tr>
<td>RIO3, II</td>
<td>+</td>
</tr>
<tr>
<td>Dip-A^{ANC1}</td>
<td>-</td>
</tr>
<tr>
<td>Dip-A^{GB1}</td>
<td>-</td>
</tr>
</tbody>
</table>

* Medium composition is as given by Hunt (1970) with the modifications described in the materials and methods section. These results are a summary of three independent experiments, each with ten replicate vials per medium per line.
by HPLC. Quantities as low as 50 pmol can be detected reliably with the o-phthaldialdehyde precolumn derivatization technique. Although some glycine was detected with glycyl-L-isoleucine, no L-isoleucine was detected. Samples of the dipeptide medium, before and after autoclaving, were also analyzed to determine if the peptide bond was being hydrolyzed as a consequence of medium preparation. No evidence for L-isoleucine liberation was found.

Another possible explanation for the survival of the null lines under the restrictive nutritional condition is the hydrolysis of the dipeptide by microbial contaminants. At the conclusion of every run of this experiment, stabs of food from every vial were plated onto mycological agar, Sabouraud dextrose agar, and tryptic soy agar. Results from contaminated vials are excluded from the summary. No reason exists to suspect the involvement of microbial contaminants in the experimental results.

The induction of DIP-A activity in the null lines by the presence of a substrate for DIP-A could be presented as an argument for their survival on the dipeptide diet. To test for this possibility, larval homogenates for each line by medium combination were assayed for DIP-A activity. Although activity differences were detected for Hochi-R and R103.11 between the amino acid and the dipeptide media, no activity could be detected for either DIP-A^{NC1} or DIP-A^{GB1} under either medium condition.

Yet another possibility in the survival of the null lines is that other enzymes may be involved in the in vivo hydrolysis of the dipeptide. Sample preparation and assay conditions employed for detecting the hydrolysis of glycyl-L-isoleucine have been optimized for homogenates from flies with DIP-A activity. The pH optimum was determined for dipeptidase activity using 0.2 M citrate buffer (pH 4.0–6.0), 0.1 M Tris-HCl buffer (pH 7.0–9.0), and 0.1 M glycine-NaOH buffer (pH 10.0). Optimum pH for the hydrolysis of L-leucyl-L-alanine was pH 8.5 (data not shown). Also, maximum hydrolysis of glycyl-L-isoleucine over the pH range of 6.0 to 8.85 was achieved at pH 8.5 (C. C. Laurie-Ahlberg, unpublished data). Glycyl-L-isoleucine-ase activity was high between pH 7.0 and pH 8.85, whereas activity levels dropped markedly below pH 7.0.

These same conditions are undoubtedly not optimal for all peptidases. The successful detection of other peptidic activities will be a function of such parameters as ionic strength, pH, and the type of components in the buffer, as well as how samples are homogenized and prepared for assays. For example, leucine aminopeptidases are membrane-bound, and the standard procedure for the preparation of samples for DIP assays would fail to solubilize LAP. Therefore, the possibility that LAP or similar enzymes actually have significant hydrolytic activity toward glycyl-L-isoleucine in DIP-A^{NC1} flies was investigated. The release of LAP activity upon treatment of membrane components with detergent is shown in Table 2. The supernatant fractions give very low LAP readings, whether treated with Triton X-100 or not. The untreated pellet fraction shows LAP activity similar to that of the supernatant fraction, but LAP of the treated pellet fraction is an order of magnitude greater. This trend is consistent among the DIP-A null lines as well as Hochi-R. The results from Hochi-R indicate that DIP-A activity is found predominantly in the supernatant, and treatment with detergent does nothing to elevate DIP-A activity levels. No DIP-A activity was detected for either null line in any of the fractions. These results suggest that LAP or other membrane-bound peptidases are not involved in the hydrolysis of glycyl-L-isoleucine.

### Relative larval viability on axenic media

The absolute viability experiment described above does not provide a quantitative measure of survival, but only indicates whether any individuals can survive from egg to adult. The relative viability experiment was performed to determine whether null activity conferred any measurable reduction in larval viability in comparison with a phenotype that has DIP-A activity. In this experiment, the segregation ratio of null versus active progeny phenotypes was measured on amino acid and dipeptide diets. If DIP-A activity is important in the acquisition of t-isoleucine for growth and development, then one might expect to find a lower ratio of null phenotype flies surviving on the dipeptide than on the amino acid medium.

Since any one of many mechanisms that cause deviation from the expected Mendelian segregation ra-

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**TABLE 2**

Comparison of LAP and DIP-A activities

<table>
<thead>
<tr>
<th>Line</th>
<th>Assay Buffer*</th>
<th>With 1% TX-100</th>
<th>Without TX-100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pellet</td>
<td>Supernatant</td>
<td>Pellet</td>
</tr>
<tr>
<td>Hochi-R</td>
<td>LAP</td>
<td>+0.0346</td>
<td>+0.0006</td>
</tr>
<tr>
<td></td>
<td>DIP-A</td>
<td>+0.0059</td>
<td>+0.0203</td>
</tr>
<tr>
<td></td>
<td>DIP-B</td>
<td>+0.0423</td>
<td>+0.1137</td>
</tr>
<tr>
<td>DIP-A^{NC1}</td>
<td>LAP</td>
<td>+0.0325</td>
<td>+0.0019</td>
</tr>
<tr>
<td></td>
<td>DIP-A</td>
<td>−0.0020*</td>
<td>−0.0004*</td>
</tr>
<tr>
<td></td>
<td>DIP-B</td>
<td>−0.0001*</td>
<td>0.0000</td>
</tr>
<tr>
<td>DIP-A^{GB1}</td>
<td>LAP</td>
<td>+0.0275</td>
<td>+0.0010</td>
</tr>
<tr>
<td></td>
<td>DIP-A</td>
<td>+0.0003*</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>DIP-B</td>
<td>−0.0004*</td>
<td>+0.0006*</td>
</tr>
</tbody>
</table>

* Letter codes are: T for Tris-HCl buffer, pH 7.2 (optimal for LAP activity) and B for boric acid-borax buffer, pH 8.5 (optimal for DIP-A activity).

* All activities are expressed as change in absorbance per minute. Each value represents the mean of at least two replicates.

* These values are not significantly different from zero.
Mean estimates of the segregation parameter for each cytogenetic deficiency by null activity line combination under axenic conditions

<table>
<thead>
<tr>
<th>Female parent</th>
<th>Diet*</th>
<th>Male parent</th>
<th>Dip-A&lt;sup&gt;BNC1&lt;/sup&gt;</th>
<th>Dip-A&lt;sup&gt;BNC2&lt;/sup&gt;</th>
<th>Female marginal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Df(2R)&lt;sup&gt;bu&lt;/sup&gt;-&lt;sup&gt;12A2Cy&lt;/sup&gt;R&lt;sup&gt;o&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0.296 (0.012)</td>
<td>0.463 (0.015)</td>
<td>0.580 (0.013)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIP</td>
<td>0.354 (0.012)</td>
<td>0.469 (0.015)</td>
<td>0.402 (0.012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVE</td>
<td>0.315 (0.009)</td>
<td>0.466 (0.010)</td>
<td>0.391 (0.009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Df(2R)M-S2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0.581 (0.011)</td>
<td>0.596 (0.011)</td>
<td>0.589 (0.008)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIP</td>
<td>0.577 (0.011)</td>
<td>0.587 (0.012)</td>
<td>0.582 (0.008)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVE</td>
<td>0.579 (0.007)</td>
<td>0.592 (0.008)</td>
<td>0.585 (0.006)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male marginal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0.439 (0.018)</td>
<td>0.530 (0.011)</td>
<td>0.484 (0.011)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIP</td>
<td>0.456 (0.016)</td>
<td>0.528 (0.012)</td>
<td>0.492 (0.010)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVE</td>
<td>0.447 (0.012)</td>
<td>0.529 (0.008)</td>
<td>0.488 (0.008)*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean estimates and their standard errors are given for each medium. Abbreviations are: AA for amino acid medium, DIP for dipeptide medium, and AVE for the mean between the two media.

Overall mean estimates and their standard errors.

Tio may be in operation in this experiment, no assumptions were made about the segregation parameter's being 0.5. The dependent variable was thus treated as a binomial variable. To minimize the heterogeneity of error variances, the data were transformed by taking the sin<sup>-1</sup> of the square root of the variance of the transformed data was performed using a linear model was employed:

\[ Y_{ijklm} = \mu + B_i + S_{ilj} + C_k + BC_{ik} + SC_{j(i)k} + D_t + BD_{it} + SD_{ijl} + CD_{ijl} + BCD_{ijlt} + SCD_{(i)kl} + R_m + BR_{m(t)} + SR_{j(i)m} + CR_{km} + BCR_{km} + SCR_{j(i)km} + DR_{km} + BDR_{km} + SDR_{(i)m} + CDR_{km} + BCDR_{km} + SCR_{j(i)km} + BCR_{km} + SCR_{j(i)km} + DR_{km} + BDR_{km} + SDR_{(i)m} + CDR_{km} + BCDR_{km} + E_{n(i)km} \]

where \( Y_{ijklm} \) is the transformed segregation parameter, \( \mu \) is the mean, \( B_i \) is the effect of the ith block (\( i = 1, 2 \)), \( S_{ilj} \) is the effect of the jth set (\( j = 1, 2 \)) within the ith block, and \( C_k \) (\( k = 1, \ldots, 4 \)), \( D_t \) (\( t = 1, 2 \)), and \( R_m \) (\( m = 1, 2 \)) are the effects associated with crosses, diet, and brood, respectively. The error term, \( E_{n(i)km} \) (\( n = 1, \ldots, 5 \)), is obtained from the replicates within each block by set by cross by food by brood combination. All other terms represent interactions. Crosses, diet, and brood were treated as fixed effects. The plot of residuals against predicted values from the above model produced no patterns.

Mean estimates of the segregation parameter for each cross by food combination are given in Table 3, and analytical results are presented in Table 4. Overall, the relative viability of the null activity progeny did not differ between the two axenic dietary conditions. Although not shown, analysis of variance of untransformed data with or without weighing, and analysis on unweighted transformed data were all consistent with these results. The three degrees of freedom associated with crosses allow for the performance of three independent orthogonal contrasts. Effects associated with differences in male parents, female parents, and their resulting combinations were either marginally significant (\( P < 0.06 \) for female parents) or highly so (\( P < 0.01 \) for male by female combinations and \( P < 0.05 \) for male parents) in contributing to the observed variation in the relative larval viability of the null activity phenotype. Orthogonal contrasts were also performed for the cross by diet interaction term. F-tests revealed that progeny produced by crosses that involved a specific male or female parent did not do differently on the two media. Seeing that crosses differed significantly in the relative viability of the null activity phenotype, analysis of variance was performed for each cross separately, as summarized in Table 5. Only in one of the crosses was a difference detected between the two media. Even in that case, the relative viability of the null activity phenotype was better on the dipeptide medium than on the amino acid medium, a direction that was not predicted.

**Relative larval viability on nonaxenic medium:**

As was the case in the previous experiment, total progeny counts per entry varied significantly, ranging from 17 to 136. The following linear model was used for the weighted analysis of variance of the transformed data:

\[ Y_{ijkl} = \mu + B_i + S_{ilj} + C_k + BC_{ik} + SC_{j(i)k} + E_{l(i)k}. \]

\( Y_{ijkl} \) is the \( \sin^{-1} \) transformation of the segregation parameter of the jth set in the ith block of the kth cross in the lth replicate. \( B_i, S_{ilj}, \) and \( C_k \) are the block, set, and the cross effects, respectively. \( BC_{ik} \) and \( SC_{j(i)k} \) are the interaction terms, and \( E_{l(i)k} \) is the error term. The cross effect can be partitioned further into the...
interaction error terms from C X Sequence of transformation or weighting of data. In this associated with the reciprocals, cytogenetic deficiencies were used as female parents and null activity phenotypes. Con- for M, F, and M X F were performed using the appropriate interaction error terms from C X B. D X C X B mean squares were used as error term to test D X C.

following components: $L_n$, $N_n$, $A_p$, $LN_{ma}$, $LA_{mp}$, $NA_{np}$ and $LNA_{mp}$, where the first three terms are effects associated with the reciprocals, cytogenetic deficiencies, and DIP-A alleles, respectively, and the other terms are their interactions. All of the cross components were treated as fixed effects.

Analytical results differed very little as a consequence of transformation or weighting of data. In this respect, the data from this experiment are similar to those that were obtained on axenic media. A summary of the weighted analysis of variance of the transformed data is given in Table 6. Neither reciprocal differences nor cross combination differences were detected. The marginal mean estimates of the segregation parameter for each line as parents were 0.506 for DIP-A<sup>NCL</sup>, 0.494 for DIP-A<sup>NGL</sup>, 0.503 for Hochi-R, 0.504 for $Df(2R)bw^{N22}Cy^R$, and 0.499 for $Df(2R)M-S2$. Under this nonaxenic condition, the relative viability of progeny that were obtained from crosses that involved either one of the two null activity parental lines did not differ from those that involved Hochi-R, a wild-type activity parental line.

### DISCUSSION

These experiments were undertaken with the idea of developing a system for investigating the adaptive potential and significance of dipeptidase activity variation by imposing a specifically directed selection pressure on DIP-A null and active phenotypes. The first task was to demonstrate fitness differences between null activity and wild type activity phenotypes. Contrary to the predicted outcome, lines that were “null” for DIP-A activity appeared to be just as viable on a restrictive dipeptide medium as lines that exhibited wild-type activity. Several explanations for this observation can be rejected readily. First, L-isoleucine was demonstrated to be an essential amino acid and crucial for the survival of Drosophila larvae. The experimental design to discern fitness differences between the activity phenotypes can thus be justified biologically. The HPLC results clearly eliminate the possibility of artifactual decomposition of the dipeptide during media preparation. Confidence can be placed in the

<table>
<thead>
<tr>
<th>Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>d.f.</th>
<th>$F$ value</th>
<th>Significance level&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
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<td>NS</td>
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<tr>
<td>S/B</td>
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<td>17.89</td>
<td>***</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>157.64</td>
<td>***</td>
</tr>
<tr>
<td>M</td>
<td>1</td>
<td>3,486.42</td>
<td>*</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
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<td>&lt;0.06</td>
</tr>
<tr>
<td>M X F</td>
<td>1</td>
<td>14,224.85</td>
<td>**</td>
</tr>
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<td>C X B</td>
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<td>NS</td>
</tr>
<tr>
<td>C X S/B</td>
<td>6</td>
<td>2.26</td>
<td>*</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>0.55</td>
<td>NS</td>
</tr>
<tr>
<td>D X B</td>
<td>1</td>
<td>1.79</td>
<td>NS</td>
</tr>
<tr>
<td>D X S/B</td>
<td>2</td>
<td>1.95</td>
<td>NS</td>
</tr>
<tr>
<td>D X C</td>
<td>3</td>
<td>0.23</td>
<td>NS</td>
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<td>NS</td>
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<td>D X F</td>
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<td>NS</td>
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<td>NS</td>
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<td>D X C X B</td>
<td>3</td>
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<td>*</td>
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<td>R</td>
<td>1</td>
<td>0.12</td>
<td>NS</td>
</tr>
<tr>
<td>R X B</td>
<td>1</td>
<td>6.85</td>
<td>NS</td>
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<tr>
<td>R X S/B</td>
<td>2</td>
<td>0.54</td>
<td>NS</td>
</tr>
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<td>NS</td>
</tr>
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<td>NS</td>
</tr>
<tr>
<td>R X C X S/B</td>
<td>6</td>
<td>1.63</td>
<td>NS</td>
</tr>
<tr>
<td>R X D</td>
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<td>0.70</td>
<td>NS</td>
</tr>
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<td>R X D X B</td>
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<td>NS</td>
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<tr>
<td>R X D X C</td>
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<td>R X D X C X S/B</td>
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<td>0.71</td>
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</table>

** Error 256

* Letter codes refer to: blocks (B), sets (S), crosses (C), male (M), female (F), diet (D), and broods (R).

<sup>a</sup> C X B mean squares were used as error term to test C. Tests for M, F, and M X F were performed using the appropriate interaction error terms from C X B. D X C X B mean squares were used as error term to test D X C.

<sup>b</sup> NS not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

### TABLE 5

Summary of weighted analyses of variance on transformed data for each cross under axenic conditions

<table>
<thead>
<tr>
<th>Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>d.f.</th>
<th>$F$ value&lt;sup&gt;c&lt;/sup&gt; with cross:</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>S/B</td>
<td>2</td>
<td>3.25 9.17*** 7.91*** 6.00***</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>3,350.40* 0.01 0.04 1.80</td>
</tr>
<tr>
<td>D X B</td>
<td>1</td>
<td>&lt;0.01 16.02 21.09* 0.07</td>
</tr>
<tr>
<td>D X S/B</td>
<td>2</td>
<td>3.30* 2.07 0.11 1.33</td>
</tr>
<tr>
<td>R</td>
<td>1</td>
<td>19.97 0.05 0.94 0.45</td>
</tr>
<tr>
<td>R X B</td>
<td>1</td>
<td>0.18 0.39 0.39 0.86</td>
</tr>
<tr>
<td>R X S/B</td>
<td>2</td>
<td>0.60 2.16 0.84 1.85</td>
</tr>
<tr>
<td>R X D</td>
<td>1</td>
<td>0.02 3.11 1.09 0.97</td>
</tr>
<tr>
<td>R X D X B</td>
<td>1</td>
<td>0.04 0.66 1.02 10.57</td>
</tr>
<tr>
<td>R X D X S/B</td>
<td>2</td>
<td>1.35 0.22 0.81 0.14</td>
</tr>
<tr>
<td>S/B</td>
<td></td>
<td>Error 64</td>
</tr>
</tbody>
</table>

<sup>a</sup> F values and their significance levels are given for each cross, where: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

<sup>b</sup> NS not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

<sup>c</sup> Cytogenetic deficiencies were used as female parents and null activity lines as male parents. Cross designations are:

11 $Df(2R)bw^{N22}Cy^P/Gla \times DIP-A^{NGL}/DIP-A^{NGL}$
12 $Df(2R)bw^{N22}Cy^P/Gla \times DIP-A^{N2F}/DIP-A^{N2F}$
21 $Df(2R)M-S2/Gla \times DIP-A^{NCL}/DIP-A^{NCL}$
22 $Df(2R)M-S2/Gla \times DIP-A^{N2F}/DIP-A^{N2F}$.
axenicity of the cultures based on the negative results from microbial contamination checks. The data point to the inescapable conclusion of in vivo hydrolysis of the dipeptide. A rather direct experiment to test this hypothesis would be to supply the null lines with radiolabeled L-isoleucine, in the form of glycyl-L-isoleucine followed by chromatography of fly extracts to assay for radiolabeled L-isoleucine. This type of study remains to be performed.

Several biological explanations can be considered. The absolute survival of DIP-A null lines on a "restrictive" dietary program raises the question of the "nullness" of these lines. Since homogenates of larvae that were grown on either the amino acid medium or the dipeptide medium exhibited no in vivo DIP-A activity, activity induction is not a reasonable explanation for the survival of null activity larvae. Perhaps these lines actually do have active forms of DIP-A in vivo, but the in vitro assay procedures simply are not sensitive enough for their detection or that a very labile enzyme is produced that is unstable upon extraction.

The absence of DIP-A activity under optimum assay conditions does not necessarily translate to a total lack of in vivo glycyl-L-isoleucine-ase activity. When Drosophila larvae are presented with a biochemical challenge for survival, it is possible that any one of a number of enzymes is capable of fulfilling the necessary metabolic functions. A small number of studies have reported the detection of various proteolytic and peptidic enzymes in D. melanogaster, including trypsin-like enzymes and carboxypeptidase A (Waldner-Stiefelmeier 1967; Waldner-Stiefelmeier and Chen 1967), cathepsin-like protease (Patterson, Dackerman and Schultz 1949), and leucine aminopeptidases (Walker, Geer and Williamson 1980; Walker, Williamson and Church 1981). No proteolytic activity toward azoalbumin and hemoglobin was found between the pH range 1.0 and 5.8 (Waldner-Stiefelmeier and Chen 1967), and proteolysis of azocasein was found to be optimal at pH 8.3 (Waldner-Stiefelmeier 1967; Waldner-Stiefelmeier and Chen 1967). To what extent these enzymes are capable of the hydrolysis of glycyl-L-isoleucine remains to be seen. The independence of the DIP system from the LAP system was confirmed by spectrophotometric measurements of DIP-A and LAP activities under assay conditions that are optimal for LAP. Even under optimum assay conditions for LAP, glycyl-L-isoleucine-ase activity was not detected among the DIP-A null lines. On the other hand, these same lines did exhibit LAP activity to the same measure as the control line, Hochi-R. The dissimilar responses by the two enzymes to detergent treatment further substantiates that DIP-A is predominantly cytosolic, whereas LAP is membrane-bound. Since the field of protein and peptide metabolism in Drosophila is virtually unexplored, a greater understanding and characterization of these enzymes would be instrumental in elucidating the metabolic alternatives that are available to developing Drosophila larvae.

Although axenic conditions were maintained throughout the course of the larval viability experiments, surface axenicity does not preclude the presence of intracellular symbionts. The transmission of endosymbionts from parent to offspring is a common feature in insects (Rockstein 1973). Such microorganisms as yeasts, bacteria, and bacterioids are often found aggregated into specialized cells called mycetocytes. The location of these cells varies among species, and may be situated in the midgut, Malpighian tubules, in the body cavity, within the gonads and the fat body (Wigglesworth 1965). In many insects, these microorganisms are intimately associated with nutrition and metabolism. When intracellular symbionts in the fat body of cockroaches, Periplaneta and Blattela, are eliminated by the addition of aureomycin in the diet or by exposure to high temperatures, cockroaches fail to grow on diets that would be adequate for normal insects (Cochran 1985). Synthesis of certain amino acids and ascorbic acid are impaired by the absence of the endosymbionts (Wigglesworth 1972, 1984). No studies have been documented on mycetocytes in D. melanogaster or on the roles of endosymbionts in meeting the nutritional requirements of fruitflies. It is worth mentioning here that under optimum assay conditions for two Drosophila peptidases, no hydrolysis of glycyl-L-isoleucine was detected among the DIP-A null activity lines. This implies that: (1) these conditions are not suitable for the assay of enzyme activity in symbionts, (2) the assay

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>F value</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>1</td>
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<tr>
<td>A</td>
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<td>L × A</td>
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</tr>
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<td>N × A</td>
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<td>1.14 NS</td>
</tr>
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<td>L × N × A</td>
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<td>1.22 NS</td>
</tr>
<tr>
<td>C × B</td>
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<td>1.35 NS</td>
</tr>
<tr>
<td>C × S/B</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>192</td>
<td></td>
</tr>
</tbody>
</table>

*a Abbreviations are: blocks (B), sets (S), crosses (C), reciprocals (L), cytogenetic deficiencies (N), and Dip-A alleles (A).

*b C × B mean squares were used as error terms to test cross differences and cross components (NS: not significant at P = 0.05 level).
procedures are not sensitive enough to detect hydro-
lytic activity that is actually present, or (3) endosym-
bionts are not responsible for the metabolism of gly-
cyl-l-isoleucine in D. melanogaster.

Certain viruses are transmitted vertically and hori-
zontally in D. melanogaster. Although work has been
done on rhabdoviruses such as sigma virus, togavi-
ruses, picornaviruses, and a reovirus (for review, see
BRUN and PLUS 1980), little is known about their
metabolic involvement in Drosophila development
and growth. Strains infected by sigma virus are made
sensitive to carbon dioxide to the point of fatality
upon a brief exposure. Flies that were used for the
axenic studies were devoid of sigma virus based on
this criterion, since carbon dioxide anesthesia of up
to ten minutes resulted in no mortality. The Drosoph-
ila C virus, a picornavirus, has been shown to affect
certain quantitative traits (THOMAS-ORILLARD 1984;
THOMAS-ORILLARD and JEUNE 1985). Lines that were
used in the larval viability studies consisted of different
cytoplasms. Although marginally significant differ-
ences were found between the two cytogenetic defi-
ciencies that were used as female parents under axenic
conditions, the failure to find reciprocal differences
under nonaxenic conditions questions the involve-
ment of extranuclear effects in these experiments. An
elaborate study in which six distinct nuclear genomes
of D. melanogaster were replaced into a series of cyto-
plasms has shown that differences in cytoplasm can
contribute significantly to the segregation of SM5, a
multiply inverted second chromosome balancer
(CLARK 1985).

The axenic larval viability studies are unique in that
the environments in which selection experiments were
performed were completely quantitative in definition
and manipulation with respect to a physiological func-
tion of an enzyme. In the relative larval viability
studies, the lack of DIP-A activity did not appear to
confer a disadvantage under a nutritional selection
pressure. A given genotype can conceivably partition
the environment in such a way as to make niches that
enhance the viability of other genotypes that would
not normally be able to survive on their own. For
example, the outcome of intraspecific competition
between flies that are deficient for amylase and those
that produce amylase was a consequence of the excr-
tion of enzymatically active amylase by amylase-pro-
ducing genotypes into the medium (HAJ-AHMAD and
HICKEY 1982). The external hydrolysis of starch in-
creased the viability of null genotypes for amylase.
Since lines that are null for DIP-A activity survive on
dipeptide medium, how this type of phenomenon
contributes to the viability of DIP-A null activity phe-
notypes is questionable.

The relative viability experiment under nonaxenic
conditions revealed that, first, reciprocal matings pro-
duced no differences in egg to adult viability, and
second, as hemizygotes the two Dip-A null alleles did
not differ from a wild-type Dip-A allele. This second
observation is consistent with null alleles at 12 other
enzyme loci (BURKHART et al. 1984). Strains made
either homozygous or hemizygous over a deficiency
for null alleles are often quite viable and fertile under
standard laboratory rearing conditions. Many of these
enzymes are involved in intermediary metabolism,
and it is conceivable that the loss of a biochemical
function is circumvented through alternative meta-
abolic networks (VOELKER et al. 1980; LANGLEY et al.

Despite the quantitative precision with which the
environment was defined and manipulated, the chro-
mosomal constitution and thus the genetic back-
ground were neither controlled nor defined. This is
a complication that is difficult to avoid in any relative
viability experiment. How many genes are present on
these chromosomes that affect viability, in general,
and what the effects of these genes are on the specific
enzyme locus under investigation are questions that
cannot be answered readily. For example, it is not
clear whether the significant genotype by en-
vironment interactions from these experiments are spec-
ific to the Dip-A locus or to the genetic background.
One method of alleviating this problem could be to clone
the coding sequence of various activity alleles and to
integrate them individually into a common genetic
background through P-element-mediated transfor-
mation (RUBIN and SPRADLING 1982; SPRADLING
and RUBIN 1982).

Aside from the larval stages of development, dipe-
tidase activity is also extremely high in the adult
female ovaries (LAURIE-AHLBERG 1982). Perhaps ac-
tivity differences confer differences in fecundity un-
der certain environmental conditions. This is an area
for future endeavor. Various components of fitness
can be estimated concurrently with net fitness when
proper procedures are applied (PROUT 1969, 1971a,
b). Before any selection experiments on any enzyme
system are conducted, careful attention must be
placed on the physiological function and the substrates
of the enzyme, alternative biochemical pathways for
the metabolism of the substrate, and the quantitative
definition and manipulation of the environment as
well as the genetic background.

As DYKHUIZEN, DEAN and HARTL (1987) pointed
out, population genetics study of naturally occurring
enzyme variants of eukaryotes is a difficult task.
LEWONTIN (1974) stated that neither the measure-
ment of genetic variation nor the measurement of
variation in fitness associated with that genetic vari-
ation is sufficient to answer the question of how much
of the existing genetic variation can actually be the
basis of adaptive evolution. The potential for adaptive
evolution must be discerned in genetic variation that currently is nonadaptive. Lewontin adds, "But such an assessment will depend on an understanding of the relation between gene and organism that far transcends any present knowledge of development, physiology, and behavior. In fact, it demands the answer to every other question that now lies open in biology."

Despite the rigorous experimental definition with which these studies were performed, the results from this paper exemplify the complexity and difficulty of addressing questions about fitness effects of allozyme differences and other smaller differences in activity level of enzymes in eukaryotes.

We thank B. W. Geer and D. Nash for their valuable suggestions concerning the axenic culturing of Drosophila. Without the technical expertise of M. Bates, D. L. Herman, M. E. Kelly and S. Spiker the HPLC work would not have been possible. We would like to extend our appreciation to C. C. Cockerham and T. H. Emigh for their statistical advice. We also thank S. Chao for her technical assistance in the biochemical work. This work was supported by U.S. Public Health Service Grant GM11546. This is paper No. 11040 of the journal series of the North Carolina Agricultural Research Service, Raleigh, North Carolina 27695-7601.

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