Zoological Studies **36**(4): 277-287 (1997)

Zoological Studies

Biochemical Comparison of Arginine Kinase Allozymes in *Drosophila melanogaster*

Yi-Chih Chien[1,*] and Glen E. Collier[2]

[1] *Department of Biology, National Changhua University of Education, Changhua, Taiwan 500, R.O.C.*
Tel: 886-4-7232105 ext. 3411 Fax: 886-4-7211156 E-mail: chien@cc.ncue.edu.tw.
[2] *Department of Biological Sciences, The University of Tulsa, Tulsa, Oklahoma 74104-3189, USA*

(Accepted May 31, 1997)

Yi-Chih Chien and Glen E. Collier (1997) Biochemical comparison of arginine kinase allozymes in *Drosophila melanogaster*. *Zoological Studies* **36**(4): 277-287. ARK^B is a rare arginine kinase allozyme found in natural populations of *Drosophila melanogaster*. To test whether the rarity of this allozyme could be due to its biochemical impairment relative to the common allozyme, biochemical properties such as catalytic efficiency and conformational stability of the rare (ARK^B) and the common (ARK^A) allozymes were compared in this study. Both allozymes were purified by ammonium sulfate fractionation, DEAE-ion-exchange column, Blue-Sepharose, and S-300 gel filtration, to yield a single coomassie-blue band on SDS-polyacrylamide gels. ARK^A has a higher *Vmax* or *Vmax/Km* than ARK^B at 18 or 29 °C, but there are no differences at 24 °C. In general, ARK^A is catalytically more efficient than ARK^B. Heat treatment of the allozymes shows that ARK^B has a lower specific activity than ARK^A, and its temperature of heat inactivation is also lower. Also, the rate of heat inactivation of ARK^B is faster. Therefore, ARK^B is more thermolabile than ARK^A. From comparisons of catalytic efficiency and thermal stability of the allozymes, we assume that ARK^B is biochemically less efficient than ARK^A, and that might partially account for the rarity of $Argk^B$ in natural populations of *D. melanogaster*.

Key words: *Drosophila melanogaster*, Arginine kinase allozymes, Catalytic efficiency, Conformational stability.

Allozymes, which are enzyme variants encoded by alleles at the same locus, were first defined by Prakash et al. (1969). Some of the enzymes can be easily detected by gel electrophoresis because they involve amino acid substitutions in the primary sequences of proteins, which alter the net charge and, therefore, electrophoretic mobility. The advantages of study of allozymes are that they can be easily analyzed by gel electrophoresis and they have a simple genetic basis. They have long been appreciated by population geneticists for the study of evolutionary processes in natural populations. Various theoretical models have been proposed in order to explain the relative contributions of such forces as selection, mutation, and drift to the distribution of allele frequencies in natural populations (Ohta and Kimura 1973, Clarke 1975, Marshall and Brown 1975, Koehn 1978).

A small number of polymorphic enzymes have been investigated with regard to the relationship between their biochemical functions and their patterns of variation in natural populations. Gene-enzyme systems in *Drosophila* species are ideally suited to this kind of approach. One of the most studied enzymes in terms of comparative biochemistry in *D. melanogaster* is ADH (alcohol dehydrogenase), because the enzyme is easy to assay, and *Drosophila* presents obvious advantages for experimental population genetics. In most natural populations of *D. melanogaster* there are 2 common alleles, "fast" (Adh^F) and "slow" (Adh^S). Biochemical studies of the 2 ADH allozymes can be summarized as follows: ADH-F is more active than ADH-S in terms of specific activity and catalytic efficiency (*Kcat*) at saturated substrate concentrations. ADH-F is less conformationally stable than ADH-S in response to increasing temperature, pH, and urea concentration (Gibson 1970, Gibson and Miklovich 1971, Gibson 1972, Vi-

*To whom correspondence and reprint requests should be addressed.

gue and Johnson 1973, Day et al. 1974a,b, Lewis and Gibson 1978, Grossman 1979, McDonald et al. 1980, Chambers et al. 1981, Thatcher and Sheikh 1981, Winberg et al. 1985).

On the basis of differences in biochemical properties between ADH-F and ADH-S, we can hypothesize that natural selection may differentiate between each allozyme in natural populations. Natural selection may distinguish between the alleles on the basis of their ADH activity levels (Wilks et al. 1980). Considering that the function of ADH is converting alcohol to its corresponding aldehyde or ketone, we can assume that ADH is a detoxifying enzyme (Clarke 1975). If this is true, then in environments containing high concentrations of alcohol, selection should favor the ADH-F allozyme because of its higher activity. Indeed, a concentration of 5%-15% ethanol produces a significant increase in the frequency of Adh^F compared to a control population (Gibson 1970, Cavenar and Clegg 1978, Van Delden et al. 1978). However, Oakeshott et al. (1980) proposed opposing modes of selection for the *Adh* locus in *D. melanogaster*. They assumed that acetaldehyde produced by ADH acting on ethanol is potentially more toxic than ethanol. Although acetaldehyde is removed by ADH as soon as it is produced, when high alcohol concentrations occur, acetaldehyde could be accumulated. Therefore, a high level of ADH activity is not favored since it allows acetaldehyde to accumulate rapidly. The laboratory experiments they performed have also shown that fitness is inversely correlated with ADH activity levels when flies are exposed to ethanol vapor.

The relationship between the levels of ADH activity and the fitness value of each allozyme is apparently complex. However, Clarke and Allendorf (1979) demonstrated that with external substrates polymorphism for enzymes, such as ADH, could be maintained by frequency- and density-dependent selections. Individuals with a "high *Vmax*" enzyme might rapidly deplete a substrate supply under certain conditions. The resulting low substrate concentrations would then favor individuals with a "low *Vmax*" enzyme. Therefore, this frequency-dependent selection is potentially capable of maintaining a balanced polymorphism.

Another hypothesis is that temperature may affect the fitness value of *Adh* alleles since ADH-F is less thermostable than ADH-S. In laboratory tests, flies homozygous for Adh^S are more likely to survive heat shock than Adh^F homozygotes (Bijlsma-Meeles and Van Delden 1974). This hypothesis is also supported by the natural population survey for distributions of Adh^F and Adh^S in the northeastern United States and in Mexico, in which the frequency of Adh^F is inversely correlated with the mean annual temperature (Vigue and Johnson 1973). But Oakeshott et al. (1982) reported that the spatial patterns of frequency variation in Adh^F and Adh^S do not consistently correlate with maximum temperature over the 3 continents of Asia, North America, and Australia. They have provided evidence that the frequency of Adh^S in all 3 continental regions is related to maximum rainfall, but the mechanisms underlying this relationship remain obscure.

Glycerophosphate dehydrogenase (GPDH) is another example of an enzyme for which biochemical properties of allozymes have been compared. Nearly all species of *Drosophila* are monomorphic at the *Gpdh* locus, except *D. subarctica*, *D. virilis*, and *D. melanogaster* (Collier and MacIntyre 1977, Narise 1980). Studies of *Gpdh* polymorphism in natural populations of *D. melanogaster* revealed that the 2 common electrophoretic variants $Gpdh^S$ and $Gpdh^F$ showed clines of frequencies with respect to seasons and latitudes (Miller et al. 1975). Biochemical analyses of these 2 allozymes showed no differences for pH optima and thermal stability. Differences were observed for 3 parameters: temperature dependence of specific activity, temperature dependence of *Km*, and reaction rate constancy over a physiological temperature range (Miller et al. 1975). In the temperature-difference dependence of *Km*, $GPDH^F$ showed temperature independence of affinity for substrate (*Km*), while $GPDH^S$ displayed a temperature-dependent pattern in which the values of *Km* increased (decreased affinity for the substrate) with increasing temperature.

These differences between $Gpdh^S$ and $Gpdh^F$ coupled with the distinctive temporal and spatial patterns of allele frequency prompted Alahiotis et al. (1977) to refer to $Gpdh^F$ as the warm-climate-adapted allele, and $Gpdh^S$ as the cool-climate-adapted allele. The temperature dependence of *Km* for GPDH from 3 tropical species of *Drosophila* was different from $GPDH^S$ and $GPDH^F$, i.e., *Km* decreases with increasing temperature (Alahiotis et al. 1977). These results suggested that natural selection is operating on the *Gpdh* locus within the genus *Drosophila*, with temperature being a mediating agent.

Electrophoretic surveys of natural populations of *Drosophila* species often reveal rare alleles. Collier and MacIntyre (1977) proposed that the rare alleles for GPDH in some species of *Drosophila* may be at low frequencies because the products of these alleles are catalytically less efficient than their "wild type" counterparts. They compared specific activi-

ties of all the available rare allozymes with those of common allozymes in 8 species of *Drosophila*. The results support the hypothesis that the specific activities of rare allozymes are lower than those of common allozymes in the species of *Drosophila* examined.

Narise (1980) compared the biochemical properties between 2 rare allozymes and the common allozyme found in natural populations of *D. virilis*. The results showed that the 2 rare allozymes ($GPDH^F$ and $GPDH^S$) were biochemically inferior to the common allozyme ($GPDH^M$). $GPDH^F$ was found to be the most thermolabile, and $GPDH^S$ was the most susceptible to inhibition by excess dihydroxyacetone phosphate. Therefore, it can be hypothesized that natural selection seems to act against the biochemically inferior alleles in natural populations.

The rate of evolution of arginine kinase is so slow that it is essentially monomorphic in most species of *Drosophila* (Collier 1990). During an extensive survey of wild-type *D. melanogaster*, 1 rare allelic variant was found. The variant (ARK^B) was isolated from a single heterozygous female, and its electrophoretic mobility was found to be slower than that of the common allozyme (ARK^A). The *Argk* locus may be evolutionarily conserved in species of *Drosophila* as a consequence of the important role this enzyme may play in energy metabolism (Collier 1990). Most newly arising *Argk* mutants are presumed to be biochemically inferior to existing common allozymes and thus are rapidly eliminated from a population by selection. In order to test this hypothesis, kinetic parameters, such as *Vmax* and *Vmax/Km*, and thermal stability of common and rare allozymes of arginine kinase were measured and compared. The results support this hypothesis.

MATERIALS AND METHODS

Drosophila stocks

Drosophila melanogaster cultures were maintained at room temperature on standard cornmeal medium. The strains used for preparation of enzyme were Oregon-R, a standard wild-type stock homozygous for the $Argk^A$ allele, and an ARK-B stock homozygous for the $Argk^B$ allozyme.

Electrophoretic assay

Individual flies were homogenized in 10 µl of electrophoresis buffer (0.05 M sodium barbital/Tris-HCl, pH 8.6), and 0.25 µl was applied to a cellulose acetate membrane in a Beckman Microzone cell. Electrophoresis was conducted at 300 V, 1.5 mA for 20 min (Collier and MacIntyre 1977). Zones of arginine kinase activity were visualized by a stain that contains 5.5 mg phosphoarginine (AP), 4.2 mg adenosine 5-diphosphate (ADP), 13 mg glucose, 3 mg nicotinamide adenine dinucleotide phosphate (NADP), 2 mg 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), 1 mg phenazine methosulfate (PM), 0.2 mg magnesium acetate, 9.5 units hexokinase, and 1.5 units glucose-6-phosphate dehyodrogenase (G-6-PDH) in 10 ml of 50 mM Tris-HCl (pH 7.5) containing 1% agar.

Arginine kinase assay

Arginine kinase activity was assayed by monitoring the reduction of NAD^+ at 340 nm. The assay mix contained 1 mM ADP, 2 mM AP, 7 mM glucose, 0.3 mM NADP, 4 mM Mg-acetate, 9.5 units hexokinase, and 1.5 units G-6-PDH in 0.05 M Tris-HCl buffer (pH 7.0). The reaction was initiated by the addition of 10 µl of enzyme in a total 1.0 ml of assay mix. Activity was monitored for 4 min.

Purification of arginine kinase

Allozymes were purified by a slight modification of the method of Wallimann and Eppenberger (1973). Frozen flies were homogenized in 10 mM $NaPO_4$ (pH 7.5), 1 mM DL-dithiothreitol (DTT), and 1 mM ethylenediamine tetraacetic acid (EDTA) in a ratio of 1 part flies: 3 parts buffer (w:v). The homogenate was filtered through glass wool, and the filtrate was centrifuged at 27 000 g for 30 min. The fraction precipitating between 60% and 90% saturation for ARK^A (40% and 70% for ARK^B) with respect to ammonium sulfate was dissolved in a minimal volume of 5 mM glycine-NaOH (pH 9.4) that was 1 mM DTT and 1 mM EDTA. This preparation was desalted by passage through a Sephadex G-25 column (3 cm x 75 cm) equilibrated with H_2O that was 1 mM DTT, and 1 mM EDTA. The desalted solution of arginine kinase was applied directly to a DEAE column (1.5 cm x 33 cm) equilibrated with 5 mM glycine-NaOH (pH 9.4), 1 mM DTT, and 1 mM EDTA. The enzyme was eluted from this column with a 0 to 0.5 M NaCl gradient in equilibration buffer. Arginine kinase eluted as a single peak near the beginning of this gradient. The pooled peak of activity was concentrated in an Amicon cell. The concentrate was diluted with 10 mM Tris-HCl (pH 7.5), 1 mM DTT, and 1 mM EDTA, and then concentrated again. This concentrate was then applied to a column of Blue

Sepharose (1 cm x 40 cm) followed by the buffer of 10 mM Tris-HCl (pH 7.5), 1 mM DTT, and 1 mM EDTA. Arginine kinase was not bound to this column under these conditions, but was retarded sufficiently to be separated from the remaining contaminating proteins. The material from this column was subjected to SDS polyacryamide gel electrophoresis to check the purity of these preparations. If contaminating proteins were still present, the preparation was concentrated and diluted with 5 mM glycine-NaOH (pH 9.4), 1 mM DTT, and 1 mM EDTA, and concentrated again. The concentrate was applied to an S-300 column (3 cm x 75 cm) which was equilibrated with 5 mM glycine-NaOH (pH 9.4), 1 mM DTT, and 1 mM EDTA. Arginine kinase eluted as a single peak separated from the remaining contaminating proteins. Again, the material from this column was subjected to SDS gel electrophoresis to check the purity of the preparation.

Throughout the preparations of arginine kinase, the amount of proteins was determined by Lowry assay (1951).

Estimation of kinetic parameters: *Vmax* and *Km*

The buffer used for kinetic measurements was 0.05 M Tris-HCl (pH 7.0) 1 mM DTT, and 1 mM EDTA because the pH of the buffer was close to the physiological pH of the organism. It is also near the pH optimum for the reaction.

1. Determination of *Vmax* and *Vmax/Km* for phosphoarginine (AP)

Vmax and *Vmax/Km* for AP were determined with various AP concentrations and fixed ADP-Mg^{+2} concentrations. The reaction mixture contained various concentrations of AP and a concentration of Mg^{+2} which was 4 times that of ADP in the buffer. ADP concentrations were 0.5, 0.25, 0.125, and 0.0675 mM.

2. Determination of *Vmax* and *Vmax/Km* for ADP-Mg^{+2}

This was done with various ADP-Mg^{+2} concentrations and fixed AP concentrations. The concentrations of Mg^{+2} were kept constant at 4 times those of ADP. The AP concentrations were 4.0, 2.0, 1.0, and 0.5 mM.

For both allozymes, *Vmax* and *Vmax/Km* were determined from secondary replots derived from double reciprocal primary plots of apparent *Vmax* or *Vmax/Km* against AP (or ADP-Mg^{+2}) at various fixed ADP-Mg^{+2} (or AP) concentrations.

Temperature of heat inactivation

Heat inactivation was determined by pre-incubating the enzymes with 5 mM glycine-NaOH (pH 9.4) at various temperatures (20, 30, 35, 40, 45, 50, 51, 52, 52.6, and 55 °C) for 1 min. Then, after cooling on ice, arginine kinase activity was measured for each treatment.

Rate of heat inactivation

This rate was determined by pre-incubating the enzymes at various temperatures (50, 51, 52, 52.6, and 55 °C) for various time intervals (1, 2, 3, 4, and 5 min). Then, activity remaining was plotted as a function of time.

Urea-gradient electrophoresis

Urea-gradient electrophoresis was performed following the method of Creighton (1979). A 10% to 8% polyacrylamide gel gradient, containing riboflavin (0.5 ml of a 0.08 mg/ml solution per 15 ml of gel mixture) and N,N'-tetramethyl-1,2-diaminoethane (10 µl per 15 ml of gel mixture) was formed in the opposite direction to the urea gradient (0 to 8 N). Bromophenol blue (0.005%) was included in the urea solution so that the linearity of the urea gradient in the polyacryamide gel could be checked visually. Then 50 to 80 µg of enzyme was layered onto the top of the gel, and Bromophenol blue was added as a marker. The electrophoresis buffer used was 0.05 M Tris-HCl (pH 8.0). The gels were run at 20 mA for 4 h. During the course of electrophoresis, the buffer temperature was kept between 15 and 20 °C. After completion of electrophoresis, the urea in the gel was leached out by soaking the gel in methanol-acetic acid-water (50:10:40 by volume) prior to staining. Then the gels were stained overnight with 0.1% (w:v) coomassie brilliant blue in 10% (w:v) trichloroacetic acid plus 10% (w:v) sulphosalicylic acid. The gels were destained by diffusion against 7.5% methanol and 5% acetic acid.

Crosses

Wild-type Oregon-R flies ($Argk^{A}$) were mated to the $Argk^{B}$ strain ($Argk^{B}$). The F_1 from this cross were self-crossed, and eggs were collected within 6 h after oviposition. The eggs were then divided into 3 broods and incubated at 20, 24, and 29 °C, respectively. Newly eclosing F_2 adults were collected at 6-h intervals and subsequently subjected to cellulose-acetate electrophoresis to determine geno-

types with respect to *Argk*. A total of 3049 F_2 flies were scored for 20, 24, and 29 °C, respectively. Developmental time was taken as the interval between the midpoints of the oviposition and eclosion periods. Average developmental time for each genotype was analyzed by one way analysis of variance.

RESULTS

Purification of arginine kinase

Arginine kinase allozymes (ARK^A and ARK^B) were purified from extracts of frozen flies of strains Oregon-R and Ark-B of *D. melanogaster* by ammonium sulfate fractionation, G-25 Sephadex filtration, DEAE-cellulose ion-exchange chromatography, Blue-sepharose, and S-300 Sephadex filtration (Tables 1, 2). The purification protocol for both allozymes was essentially the same, except that ARK^A precipitated between 60% and 90% saturation with respect to ammonium sulfate, while ARK^B precipitated between 40% and 70% saturation. The difference might be due to the different charges of the allozymes. The specific activities measured for each step in the preparation of ARK^B were much higher than those in the preparation of ARK^A, because the concentrations of Mg^{+2} ion were different in the arginine kinase assay mix used for the ARK^B preparation. Mg^{+2} is required for arginine kinase activity (Blethen 1972, Cheung 1973, Eppenberger and Wallimann 1973). Therefore, we measured the effect of Mg^{+2} ion concentration on arginine kinase activity. The maximum arginine kinase activity was reached when the molar ratio of Mg^{+2}: ADP was 4:1 (data not shown). The Mg^{+2} ion concentration in the arginine kinase assay mix for the preparation of ARK^A was 0.1 mM, which is far below the optimal Mg^{+2} ion concentration (4 mM) used in the arginine kinase assay mix for the preparation of ARK^B. In all following experiments, the molar ratio of Mg^{+2}: ADP was kept at 4:1. Filtration through Sephadex G-300 was necessary for preparations of both allozymes, as the results of SDS-polyacrylamide gels indicated that there were more than 2 coomassie blue staining bands after the Blue-sepharose step. After the filtration of Sephadex G-300, the pool was again subjected to SDS polyacrylamide gel electrophoresis. These preparations were then judged pure as there was only a single coomassie blue staining band on the SDS polyacrylamide gels (data not shown).

Vmax and *Vmax/Km* of arginine kinase allozymes measured at various temperatures

The kinetic parameters, *Kcat* and *Kcat/Km*, are theoretically considered as the true estimators of catalytic efficiency of an enzyme with respect to a specific substrate (Fersht 1985). They are not often determined because of the requirement of pure enzymes, and/or the inability to determine the number of active sites (Fersht 1985). In this study, *Vmax* and *Vmax/Km* were normalized for enzyme specific activity and are assumed to correspond to *Kcat* and *Kcat/Km* for comparative purposes.

At 24 °C, no apparent differences were observed in values of *Vmax* and *Vmax/Km* for both allozymes (Fig. 1; Table 3). However, ARK^A apparently has higher *Vmax* or *Vmax/Km* than ARK^B at 18 or 29 °C, respectively (Fig. 1; Table 3). As a result,

Table 1. Purification table of *Drosophila melanogaster* ARK^A

Step	Total[a] protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Yield (%)
Crude extract	3752.0	47 000	12.52	100
60%-90% Ammonium sulfate cut, after filtration through Sephadex G-25	361.0	13 000	33.24	28
DEAE-cellulose	77.5	6000	77.42	13
Blue-sepharose	12.4	3600	290.32	8
Sephadex G-300	5.2	2800	538.46	6

[a]Protein was determined by the method of Lowry et al. (1951).

Table 2. Purification table of *Drosophila melanogaster* ARK^B

Step	Total[a] protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Yield (%)
Crude extract	2613.0	131 200	50.21	100
40%-70% Ammonium sulfate cut, after filtration through Sephadex G-25	300.0	41 100	136.63	31
DEAE-cellulose	64.8	27 800	429.01	21
Blue-sepharose	10.3	13 300	1291.26	10
Sephadex G-300	7.6	10 400	1368.42	8

[a]Protein was determined by the method of Lowry et al. (1951).

Table 3. Values for *Vmax* and *Vmax/Km* in the direction of ATP synthesis for *Drosophila melanogaster* ARK^A and ARK^B, calculated from secondary plots of variations of intercepts and slopes of primary plots

Temperature	Allozyme	*Vmax* ($IU \cdot min^{-1} \cdot mg^{-1})10^{-2}$		*Vmax/Km* ($IU \cdot min^{-1} \cdot mM^{-1})10^{-1}$	
		ADP	AP	ADP	AP
29 °C	ARK^A	1.34	1.26	1.43	0.49
	ARK^B	1.24	1.36	0.82	0.25
24 °C	ARK^A	1.24	1.20	1.05	0.31
	ARK^B	1.24	1.27	0.95	0.24
18 °C	ARK^A	1.32	1.43	1.02	0.20
	ARK^B	1.08	1.02	0.96	0.12

ARK^A is catalytically more efficient than ARK^B at saturated substrate concentrations, at a temperature of 18 °C. Moreover, at 29 °C, ARK^B is catalytically less efficient than ARK^A at a low substrate concentration. Fig. 2 shows sets of Lineweaver-Burk plots for both ARK^A and ARK^B. These plots are consistent with those of arginine kinase from other organisms, in which the reaction of the enzyme has been identified to be of the random-order rapid-equilibrium type (Smith and Morison 1969, Blethen 1972, Cheung 1973).

Temperature of heat inactivation

The activity of ARK^A dropped to about 90% of the original activity when heated to 50 °C (Fig. 3). The activity of the heat-treated samples (up to 40 °C) was similar to that of the untreated control. In contrast, the activity of ARK^B varied at temperatures from 20 °C to 50 °C. The activity increased with increasing temperature reaching a peak at 40 °C. Then it declined as temperatures increased to 50 °C (Fig. 3). Further, the activity of the heat treated samples of ARK^B was significantly lower than the activity of the untreated control. The specific activity of ARK^A was greater than that of ARK^B at equivalent temperatures throughout the experiment.

Rate of heat inactivation

Differences in the rate of heat inactivation were greatest at 52.6 °C (Fig. 4). ARK^B was totally inactivated after incubation at 52.6 °C for 3 min, while ARK^A still retained 8% of its original activity after 5 min incubation. Both enzymes were totally inactivated at a temperature of 55 °C for 1 min.

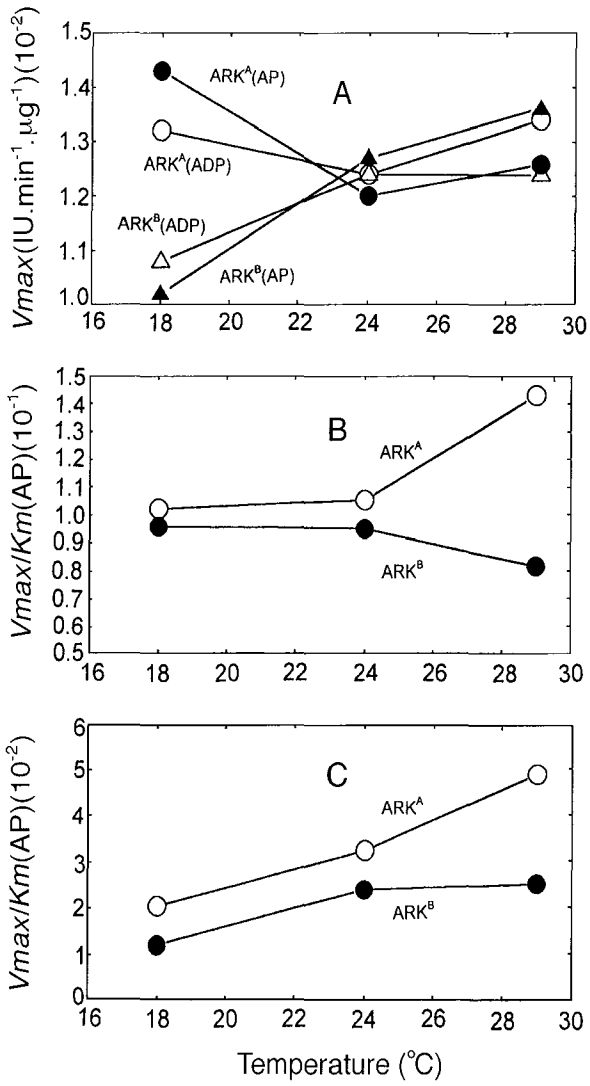

Fig. 1. Values for *Vmax* (A) and *Vmax/Km* (B and C) in the direction of ATP synthesis for *Drosophila melanogaster* ARK^A and ARK^B in relation to temperature (18, 24, and 29 °C).

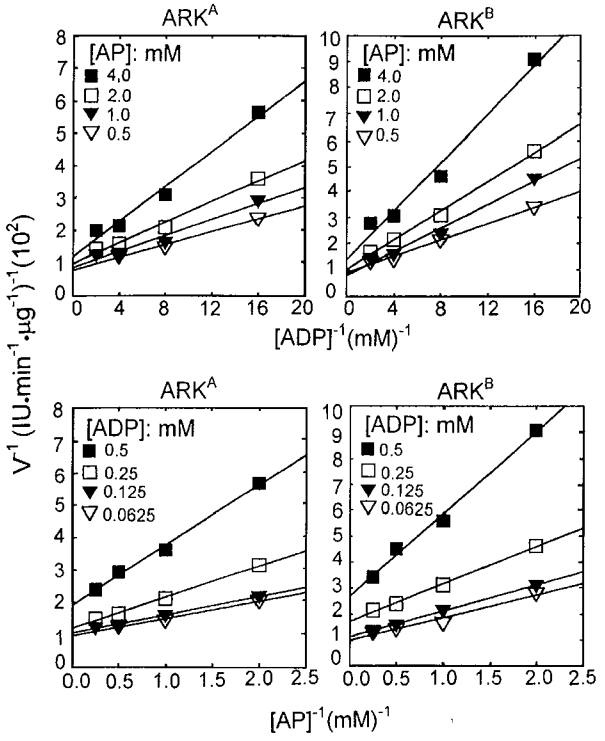

Fig. 2. Primary plots of *Drosophila melanogaster* ARK^A and ARK^B at pH 7.0 and 29 °C. Variations of the reciprocal of the specific initial rate, *v*, with the reciprocal of the ADP (or phosphoarginine) concentration for several constant phosphoarginine concentrations (or ADP) (mM) are shown.

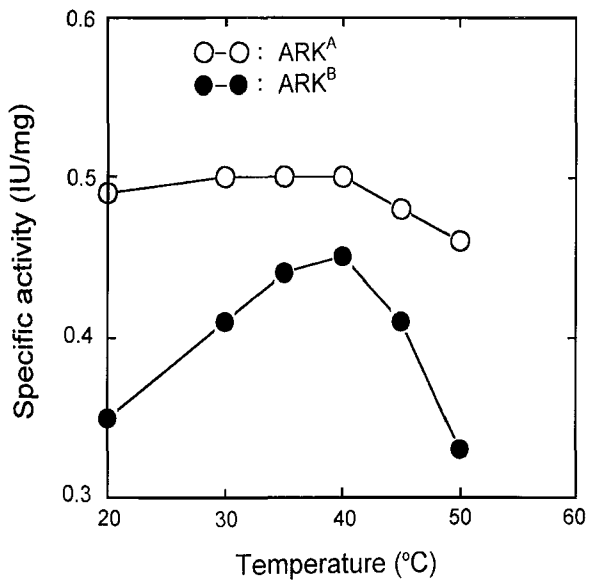

Fig. 3. Temperature of heat inactivation of *Drosophila melanogaster* arginine kinase. The enzyme was preincubated in 5 mM glycine-NaOH, pH 9.4, at various temperatures for 1 min. After cooling on ice, arginine kinase activity was measured as described in Material and Methods.

Urea-gradient electrophoresis

This experiment of urea gradient electrophoresis for both allozymes was performed in order to further confirm the differential conformational stability suggested by the differential rates of heat inactivation. Unexpectedly, ARK^A is conformationally slightly less stable than ARK^B (Table 4). It is interesting that both allozymes denature at very low urea concentrations (Table 4), which is unusual when compared to the denaturing points of various proteins studied by Creighton (1979 1980).

Crosses

This experiment of crosses was performed in order to see if temperature would affect the developmental rate of the 3 *Argk* genotypes. F_2 embryos from the cross of wild type Oregon-R and Ark-B were divided into 3 broods and incubated at 20, 24, and 29 °C, respectively. F_2 adults were collected every 6 h and subjected to cellulose-acetate electrophoresis to determine $Argk^{A/A}$, $Argk^{A/B}$, and $Argk^{B/B}$. The developmental rates for each genotype at each temperature are summarized in Table 6. The cumulative percentage of each genotype at 20, 24, and 29 °C are plotted in Fig. 5. At 29 °C, the average developmental time (228 h) for $Argk^{A/A}$ is significantly ($p < 0.01$) different from that for $Argk^{B/B}$ (237 h). At 20 °C, the developmental time (437 h) for $Argk^{A/A}$ is also significantly ($p < 0.01$) different from that for $Argk^{B/B}$

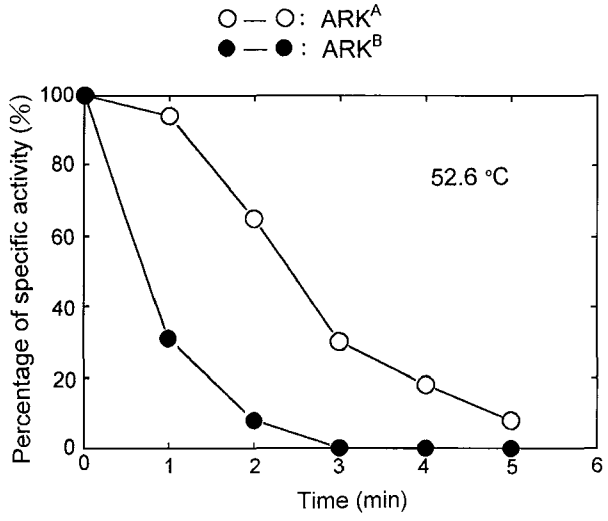

Fig. 4. Rate of heat inactivation of *Drosophila melanogaster* arginine kinase. The enzymes were pre-incubated in 5 mM glycine-NaOH, pH 9.4, at 52.6 °C for various time intervals (1, 2, 3, 4, and 5 min). The activity remaining was plotted as a function of time.

(451 h). However, there is no significant ($p > 0.01$) difference between the developmental rates of $Argk^{A/A}$ and $Argk^{B/B}$ at 24 °C. At each temperature, the developmental rate of the heterozygote ($Argk^{A/B}$) was indistinguishable from that of the homozygote $Argk^{A/A}$.

DISCUSSION

Biochemical models which attempt to define functional differences between allozymes (Clarke 1975, Koehn 1978) may be the best and the most reasonable bases for explaining the patterns of distributions of enzyme variants. We can attempt to assign adaptive significance in different environments to differences in biochemical properties of variants. Such a hypothesis is testable since it can predict the direction of change in allele frequencies in captive populations under manipulated experimental environments. Further it should explain the geographic distributions of the enzyme variants in relation to such environmental variables as temperature.

A variation of this hypothesis states that the gene products of rare alleles are biochemically inferior to the product of common alleles, thus, natural selection would act against them in natural populations. This hypothesis has been tested and supported for *Gpdh* in several *Drosophila* species (Collier and MacIntyre 1977, Narise 1980).

One of the biochemical properties examined in this study was relative catalytic efficiency. Two kinetic parameters of enzymes, *Vmax* and *Vmax/Km*, were considered as estimators of catalytic efficiency. The fact that ARK^A has a higher *Vmax* or *Vmax/Km* than ARK^B at 18 or 29 °C, respectively (Fig. 1; Table 3), may reflect that ARK^A is superior to ARK^B in catalyzing the transfer of phosphoryl groups between arginine-phosphate and ADP to form arginine and ATP at physiological conditions. However, the result that there is no significant difference in both *Vmax* and *Vmax/Km* between these 2 allozymes at 24 °C is consistent with that of the developmental rate experiment (Fig. 5; Table 5) which will be discussed later. In general, we may conclude that ARK^A has a higher catalytic efficiency than ARK^B.

Another biochemical property examined was the relative thermal stabilities of ARK^A and ARK^B. In the experiment designed to determine the temperature of heat inactivation, ARK^B was similar to ARK^A

Table 4. Denaturing points of both *Drosophila melanogaster* ARK^A and ARK^B in terms of urea concentration

Enzyme	Denaturing concentration (M)[a]
ARK^A	1.28[b]
ARK^B	1.59[c]

[a]The denaturing points in terms of urea concentration were calculated from the length of the undenatured enzyme band on the gel, i.e., [length of undenatured band (cm)/total length of the gel (cm)] x 8 M.
[b]Average of values determined from 2 gels.
[c]Average of values determined from 3 gels.

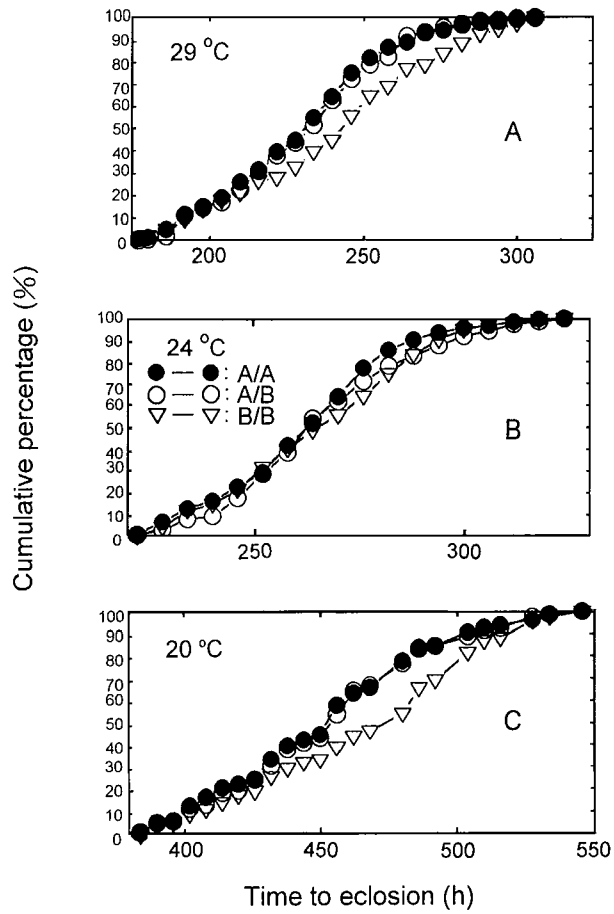

Fig. 5. Wild-type Oregon-R flies ($Argk^A$) were mated to the ARK-B strain ($Argk^B$). The F_1 progeny from this cross were self-crossed, and eggs were collected within 6 h after oviposition. The eggs were then divided into 3 broods and incubated at (A) 20 °C, (B) 24 °C, and (C) 29 °C, respectively. Newly eclosing F_2 adults were collected at 6 h intervals and subsequently subjected to cellulose-acetate electrophoresis to determine genotypes with respect to *Argk*. A total of 3049 F_2 flies were scored for 20, 24, and 29 °C, respectively. Developmental time was taken as the interval between the midpoints of the oviposition and eclosion periods. Average developmental time for each genotype was analyzed by one way analysis of variance.

Table 5. Development time for $Argk^{A/A}$, $Argk^{A/B}$, and $Argk^{B/B}$ at 20, 24, and 29 °C

Temperature	Genotypes					
	A/A		A/B		B/B	
	Number of flies	Average[a] development time (h)	Number of flies	Average development time (h)	Number of flies	Average development time (h)
20 °C	287	437	578	436	265	451
24 °C	229	251	520	247	212	250
29 °C	230	228	494	226	234	237

[a]Development time was taken as the interval between the midpoints of the oviposition and eclosion periods.

in terms of the temperature at which each was totally inactivated. However, ARK^B differed from ARK^A in that its activity fluctuated upon heat treatment and was apparently lower when compared at equivalent temperatures (Fig. 3). Further, at 52.6 °C (Fig. 4) ARK^B was inactivated faster than ARK^A. These results suggest that ARK^B is more thermosensitive and thermolabile than ARK^A.

Thatcher and Sheikh (1981) found that differences in thermal stability of ADH allozymes were reflected in differential sensitivity to denaturation by urea. When similar experiments were done with the ARK allozymes, ARK^A was found to be slightly more sensitive to urea than was ARK^B (Table 4). However, both allozymes were denatured at very low urea concentrations (Table 4) compared to the denaturing points of other proteins (Creighton 1979 1980). In other words, both allozymes are conformationally unstable.

The reason for this conformational instability is unclear. However, it may be related to the multiple functions of the *Argk* gene product. Two isozymes of arginine kinase are encoded by a single gene in *D. melanogaster* (Munneke and Collier 1988), one a myofibrillar form and the other a mitochondrial form. But, the molecular environment of the myofibril is presumed to be quite different from that of mitochondria. The isozymes of arginine kinase may have different conformations to accommodate these different molecular environments. The property of a protein to assume different conformational endpoints may correlate with general conformational instability. This possibility deserves further investigation.

Are these differences in biochemical properties reflected in differences in fitness of *Argk* genotypes? In other words, how would natural selection distinguish between *Argk* alleles? To answer this question, we must consider the functions of arginine kinase. The phosphagen kinase of vertebrates, creatine kinase, has been proposed to play a major role in the targeting of energy during normal muscle contraction (Bessman and Carpenter 1985). Creatine kinase is also implicated in the targeting of energy for protein and lipid synthesis as well. Further, it is associated with the sarcoplasmic reticulum and plasma membrane and, therefore, may help in providing energy for calcium transport (Bessman and Carpenter 1985). In non-muscle cells, creatine kinase may play a role in cell movement (Bessman and Carpenter 1985). We assume that arginine kinase plays the same role in invertebrates as creatine kinase plays in vertebrates. Furthermore, arginine kinase may play an important role in the development of fruit flies. James and Collier (1990) have measured arginine kinase activity in the developmental stages of *D. melanogaster*. There are 2 peaks of arginine kinase activity, one at the prepupal stage and the other near eclosion. The peak near eclosion is presumed to be due to synthesis of arginine kinase in developing indirect flight muscles. The prepupal peak is associated with an increase in activity in imaginal disks. Thus, it is assumed to provide energy for differentiation of imaginal disks. Given these considerations, we can assume that natural selection would favor individuals with high *Argk* catalytic efficiency and high thermal stability. From the differences in catalytic efficiency and thermal stability between the 2 allozymes, we predicted that the development rates among genotypes in captive populations of *D. melanogaster* should be different in relation to environmental variables such as temperature. As expected, the flies that are homozygous for $Argk^A$ have a significantly faster developmental rate than those homozygous for $Argk^B$ at temperatures of 20 and 29 °C (Table 5; Fig. 5A, C), and no difference in developmental rates was observed between the 2 genotypes at 24 °C (Table 5;

Fig. 5B). In Table 3, both *Vmax* and *Vmax/Km* between ARK^A and ARK^B reveal no significant differences at 24 °C. It is possible that at the environmental temperature of 24 °C, the in vivo level of activity of ARK^B is the same as that of ARK^A. This would enable the flies that were $Argk^{B/B}$ to compete in development with flies that were $Argk^{A/A}$. Significant differences in developmental rates between genotypes at 20 and 29 °C may be due to differences in catalytic efficiency and thermal stability between *Argk* allozymes. We can not exclude the possibility that they are due to the effects of genes closely linked to *Argk* rather than *Argk* itself. It is interesting that heterozygotes, $Argk^{A/B}$, have developmental rates similar to homozygotes, $Argk^{A/A}$. It is generally thought that the enzyme activity produced by heterozygotes should be intermediate between the 2 homozygotes. Therefore, the developmental rate of heterozygotes also should be intermediate. But Kacser and Burns (1981) pointed out that a reduction of 50% in enzyme activity in a heterozygote has only a slight influence, usually not detectable, on the phenotype. Consequently, the heterozygote usually differs very little from the homozygote in viability. It is possible that the level of arginine kinase activity produced by flies homozygous for $Argk^A$ is in excess of that needed for development, and the level of arginine kinase activity produced by the flies heterozygous for *Argk* is enough for normal development.

From the survey of biochemical properties of the 2 *Argk* allozymes, we conclude that the rarity of $Argk^B$ can be accounted for at least partly by the relative impairment in catalytic efficiency and thermal stability of $Argk^B$ relative to the common variant, $Argk^A$.

REFERENCES

Alahiotis S, S Miller, E Berger. 1977. Natural selection at the α-GDH locus in *Drosophila*. Nature **269:** 144-145.

Bessman SP, CL Carpenter. 1985. The creatine-creatine phosphate energy shuttle. Ann. Rev. Biochem. **54:** 831-862.

Bijlsma-Meeles E, W Van Delden. 1974. Intra- and inter-population selection concerning the alcohol dehydrogenase locus on *Drosophila melanogaster*. Nature **247:** 369-371.

Blethen SL. 1972. Kinetic properties of the arginine kinase isoenzymes of *Limulus polyphemus*. Arch. Biochem. Biophys. **149:** 244-251.

Cavenar DR, MY Clegg. 1978. Dynamics of correlated genetic systems. IV: Multilocus effects of ethanol stress environments. Genetics **90:** 629-644.

Chambers GK, AV Wilks, JB Gibson. 1981. An electrophoretically cryptic alcohol dehydrogenase variant in *Drosophila melanogaster*. III. Biochemical properties and comparison with common enzyme forms. Aust. J. Biol. Sci. **34:** 625-637.

Cheung AC. 1973. Kinetic properties of arginine phosphokinase from honeybees, *Apis mellifera* L. (Hymenoptera, Apidae). Arch. Biochem. Biophys. **154:** 28-39.

Clarke B. 1975. The contribution of ecological genetics to evolutionary theory: detecting the direct effects of natural selection on particular polymorphic loci. Genetics **79:** 101-113.

Clarke B, FW Allendorf. 1979. Frequency-dependent selection due to kinetic differences between allozymes. Nature **279:** 732-734.

Collier GE. 1990. Evolution of arginine kinase within the genus *Drosophila*. J. Heredity **81:** 177-182.

Collier GE, RJ MacIntyre. 1977. Low specific activity of rare allozymes of α-glycerophosphate dehydrogenase in *Drosophila*. Nature **267:** 839-840.

Creighton TE. 1979. Electrophoretic analysis of the unfolding of proteins by urea. J. Mol. Biol. **129:** 235-264.

Creighton TE. 1980. Kinetic study of protein unfolding and refolding using urea gradient electrophoresis. J. Mol. Biol. **137:** 61-80.

Day TH, PC Hillier, B Clarke. 1974a. Properties of genetically polymorphic isozymes of alcohol dehydrogenase in *Drosophila melanogaster*. Biochem. Genet. **11:** 141-153.

Day TH, PC Hillier, B Clarke. 1974b. The relative quantities and catalytic activities of enzymes produced by alleles at the alcohol dehydrogenase locus in *Drosophila melanogaster*. Biochem. Genet. **11:** 155-165.

Fersht AR. 1985. Enzyme structure and mechanism. 2nd ed. San Francisco: WH Freeman.

Gibson JB. 1970. Enzyme flexibility in *Drosophila melanogaster*. Nature **227:** 959-960.

Gibson JB. 1972. Differences in the number of molecules produced by two allelic electrophoretic enzyme variants in *Drosophila melanogaster*. Experientia **28:** 975-976.

Gibson JB, R Miklovich. 1971. Modes of variation in alcohol dehydrogenase in *Drosophila melanogaster*. Experientia **27:** 99-100.

Grossman A. 1980. Analysis of genetic variation affecting the relative activities of fast and slow ADH dimers in *Drosophila melanogaster* heterozygotes. Biochem. Genet. **16:** 765-780.

James JM, GE Collier. 1990. Hormonally regulated expression of arginine kinase in *Drosophila melanogaster*. Roux's Arch. Dev. Biol. **198:** 474-478.

James JM, GE Collier. 1992. Early gene interaction during prepupal expression of *Drosophila* arginine kinase. Dev. Genet. **13:** 302-305.

Kacser H, JA Burns. 1981. The molecular basis of dominance. Genetics **97:** 639-666.

Koehn RK. 1978. Physiology and biochemistry of enzyme variation: the interface of ecology and population genetics. *In* P Brussard, ed. Ecological genetics: the interface. New York: Springer, pp. 51-72.

Lewis N, J Gibson. 1978. Variation in amount of enzyme protein in natural populations. Biochem. Genet. **16:** 159-170.

Lowry OH, NJ Rosebrough, AL Farr, RJ Randall. 1951. Protein measurement with the folin phenol reagent . J. Biol. Chem. **193:** 265-275.

Marshall DR, AHD Brown. 1975. The charge-state model of protein polymorphism in natural populations. J. Mol. Evol. **6:** 149-163.

McDonald JF, SM Anderson, M Senios. 1980. Biochemical differences between products of the *Adh* locus in *Drosophila*. Genetics **95:** 1013-1022.

Miller S, RW Pearcy, E Berger. 1975. Polymorphism at the α-glycerophosphate dehydrogenase locus in *Drosophila mela-*

nogaster. I: Properties of adult allozymes. Biochem. Genet. **13:** 175-188.

Munneke LR, GE Collier. 1988. Cytoplasmic and mitochondrial arginine kinases in *Drosophila*: evidence for a single gene. Biochem. Genet. **26:** 131-141.

Narise S. 1980. Purification and biochemical properties of allelic forms of cytoplasmic glycerol-3-phosphate dehydrogenase from *Drosophila virilis*. Biochem. Biophys. Acta **615:** 289-298.

Oakeshott JG, DG Anderson, PR Anderson, GK Chambers, JB Gibson, WR Knibb. 1982. Alcohol-dehydrogenase and glycerol-3-phosphate dehydrogenase clines in *Drosophila melanogaster* on different continents. Evolution **36:** 86-96.

Oakeshott JG, PR Anderson, A Champ, JB Gibson. 1980. Opposing Modes of Selection on the Alcohol-dehydrogenase locus in *Drosophila melanogaster*. Aust. J. Biol. Sci. **33:** 105-114.

Ohta T, M Kimura. 1973. A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. Genet. Res. Cambridge **22:** 201-204.

Prakash S, RC Lewontin, JC Hubby. 1969. A molecular approach to the study genic heterozygosity in natural populations. IV: Patterns of genic variation in central marginal and isolated populations of *Drosophila pseudoobscura*. Genetics **61:** 841-858.

Smith E, JF Morrison. 1969. Kinetic studies in the arginine kinase reaction. J. Biol. Chem. **244:** 4224-4234.

Thatcher DR, R Sheikh. 1981. The relative conformational stability of alcohol dehydrogenase alleloezymes purified from *Drosophila melanogaster*. Biochem. J. **197:** 111-117.

Van Delden W, AV Boerema, A Kamping. 1978. The alcohol dehydrogenase polymorphism in populations of *Drosophila melanogaster*. I: Selection in different environments. Genetics **90:** 161-191.

Vigue CL, FM Johnson. 1973. Isozyme variability in species of the genus *Drosophila*. VI: Frequency-property-environment relationships of allelic alcohol dehydrogenase in *Drosophila melanogaster*. Biochem. Genet. **9:** 213-227.

Wallimann T, HM Eppenberger. 1973. Properties of arginine kinase from *Drosophila melanogaster*. Eur. J. Biochem. **38:** 180-184.

Wilks AV, JB Gibson, JG Oakeshott, GK Chambers. 1980. An electrophoretically cryptic alcohol dehydrogenase variant in *Drosophila melanogaster*. II: Post-electrophoresis heat-treatment screening of natural populations. Aust. J. Biol. Sci. **33:** 575-580.

Winberg JO, R Hovik, JS McKinley-McKee. 1985. The alcohol dehydrogenase allelozymes Adh^s and Adh^f from the fruitfly *Drosophila melanogaster*. An enzymatic rate assay to determine the active-site concentration. Biochem. Genet. **23:** 205-216.

黃果蠅(*Drosophila melanogaster*)精氨酸激酶異構酶之生化特性比較

簡一治[1] Glen E. Collier[2]

黃果蠅(*Drosophila melanogaster*)的自然族群中存在一種稀少的精氨酸激酶異構酶(arginine kinase allozyme)叫ARK^B，而其對偶基因分佈頻率的稀少性(rarity)可能由於其生化能力劣於另一種對偶基因分佈普遍的異構酶叫ARK^A，因此自然選汰的壓力作用其上而無法擴散。為測驗此假設，本文比較了此二異構酶的催化能力(catalytic efficiency)及結構的穩定度(conformational stability)等。

此二種異構酶均先被純化(protein purification)，然後才進行生化反應的試驗。酵素動力學(kinetics)的結果顯示在 18 ℃及 29 ℃時，ARK^A有較高的 *Vmax* 或 *Vmax/Km*，但在 24 ℃時則無顯著差異，然而綜合而言，此結果建議ARK^A的催化能力較ARK^B為強。另外，熱處理的實驗顯示ARK^B活性(specific activity)下降速率較快，且活性被完全抑制的溫度也較低，以及速率也快很多。此結果建議ARK^B對熱的穩定性較ARK^A為差。從對此二異構酶在催化能力及熱的穩定度的比較，本文建議ARK^B的生化能力劣於ARK^A，而此建議可能部份解釋為什麼$Argk^B$在黃果蠅的自然族群中的分佈那麼少。

關鍵詞： 黃果蠅(*Drosophila melanogaster*)，異構酶，催化能力，結構的穩定度。

[1] 國立彰化師範大學生物學系

[2] Department of Biological Sciences, University of Tulsa, Tulsa, Oklahoma 74104-3189, USA