ADAPTATION OF DROSOPHILA ENZYMES TO TEMPERATURE—I. ACETYLCHOLINESTERASE AND NADP-DEPENDENT ISOCITRATE-DEHYDROGENASE

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Abstract—1. The relationship between $K_m$ and assay temperature was examined in three tropical and two temperate Drosophila species, and in the cosmopolitan species, D. melanogaster, for isocitrate dehydrogenase and acetylcholinesterase.

2. For both enzymes $K_m$ patterns were similar among species from the same habitat, and different between habitats. No such parallelism was seen with respect to thermal inactivation.

3. The $Q_{10}$ values in general reflected temperature dependent changes in $K_m$, but exceptions were noted.

INTRODUCTION

In a number of studies on aquatic poikilotherms the importance of temperature dependent changes in the catalytic properties of enzymes, for immediate and evolutionary temperature compensation, has been documented (Hochachka & Somero, 1973). From these studies one mechanism in particular stands out as ubiquitous for metabolic rate compensation. This scheme, decreases in temperature through most of an organism’s physiological temperature range, which would normally reduce enzyme reaction rates, are compensated by an immediate increase in enzyme substrate affinity. This leads to reaction rate constancy, presumably buffering the individual against changes encountered during daily, or seasonal temperature fluctuations. Indeed, minimal $K_m$ values typically occur at the lower limits of habitat temperature, indicating the likely expression of this mechanism in vivo.

The present work deals with the temperature dependent kinetic parameters of two enzymes, NADP dependent isocitrate dehydrogenase (IDH), and acetylcholinesterase (ACHE) in several temperate and tropical species of Drosophila. The purpose of the investigation was, first, to determine whether patterns of positive thermal modulation exist for enzymes in terrestrial poikilotherms as well, and, if so to find out whether correlations (or distinctions) could be found among (or between) temperate and tropical species. These two enzymes have been studied extensively in aquatic forms (Baldwin & Hochacha, 1970; Moon & Hochacha, 1971; Baldwin, 1971). In addition, we hoped to examine and compare temperature dependent kinetic parameters for genetic or allozymic variants of one enzyme, IDH, found in one species, D. melanogaster, in order to determine whether any-thing would be learned of the adaptive significance of genetically based protein polymorphism (Lewontin, 1974).

MATERIALS AND METHODS

Experimental organisms

All Drosophila species (D. willistoni, D. arizonensis, D. equinoxialis, D. virilis, D. americana and D. melanogaster) examined in this study were maintained at 25°C standard banana-agar food medium. The strain of D. melanogaster used for enzyme preparation was Swedish-C, a stock homozygous for the electrophoretically slow allele of IDH, and Samarkand, a strain homozygous for the fast allele.

Enzyme preparation and assay techniques

(i) Acetylcholinesterase ACHE (E.C. 3.1.1.7.). Flies (150 mg; 4–10 days old) were homogenized in 3 ml of 0.1 M phosphate buffer pH 7.5 containing 0.5 mM NaCl, 0.25 mM EDTA, and 0.5% Triton-X100. After incubation for 20 min on ice, extracts were centrifuged for 10 min at 20,000 g in a Beckman J-21B centrifuge at 2°C. A 20–50% ammonium sulfate fraction was obtained. The ammonium sulfate was then removed by chromatography on a small (1 × 25 cm) column of Sephadex G-25 made up in 0.1 M phosphate buffer pH 8.0. The partially purified enzyme was used immediately. The ACHE spectrophotometric assay was that described by Cherbas et al. (1977) except that the reaction kinetics were followed for 5 min in a Guildford-2400S recording spectrophotometer, and activity measured by slope calculation. For ACHE electrophoresis we followed Cherbas et al.’s (1977) method.

(ii) Isocitrate dehydrogenase-NADP (E.C. 1.1.1.42.). Flies (150 mg; 4–10 days old) were homogenized with mortar and pestle in 3 ml of 0.2 M sodium phosphate buffer pH 6.0 saturated with 0.1 phenylthiourea. The crude extracts were centrifuged for 25 min for 15,000 g at 2°C, in a Beckman J-21B centrifuge. A 30–60% ammonium sulfate fraction was obtained. The ammonium sulfate was then removed by Sephadex G-25 column chromatography in 0.1 M Tris-HCl buffer at pH 8.5. Protein was assayed according to Lowry et al. (1951) using bovine serum albumin in homogenization buffer as the standard. We obtained 3-fold purified enzyme. This partially purified enzyme was used immediately. The assay mixture contained, in a total volume of 1 ml, 0.1 mM NADP, 0.84 mM
MgSO₄, 0.1 M Tris-HCl buffer pH 8.5, varying levels of D,L-isocitrate (Na₃l) and enzyme. Starch gel electrophoresis carried out according to Alahiotis & Berger (1977).

For the thermal stability studies, crude extracts were incubated at 49 and 50°C for ACHE and IDH, respectively, in a water-bath. Samples were removed from the water-bath, cooled on ice, and assayed for activity. The results were plotted as percent of original activity vs time of incubation. In order to estimate kinetic parameters, four to six substrate concentrations were assayed, in duplicate, at each temperature. \( K_m \) and \( V_{max} \) values were determined from \( 1/S \) vs \( 1/V \) plots using a computer programmed regression analysis.

RESULTS

(A) Isocitrate dehydrogenase

Electrophoresis of extracts from six Drosophila species, followed by specific staining for IDH activity, revealed a single activity band in each species. With the exception of the \( D. \) equinoxialis-\( D. \) willistoni pair, IDH from each species had a unique electrophoretic mobility rate. Within \( D. \) melanogaster two allozymic variants, designated IDH¹ and IDH² could be distinguished.

The activities of partially purified IDH were examined with respect to heat stability. As seen in Fig. 1, the pattern of heat inactivation was species specific, indicating, as well, that the primary sequence of even the \( D. \) equinoxialis and \( D. \) willistoni IDHs were different. No particular association between inactivation rate and natural habitat temperature could be seen.

We next examined some of the kinetic properties of IDH. Figure 2 summarizes the relationship between \( K_m \) and assay temperature for IDH obtained from the three possible IDH genotypes of \( D. \) melanogaster. In general, \( K_m \) values remained fairly constant for the two homozygous genotypes, but marked increases in \( K_m \) with increased temperature were seen with the heterozygote. We are unable to account adequately for the uniqueness of the heterozygote pattern, but since this enzyme is dimeric the novel properties may reflect the contribution of heterodimeric enzyme molecules—that is, those IDH molecules composed of subunits determined by the two different alleles.

Figure 3 summarizes data comparing the three tropical species, \( D. \) arizonensis, \( D. \) willistoni and \( D. \) equinoxialis, and the two temperate species, \( D. \) virilis and \( D. \) americana. At temperatures below 25°C, the \( K_m \) values of all five species were quite comparable, showing, if at all, only a modest increase with elevated temperature. At 35°C, however, a marked increase in \( K_m \) was noted among the temperate species, while only a two-fold increase appeared among the tropical species.

From the kinetic data we were able to determine \( Q_{10} \) values over three 10°C intervals, at substrate concentrations below the \( K_m \) (Hochachka & Somero, 1973). These data are summarized on Table 1. As

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Fig. 1. Thermal inactivation of isocitrate dehydrogenase at 50°C. Plots designate \( D. \) equinoxialis (△); \( D. \) arizonensis (x); \( D. \) willistoni (○); \( D. \) virilis (●); \( D. \) americana (▲); \( D. \) melanogaster IDH¹⁺⁺ (□); \( D. \) melanogaster IDH²⁺⁺ (△).

Fig. 2. Apparent \( K_m \) for D,L-isocitrate, as a function of temperature for \( D. \) melanogaster IDH¹⁺⁺ (●); IDH²⁺⁺ (○); IDH¹⁺⁺ (△).

Fig. 3. Apparent \( K_m \) for D,L-isocitrate, as a function of temperature for \( D. \) virilis (△); \( D. \) americana (▲); \( D. \) arizonensis (○); \( D. \) willistoni (x); \( D. \) equinoxialis (○).
Adaptation of *Drosophila* enzymes to temperature

Table 1. Temperature coefficients \(Q_{10}\) determined for IDH and ACHE, over three temperature intervals

| Species                  | Temperature range |        |        |
|--------------------------|-------------------|--------|--------|
|                          | 5-15°C  | 15-25°C | 25-35°C|
| (A) IDH                  |         |        |        |
| *D. willistoni*          |         | 1.75   | 1.69   |
| *D. equinoxialis*        | 1.73    | 1.90   | 1.52   |
| *D. arizonensis*         | 2.10    | 1.79   | 1.37   |
| *D. melanogaster F/F*    | 1.88    | 1.81   | 1.50   |
| *D. melanogaster S/S*    | 1.90    | 1.63   | 1.46   |
| *D. virilis*             | 1.63    | 1.99   | 1.27   |
| *D. americana*           | 2.07    | 1.46   | 1.48   |
| (B) ACHE                 |         |        |        |
| *D. willistoni*          | 1.70    | 1.37   | 1.26   |
| *D. equinoxialis*        | 1.56    | 1.35   | 1.24   |
| *D. arizonensis*         |         | 1.60   | 1.38   |
| *D. melanogaster*        |         | 1.30   | 1.39   |
| *D. virilis*             |         | 1.43   |        |
| *D. americana*           | 1.43    | 1.24   | 1.35   |

The values represent an average of two separate experiments, and were determined at the following concentrations of substrate: d,l-isocitrate at 12.5 μM; acetylthiocholine iodide at 8 μM.

might be predicted from the model or positive thermal modulation, \(Q_{10}\) values in the 5–15°C temperature range were invariably greater than those in the 25–35°C range, for each species. Surprisingly, however, the absolute \(Q_{10}\) values in *D. virilis* and *D. americana* in the high temperature range were comparable to those of the other species, in spite of the divergence in \(K_m\) values. Activation energies were calculated for all species and genotypes according to the formula in Robert & Gray (1972) from Arrhenius plots of log \(V_{max}\) vs 1/T. The values in all cases were quite similar (15.47 ± 0.43).

(B) Acetylcholinesterase

A similar analysis was carried out for partially purified *Drosophila* acetylcholinesterase. In this case the electrophoretic mobilities of ACHE in all five species were indistinguishable. Heat inactivation studies, however, revealed striking differences between many of the species (Fig. 4) again indicating the insensitivity of electrophoresis in detecting protein sequence differences (Bernstein et al., 1973). As with IDH, the order of heat stability among the species was not related to habitat temperature.

The relationship between \(K_m\) for acetylthiocholine and assay temperature was examined for each species and these results are summarized in Fig. 5. *D. virilis*, *D. americana*, and to some extent *D. melanogaster*, show characteristic patterns of positive thermal modulation: as temperatures increased, substrate affinity decreased. In contrast, profiles for the three tropical species show an unusual pattern in which both temperature and substrate affinity increase together. Activation energies were calculated for all species and found to be similar (15.7 ± 0.8).

Once again temperature coefficients were determined at three 10°C temperature intervals, at substrate concentrations below \(K_m\) in order to see the extent to which the \(K_m\) patterns were reflected in rate. Taken as a whole the data show very little if any difference between \(Q_{10}\) values of tropical and temperate species, in spite of the marked differences in the \(K_m\) vs temperature data.
DISCUSSION

We began this study with two questions in mind. First, we wondered whether patterns of positive thermal modulation, which are so commonly found in aquatic poikilotherms, occur in terrestrial species. The answer, it seems, is yes and no. The $K_m$ vs temperature pattern for IDH, in four of the five species, showed this pattern, particularly at higher temperatures. In D. melanogaster, $K_m$ values remained roughly constant with varying temperature, with the exception of IDH$^{186}$ enzyme.

In the case of acetylcholinesterase, three species showed $K_m$ values increasing with temperature, and three species showed the reverse. The unusual pattern in which $K_m$ decreases with increasing temperature has been termed "negative thermal modulation" and is rarely encountered in aquatic forms (Hochachka & Somero, 1973). According to the biochemical reasoning, a simultaneous reduction of enzyme-substrate affinity and kinetic energy should act synergistically to reduce reaction rates precipitously. One would anticipate enormous $Q_{10}$ values resulting. Hochachka & Somero (1973) point out that for ACHE the molecular basis of negative thermal modulation may involve the hydrophobic interactions stabilizing the enzyme-substrate complex. It is known that hydrophobic interactions weaken as temperature is lowered, and thus one can possibly account for this effect in physical terms. For a third enzyme we have studied (Alahiotis, Miller & Berger, 1977) a similar situation was observed in which species showed a pattern of positive thermal modulation while others showed a pattern of negative thermal modulation. Although we cannot explain why, it has become clear from the data that the relationship between $Q_{10}$ and $K_m$ changes is not always direct. We have seen for ACHE especially, a situation in which $Q_{10}$ values for different species were quite comparable, and yet the $K_m$ versus temperature patterns differed markedly.

The second question of interest concerned parallelism of enzymatic function among species inhabiting similar habitats. Here we compared the behavior of enzymes extracted from three tropical, and two temperate species. For both ACHE and IDH the general patterns of $K_m$ vs temperature correlated well with geographic origin, and not phylogeny. This parallelism was also found in earlier work on another enzyme of Drosophila $\alpha$-glycerophosphate dehydrogenase. While we cannot say with any certainty how these kinetic differences translate into physiological differences, $in vivo$, the habitat correlated similarities strongly suggest that natural selection is operating on these loci, with temperature being one mediating agent.

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