

ENZYME CHANGES DURING DEVELOPMENT OF HOLO- AND HEMI-METABOLIC INSECTS

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(Received 12 November 1974)

Abstract—1. Several carbohydrate metabolizing enzymes were examined spectrophotometrically and electrophoretically in four life stages of the holometabolic fruit fly, *Drosophila pseudoobscura*, and in three life stages of the hemimetabolic pea aphid, *Acyrtosiphon pisum*.

2. *Drosophila* pupae exhibit significantly lower enzyme activities than third instar larvae. Most enzymes recover to larval levels or higher in adults, but some continue to decrease following eclosion.

3. Enzyme activities remain relatively unchanged during development of *Acyrtosiphon*.

4. The same isozymic forms of each enzyme are usually represented in all life stages of *Drosophila*, and in all life stages of *Acyrtosiphon*.

5. Enzyme levels parallel the distinct morphological and physiological changes characterizing development of representative holo- and hemi-metabolic insects.

INTRODUCTION

THE IMAGOS of most holometabolous insects are morphologically and ecologically very different from their respective larvae. The transition from larva to adult requires a unique pupal stage during which larval structures are histolysed and reorganized into adult tissues. "The metamorphosis of the higher insects embodies the most profound reorganization of a grown animal that is known" (Wyatt, 1968). In contrast, far fewer morphological and ecological differences are observed between life stages of some hemimetabolous insects. Development proceeds through a series of molts and culminates in adults which may closely resemble the instars except in size and reproductive capacity. Although these gross developmental differences between holo- and hemi-metabolous insects have long been recognized, relatively few comparative studies have been made of the underlying biochemical and genetic bases of these differences. In this study, we quantify activity levels of the isozymic forms of several carbohydrate-metabolizing enzymes during life stages of insects exhibiting holometabolic and hemimetabolic development. Our objective is to quantify changes in components of central metabolic pathways, and to associate these changes with distinct patterns of insect ontogeny.

Since higher Diptera exhibit the most pronounced metamorphosis among insects, we have examined *Drosophila pseudoobscura* as an extreme representative of holometabolic development. Larvae are fusiform grubs passing through three instars prior to pupation. The winged adults bear little morphological or ecological resemblance to the larvae. Hemimetabolic development of the pea aphid, *Acyrtosiphon pisum*, proceeds through four instars to adult. All stages feed in phloem of legume plants, and, except for size differences, are morphologically nearly indistinguishable. Thus *Drosophila* and *Acyrtosiphon* provide appropriate material for studying the genetic basis of contrasting developmental strategies.

MATERIALS AND METHODS

Activity levels

We have found the following to be a simple and repeatable procedure for measuring levels of enzyme activity in individual organisms. A fly or aphid is placed in a small mortar with 0.02 ml of deionized water and is thoroughly homogenized with a glass rod. Another 0.13 ml of water is added and stirred. From this homogenate, 0.10 ml is pipetted through gauze to a spectrophotometer microcuvette containing appropriate buffer, substrate, ions, and coenzymes. Since each of the catalyzed reactions involves formation of either NADH or NADPH, enzyme activity is measured by the rate of change in absorbance at 340 m μ over a 3-min period. Assays were made at room temp on a Zeiss PMQII spectrophotometer.

Stock solutions of the following were prepared: Buffers—(A) 0.05 M Tris-HCl, pH 8.5; (B) 0.25 M Tris-HCl, pH 7.1; (C) 0.10 M Tris-HCl, pH 7.1. Substrates—(D) 370 mM DL- α -glycerophosphate; (E) 173 mM DL-isocitric acid; (F) 500 mM α D(+) glucose; (G) 6.6 mM D-fructose-6-phosphate; (H) 64 mM L-malic acid; (I) 330 mM α D glucose-1-phosphate; (J) isopropanol. Cofactors—(K) 53 mM ethylenediamine tetraacetic acid; (L) 3.8 mM nicotinamide adenine dinucleotide; (M) 1.3 mM nicotinamide adenine dinucleotide phosphate; (N) 227 mM manganese chloride; (O) 50 mM magnesium chloride; (P) 8.5 mM adenosine 5'-diphosphate; (Q) 1000 units glucose-6-phosphate dehydrogenase per 100 ml water; (R) 1400 units hexokinase per 50 ml water; (S) 1% histidine.

Assay mixtures for the various enzymes were: (1) alcohol dehydrogenase (ADH): 0.75 ml of (A), 0.05 ml of (J), and 0.10 ml of (L); (2) α -glycerophosphate dehydrogenase (α GPD): 0.6 ml of (A), 0.1 ml of (D), 0.1 ml of (K), and 0.1 ml of (L); (3) isocitric dehydrogenase (IDH): 0.58 ml of (A), 0.1 ml of (E), 0.2 ml of (M), and 0.02 ml of (N); (4) phosphoglucose isomerase (PGI): 0.50 ml of (C), 0.10 ml of (G), 0.1 ml of (K), 0.1 ml of (M), 0.02 ml of (O), and 0.08 ml of (Q); (5) phosphoglucomutase (PGM): 0.63 ml of (C), 0.06 ml of (I), 0.1 ml of (M), 0.02 ml of (O), 0.08 ml of (Q), and 0.01 ml of (S); (6) malic enzyme (ME): 0.64 ml of (A), 0.05 ml of (H), 0.20 ml of (M), and 0.01 ml of (O); (7) adenylate kinase (ADKIN): 0.26 ml of (B), 0.2 ml of (F), 0.25 ml of (M), 0.01 ml of (O), 0.05 ml of (P), 0.08 ml of (Q), and 0.05 ml of (R). In each case, addition of 0.10 ml of tissue

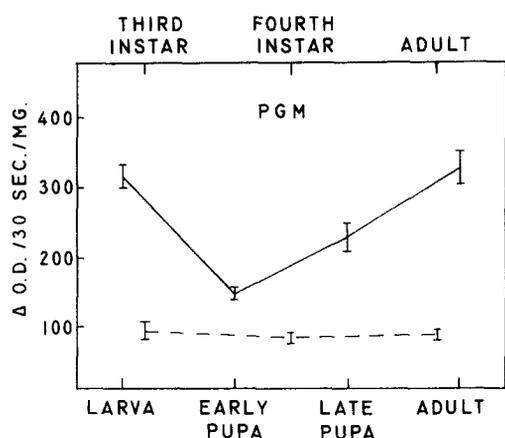


Fig. 1. Levels of phosphoglucumutase activity in third and fourth instars and adult *Acyrtosiphon*, and in larvae, early and late pupae, and adult *Drosophila*. Activities expressed as initial change in optical density ($\times 1000$) per 30 sec/mg wet body wt. Mean \pm one S.E. is indicated.

homogenate to the microcuvette brought the final assay vol to 1 ml.

Flies and aphids were weighed in groups of ten to the nearest 0.01 mg, and each individual assigned a wt equal to the mean value for the group. Thus in the graphs that follow, the variance about the mean enzyme activity/unit wt of insect represents a function of the total interorganism variance, a component of which may be due to variability in weight among organisms.

Isozyme analysis

Horizontal starch gel electrophoresis was carried out according to standard techniques described by Ayala *et al.* (1972). Enzymes were localized with staining mixtures comparable to those given above (for exact recipes, see Ayala *et al.*, 1972).

In both the spectrophotometric and electrophoretic studies, four developmental stages of *Drosophila pseudoobscura* were used—third instar larvae, early pupae, late pupae, and adults. For our purposes, early pupae are defined as those prior to visible development of wings. Adults include only sexually mature flies, older than 12 hr post eclosion. For each life stage in *Drosophila* and *Acyrtosiphon*, a minimum of ten individuals were assayed individually for each enzyme.

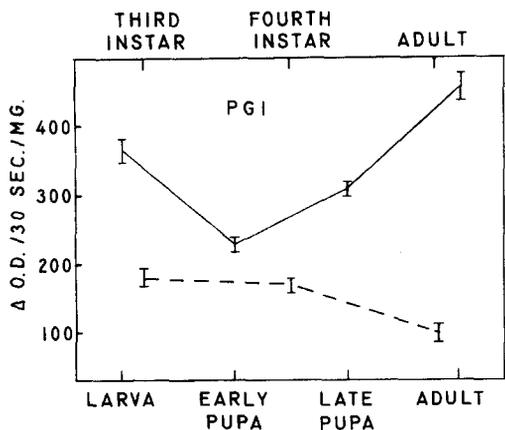


Fig. 2. Levels of phosphoglucose isomerase activity during development of *Acyrtosiphon* and *Drosophila*. Explanation as in legend to Fig. 1.

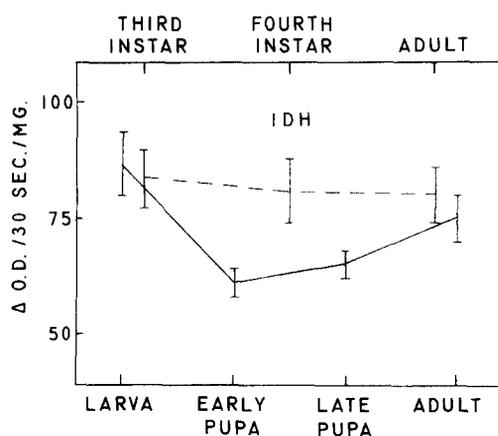


Fig. 3. Levels of isocitrate dehydrogenase activity during development of *Acyrtosiphon* and *Drosophila*. Explanation as in legend to Fig. 1.

RESULTS

Holometabolic development

Activity levels of seven enzymes during development of *Drosophila pseudoobscura* are presented in Figs. 1-6. Since overall mean wt of larvae, pupae, and adults were nearly identical (1.38 mg, 1.32 mg, and 1.31 mg per individual, respectively), the curves represent both mean activity/organism and mean activity/unit wt. Two basic patterns are evident. Following a significant drop in pupae, enzyme levels either recover to near larval levels or higher (PGM, IDH, PGI, α GPD, ADKIN) or else continue to drop in adults (ADH, ME).

Developmental levels of some of these enzymes have previously been reported in *Drosophila*. In accord with our results, IDH shows a U-shaped curve (pupae lower than late third instar larvae and adults) in *Drosophila melanogaster* (Fox, 1971), as does α GPD (Rechsteiner, 1970; Karlson & Sekeris, 1964). We did not observe U-shaped variation in alcohol dehydrogenase in *Drosophila pseudoobscura* although U-shaped curves have been reported for ADH in *D. melanogaster* (Hewitt *et al.*, 1974) and in *D. hydei* (Imberski & Strommen, 1972). In order to confirm our

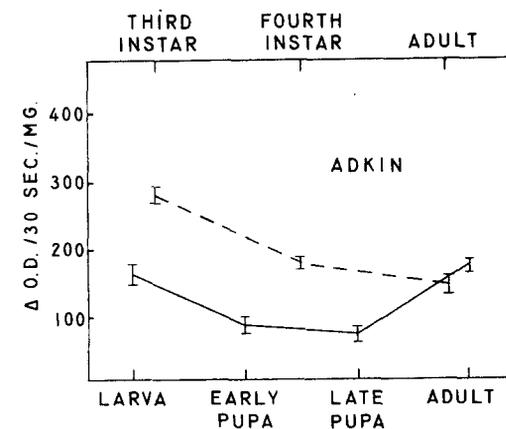


Fig. 4. Levels of adenylate kinase activity during development of *Acyrtosiphon* and *Drosophila*. Explanation as in legend to Fig. 1.

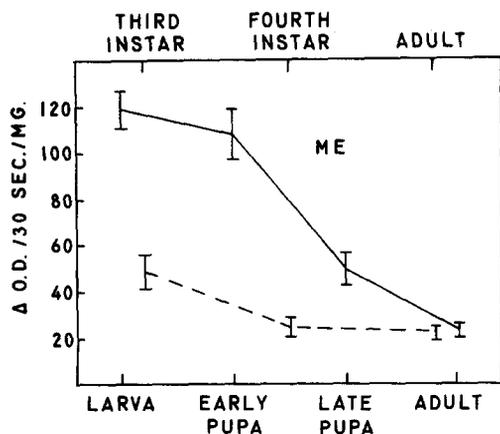


Fig. 5. Levels of malic enzyme activity during development of *Acyrthosiphon* and *Drosophila*. Explanation as in legend to Fig. 1.

results, we assayed ADH in *D. persimilis* (a very close relative of *D. pseudoobscura*) and *D. melanogaster*. Although *D. persimilis* shows a nearly identical developmental pattern to *D. pseudoobscura*, *D. melanogaster* does show a strong U-shaped curve. ADH developmental patterns thus appear to be variable between (and perhaps within) *Drosophila* species and we are presently investigating this phenomenon.

The activity of α -glycerophosphate dehydrogenase is much higher in adults than in larvae and early pupae (by at least 100 and 400%, respectively). A great deal of attention has been paid to the high levels of α GPD in winged insects, and it is now known that this enzyme plays a critical role in the metabolic processes of energy requiring flight muscle (Wyatt, 1968; Sacktor, 1965). α -Glycerophosphate dehydrogenase forms part of a cytosol-mitochondrial shuttle system which allows oxidation of extramitochondrial NADH and hence continuance of the glycolytic cycle as well as transfer of electrons to the oxidative phosphorylation chain (Hochachka & Somero, 1973). This enzyme thus represents changing levels which are closely tied to the physiological requirements of an organism.

In most cases, the striking changes in activity levels during development appear to be the result of changing quantities of the same isozymic products. In fact,

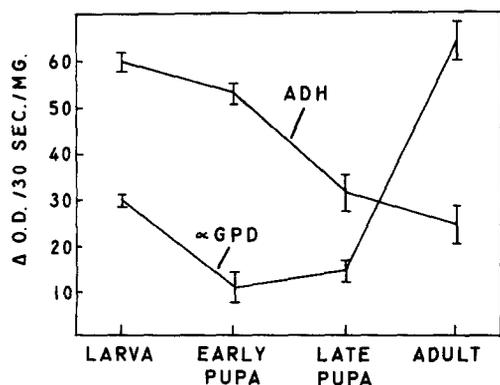


Fig. 6. Levels of alcohol dehydrogenase and α -glycerophosphate dehydrogenase activity during development of *Drosophila pseudoobscura*. Explanation as in legend to Fig. 1.

for the enzymes electrophoretically examined, we have no evidence of different loci being completely turned on or off in *Drosophila pseudoobscura*. Two sharp bands of very different mobility appear on gels stained for PGM. The furthest anodal band is darker in larvae and early pupae than in adults, but in all stages the less anodal band is the most intense. A sharp band appears for ME in all life stages, and is most intense in larvae. The locus encoding this zone (ME-1) is known to be polymorphic in *D. pseudoobscura* (Ayala & Powell, 1972), as is a very much lighter product of a possible second ME locus (ME-2) on their gels. We did not observe this second light zone of activity. Isocitric dehydrogenase exhibits a single sharp band in all life stages.

We could not resolve PGI into sharp bands, although a wide blur consistently migrated slightly toward the anode in larvae, pupae and adults. A single sharp band, darkest in adults, appears on gels stained for α GPD. Using very different electrophoretic techniques, Hubby & Lewontin (1966) show two close zones of α GPD activity in *D. pseudoobscura*. Since no zymogram variability was observed between individuals or strains (Lewontin & Hubby, 1966), these bands may simply represent post-translational changes in products of a single locus acting throughout development.

The zymogram of ADH shows three sharp bands, two of which migrate cathodally under our conditions, and one which migrates slightly anodally. All bands are darkest in larvae and early pupae, and lightest in adults. Although a multiple locus model could be proposed for this zymogram pattern, two of the bands very likely represent subbands of the primary gene product (Jacobson *et al.*, 1972). At any rate, there is presently no conclusive evidence for different ADH loci being active during the life stages of *Drosophila pseudoobscura*.

The adenylate kinase zymogram is more complex. There are five major zones of activity, each present in all life stages. Lack of variability in the banding pattern of the individuals we surveyed makes it presently impossible to know whether these bands represent products of the same or different ADKIN loci.

In summary, the ontogeny of *D. pseudoobscura* is characterized by dramatic changes in levels of glucose metabolizing enzymes. Whole body enzyme levels invariably drop significantly in pupae, and usually, although not always, recover to larval levels or higher in adults. In most cases, changing levels of enzyme activity appear not to be a consequence of the complete turning on and off of different genes during development, but rather appear to represent changing concentrations or activations of molecular products of the same loci.

Hemimetabolic development

Activity levels of five enzymes during development of *Acyrthosiphon* are presented in Figs. 1-5. The weight of individual pea aphids increases greatly during development (about 0.95 mg, 2.60 mg, and 3.85 mg apiece for third instars, fourth instars, and adults, respectively). Thus we have corrected for weight, and the graphs represent activities per unit wt of organism. (Expressed on a per organism basis, activity of each enzyme increased through development.)

Again, two patterns are evident, although the patterns are quite different from those in *Drosophila*. The enzymes examined either maintain a relatively constant level of activity per unit wt during development (PGM, IDH), or else decrease somewhat in later life stages (ME), particularly in the adult (PGI, ADKIN).

The zymogram patterns appear very similar in the three life stages. A single sharp band is expressed for PGM. A single band also appears on gels stained for ME and for IDH. Phosphoglucose isomerase exhibits one darkly staining band in all life stages examined. In addition, a much slower migrating, very faint band appears in fourth instars and adults. This band may represent the product of a separate locus, although its faint appearance suggests that it contributes very little to the activity levels shown in Fig. 2.

(Note: In both *Acyrtosiphon* and *Drosophila*, the relative intensities of the bands appearing on the gels support in a general sense the curves depicted in Fig. 1 through 6. Thus, for example, the α GPD band in *Drosophila* zymograms appears very intense in adults, less intense in larvae, and faintest in early and late pupae. Nonetheless enzyme levels are not readily or accurately quantified from starch gels alone.)

As in *Drosophila*, the adenylate kinase zymogram is complex. Three major zones of activity appear on gels in adults, and the least anodal of the zones cannot be seen in third and fourth instars. The appearance of the bands and their wide separation on the gels suggests that they represent products of different loci, one of which may be turned on primarily in adults. However, until genetic variants are observed this interpretation must remain tentative.

DISCUSSION

All of the enzymes we have examined in *Drosophila* pupae show a significant decrease in activity from third instar larvae. Although protein molecules are several steps removed from the genes, and need not directly reflect patterns of gene induction or repression, they do generally reflect changing metabolic capabilities of organisms. One of the best known metabolic observations on metamorphosing insects is the depression of oxygen utilization during pupation (Agrell, 1964; Wigglesworth, 1965). Accompanying this decrease in respiratory rate is a corresponding decrease in the terminal oxidases of the cytochrome system (Agrell, 1964), and previously reported decreases in certain dehydrogenases in pupating mealworms and houseflies (Ludwig & Barsa, 1958, 1959). Nonetheless, the rate of metabolism may not be limited by levels of respiratory enzymes but rather by the energy requirements of protein and tissue synthesis in the pupa (Wyatt, 1968). The stimulation of oxygen uptake by certain chemicals or by mechanical injury of silk moth pupae indicates that respiratory capacity normally exceeds demand.

Among the higher Diptera, metamorphosis entails a major degree of histolysis of larval tissues (Whitten, 1968). We have examined only whole body enzyme levels and do not know how much activity is contributed by various tissues. Muscles, fat body, and intestine make up much of the larval weight, and all of these organs and tissues apparently completely histolyze during metamorphosis (Bodenstein, 1965). Thus

decreasing enzyme activity could simply reflect protein destruction during histolysis. However, histolysis probably does not result in a depression in whole body levels of all proteins. Proteinase activity (measured at constant pH) shows very little variation during development (Agrell, 1964). Also, histolysis and histogenesis are not consecutive but rather concurrent, and thus total cellular tissue may not change much (Wyatt, 1968).

In summary, biochemical, physiological, and morphological changes all parallel one another during *Drosophila* metamorphosis. Enzyme levels decrease, respiration decreases, and tissues histolyze. Although these processes clearly comprise an integrated and adaptive developmental strategy, it is less clear which of the processes are causally related.

The changes in levels of enzyme activity during development are much less pronounced in *Acyrtosiphon*. Enzyme levels per unit body wt either remain constant or else decrease somewhat in later life stages. There is no evidence of U-shaped developmental patterns of enzyme levels. Again, in most cases, the same isozymic forms of each enzyme are present in third and fourth instars and adults. α GPD, an enzyme which is known to be essential to flying insects, was not detected in our assays of the flightless pea aphid.

The decrease in levels of ADKIN, ME, and PGI in later life stages was not expected. This pattern may reflect changes in body composition. For example, much of the body cavity of adults is occupied by developing embryos, and, after reproductive age is passed, the cavity becomes filled with fat deposits and water (Marv Kinsey, pers. comm.). However, if this is the cause of the decrease in some enzymes per unit body wt, the decrease should be expressed in all functionally related enzymes. Furthermore, the drop from third instars to fourth instars is not explained. At any rate, the magnitude of the activity changes are not nearly as pronounced as in *Drosophila* for most enzymes examined.

Wyatt (1968) argues that the major metabolic pathways during pupation differ quantitatively but not qualitatively from those of normal tissues. Our results agree with this conclusion. Not only are the same enzymes present in late larvae, pupae and adult *Drosophila*, but usually the same isozymic form is represented, though in different concentrations. Decreasing enzyme levels in pupae may reflect gene repression, increased degradation (or inactivation) of existing enzyme molecules, or both, but the rise in activity levels in adults must largely represent renewed enzyme synthesis. This renewed synthesis in most cases appears to result from induction of the same genes rather than a completely different cohort of loci.

Two of the enzymes we have examined in *Drosophila* did not recover to larval levels in adults. Malic enzyme catalyses the interconversion of pyruvic acid and malic acid, and is thought to be significant for its capacity to generate NADPH for biosynthetic pathways. It is not clear why levels are low in adults and late pupae. Alcohol dehydrogenase is unique among the enzymes examined because its substrates are probably of external origin. ADH may play a critical role in allowing flies to tolerate and/or utilize alcohols in the environment. Thus we might expect ontogenic patterns of ADH to differ among species

or genotypes depending on ecological requirements. We are currently investigating ADH levels in a variety of *Drosophila* species. If the major physiological function of ADH is indeed environmentally related, the lack of significant quantities of alcohol in the pea aphid environment may explain our inability to detect ADH activity in those individuals we examined.

Some other enzymes (for example, lactate dehydrogenase—Rechsteiner, 1970) also decrease in activity in adult *Drosophila*. The increase in enzyme synthesis in adults thus does not extend to all loci, but as expected, reflects selective control.

Acknowledgements—Drs. F. J. Ayala, G. J. Edlin, and C. L. Judson were all very kind in allowing us free use of their laboratory facilities. We also wish to thank Marv Kinsey for supplying the pea aphids used in this study. Research was supported by NIH training grants in genetics.

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