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THE EFFECT OF TEMPERATURE ON THE EXPRESSION OF ENZYMES IN THE VESTIGIAL MUTANT OF *DROSOPHILA MELANOGASTER*

by

Rosevelt L. Pardy

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I hereby recommend that this dissertation prepared under my direction by Rosevelt L. Pardy

entitled The Effect of Temperature on the Expression of Enzymes in the Vestigial Mutant of Drosophila melanogaster

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

Peter E. Pickens — Dissertation Director

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After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

Peter E. Pickens	July 29, 1969
Ronald Koch	July 29, 69
Robert W. Hoshaw	July 30, 1969
Paul G. Bartels	July 30, 1969
W. Toews	July 30, '69

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ABSTRACT

Homozygous vg mutants of the fly Drosophila melanogaster, which possesses vestigial wings, exhibits a two-band alkaline phosphatase pattern as demonstrated by electrophoresis. This pattern may be converted to the single band, wild-type pattern by rearing the vg larvae at 32°C. The conversion takes place at a critical period, 30 hours after hatching of the larvae and 35 hours before conversion of the vestigial to wild-type wings takes place. Neither azauracil nor sodium lactate, agents reported to affect wing development, had any effect on wing or enzyme patterns. Studies of pH optima and kinetic properties show that vg alkaline phosphatases have the same activity characteristics as those of the wild-type. Histochemical evidence indicates no differences between vg and wild-type flies in the distribution of alkaline and acid phosphatases which are limited to the hind gut and malpighian tubules. This along with the evidence from genetic crosses suggests that the two band, temperature convertible pattern of vg flies is a result of a separate mutation not related to wing formation.

CHAPTER 1

INTRODUCTION

In 1912 Morgan reported for the first time the existence of a mutation in Drosophila melanogaster which when in the homozygous condition gave rise to a mutant with highly reduced wings. This mutation was named vestigial (vg). Subsequent work on the anatomy and development of the vestigial mutant (Chen, 1929; Cuenot, 1923; Auerbach, 1936) revealed that in addition to the wing defect, the mutant exhibited a larva smaller than wild-type, delayed pupation, reduced viability, abnormal muscles, smaller wing discs, and smaller haltere discs.

While engaged in a statistical analysis of the size of the vg mutant wings, Roberts (1918) observed a sudden and unexpected irregularity in the data. Upon further investigation it was revealed that as a result of a local heat wave some of the experimental flies had been reared at temperatures in excess of 30°C. Roberts found that vg larva reared at these elevated temperatures developed nearly normal or wild-type wings. These findings lay dormant until Harnly (1936) embarked on a detailed study of this temperature phenomenon. Harnly (1936) showed

that there is a critical period in the development of the larva where temperatures in the range of 30°C-32°C are effective in converting the vg wings to normal size wings. This critical period begins about 64 hours after hatching of the egg and lasts to pupation. In subsequent studies Harnly (1942) discovered nine temperature sensitive vg mutants which under appropriate temperatures could produce normal size wings. Among these genotypes was a mutant which Harnly named vestigial pennant (vg^p). This mutant was morphologically like the wild-type. A cross between vg^p and vg flies resulted in heterozygous offspring which had vestigial wings. If larvae from such a cross are reared at either 16°C or 32°C they develop with enlarged wings. Larvae reared at 25°C develop typical vestigial wings. From this work on the temperature sensitive mutants Harnly (1951) developed the following hypothesis to explain the effect of temperature on the wing size of the vg and vg^p mutants: There is a critical step or reaction in the development of wing discs in the larval fly. This critical step in the wild-type larva may take place at any temperature between the upper and lower lethal temperature for the organism (16°C-32°C). In the case of the wing mutants the temperature optima for this critical step is displaced beyond the lethal temperatures, above 32°C in the case of the vg mutant and below 16°C in the case of the vg/vg^p heterozygote. This hypothesis concerning reaction optima suggests that these may

be temperature optima of discrete biochemical pathways or components of biochemical pathways such as single enzymatic reactions.

The observation that different mutants exhibit different biochemical properties is now better understood in the light of our current knowledge of the molecular events associated with or related to gene activity. According to the central dogma, (Crick, 1958), genes may be composed of a linear arrangement of nucleotides in DNA which through the processes of transcription and translation specify the linear sequence of amino acids in the polypeptides which make up proteins such as enzymes. A change in the amino acid sequence of the polypeptide components of an enzyme as a result of mutation may affect its enzymatic or catalytic properties by altering essential steric or conformations relationships affecting the catalytic, binding, or allosteric sites. Such changes may reduce the enzyme's catalytic ability or may destroy it completely (Wagner and Mitchell, 1965). It is conceivable that if an enzyme's catalytic ability has only been slightly altered through genetic mutation, it might be possible to restore some of its original activity by changing one or more of the environmental parameters such as temperature, pH, substrate concentration, or by the addition of certain co-factors. Work with Neurospora (Horowitz et al, 1960) for instance, has shown that the temperature sensitivity of certain

mutants may be overcome through treatment with riboflavin. In this context, some of the fine structural changes that may have occurred as a consequence of mutation could be further explored by testing for differences in electrophoretic mobility.

Current work has demonstrated, in general, two major phenomena: the electrophoretic pattern of isozymes changes with developmental stage (Markert and Møller, 1959; Laufer, 1961; Moorjani, 1967; Wallis and Fox, 1968), and the electrophoretic patterns may be species, organ, or tissue specific (Markert and Møller, 1959; Paul and Fottrell, 1961). In addition, it is strongly suggested that the different isozymes play an intimate role in the development of the organism (Laufer, 1961) though the exact nature of this role in any one case has yet to be unequivocably defined.

It is not known which enzymes, if any, have been altered so as to affect wing formation in the vg mutants. Consequently the choice of enzymes to be studied is affected by two factors: the possible role of the enzyme based on what is known about its activity in other biological systems, and the availability of a sensitive, reliable assay method.

Among the enzymes that meet the above criteria are the phosphate hydrolyzing enzymes such as the alkaline and acid phosphatases, and the dehydrogenase enzymes such as

lactate dehydrogenase, malate dehydrogenase, α-glycerophosphate dehydrogenase, and the nonspecific esterases.

The acid and alkaline phosphatases are involved with manifold metabolic processes and hence alteration of these enzymes could conceivably result in biochemical or morphological deformities of the organism. The alkaline phosphatases are those enzymes which are active at pH 9.3-9.6 and which catalysize the removal or transfer of orthophosphate from phosphorylated alcohols, sugars, gylcerophosphate and phosphomononucleotides (Stdatman, 1961). On the other hand, the acid phosphatases are enzymes which are active at pH 4.0-6.0 and catalytically remove orthophosphate from phosphoproteins, 2'3' cyclic ribonucleotides or phosphodiesters (Schmidt, 1961). Isozymes of these enzymes have been demonstrated in Drosophila pseudeoboscura (Hubby and Lewontin, 1966), and in Drosophila melanogaster (Beckman and Johnson, 1964; Johnson, 1966; Wallace and Fox, 1968) as well as in many other organisms (see review by Shaw, 1965). In addition it has been shown that alkaline phosphatase patterns in vg mutants may be altered in vg flies reared at 32°C (Pardy, unpublished observations).

The esterases are another category of enzymes which have been shown to exist in different isozymes in a variety of organisms (Paul and Fottrell, 1961; Shaw 1965). Esterases are those enzymes which catalyze the hydrolysis of ester linkages (Fruton and Simmonds, 1958). Isozymes of esterases

have been demonstrated in Drosophila aldrichi and D. mulleri (Johnson et al, 1964) and in Drosophila melanogaster (Wright, 1963).

The nicotinamide adenine dinucleotide (NAD) linked-dehydrogenases, particularly malate dehydrogenase, -glycerophosphate dehydrogenase and lactate dehydrogenase, have been shown to exist in isozymic form. (Markert and Møller, 1957; Hubby and Lewontin, 1966). These enzymes catalyze the removal of hydrogen from substrates involved in intermediary metabolism. One of these enzymes (lactate dehydrogenase) may be implicated in the conversion of the wings of vg flies because Tanaka (1967) has shown that vg larva grown at room temperature on a medium containing ammonium lactate develop normal-type wings. In this instance lactate might be serving as an activator, inhibitor or as an energy source in a lactate dehydrogenase mediated pathway.

The importance of α-glycerolphosphate dehydrogenase in the energy metabolism of insect muscle has been shown (Sacktor, 1965) and isozymes of this enzyme have been demonstrated in Drosophila (Hubby and Lewontin, 1966). Apparently -glycerolphosphate dehydrogenase in insects serves a similar function to lactate dehydrogenase in higher vertebrates during anerobic metabolism.

Malate dehydrogenase isozymes have been demonstrated in Drosophila (Hubby and Lewontin, 1966) and have been shown to undergo modifications of electrophoretic mobility during

different stages of development in other invertebrates, such as starfish (Patton, Mets, and Villee, 1967). It would be of interest to study other wing mutants to see if similar mechanisms are involved in wing conversions. Enzymatic analysis might reveal similarities to the vg system. Vg mutants are not the only wing mutations of Drosophila melanogaster to exhibit inducable conversions. Rizki and Rizki (1965) have shown that when 67 hour larvae of the mutant dumpy are placed in a solution containing 6-azauracil, flies with normal, wild-type wings are produced. Azauracil is a pyrimidine analog which functions as an inhibitor of nucleic acid synthesis (Mahler and Cordes, 1966). As in the case of the vg flies, the effect is induced at a critical time during larval development (approximately 56 hours after hatching).

This investigation, therefore, sought to determine the effect of temperature, certain chemicals and experimental genetic crosses on the isozymic patterns of selected enzymes of vg, vg^p, dp wing mutants. In addition, certain kinetic properties and histochemical localization of the alkaline and acid phosphatases were determined.

CHAPTER 2

MATERIALS AND METHODS

Collection and Maintenance of Flies

Drosophila melanogaster mutants used in this study were obtained from the following sources: Drosophila Laboratory, University of Arizona (wild-type), California Institute of Technology, Pasadena, California (vestigial, dumpy), University of Groningen, Groningen, Netherlands (vestigial pennant). Stocks of these flies were reared at 25°C on standard agar-cornmeal-kayro medium. In order to raise flies at various temperatures and in relative synchrony, fertilized females were allowed to lay eggs for three to four hours on heavily yeast supplemented media in 1/2 pint bottles. These bottles were then incubated in temperature regulated incubators.

To facilitate the collection of larvae, a modification of the above procedure was devised. Media was prepared in 9 x 1 cm petri dishes. After cooling and yeasting these dishes were placed in a 12 x 18 cm battery jar containing 200-300 adult flies (ca. 50% females). These jars were covered with several layers of gauze to prevent the escape of the adult flies. In order to prevent drying of the media,

the battery jars were placed in a covered aquarium containing about 1 inch of water.

When the surface of the media in the petri dishes was well covered with eggs, the dishes were removed and incubated with covers until the desired larval stage had developed. Larva were easily removed from the surface of the media with a dissecting needle.

Preparation of Enzyme Extracts

One half gram of flies (about 500 flies) was ground in a tissue grinder with 2 mls of chilled buffer appropriate to the type of enzymatic analysis to be performed. The crude homogenate was centrifuged at 3500 x g for 5 minutes. The supernatent was decanted and centrifuged under the same conditions. The final supernatent layer contained 1.5 mg/ml of protein as measured by the Lowry method (Lowry et al, 1953) and compared to a standard curve prepared from bovine serum albumin.

For semi-micro electrophoresis, flies or parts of flies (head, thorax, abdomen) were ground in a semi-micro mortar and pestle in 0.05 ml of buffer. Crude homogenate was extracted from the grindings by absorption on 1 x 2 mm strips of Whatman 3 mm filter paper.

Electrophoresis

At first, proteins were separated by electrophoresis according to a slightly modified version of the

method of Hubby and Lewontin (1966) using a vertical gel apparatus (E.C. Corporation, University City, Philadelphia, Pennsylvania). Separation of proteins was carried out on a 6% polyacrylamide gel prepared in 0.1M tris-borate pH 9.0 buffer and polymerized by ammonium persulfate. Previous to electrophoretic separation a 30-minute pre-run at 400 volts was performed to remove excess persulfate catalyst. Following the pre-run, 50 ml of supernatent were introduced into the sample slots and allowed to settle for 10 minutes. At the end of the 10 minutes settling period electrophoresis was carried out for 2 hours at 400 volts. During the electrophoretic separation the gel was maintained at 5°C by circulating refrigerated water through the gel apparatus.

In addition to the vertical gel electrophoresis system a semi-micro electrophoresis apparatus was employed. Three mm gels of 6% polyacrylamide were poured on 8 x 10 cm glass plates. Following polymerization, the gels were stored at 5°C for 24 hours in buffer which was changed periodically. This procedure allowed diffusion and subsequent removal of the persulfate catalyst. For electrophoresis, electrical contact was made between the gel and buffer tanks by means of filter paper wicks. Following a 10 minute pre-run, sample homogenates were introduced into the gel on 1 x 2 mm strips of Whatman 3 mm filter paper. In order to

accomodate insertion of the strips, a slot 1.5 cm from the edge and extending the length of the gel was cut with a razor blade.

To prevent excessive heating during electrophoresis, the buffer tanks and glass plate supporting the gel were surrounded by crushed ice and the separation was carried out in the cold room.

Enzyme Staining

Phosphatases and esterases were detected using azopost coupling methods (Nachlas and Seligman, 1950; Pearse, 1960; Hubby and Lewontin, 1966). This assay depends upon the enzymatic cleavage of a phosphate or acid moiety from the substrate a-naptholphosphate (phosphatases) or a-napthol acetate (esterases). The reaction releases free napthol which remains at the site of activity. At some time after the reaction, a dye coupler, Fast Blue RR, is added which reacts with the napthol to form a colored, insoluble formazan dye. This dye is deposited at the site of the reaction and thus serves to mark the location of the enzyme in question.

In practice, alkaline phosphatases were detected by placing electrophoresis gels in 100 ml of 0.2M tris-HCl buffer, pH 9.0, containing 20 mg a-napthol phosphate and 10 ml of 0.001M magnesium chloride. After 1 to 2 hours of incubation at room temperature in this solution, the gel was

placed in a fresh solution of buffer containing 50 mg/100 ml of Fast Blue RR salt until the sites of activity were developed. Acid phosphatases were detected as above with the exception that the buffer used was 0.2M acetic acid-sodium acetate pH 5.0.

For esterase detection following electrophoresis, the gels were incubated at 5°C in 100 ml of a 0.5M solution for one hour to lower the pH of the gel. The gels were then incubated for 1-2 hours at room temperature in 100 ml of 0.2M phosphate buffer containing 20 mg a-napthyl acetate. Following this incubation the gels were transferred to fresh buffer containing 50 mg/100 ml of Fast Blue RR salt until the enzyme bands were developed.

In addition to the phosphatases and esterases gels were also assayed for the NAD-linked lactate, malate and α-glycerophosphate dehydrogenases. In this assay the particular dehydrogenase enzyme transfers hydrogen from the substrate (lactic acid, malic acid, or α-glycerophosphate) to the acceptor molecule NAD forming reduced NAD (NADH). NADH then transfers the electron to another electron acceptor, phenazine methosulfate (PMS). PMS, in turn, reduces nitroblue tetrazoleum (NBT). Upon reduction NBT precipitates as an insoluble blue dye which serves to indicate the position of the gel-bound dehydrogenases.

To demonstrate the presence of lactate dehydrogenase the technique of Knowles and Fristrom (1966) was used.

Gels were incubated at 37°C for one hour in 100 ml of 0.3M tris-HCl pH 7.4 buffer containing 15 mg NAD, 50 mg nitro blue tetrazoleum, 0.1 millimoles KCN, 36 millimoles sodium lactate. Following incubation, 2 mg of PMS were added to the above solution and incubation continued until the enzyme bands developed.

Malic dehydrogenase and α-glycerophosphate dehydrogenases were detected in a similar manner (Hubby and Lewontin, 1966) except that for malic dehydrogenase the incubation media was prepared in 0.2M tris-HCl, pH 8.5, and contained 15 millimoles of sodium malate. -glycerophosphate dehydrogenase was assayed by substituting 15 millimoles of α-glycerophosphate as substrate in the above buffer. To determine the possible presence of a "nothing dehydrogenase" (Pearse, 1960) a test gel was incubated in the above solution in the absence of any substrate.

At the completion of all enzyme assays the gels were placed in a wash solution consisting of glacial acetic acid-methanol-water (1:5:5). This wash immediately stopped the enzymatic reaction, fixed the protein in the gel, and removed most of the background staining.

In addition to the assays for enzyme activity, gels were also stained for general proteins. Following electrophoresis, gels were placed in 1% amido black protein stain prepared in the previously described wash solution.

Background and unbound stain was removed by repeated washing with the acetic acid-methanol-water wash.

Determination of Enzyme Activity

To determine the activity of acid and alkaline phosphatases in supernatent from tissue homogenates the Taussky and Shore (1954) assay for inorganic phosphate was used. In this method orthophosphate, released from glycerophosphate, is complexed with ammonium molybdate and the resultant heteropoly acid is subsequently reduced to molybdenum blue by ferrous sulfate. Molybdenum blue absorbs light at 740 mu so that the amount of inorganic phosphate released by the enzymatic reaction can be assayed in the spectrophotometer. The following procedure was used for the determination of the pH optima of the alkaline phosphatase activity in homogenates. For each pH 2 mls of 0.2M tris-HCl buffer was pipetted into a 15 ml test tube. To this was added 0.1 ml 0.001M magnesium chloride and 50 ml of supernatent. At time zero 0.1 ml of 1M sodium , glycerophosphate was added to start the reaction. At the end of 20 minutes the reaction was stopped by adding the enzyme-substrate preparation to a 12 ml centrifuge tube containing 0.5 ml 20% trichloroacetic acid. The tubes were shaken for 30 seconds and then placed on ice to allow complete precipitation of the proteins. After 2 hours the tubes were centrifuged for 10 minutes at 3500 x g. Following centrifugation

2 mls of the supernatent material was transferred to 15 ml test tubes and 1 ml of Taussky reagent (Taussky and Shorr, 1954) was added. After 30 minutes the reaction mixture was placed in a cuvette having a 1 cm light path and the optical density at 740 mu was determined using a Beckmen DU-2 spectrophotometer. All reactions were carried out at room temperature (25°C). The pH of the supernatent-substrate mixture was measured to guard against pH changes occurring after the addition of the homogenate.

In addition to pH curves, time curves of alkaline and acid phosphatases were prepared by following the rate of reaction in question over a period of 25 minutes. This was accomplished by incubating 1 ml of the supernatent in 20 ml of buffer (0.2M acetate, pH 5.3 for acid phosphatases; 0.2M tris-HCl pH 9.0 plus 0.1 ml of 0.001M magnesium chloride for alkaline phosphatases. At time zero 0.5 ml of 1M α,β-glycerophosphate was added to start the reaction. At five minute intervals 2 ml of the enzyme-substrate mixture was removed and added to 0.5 ml of 20% trichloroacetic acid contained in 12 ml centrifuge tubes and the same procedure as previously described was performed to assay for the released inorganic phosphate. In all cases a blank consisting of the supernatent and reagents was prepared and immediately assayed for background orthophosphate.

Histochemical Localization of Enzymes

To determine the distribution of acid and alkaline phosphatases, several procedures were employed. For alkaline phosphatases both the calcium cobalt and the azo dye technique (Pearse, 1969) were used as a cross-check of the specificity of the reaction. In the first case the phosphate enzymatically released from glycerophosphate is combined with calcium ions to form an insoluble precipitate which is bound at thesite of the reaction. Subsequent treatment with cobalt forms cobalt phosphate. Finally the cobalt salt is made visible by conversion to black cobalt sulfide through treatment of the preparation with ammonium sulfide.

Adult or larval flies were fixed in acetone for 24 hours at 5°C. The tissue was then dehydrated in ethanol and embedded in 56°C tissumat for 30 minutes. Following embedding 10 u sections were cut and affixed to slides using Myers egg albumin.

For analysis of alkaline phosphatases, sections were incubated in 50 ml of a solution containing 20 ml of 0.2M tris-HCl, pH 9.0, 20 ml of calcium chloride (2%), 10 ml of -glycerophosphate (2%) and 0.1 ml of 2% magnesium sulfate at 37°C. After 45 minutes the sections were rinsed in distilled water and placed in 50 ml of 2% cobalt nitrate for 5 minutes. Following this the sections were rinsed 3 times

in distilled water and transferred to distilled water containing 3 drops of ammonium sulfide. After inspection of the sections for the black cobalt sulfide particles the sections were counter-stained with either light green or hematoxylin, and then dehydrated and mounted.

Sections were also incubated in 50 ml of 0.2M tris-HCl, pH 9.0 buffer containing 20 mg of a-napthyl phosphate and 40 mg of Fast Red Salt. Deposition of red formazan dye via the reaction previously described under Enzyme Staining marked the location of alkaline phosphatase activity. Sections so treated were counter-stained with hematoxylin and mounted in glycerine jelly.

Acid phosphatases were detected by using the same routine as described for the calcium-cobalt method except that 2% lead nitrate was substituted for calcium chloride in 0.2M pH 5.0 acetate buffer. The localization of enzyme activity was made visible by the formation of black lead sulfide. Similarly, the azo dye procedure was used in the method described for alkaline phosphatase detection except that the reaction was carried out in 0.2M pH 5.0 buffer.

CHAPTER 3

RESULTS

Alkaline Phosphatase Patterns of Adult Flies

The normal wing morphology of wild-type, vg, reared at 25°C, and vg flies reared at 32°C is shown in Fig. 1. The wing phenotypes obtained from experimental crosses of 9 genotypes reared at three different temperatures are indicated in Table I. These data confirm the results of crosses by other workers (Harnly, 1935; Southin and Carlson, 1962).

The alkaline phosphatase pattern of wild-type and vestigial flies are shown in Figs. 2,3, and 4. The alkaline patterns for the other flies listed in Table I are the same as that of the wild-type. The wild-type enzyme pattern, hereafter referred to as the standard pattern, consists of one heavily stained band. The vestigial pattern, on the other hand, is comprised of two densely stained bands.

Adult vestigial flies reared from larvae kept at 32°C for 60 hours and then transferred to 25°C for pupation, develop wings which approach the wild-type wing in length and area (Fig. 1) as described by Harnly (1935). Homogenates prepared from these adults exhibit the standard

pattern of alkaline phosphatases. Wild-type flies reared under the same conditions develop normal wings and homogenates from these flies show no alteration in the standard alkaline phosphatase pattern. Apparently raising vg flies at 32°C not only causes near-normal wings to develop, but also alters the alkaline phosphatase pattern to the standard pattern.

It is known (Harnly, 1935) that the critical effective period for wing enlargement of the vg flies at 32°C begins at 64 hours after hatching of the larvae. To determine if the vg alkaline phosphatase pattern conversion also has a critical effective period and to see if this period might coincide with critical period for wing enlargement, the following set of experiments were performed. Vg larvae were reared at 32°C from the time of hatching. Every 5 hours, commencing 5 hours after hatching and continuing through 70 hours, larvae were removed and allowed to continue development through pupation at 25°C. The alkaline phosphatase patterns of adults reared in this manner were then determined. A critical period of about 32 hours was evident in all experiments. Thus, the alkaline phosphatase patterns of adults exhibited the typical 2-banded vestigial pattern when the larvae were reared at 32°C for any number of hours up to 30. Adult flies raised from larvae kept at this higher temperature just 30 hours exhibited the alkaline phosphatase pattern shown in Fig. 5, which consisted

of a single elongated band spanning to range covered by the two distinct bands. Beyond 30 hours, through the remainder of larval development, the vestigial flies raised at 32°C exhibited the standard pattern. The alkaline phosphatase patterns of wild-type flies raised under the same conditions also were not different from the standard pattern.

Alkaline Phosphatase Patterns of Larvae

In order to determine whether changes in alkaline phosphatase patterns can be detected before the fly becomes an adult, larvae raised under identical conditions as above were analyzed for changes in pattern. The semi-micro method of electrophoresis was used as it allowed the use of a smaller amount of material.

Wild-type larvae reared and assayed before and after 30 hours at 32°C exhibit the standard pattern. Vg larvae assayed before 30 hours exhibit the typical vg pattern while larvae assayed after 30 hours had the standard pattern. As in the case of the adults, those larvae assayed at 30 hours of incubation had the intermediate alkaline phosphatase pattern.

Enzymatic Activity and pH Optima of Phosphatases

Although the alkaline phosphatase patterns of the vg mutant is different from the wild-type, the acid phosphatase pattern is not. (Fig. 6, Table II). Since similarities in

pattern might not reflect differences in enzymatic activity and the two types are somewhat related, it seemed that a comparative study of the activity of the two enzyme systems might be revealing. Consequently, homogenates from wild-type, vg, vg^p, $vgvg^p$ and dp flies reared at 16°C, 25°C and 32°C were assayed for the pH optima and relative enzymatic activity of acid and alkaline phosphatases. There were no significant differences in either the acid or alkaline phosphatases from any of the mutants examined. All mutants exhibited the same rate and pH optima. The values shown are average values derived from data pooled from five experiments. The phosphatases exhibited peaks of activity at pH 3.9, 7.5, and 9.3 (Fig. 7). In all cases the alkaline phosphatase activity of the homogenates exceeded that of the acid phosphatases by 60% (120 ugP/mg/hr vs. 36 ugP/mg/hr). (Fig. 8, 9, Table III).

In order to localize the phosphatases within the fly, semi-micro electrophoresis was performed on homogenates prepared from separated heads, thoraxes and abdomens of vg and wild-type flies. Phosphatase patterns could be detected only from homogenates prepared from abdomens; no activity was observed in heads or thoraxes. The acid and alkaline phosphatases patterns obtained from the abdomen were identical to the normal vg and wild-type patterns previously described from whole animal homogenates. To further localize the phosphatases within the abdomen of vg

and wild-type flies, histochemical staining was used. It was observed that the hind gut of both adult and larva exhibited alkaline and acid phosphatase activity. (Fig. 10, 11). There appeared to be a progressive increase in the activity proceeding posteriorly to the anal gland.

In addition to occurring in the hind gut, alkaline phophatase activity was found in the malpighian tubules (Figs. 10, 12, and 13). No phosphatase activity occurred in sections from either the hear or thorax. Identical results were obtained from both the cobalt and azo dye (Pearse, 1960) techniques. No differences in localization of phosphatase activity occurred between either vg or wild-type flies or larva.

The Effect of a Genetic Cross on Enzyme Patterns

Because the vg alkaline phosphatase pattern, like the wings, appears to be modified by raising the flies at elevated temperatures, it was felt that some relationship between the enzyme and wing formation might exist. Furthermore, since the enzyme critical period is not the same as the wing critical period (30 vs. 64 hours), the possibility existed that the alkaline phosphatases might have to be altered to the standard type, during the interval between the two critical periods, before normal wings could be produced. On the other hand, a separate mutation, not related to wing formation could account for the differences in vg alkaline

phosphatases and their thermal alterability. To examine these two possibilities, another mutant, vestigial-pennant (Harnly, 1948) was used in making experimental crosses. Vestigial-pennant resembles the wild-type fly morphologically in that it has normal wings. Genetically, the vestigial-pennant carries a gene which when heterozygous with vg gives rise to flies having vestigial wings (Harnly 1942). When larvae from a vg^p x vg cross are reared at 32°C or 16°C they develop enlarged wings (Table I). Flies reared at 25°C have the typical vestigial wings. Homogenates from heterozygous flies ($vgvg^p$) produce the standard alkaline phosphatase pattern regardless of the temperature at which they are raised. Similarly the vg homozygotes also give the standard or wild-type pattern.

Other Enzyme Patterns

To determine if there were intrinsic differences in other enzymes or if enzymatic patterns might be altered by changes in temperature, homogenates of all flies listed in Table I were assayed for esterase, acid phosphatase, lactate dehydrogenase, α-glycerophosphate dehydrogenases and malate dehydrogenase. In addition enzymes from an eye mutant, bar eye, were also assayed to see if a mutant without wing defects might exhibit enzyme patterns that are different from those found in flies with modified wings. The standard enzyme patterns obtained for all enzymes listed above are

shown in Fig. 14. The results of these experiments are listed in Table II. As is indicated, all patterns were similar to the standard patterns of the wild-type fly except for those of the acid phosphatases of the dp mutant. (Fig. 6). No lactate dehydrogenase activity could be detected in any of the homogenates tested. No homogenates exhibited any "nothing dehydrogenases" activity.

The Effect of Wing Transforming Agents on Enzyme Patterns

In addition to reports of modifications of wing size in Drosophila as a result of changes in temperature, there are two recent papers in which investigators have claimed that wings can be altered by chemical agents. Thus, Tanaka (1968) has stated that feeding vg larvae lactate causes the adults to emerge with enlarged wings. However, in spite of a number of experiments in which larva were reared in media containing sodium lactate (0.5M), Tanaka's results could not be confirmed in this laboratory. That is no wing enlargement of the adults was observed, nor was any typical vg enzyme pattern altered. As expected, lactate had no effect on wild-type controls.

In another paper Ritzki and Ritzki (1967) demonstrated that azauracil would promote the development of normal wings in the mutant, dumpy. To see if this agent might elicit a similar effect in the vg mutant, third instar

larvae were reared on filter paper moistened with a solution of azauracil (1mg/ml) according to the method used by the authors. Larvae were also raised on filter paper moistened with distilled water as a control. Under both conditions the adults emerged with vg wings. Enzymes from adults reared on the azauracil and the controls exhibited the typical alkaline phosphatase pattern and the standard patterns for all other enzymes. In a similar experiment wild-flies were also reared on azauracil. No effect on either wings or enzyme patterns was detected.

Repetition of the experiments performed by Ritzki and Ritzki (1967) did confirm that dumpy wings were enlarged by azauracil. However enzyme patterns (Table II) were not altered by treatment with azauracil or by rearing dumpy larvae at 16°C or 32°C. However, as noted above the acid phosphatases of dumpy are normally different from those of the wild-type fly (Fig. 6).

An additional set of experiments were performed to see if the dumpy allele might affect the expression of the vg mutant. Dumpy flies were crossed with vestigials and the larvae were reared at 16°C, 32°C, and 25°C. The heterozygous adults resulting from such a mating developed typical dumpy wings (Table I). Subsequent analysis of the enzymes of these flies revealed the standard alkaline phosphatase pattern and the dumpy acid phosphatase pattern. The other

enzymes tested all gave the standard patterns. No temperature effects (enzymatic or morphological) could be detected in these experiments.

As shown in Fig 3b, wild-type, vg, and dp larvae all exhibited the same protein patterns. No differences under any condition of temperature were noted in this pattern. These patterns were similar to those reported by Hubby (1965).

Finally, an attempt was made to bring about the enzyme conversion in vitro. Homogenates from vg flies were incubated at 32°C for 5 and 10 minutes in a water bath. Following this incubation the homogenates were assayed electrophoretically for alkaline phosphatase bands. The result of this experiment were inconclusive in that the electrophoretic pattern obtained from such homogenates was a broad, streaked, nonspecific area of staining. No distinct bands could be detected. Protein denaturation or bacterial degradation as a result of the crude method employed probably accounts for the results obtained.

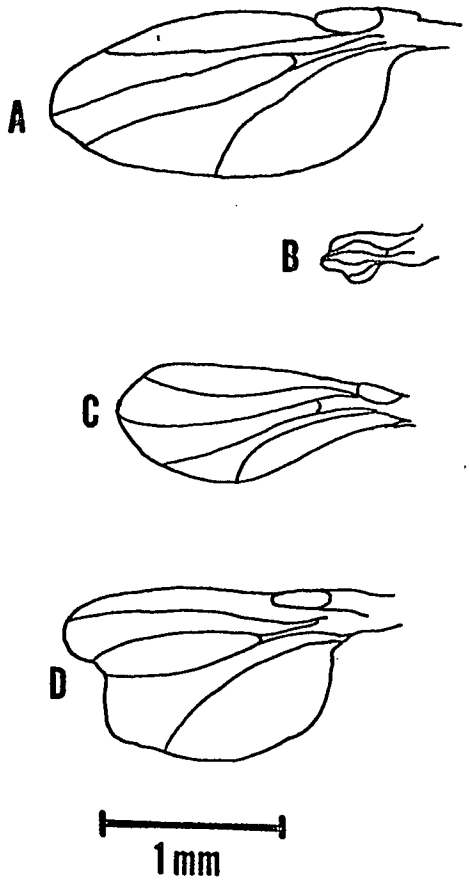

Figure 1. A comparison of the wing morphology of three different mutants of D. melanogaster.
A. wild-type, B. vh, C. vh reared at 32°C,
D. dp.

Table I. Comparison of the wing phenotypes of genotypes reared at 16°C, 25°C, and 32°C. Wild-type=+ vestigial=vg, bestigial-pennant-vg^p, dumpy=dp.

Genotype	Wing phenotype at temperature indicated		
	16°C	25°C	32°C
++	+	+	+
vgvg	vg	vg	enlarged
dpdp	dp	dp	dp
vg^pvg^p	+	+	+
+vg	+	+	+
$+vg^p$	+	+	+
+dp	+	+	+
$vgvg^p$	enlarged	+	enlarged
vgdp	dp	dp	dp
vg^pdp	+	+	+

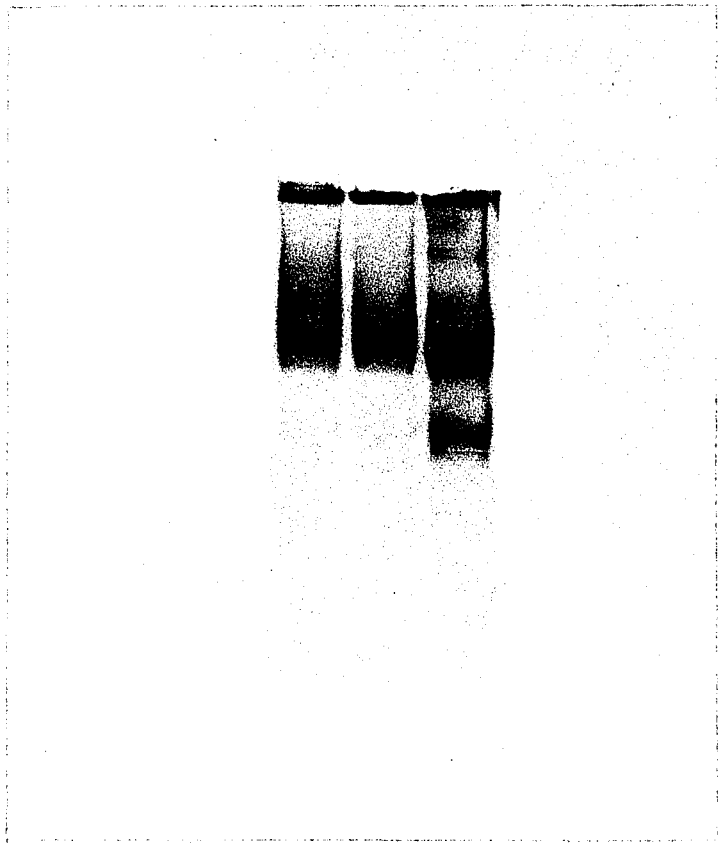

Figure 2. Acrylamide gel showing alkaline phosphatase activity. Left to right: wild-type, vg reared at 24°C, vg reared at 32°C. Migration from top to bottom, anode at bottom.

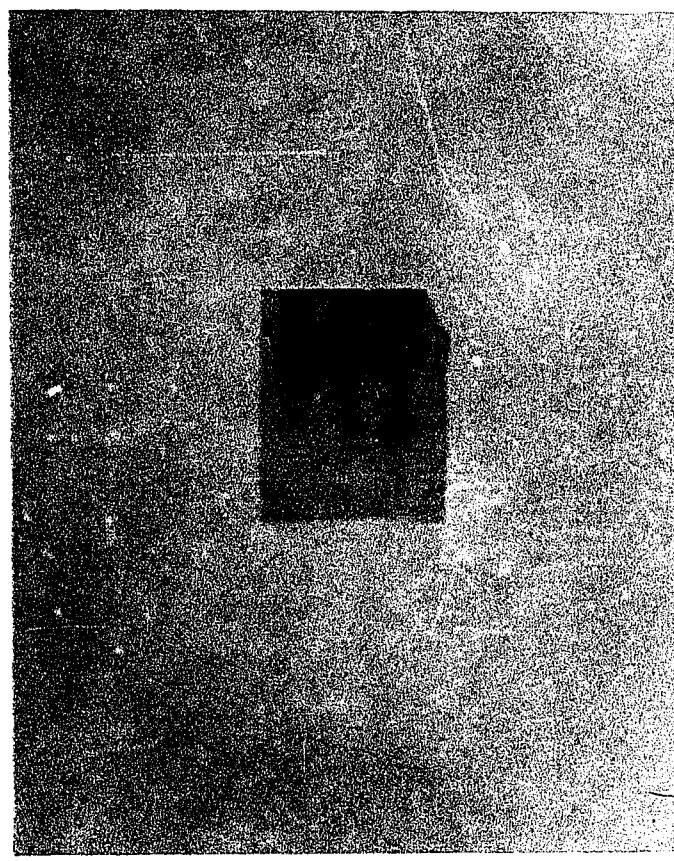

Figure 3. Acrylamide gel from semi-micro electrophoresis showing alkaline phosphatase activity. Left to right: wild-type, vg reared at 32°C, vg reared at 25°C. Anode at bottom.

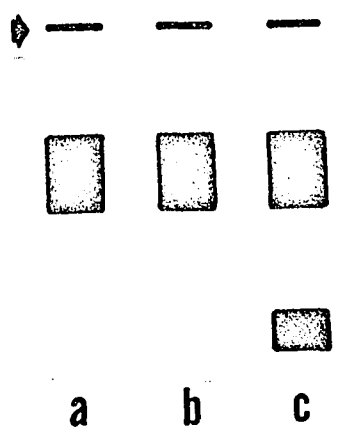

Figure 4. Diagram of acrylamide gel comparing principle bands of alkaline phosphatase activity. a. wild-type, b. vg reared at 32°C, c. vg reared at 25°C. Arrow indicates origin. Anode at bottom.

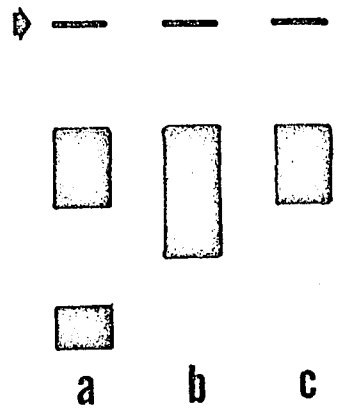

Figure 5. Diagram of alkaline phosphatase pattern of vg flies reared at 32°C and then transferred to 25°C after the following intervals: a. 25 hours, b. 30 hours, c. 35 hours. Anode at bottom.

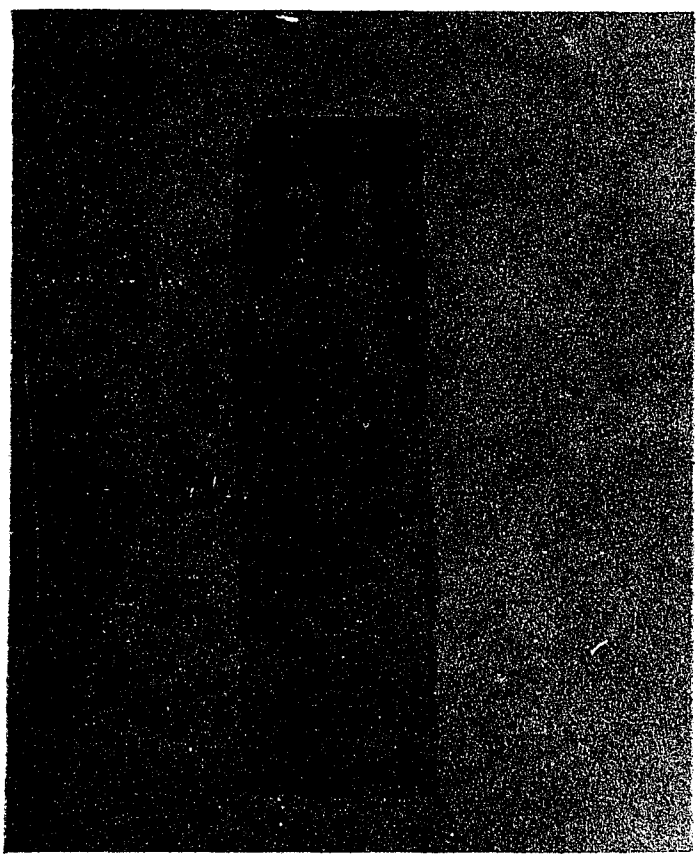

Figure 6. Acrylamide gel from semi-micro electrophoresis showing acid phosphatase activity. Left to right: wild-type, vg, dp. Anode at bottom.

Table II. Comparison of enzyme bands in 11 genotypes of *Drosophila melanogaster*.

Genotype	Alp	AP	Est.	MDH	GPDH
++	std	std	std	std	std
vgvg	vg(1)	std	std	std	std
$vg^{p}vg^{p}$(2)	std	std	std	std	std
dpdp(3)	std	dp	std	std	std
+vg	std	std	std	std	std
$+vg^{p}$	std	std	std	std	std
+dp	std	std	std	std	std
$vgvg^{p}$(2)	std	std	std	std	std
vgdp	std	std	std	std	std
vg^{p}dp	std	std	std	std	std
bb(4)	std	std	std	std	std

1. Standard band obtained when flies are reared at 32°C
2. Standard bands obtained when flies are reared at 16°C, 25°C, or 32°C.
3. Patterns do not differ when flies are reared at 16°C, 25°C, or 32°C.
4. Bar-eye

ALP=alkaline phosphatase, AP=acid phosphatase,

Est.=esterase, MDH-malate dehydrogenase,

GPDH= -glycerophosphate dehydrogenase

Table III. A comparison of alkaline and acid phosphatase activity in homogenates of Drosophila. Values from average of five experiments.

	O.D change/min	ugP/mg/hr
Alkaline phosphatase	1.40	120
Acid phosphatase	0.60	36

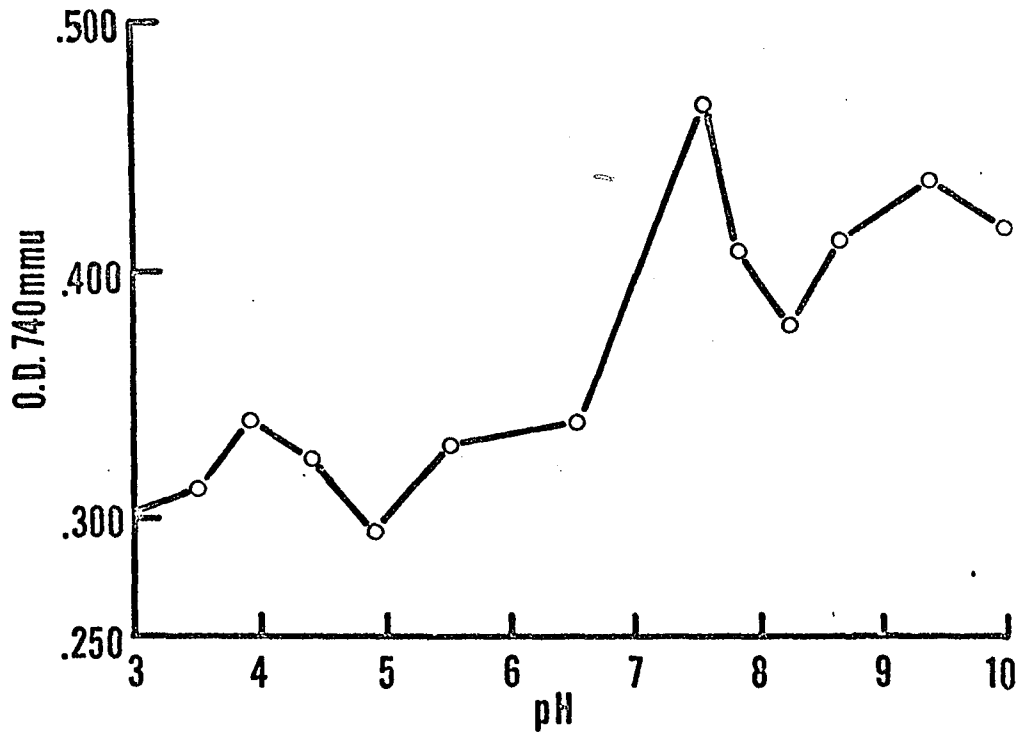

Figure 7. pH activity curve of phosphatase activity from homogenates of Drosophila. Values computed from average of five experiments.

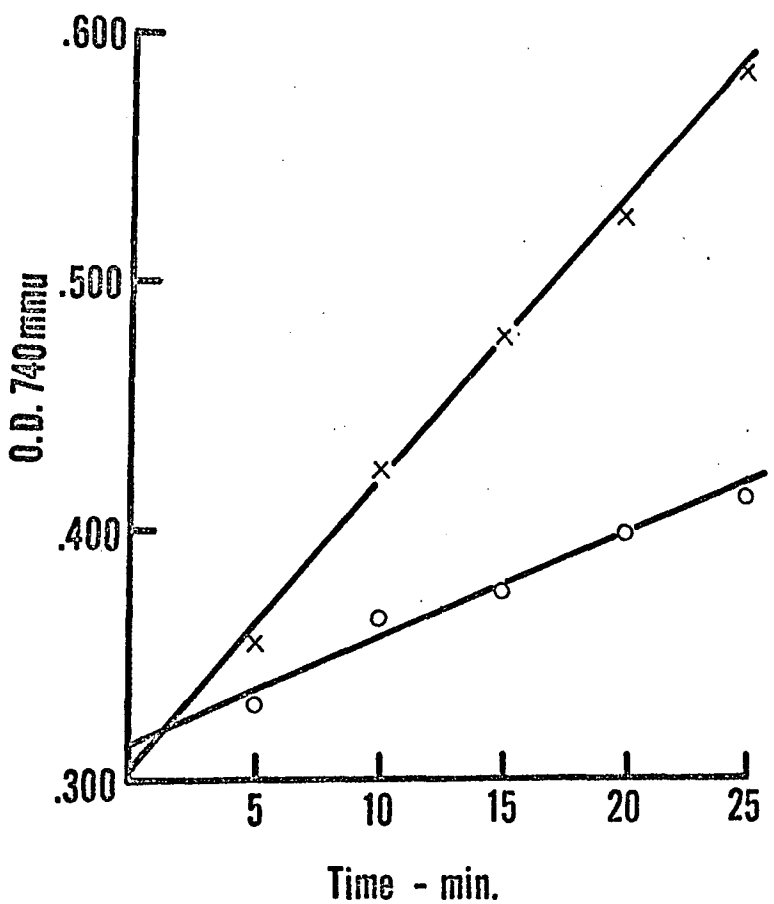

Figure 8. Rate of substrate hydrolysis by (X) alkaline phosphatase and (O) acid phosphatase. Both curves begin at the background level of inorganic phosphate.

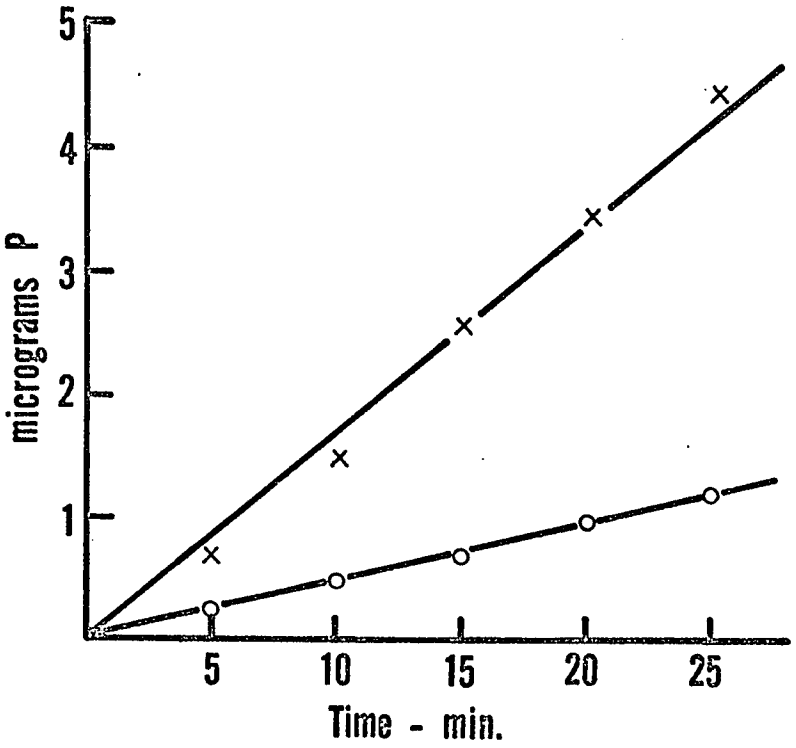

Figure 9. Plot of micrograms of inorganic phosphorus released vs. time. (X) alkaline phosphatase, (O) acid phosphatase.

Figure 10. Longitudinal section of wild-type *Drosophila* abdomen showing alkaline phosphatase activity, using the cobalt method, in the adult hind gut. Note activity in malpighian tubules (upper left). 200X

Figure 11. Demonstration of acid phosphatase activity in a cross section (right) and a longitudinal (left) of the hind gut, using the azo dye technique. 660X

Figure 12. Cross section of wild-type adult malpighian tubules showing alkaline phosphatase activity, using the azo dye technique. 620X

Figure 13. Cross section of vg adult malpighian tubules showing alkaline phosphatase activity in nuclei, made possible by a short (5 minute) incubation time, using the cobalt method. 620X

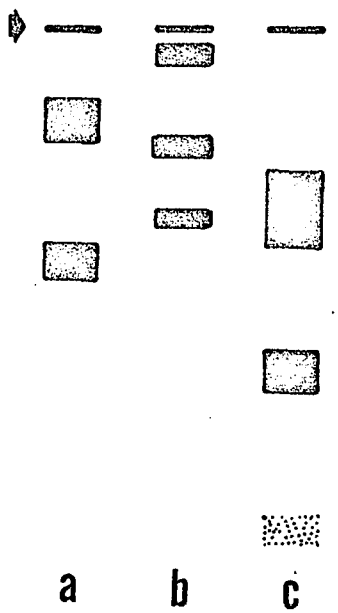

Figure 14. Composite diagram of acrylamide gels stained for various patterns of enzyme activity in wild-type flies. a. malate dehydrogenase, b. -glycerophosphate dehydrogenase, c. esterase.

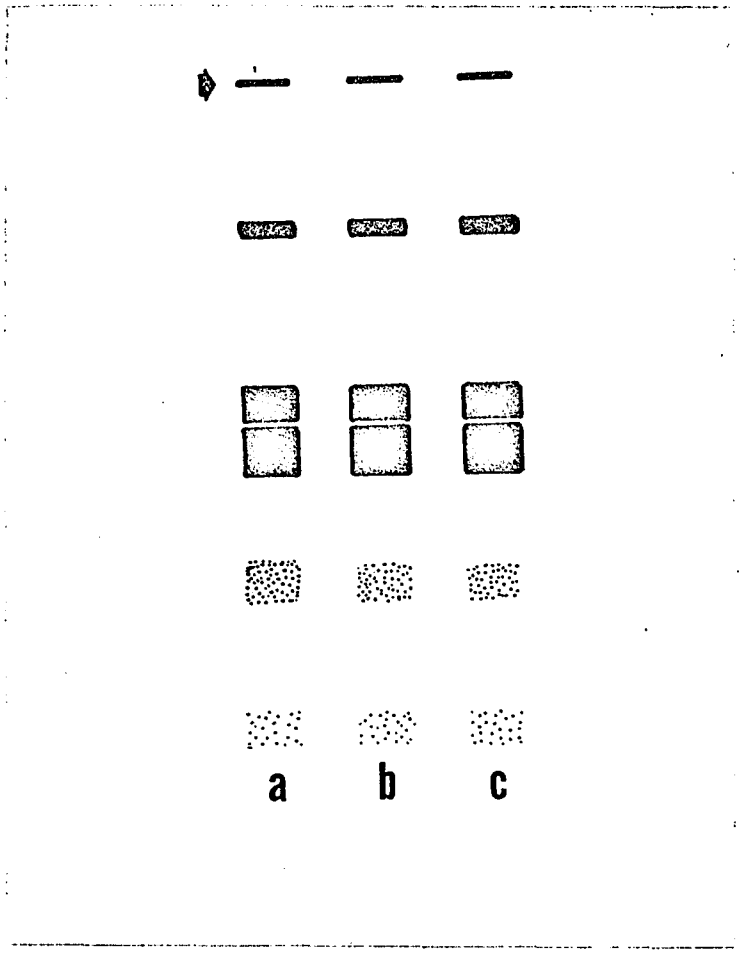

Figure 15. Diagram of acrylamide gel stained for larval proteins. a. wild-type, b. vg, c. dp.

CHAPTER 4

DISCUSSION

The existence of variations in electrophoretic mobility of alkaline phosphatases in Drosophila are not without precedent. Indeed, recent work by Schneiderman, Young, and Childs (1966) has shown the existence of a mutant which affects the mobility of the characteristic single band alkaline phosphatase in wild-type Drosophila melanogaster. To date, the two electrophoretic varieties of alkaline phosphatase of vestigial mutants has not been reported. The normal two-band configuration of the vg mutants might be explained by analogy to the sub-unit hypothesis advanced for lactate dehydrogenase (Markert, 1962). It is admitted that such a comparison may be risky for two reasons: First, it is conceded that the alkaline phosphatases represent a class of enzymes specific for a variety of substrates whereas lactate dehydrogenase is a single enzyme with narrow specificity. Second, unlike LDH, the alkaline phosphatases have not yet been prepared in a crystallized, purified form. Even the purest form of alkaline phosphatase is associated with microsomal particles or other subcellular material (Stadtman, 1961).

Such contamination precludes accurate physical-chemical characterization. In spite of these difficulties and concommittant dangers, comparison of alkaline phosphatases with LDH is useful in establishing a theoretical framework in which to postulate the mechanism for the two banded alkaline phosphatase pattern observed in the vg mutant and its alteration by elevated temperature.

According to Markert and Ursprung (1962), LDH is a tetramer composed of two subunits, A and B, derived from two different gene loci. The subunits represent two distinct polypeptides. Random association of these two subunits leads to the production of five distinct enzyme species (AAAA, AAAB, AABB, ABBB, BBBB) each of which has its own unique electrophoretic mobility. In fact the five different forms are widely distributed in many different animals (Shaw 1965) though within any individual, certain tissues are specified for one particular configuration. Thus certain tissues have the ability to produce just one tetramer or isozyme compared to the 5 possible types that would result from a simple, random association of subunits (Markert and Ursprung 1962).

It is theorized (Stadtman 1961) that some alkaline phosphatases may be composed of two subunits, thus being dimers. If this were indeed the case in Drosophila, it is obvious that three configurations are possible (AA, AB, BB) if random association takes place. Since only one band of

activity is normally present in wild-type Drosophila this would indicate that only one configuration, for example AB, was produced. It is conceivable that in the vg mutant, a mutation in one of the loci producing a subunit, allows the production of a subunit which will couple not only with the other subunit giving AB, but also with itself to yield two distinct dimers -- AB and BB or AB and AA. Such a condition might give rise to the two electrophoretically distinct isozymes as observed in vg reared under normal conditions.

The effect of temperature on the conversion of the two-banded pattern to the single band in vg mutants might be explained in the following manner. At 25°C, during the production of A and B subunits, formation of both AB and BB isozymes is kinetically and thermodynamically possible. However, at 32°C only one form, AB is thermodynamically stable and hence only one isozyme band is evident. The fact that the conversion from AB and BB to all AB takes place at a specific period during the larval development might be explained on the basis of the isozymes being in a different steric configuration or in an unbound state thus allowing spontaneous disassembly of the BB form. The broad band observed at 32°C after 30 hours of incubation might represent a continuum of successive steps in this unbinding, disassembly and reassembling process.

An alternative explanation, gene duplication, might account for the two-band alkaline phosphatase pattern observed in vg flies reared at 25°C. Duplication of a region of a chromosome would result in a doubling of a gene or several genes. If subsequent to this duplication, a mutation occurs in one of the duplicated segments, the product of one gene will differ from that of the other duplicate. It is possible that such a condition might occur in the vg flies. In this case the alkaline phosphatase region might have duplicated and one of the cistrons mutated thus giving rise to two alkaline phosphatases distinct in their electrophoretic mobility. Under normal conditions both of these phosphatases are thermodynamically stable insofar as their steriochemical conformation is concerned. At elevated temperature, only one conformation, i.e. the wild-type or standard, is stable and thus conversion to this conformation takes place.

Finally it is possible that the alkaline phosphatase pattern is a result of two, separate, unrelated genes. One of these genes produces an enzyme which only incidently possesses alkaline phosphatase activity. At elevated temperature this enzyme is inhibited, bound to some other substrate, or otherwise not active as an alkaline phosphatase.

The first hypothesis is favored over the others because an intermediate band is formed during conversion;

heterozygous vg flies have only one band thus reducing the possibility of a duplication which would be expected to manifest itself in the heterozygote; and finally because a subunit configuration has been advanced for alkaline phosphatases (Stadtman, 1961).

Because the enzyme conversion exhibited a critical period during larval development and conversion could not be brought about in adult flies, it is assumed that alkaline phosphatase produced in the larval is carried over into the adult. Further, it is assumed on the basis of the current work, that de novo synthesis of alkaline phosphatase in the adult either does not take place or is produced at such a low rate as not to be detected with the methods used.

The fact that all of the other mutants tested had the standard patterns of alkaline phosphatase, malate dehydrogenases, -glycerophosphate dehydrogenases and esterases means that these mutants all possessed the same genetic make-up insofar as the enzymes tested are concerned. The dumpy mutants (dp) exhibited a different pattern (Fig. 6) of acid phosphatases. Subsequent crosses with wild-type (Table II) yielded heterozygotes which had the standard pattern. This indicated that dp, in addition to mutant wings, also has a mutant acid phosphatase which on the basis of the above test is a simple recessive. Such phosphatase mutants of Drosophila are not uncommon andthe number of such

mutants reported increases each year as more flies are tested electrophoretically (Schneiderman 1966; Wallis and Fox 1968).

The esterase pattern found in the Drosophila examined in this study differed from the patterns described by Kambysellis, Johnson and Richardson (1969) for D. aldrichi and D. mulleri. These two species had two principal esterase bands compared to the 3 bands for D. melanogaster detected in this study. The most extensive study of D. melanogaster esterases is that of Wright (1963). Results of Wright's work are not directly comparable because he used starch gel electrophoresis thus giving an over-all esterase profile somewhat different from that obtained with the polyacrylamide technique.

The enzyme -glycerophosphate dehydrogenase has been shown to be of great importance in the metabolism of flight muscle of a variety of insects (Brosemer et al, 1967; Balboni, 1967). Isozymes of these enzymes have been found in several species of Drosophila (Hubby and Lewontin (1966). According to Grell (1967) there are several allelic forms of α-glycerophosphate dehydrogenase in D. melanogaster. While no such allelism was noted in the present work, it is likely that this can be accounted for on the basis of the different strain of D. melanogaster used by Grell. In his work, Grell examined the Swedish and Oregon RC strains while in the present work Oregon R was not used.

Malate dehydrogenase has been shown to exist as isozymes in Drosophila (Hubby and Lewontin, 1966). In general the patterns obtained for the enzyme in the D. melanogaster mutants used in this study were similar to those obtained by Hubby and Lewontin. In all probability the same genetic systems are at work in the two cases.

As stated previously, the level of lactate dehydrogenase was so low as to be undetectable. This is in line with the findings of other authors (Knowles and Fristrom, 1966). Very faint activity was found in the larval integuments of D. melanogaster by Knowles and Fristrom (1966) but this was only after gram quantities of larval tissue had been extracted. As shown by Sacktor (1965) lactate dehydrogenase activity is in general low in most insects.

The values for the pH optima of the alkaline and acid phosphatases agree roughly with those reported by Stadtman (1961), with the exception that the Drosophila homogenates exhibited a peak of activity at pH 7.4. Since this peak of activity is on the alkaline side of neutrality, by definition, it represents an alkaline phosphatase (Stadtman, 1961). While this might represent a distinct enzyme species, it was not possible to localize its activity electrophoretically. This suggests that either the enzyme had the same electrophoretic mobility as the other alkaline phosphatases, or that this is not a separate enzyme and represents just another pH optima of the existing alkaline

phosphatases. As individual enzymes generally do not exhibit more than on pH optima (Mahler and Cordes, 1966), the first explanation is probably the most likely one.

Comparison of the activity of the alkaline and acid phosphatases showed that there were no differences between enzymes from any of the mutants tested. This is not surprising in view of the fact that, with the exception of the vg mutant, all mutants exhibit the standard pattern. However, while vg flies raised at 25°C have a different alkaline phosphatase pattern, no difference in over-all activity was noted. Apparently the two isozymes of the vg alkaline phosphatase differ in their electrophoretic mobility, but this does not affect the overall catalytic ability of the enzyme system. This would seem to indicate that the mutation giving rise to the two bands in the vg mutants affects only electrophoretic mobility and not catalytic properties--at least not as measured in this work.

The most extensive report of the distribution of phosphatase enzymes in insects in that of Day (1949). Working with several members of the Locustidae and Blattidae be determined the sites of activity of the acid and alkaline phosphatases in various tissues of these insects. Day's results showed that alkaline phosphatase activity is abundant in the cells of the hind gut. Activity of this enzyme was also noted in the malpighian tubules of all the insects that he studied. The association of alkaline phosphatase

enzyme with absorptive tissue, especially that concerned with water balance, occurs widely in many animals--including mammals (Pearse, 1961). Thus its occurrence in the malpighian tubules of Drosophila is not surprising, as these structures are concerned with the reabsorption of water.

The findings of the present study agree in general with those of Day (1949), with one possible exception. Day found acid phosphatase activity in the mid gut of locusts and cockroaches. No such localization was evidenced in Drosophila. Acid phosphatase activity, like the alkaline phosphatases appears to be confined to the hind gut. It is quite possible that this difference may be related to the systemic differences between the locusts and cockroaches belonging to the order Orthoptera, and Drosophila, belonging to the order Diptera.

The failure of the present worker to induce the wing enlargement by lactate in vg mutants as reported by Tanaka (1967) might be explained in several ways. Tanaka may have had a strain of vg mutant different from those used in the present study and thus the lactate effect might be strain specific. On the other hand, non-specific effects, not mentioned by Tanaka might have played a role in the lactate conversion phenomenon. It is also possible that lactate could serve as a substrate for some micro-organism which when ingested by the larvae might induce wing enlargement.

As lactate caused no detectable increase in lactate dehydrogenase activity as measured by electrophoresis, there is an indication that if lactate is serving some biochemical function, it is probably being metabolized by a pathway other than one mediated by lactate dehydrogenase.

While the results of Ritzki and Ritzki (1965), in which wild-type wings are induced in dp flies by azauracil, has been confirmed, no effect of this agent on vg mutants could be detected. There is evidence (Mahler and Cordes, 1966) that some pyrimidine analogs, such as azauracil, may be incorporated into RNA. This process in a few cases leads to altered enzymes. The activity of enzymes so altered is usually reduced (Mahler and Cordes, 1966). Azauracil did not affect the activity or isozymic patterns of any of the enzymes tested from vg or dp mutants. Perhaps enzymes other than those tested might reveal alterations or, and more likely, azauracil operates at some other level in the dp wing conversion. That azauracil and not temperature affects the dp wings and temperature and not azauracil affects the vg wings, strongly suggests that the mechanisms involved in the two systems are not the same.

The fact that the vg mutant has temperature sensitive alkaline phosphatase patterns in addition to a temperature sensitive morphological feature, strongly hints at a relationship between the two phenomena. This idea is strengthened by the observation that the alkaline phosphatase pattern is

altered prior to the wing conversion critical period. It could be argued that if the alkaline phosphatases were involved directly in wing formation and that only the standard pattern represented the active form (as far as wing formation is concerned) then conversion to the standard or active form should precede the time when wing development begins. Three important pieces of data argue against this hypothesis. First, there is no significant difference in the pH optima or catalytic activity of the alkaline phospatases in either wild-type or vg mutants, or between vg mutants reared at 32°C compared with those reared at 25°C. Second, alkaline phosphatase activity has not been found to be localized in the wing imaginal discs or in the thorax where one would expect it to be if it was involved directly with wing formation. This is not to say that some product of alkaline phosphatase activity could not be transported from its locale in the gut or malpighian tubules to the site of wing development. However, if alkaline phosphatase were directly involved with wing formation one might expect it to be found more intimately associated with those structures concerned with wing development. Finally, the genetic crosses between vestigial (vg) and vestigial pennant (vg^p) give rise to temperature sensitive $vgvg^p$ heterozygotes which have the standard pattern of alkaline phosphatase. It will be recalled that $vgvg^p$ heterozygotes exhibit wing enlargement

when reared at either 16°C or 32°C. (Table I). If indeed the alkaline phophatase were involved directly with wing formation, then iso-enzyme conversion should be noted at both temperatures. Since only the standard pattern emerges from the $vgvg^{p}$ heterozygotes, it is suggested that the vg double banded pattern is a result of a mutation giving rise to a temperature sensitive set of isozymes which do not play any direct role in wing formation of the wing conversion in vg mutants raised at 32°C. That this mutation is recessive has been shown by the fact that the two banded alkaline phosphatase pattern manifests itself only in homozygous flies.

CHAPTER 5

SUMMARY

By using electrophoresis to separate enzyme proteins, it has been shown that the vestigial wing mutants of Drosophila melanogaster possess two bands of alkaline phosphatase activity. The conversion of the vg alkaline phosphatase pattern to the single band, wild-type pattern may be brought about by rearing the vg larva at 32°C. The conversion has been found to take place about 30 hours after hatching of the larvae, and precedes conversion of the vestigial wings to wild-type wings by 35 hours. Three hypothesis for the enzyme conversion are presented and discussed.

In contrast to changes in alkaline phosphatases, there are no modifications of acid phosphatases in the mutant and they are the same as those of the wild-type under all conditions. Furthermore, histochemical studies and enzyme activity analyses indicate that alkaline and acid phosphatases have the same distribution and activity in vg and wild-type flies. Alkaline and acid phosphatases have been shown to be limited to the hind gut of both larvae and adult Drosophila, although alkaline phosphatase activity has been demonstrated to occur also in the malpighian tubules.

Azauracil and sodium lactate, agents which have been shown by other workers to affect wing morphology, were ineffective in altering either the wing or electrophoretic patterns of enzymes of vg flies.

Experimental genetic crosses with other wing mutants strongly suggest that the vg alkaline phosphatase pattern and its thermal convertability is the result of a separate gene not related to the temperature-sensitive wing conversion phenomenon of vg flies.

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