THE UNIVERSITY OF CALGARY

# XANTHINE DEHYDROGENASE IN *Drosophila melanogaster*: ROLE OF THE *cinnamon*$^+$ GENE AND THE TIME OF ACTIVATION OF THE *rosy*$^+$ GENE IN DEVELOPMENT

by

JOSEPH CHRISTOPHER ROBINSON

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements for the

Degree of Master of Science

Department of Biology

Calgary, Alberta

May, 1977

THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled XANTHINE DEHYDROGENASE IN *Drosophila melanogaster*: ROLE OF THE *cinnamon*$^{+}$ GENE AND THE TIME OF ACTIVATION OF THE *rosy*$^{+}$ GENE IN DEVELOPMENT submitted by JOSEPH CHRISTOPHER ROBINSON in partial fulfillment of the requirements for the degree of Master of Science.

Leon Browder

L. W. Browder, Supervisor
Department of Biology

John H. Williamson

J. H. Williamson
Department of Biology

Gustav A. Schultz

G. A. Schultz
Division of Medical Biochemistry

Date 6 May, 1977

## ABSTRACT

Antiserum to xanthine dehydrogenase (XDH) was prepared and used to determine the XDH cross-reacting material (XDH-CRM) content of *Drosophila* extracts. The *cinnamon* (*cin*) mutation does not affect synthesis of XDH protein as extracts of three *cin* alleles were found to contain wild-type amounts of XDH-CRM. This indicates that the $cin^+$ gene is involved in activation of XDH protein. An attempt was made to characterize the role of the $cin^+$ factor by demonstrating *in vitro* complementation between extracts of *cin*, *rosy* and *maroon-like* flies. The results were equivocal. XDH-CRM synthesis, directed by the embryonic genome, was detected in gastrulating embryos. This result indicates that the XDH structural gene ($rosy^+$) becomes active at or before this stage in development.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Dr. Leon Browder for his patience and help throughout the course of this research. I am grateful to Dr. John Williamson and Dr. Gil Schultz for their technical advice and to my fellow students for their support compos mentis. The National Research Council of Canada and the Department of Biology are acknowledged for their support.

TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# INTRODUCTION

In order to understand how organismic development proceeds through cellular differentiation, one requires knowledge of the mechanisms of genetic control of protein synthesis. Analysis of the procaryote operon was accomplished by investigating mutants which functionally affect certain enzymes (Monod and Jacob, 1961). It is presumptuous to assume that homologies exist between the mechanisms of genetic regulation in procaryotes and eucaryotes, but such an approach could be suitable for elucidation of these mechanisms in higher organisms. Any model concerning eucaryotic gene control must possess features that take into consideration the unique aspects of the eucaryotic genome such as lack of clustering of functionally related genes, the presence of chromosomal proteins and the existence of repetitive and non-repetitive nucleotide sequences (Lewin, 1974).

Various mutations that affect the appearance or activity of specific enzymes can be identified. If the manner in which a particular mutation causes its effect can be determined, then possible functional roles for that gene may be suggested. Following the model proposed by Britten and Davidson (1969; also, Davidson and Britten, 1973), several classes of mutants are theoretically possible. Within a particular gene-enzyme system, the structural gene would be the nucleotide sequence coding for a

polypeptide which is an integral part of the enzyme. Mutations at such a locus could be characterized by an absence of the enzyme, or a modification of the enzyme in terms of kinetic or physicochemical properties (Courtright, 1976). According to the Britten-Davidson model, transcription of structural genes is directly regulated by contiguous regions composed of moderately repetitive nucleotide sequences. These regulatory genes are thought to serve as receptor sites for cellular factors that coordinate transcription by binding to the regulatory sites, thus activating the adjacent structural genes. Aberrations at these loci (regulator gene mutations) could affect the quantity of enzyme protein produced rather than its quality in terms of kinetic or physicochemical properties (Courtright, 1976). There is evidence for another class of gene whose products are involved in the post-translational activation of the products of structural genes. Mutations at an activator gene could be characterized as causing a change in the activity of an enzyme but not affecting the quantity of enzyme protein produced (Courtright, 1976).

The wealth of genetic information available for Drosophila melanogaster has contributed to the partial characterization of several gene-enzyme systems (Dickinson and Sullivan, 1975; O'Brien, 1975; Courtright, 1976). The extensively described xanthine dehydrogenase (XDH) system shows considerable promise as an example for study. Possible representatives of all three types of mutations have been

found affecting XDH.

The first description of the XDH gene-enzyme system came when the absence of the eye pigment isoxanthopterin (IXP) in certain eye colour mutants was linked to the absence of XDH-catalysed conversion of 2-amino-4-hydroxypteridine (AHP) to IXP (Forrest *et al.*, 1956). Several unlinked loci are now known to affect XDH activity: *rosy* (*ry*), located at 3-52.0, *maroonlike* (*mal*) at 1-64.8, *cinnamon* (*cin*) at 1-0.0 and *low xanthine dehydrogenase* (*lxd*) at 3-33 (Schalet, 1963; Baker, 1973; Keller and Glassman, 1964b).

Since null and electrophoretic mutants for XDH map at the *ry* locus, which also demonstrates dosage dependent expression, it is believed to be the XDH structural gene (Yen and Glassman, 1965; Grell, 1962). Homogenates of $ry^2$ flies contain no XDH activity or cross-reacting material (CRM) to anti-XDH antiserum (Glassman and Mitchell, 1959). The absence of material which will bind anti-XDH antibodies in this null mutant indicates that the mutation prevents production of normal XDH protein. The *ry* locus has been the object of intensive genetic analysis using the numerous *ry* alleles which have been identified (Chovnick *et al.*, 1964, 1971; Gelbart *et al.*, 1974; McCarron *et al.*, 1974). The limits of the $ry^+$ structural element are now known and all null-XDH mutants fall within this region. Approximations of the length of the structural element using the information provided by genetic analysis indicate that the

*ry*$^+$ locus is a single cistron coding for a single XDH subunit (Gelbart *et al.*, 1976). Purified XDH of molecular weight approximately 300,000 can be dissociated into two electrophoretically identical subunits of about 140,000 daltons (Seybold, 1974). Recently, a *ry* variant has been mapped just outside the structural region of the locus (Chovnick *et al.*, 1976). This mutant gene is apparently responsible for an increase in transcription and/or translation of the structural element when located adjacent to it. Such evidence indicates that this may be a regulatory site since it affects the quantity and not quality of the structural gene product.

Expression of the *mal*$^+$ locus is necessary for the activity of XDH (Forrest *et al.*, 1961). The *mal*$^+$ gene product has not been identified. However, it affects XDH activity rather than synthesis since extracts of the mutant *mal* contain wild-type amounts of XDH-CRM (Glassman and Mitchell, 1959). The presence of *mal* prevents the activity of several other enzymes including aldehyde oxidase (AO) and pyridoxal oxidase (PO) (Courtright, 1967; Dickinson, 1970; Glassman *et al.*, 1964). For XDH and AO activities there are no dosage effects since *mal* heterozygotes have wild-type levels of these enzymes (Grell, 1962). The apparent activator role of the *mal*$^+$ factor can be illustrated by *in vitro* complementation of *ry* and *mal* extracts. Mixing and incubation of the two produce small amounts of enzymatically active XDH; *mal* flies contain the *ry*$^+$ gene

product and ry flies contain active $mal^+$ product (Glassman, 1962). The large size of the $mal^+$ complementing factor (approximately 250,000 daltons) precludes the possibility that it is an integral part of the active XDH molecule (Glassman et al., 1966). Activation by the $mal^+$ factor does not involve any appreciable change in the molecular weight of the XDH protein or incorporation of the FAD, molybdenum or iron cofactors associated with XDH (Andres, 1976). This mutant is also subject to a maternal effect, since mal progeny from heterozygous mothers possess low levels of XDH activity in larval and pupal stages and have wild-type eye colour as adults (Glassman and McLean, 1962; Browder and Williamson, 1976). This maternal effect or rescue from the mutant phenotype occurs at least in part because of the $mal^+$ factor deposited in the unfertilized oocyte by the +/mal mother (Sayles et al., 1973).

The cin mutant bears many similarities to mal. Flies that are cin lack XDH, AO and PO activities, and maternally affected flies have wild-type eye colour (Baker, 1973; Browder and Williamson, 1976). Enzymatically active XDH can be detected from early larval stages through to newly eclosed adults in maternally affected cin. The duration of this maternal effect as well as the level of activity found at any particular stage is much greater than in maternally affected mal, raising the possibility that the maternally derived $cin^+$ factor is causing synthesis of enzymatically active XDH to occur in these cin progeny (Browder and

Williamson, 1976). Another dissimilarity with mal is that the majority of cin progeny from maternally affected cin mothers die during embryogenesis (Baker, 1973). Absence of XDH, AO and PO activities have not been seen to be lethal in other instances. Whether the $cin^+$ gene is regulatory in that it affects synthesis of XDH, AO and PO or is an activator gene providing for the activity of these enzymes is yet to be established. One must first determine if the presence of cin causes a change in the synthesis of these enzymes.

The gene product of the $lxd^+$ locus also has not been identified, but flies homozygous for lxd have reduced levels of XDH, AO and PO activities (Keller and Glassman, 1964b: Courtright, 1967; Glassman et al., 1964). There is no lxd dosage effect on XDH or AO activity (Keller and Glassman, 1964b; Browder and Williamson, 1976). The XDH found is normal with respect to kinetics as is the size and electrophoretic mobility of the AO (Yen and Glassman, 1967; Courtright, 1967). Flies that are lxd contain wild-type amounts of XDH-CRM (Glassman, 1965). The $lxd^+$ gene is apparently involved in activation of the XDH molecule since lxd flies are normal with respect to quantity and quality of XDH; only the number of active molecules seems to be in variance. Control by this locus is probably exerted via the $mal^+$ and $cin^+$ factors since lxd flies will not complement in vitro with ry flies and thus apparently lack the $mal^+$ factor. In

addition, cin and mal flies from $cin^+/cin$ and $mal^+/mal$ mothers which are lxd have mutant eye colour. The presence of the lxd gene appears to prevent transmission of $cin^+$ or $mal^+$ factors from the mother to the mutant progeny thus blocking the maternal effects (Glassman et al., 1964; Courtright, 1975).

The preceding information concerning these interrelated gene-enzyme systems has been summarized in Table 1. A working hypothesis derived from this evidence, with respect to XDH, is that the structural polypeptide produced by the $ry^+$ gene is activated by the $mal^+$ factor after translation. This factor, necessary for assembly or activation of AO and PO as well, does not become active or is produced in low quantities unless the $lxd^+$ gene product is present. The $cin^+$ factor may be involved in either regulation of transcription or translation of the $ry^+$ gene or activation of the $ry^+$ gene product.

Mutations affecting XDH synthesis and activity have proven to be useful tools for the study of gene expression during development, particularly in analyzing maternal and embryonic nuclear contributions to development (Sayles et al., 1973; Browder and Williamson, 1976). Initial development in most organisms is at least partially supported by products of oogenesis. After fertilization, the embryonic genome is apparently differentially activated and development occurs with the progressive appearance of new gene products (Davidson, 1968). The maternal effects

TABLE 1

Properties of XDH mutants. The symbols used are: (+) wild-type levels; (N.D.) not determined; (yes) indicates presence with no reference to quantity.

| ENZYMATIC PROPERTY | MUTANT | | | |
|---|---|---|---|---|
| | ry | mal | cin | lxd |
| XDH activity | 0[1] | 0[1] | 0[2] | 20-25%[3] |
| XDH-CRM | 0[4] | +[4] | N.D. | +[5] |
| AO activity | +[6] | 0[6] | 0[2] | 5-10%[6] |
| AO-CRM | +[6] | yes[7] | N.D. | yes[6] |
| PO activity | +[8] | 0[8] | 0[2] | 0[8] |
| PO-CRM | N.D. | N.D. | N.D. | N.D. |

1. Forrest et al., 1961.
2. Browder and Williamson, 1976.
3. Keller and Glassman, 1964b.
4. Glassman and Mitchell, 1959.
5. Glassman, 1965
6. Courtright, 1967.
7. Dickinson, 1970.
8. Glassman et al., 1964.

associated with the *mal* and *cin* mutants of *Drosophila* are excellent examples of the enduring control exerted by maternally derived materials. An examination of the mechanisms of the activation of the embryonic genome may provide insight into the means by which regulatory genes function. Determination of the time at which specific embryonic genes become active could facilitate such a study. Activation of embryonic genes in *Drosophila* is usually found to occur quite late in development. The appearance of an active enzyme in heterozygous progeny of null mutant mothers or an allozyme related to the genotype of the father indicates the time of activation of an embryonic gene. Most ontogenic surveys of *Drosophila* enzymes such as AO do not show detectable activity of paternal origin until early larval stages (Dickinson, 1971). There are some indications that earlier activation of embryonic genes does occur such as the observation of increases in activity of lactate dehydrogenase and $\alpha$-glycerolphosphate dehydrogenase ($\alpha$-GPDH) in *Drosophila* embryos. In addition the production of $\alpha$-GPDH allozymes related to the paternal genotype and not the mother's has been detected in late embryos and early first instar larvae (Rechsteiner, 1970; Wright and Shaw, 1969). By contrast, Sayles *et al.*, (1973) detected initial production of enzymatically active XDH in $ry^{+}/ry^{2}$ progeny of $ry^{2}/ry^{2}$ mothers at the time of gastrulation. The differences in time at which these various activities have been detected may be

due to either different times of activation of individual embryonic genes or different levels of sensitivity in the various experimental approachs.

The experiments presented in this thesis have been designed to help clarify the role of the $\underline{cin}^{+}$ gene in the XDH gene-enzyme system and to expand upon our knowledge of gene expression during development using the ry mutant. In order to determine if the cin mutation affects XDH synthesis, anti-XDH must be used to demonstrate if cin flies contain XDH-CRM. Use of this antiserum also allows one to monitor the activity of the $\underline{ry}^{+}$ gene during development without relying upon the activity of other genes or maternal substances which would be necessary for enzymatically active XDH to be detected. Discovery of the first appearance of XDH-CRM of embryonic origin could help clarify the conflicting data on initial expression of the embryonic genome.

# MATERIALS AND METHODS

## A) *Drosophila melanogaster* Stocks

All cultures were kept at 25±5°C in 120 ml milk bottles containing a standard cornmeal-yeast-sugar medium (Lewis, 1960).

| Genotype | Description |
|---|---|
| 1. Oregon-R (Ore-R) | - a wild-type strain with normal XDH activity. |
| 2. *ry*$^{2}$ | - *rosy* eye colour; an allele of *ry*, totally deficient in XDH activity. |
| 3. *mal* | - *maroon-like* eye colour; totally deficient in XDH activity. |
| 4. *ru;lxd;by* | - *low xanthine dehydrogenase* (*lxd*) accompanied by the third chromosome markers *roughoid* (*ru*) and *blistery* (*by*). |

5. $ry^2$; mal - a double mutant for rosy and maroon-like

6. C(1)DX, y f / y $cin^1$ / Y
   - a reversed acrocentric compound-X chromosome stock; females possess yellow (y) and forked (f) phenotypes; males are yellow and maternally affected cin. This will be referred to as the $cin^1$ stock.

7. C(1)RA, y cin / y cin / $y^+$ $cin^2$ Y
   - a reversed acrocentric compound-X chromosome stock; males and females are heterozygous for yellow and the $cin^1$ and $cin^2$ alleles because of the translocated $y^+$ and $cin^2$ loci on the Y chromosome. This is the $cin^2$ stock.

8. C(1)DX, y f / y $cin^3$ v f / Y
   - in this compound-X chromosome stock, females are yellow and forked while males are yellow, forked, vermillion eyed (v) and maternally affected $cin^3$. This is the $cin^3$ stock.

All chromosomes and mutations are described by Lindsley and Grell (1968) with the exception of cin (Baker, 1973). The $cin^2$ allele occurred spontaneously in a stock culture of C(1)RA, y $cin^1$/ y $cin^1$/ $sc^8$ Y. The $sc^8$ translocation contains the $y^+$ and $cin^+$ loci. The $cin^3$ allele was induced by ethyl methanesulfonate and was originally designated a female sterile (fs(1)M50; Mohler, 1977).

The balanced $cin^1$ and $cin^3$ stocks provide maternally affected cin males since the compound-X chromosomes are inherited from female parent to female progeny while the mutant-X chromosome in males is derived from the father. Flies which are $cin^1$ and $cin^3$ must be maternally affected in order to survive. However, $cin^2$ flies do survive as progeny of $cin^2$ mothers.

B) Embryo Collection Techniques

Precisely timed embryos were obtained by mating aged males with aged virgin females. The parents were allowed to mate for 1.5 hours in 120 ml milk bottles containing standard medium. The small volume of these containers increased the probability that mating would occur. The adults were transferred to covered 5" x 8" trays containing a yeast-agar-grape juice medium (Kriegstein and Hogness, 1974). The trays were kept in the dark at 25°C until a synchronous burst of egg laying was observed 8 - 9 hours later. After discarding the adults, the eggs, which were easily seen on the purple food, were removed with a small camel hair brush

and placed in Drosophila Ringer solution (Ephrussi and Beadle, 1936). The eggs were collected on a glass-fiber filter set in a 15 ml sintered glass funnel, drained, then washed for 2 minutes with 4.6% sodium hypochlorite to remove the chorions. The embryos were washed with Ringer solution, then submerged in a small petri dish containing Ringer solution. The embryos were then incubated at 25°C and observed at intervals with a dissecting microscope and transmitted light. Embryos of various developmental stages were identified according to the descriptions of Bownes (1975), counted and removed by drawing them into a 0.2 ml micropipet. They were allowed to settle in a 1.5 ml Eppendorf microcentrifuge tube and the excess Ringer solution decanted. The embryos were immediately homogenized in the microcentrifuge tube using a motorized teflon pestle in 0.1 M Tris-Cl(pH 8.0).

C) Assays for XDH Activity

1. Fluorimetric Assay:

The procedure used to assay XDH activity is a modification of the technique described by Glassman and Mitchell (1959). An Aminco-Bowman spectrophotofluorimeter (365 nm excitation, 405 nm emission wavelengths) was used to monitor the increase in fluorescence as the substrate AHP is converted to IXP at 30°C. The assay mixture consisted of 0.97 ml extract (suitably diluted), 1.0 ml 0.1 M Tris-Cl(pH 8.0), 0.01 ml B-nicotinamide adenine dinucleotide (NAD; 2mg/ml buffer) and 0.02 ml AHP ($3 \times 10^{-4}$ M in same buffer). The fluorimeter was calibrated such that $1.0 \times 10^{-6}$ quinine sulphate in 0.1 M sulphuric acid solution had a reading of 30 units. Increase in fluoresence was correlated with units ($10^{-6}$ mole/min) of AHP oxidized by using a mixed dilution curve representing decreasing increments of AHP and equivalent increases of IXP. A change of one fluoresence unit represented $0.037 \times 10^{-6}$ moles of AHP oxidized. Protein determinations were made using the method of Lowry et al. (1951) with Bovine serum albumin as a standard.

2. XDH Assay Using Thin Layer Chromatography (TLC)

This procedure, used in XDH-CRM determinations, is a modification of the technique described by Browder and Williamson (1976). The sample to be assayed (0.02 - 0.025 ml) was placed into a Falcon Microtest plate well

containing 0.01 ml of a reaction mixture consisting of either $9 \times 10^{-5}$ M AHP and 0.03% NAD in 0.1 M Tris-Cl(pH 8.0) or an identical solution but lacking AHP. Water soaked tissue paper was used to maintain humidity in the Microtest plates which were incubated at 30°C for 3 hours. The incubation was performed in the dark to prevent any photo-oxidation of the AHP. After incubation, 0.005 ml of 95% ethanol was added to precipitate some of the protein and facilitate spotting of the samples for TLC. The samples were drawn into 0.05 ml micropipets, sealed with Dade mini-seal and centrifuged at 5,000 x g for 10 minutes in a centrifuge equipped with a microhematocrit head. Following centrifugation, the open ends of the micropipets were drawn to a point in a flame and the sealed ends, with any pellet present, were broken off. The contents were spotted through the drawn tips onto Brinkmann Polygram CEL-300 TLC sheets that were developed for 10 cm. in Gelman Instant TLC chambers with 5% acetic acid as solvent. After drying, the sheets were observed and photographed under long wave-length (365 nm) ultraviolet light. The AHP and IXP present in a sample could be seen as a blue ($Rf \cong 0.62$) and a purple ($Rf \cong 0.45$) fluorescent spot respectively. The amount of XDH-catalyyzed conversion of AHP to IXP was judged by the presence and relative intensity of the flour-escent IXP spot. In instances where no AHP was added to the reaction mixture, any detected AHP or IXP is endogenous to the extract used.

D) Preparation of Anti-XDH Serum

In order to detect XDH-CRM, anti-XDH had to be prepared. The procedure outlined by Seybold (1974) was modified and used to purify XDH antigen from wild-type flies. However, since the XDH invariably lost its activity during the preparation, impure XDH was used for immunization of rabbits. The resulting antiserum contained antibodies to a number of Drosophila proteins (see below).

1. Purification of XDH

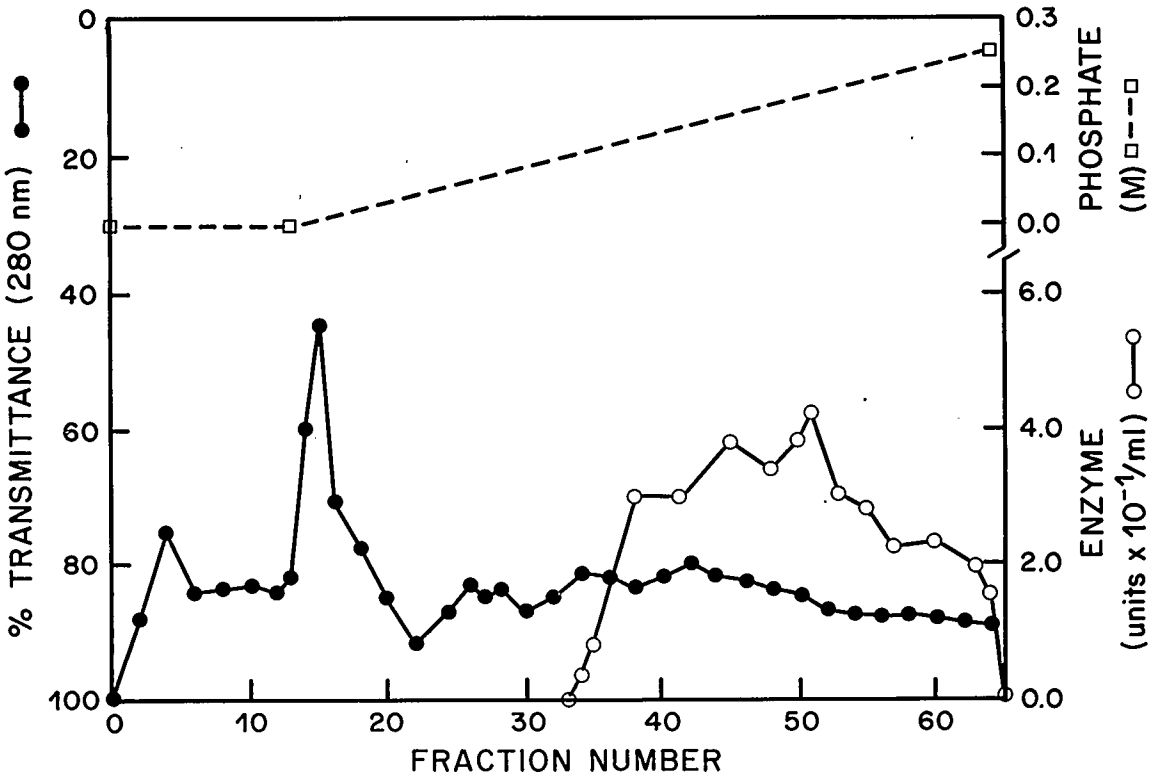
All steps in this procedure, unless otherwise indicated, were carried out at 4°C. Whole, live Ore-R flies in separate batches of 60 g and 45 g were homogenized at full speed in a Waring blendor for two minutes with 7 volumes (w/v) of 0.1 M Tris-Cl(pH 8.0) containing 1mM dithiothreitol for stabilization of the XDH. The homogenates were centrifuged at 15,000 x g for 20 minutes and the supernatants separately processed. The first homogenate was treated for 10 minutes with Norit-A (1g/4g flies) and centrifuged as above. Ammonium sulphate was added to the preparation, and the proteins that precipitated between 40 - 60% saturation were collected by centrifugation as above. The supernatant was then heat treated at 59°C for 5 minutes and quickly cooled in ice water. This temperature was selected since heat treatment performed at 68°C as described by Seybold caused considerable loss of XDH activity. The yeild was increased by employing the lower temperature.

However, the extent of purification obtained at this step was decreased in comparison to Seybold's results. The heat treated extract was centrifuged as above and the supernatant desalted on a Sephadex G-25 column equilibrated to 0.1 M Tris-Cl(pH 8.0). The second extract was treated with Norit-A, centrifuged and combined with the partially purified and desalted first extract. The combined extracts were $(NH_4)_2SO_4$ fractionated, heat treated and desalted as described above. This preparation was run through a DEAE-Sephadex A-25 column equilibrated to 0.1 M Tris-Cl(pH 8.0). In contrast to Seybold's results, XDH did not bind to the ion exchange resin at pH 8.0. Hence, when the sample was run through the column, all the XDH activity was eluted with the exclusion proteins. In comparison to Seybold's results, yield for this step was excellent, but extent of purification was low as actual chromatography of the sample was not performed. The exclusion sample was then concentrated under nitrogen pressure using an Amicon XM-100 membrane and was loaded onto a hydroxyapatite (Bio-Gel HTP) column equilibrated to 1mM sodium phosphate (pH 6.7). The sample was eluted with a 500 ml linear gradient of 1 mM - 0.25 M sodium phosphate (pH 6.7) at 45 ml/hr. The elution profile is presented in Figure 1. Peak fractions for XDH activity were pooled and concentrated as above. In three attempts at this procedure, the XDH quickly lost its activity at this final concentration step. Therefore, chromatography on Sephadex G-200 as prescribed by Seybold was not

FIGURE 1

Hyroxyapatite chromatography of XDH. The closed circles represent percent transmittance (280 nm), the open circles represent enzyme activity and the broken line denotes elution buffer molarity.

1


0
20
40
60
80
100
% TRANSMITTANCE (280 nm)
0.3
0.2
0.1
0.0
PHOSPHATE (M)
6.0
4.0
2.0
0.0
ENZYME (units x $10^{-1}$/ml)
0
10
20
30
40
50
60
FRACTION NUMBER

done and this impure preparation was used to immunize rabbits. Glassman and Mitchell (1959) reported that partially purified XDH was unstable and Seybold described electrophoretically pure XDH as having a half life of only 12 hours. The extent of purification of XDH at the various steps is listed in Table 2. In comparison to Seybold's results, the overall yield was better, but the fold purification poor. This is almost entirely due to the modifications employed at the heat treatment and DEAE chromatography steps, as the results for steps common to those of Seybold were very similar. Ultrafiltration using the XM-100 membrane provided some purification in itself. The relatively large pore size seemed to permit the passage of many protein molecules while retaining the XDH. The specific activity at each stage was much lower than that measured by Seybold. The difference may be partially due to the use of Ore-R flies of indeterminate age whereas Seybold used 3 day old flies of the Sevelen strain. XDH activity declines in older flies (Glassman and Mitchell, 1959) and there is some variation in the amount of XDH activity detectable in different strains (Keller and Glassman, 1964 a).

2. Antibody Stimulation and Preparation of Antiserum

Two Belted Belgian Dutch rabbits were each injected subcutaneously at several sites along the back with 0.75 ml (0.2 mg protein) of the partially purified XDH mixed with equal parts of Freund's complete adjuvant. Immunization

TABLE 2

XDH Purification Data

| Step | Protein (mg) | Activity (units) | Specific Activity (units/mg) | Fold Purification | Yield (%) |
|---|---|---|---|---|---|
| Batch A[1] | 168 | 100 | (0.60) | - | - |
| Batch B[2] | 1056 | 225 | (0.21) | - | - |
| Mixture(A+B) | 1224 | 325 | 0.27 | - | - |
| $(NH_4)_2SO_4$ Fractionation | 382 | 217 | 0.57 | 2.1 | 66.7 |
| Heat Treatment | 246 | 211 | 0.86 | 3.2 | 64.9 |
| G-25;DEAE | 186 | 198 | 1.00 | 3.7 | 60.9 |
| XM-100 Concentration | 99 | 198 | 2.00 | 7.4 | 60.9 |
| Hydroxyapatite | 19 | 99 | 5.20 | 19.3 | 30.5 |
| XM-100 | 4 | - | - | - | - |

1. From 60 g flies; Norit-A, $(NH_4)_2SO_4$, heat, G-25-treated.
2. From 45g flies; Norit-A-treated.

was performed weekly for 7 weeks. The rabbits were bled by cardiac puncture and the blood was allowed to clot for 2 hours at room temperature. After permitting the clots to contract by storing at 4°C, the serum was decanted and centrifuged at 10,000 x g for 20 minutes then stored at -20°C. When thawed for use in subsequent experiments, the antiserum was treated for 10 minutes at 4°C with Norit-A (50mg/ml) then centrifuged as above. The Norit-A removes any inhibitory factors such as purines and pteridines which would affect XDH activity (Glassman and Mitchell, 1959).

E) Characterization of the Antiserum

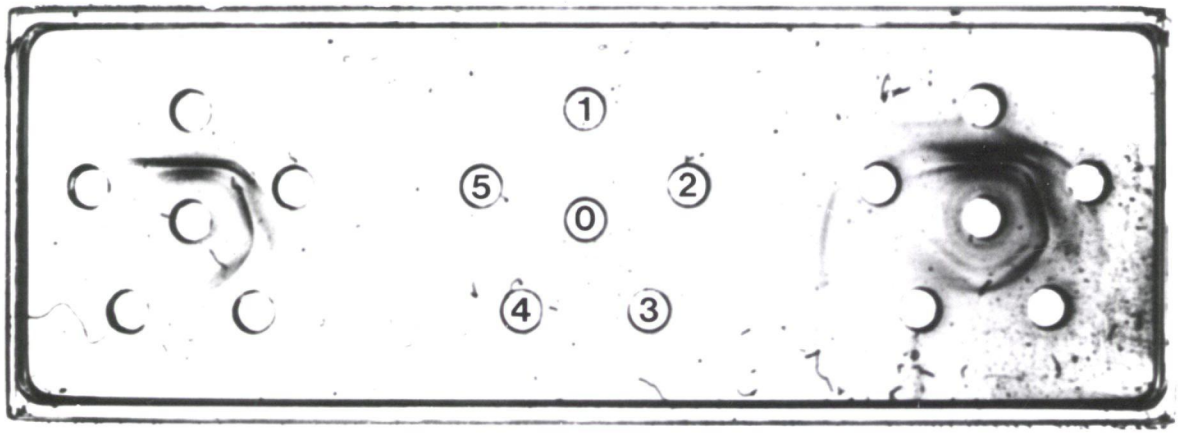
Evidence that the serum contained antibodies to Drosophila proteins was obtained by observing precipitin bands formed in Ouchterlony plates when the antiserum was tested by double-diffusion against various dilutions of the antigen mixture (Figure 2). No precipitin bands were formed when control serum was used.

Determination of the anti-XDH content of the serum was accomplished by an XDH inhibition experiment. The XDH molecules in a Drosophila extract would be effectively removed by immunoprecipitation when the extract is incubated with anti-XDH. Following centrifugation to remove the precipitate, an XDH assay of the supernatant would show decreased XDH activity. The anti-XDH was titrated by performing this experiment with different concentrations of

FIGURE 2

Ouchterlony double-diffusion test. The center wells contain: (A) serum from immunized rabbit #1; (B) control serum from an unimmunized rabbit; (C) serum from immunized rabbit #2. The outer wells contain dilutions of the antigen: (1) undiluted; (2) 1:2, (3) 1:4, (4) 1:10; (5) 1:100. The numbered labels given for (B) can be directly applied to (A) and (C).

2




A B C

antiserum and determining the minimum quantity required to completely inhibit the XDH activity of a given Ore-R extract. Ore-R flies were homogenized in a Ten Broeck tissue grinder with 0.1 M Tris-Cl(pH 8.0) buffer (200 flies/ml). The extract was treated for 10 minutes with Norit-A(0.4mg/fly) and centrifuged at 15,000 x g for 20 minutes. Dilutions of the antiserum (1:27; 1:53; 1:106; 1:213) were made and 0.16 ml of each was mixed with 0.05 ml aliquots (10 fly equivalents) of the Ore-R extract in 1.5 ml Eppendorf micro-centrigue tubes. The mixtures were incubated at $4^{o}C$ for 12 hours, after which they were centrifuged at 30,000 x g for 20 minutes. Aliquots of 0.02 ml were removed from each sample and assayed for XDH activity by the TLC technique.

F) XDH-CRM Determinations

XDH-CRM was assayed by a two-step immunoabsorbtion technique similar to that of Glassman and Mitchell (1959). In this procedure, various Drosophila extracts were incubated with antiserum. If XDH-CRM is present in the test extract, it would complex with and remove the anti-XDH from solution by precipitation. In the second step, the solution is incubated with an Ore-R extract. If no anti-XDH remains in the solution, the Ore-R XDH is unaffected and subsequent TLC assay reveals XDH activity. If, however, XDH-CRM was missing in the test extract, anti-XDH remains in solution and the Ore-R XDH is

precipitated. Therefore, the TLC assay for XDH activity is negative.

Test extracts were prepared by homogenizing organisms in 0.1 M Tris-Cl(pH 8.0), treating with Norit-A and centrifuging at 15,000 x g for 20 minutes. Adults were homogenized in Ten Broeck tissue grinders while larvae and embryos were processed in 1.5 ml microcentrifuge tubes using a motorized teflon pestle. For adults and larvae, 0.4 mg Norit-A/organism was used, while extracts of 250 - 600 embryos each were all treated with 15 mg in order to remove any inhibitory factors. The test extracts were mixed with the minimum amount of antiserum required to completely inhibit the XDH activity of an Ore-R extract. These mixtures were incubated for 12 hours at $4^{o}C$ in sealed microcentrifuge tubes. A small amount of Norit-A ($\cong$ 5mg) was added to the samples, which were immediately centrifuged at 30,000 x g for 20 minutes. The charcoal helped to localize and consolidate the small pellet formed. The supernatants were transferred to fresh microcentrifuge tubes and the predetermined amount of Norit-A-treated Ore-R extract added. Following incubation for 12 more hours at $4^{o}C$, the mixtures were centrifuged as above and samples removed from the supernatant were assayed for XDH activity by the TLC technique. Controls consisted of:

1. Test extract incubated with Norit-A-treated control serum, then with Ore-R extract (uninhibited Ore-R XDH activity).

2. Test extract incubated with buffer (equivalent to volume of serum plus Ore-R) demonstrating the level of XDH activity in the test extract.
3. Buffer (test extract volume) and antiserum incubated with Ore-R extract (complete inhibition of XDH activity for comparison to XDH-CRM determinations using extracts of mal/mal; +/$ry^2$ embryos).

In addition, samples of the XDH-CRM determination mixtures were checked for endogenous AHP or IXP by the TLC technique. The scheme of quantities and concentrations of the components used in the various XDH-CRM determinations is presented in Table 3.

G) In Vitro Complementation

Extracts of $cin^1$ males, $ry^2$ and mal stock adults were made using Ten Broeck tissue grinders in 0.1 M Tris-Cl (pH 8.0). The $cin^1$ males were aged 8 - 15 days to eliminate any maternal effect XDH activity. Aliquots (0.15ml/40 flies) of an extract from one genotype were mixed with an equivalent amount of extract from another genotype. Controls consisted of a single mutant extract mixed with buffer and also double aliquots of the same extract. The mixtures were incubated for 4 hours at 30°C with 100 units of penicillin and $10^{-4}$mg of streptomycin. Following this, the complementation mixtures were treated for 10 minutes with 30 mg Norit-A and centrifuged at 15,000 x g for 20 minutes. From the supernatants, 0.025 ml samples were removed and

TABLE 3

XDH-CRM Determination Scheme

| Test Extract Source | Test Extract (Vol./number flies) | Serum (diluted 1:3) | Ore-R Extract (vol./number flies) | Assay Samples |
|---|---|---|---|---|
| Stock adults | 0.15 ml/20<br>0.15 ml/10[1]<br>0.15 ml/5<br>0.15 ml/2.5 | 0.01 ml | 0.05 ml/10 | 0.02 ml |
| *mal* larvae | 0.15 ml/20<br>0.15 ml/10[1]<br>0.15 ml/5 | 0.01 ml | 0.05 ml/10 | 0.02 ml |
| *cin* larvae from *cin* | 0.1 ml/10<br>0.1 ml/5[1]<br>0.1 ml/2.5 | 0.005 ml | 0.025 ml/5 | 0.025 ml |
| *cin* adults from *cin* | 0.1 ml/5[1]<br>0.1 ml/2.5<br>0.1 ml/1.25 | 0.005 ml | 0.025 ml/5 | 0.025 ml |
| *mal*/*mal*; +/*ry*[2] embryos: | | | | |
| -staged | 0.1 ml/175-200[1] | 0.005 ml | 0.025 ml/5 | 0.025 ml |
| -unstaged | 0.1 ml/500 | | | |

1. Concentration of test extract used in controls.

checked for endogenous AHP or IXP by the TLC technique. The remainder of each solution was added to a reaction mixture consisting of $2 \times 10^{-4}$M AHP, 0.07% NAD and $2 \times 10^{-2}$M dithiothreitol in 0.1M Tris-Cl(pH 8.0). These mixtures were incubated in the dark at 30°C for 36 - 60 hours in sealed microcentrifuge tubes. Samples of 0.025 ml were removed, mixed with 0.02 ml of 95% ethanol and drawn up into 0.05 ml micropipets. These were centrifuged at 5,000 x g for 10 minutes, the tips drawn and the contents chromatographed as previously described. The remainder of each complementation mixture was streaked on brain-heart infusion (BHI) medium and incubated for 48 hours at 37°C as a check for bacterial contamination.

# RESULTS

## A) Anti-XDH Properties of Immune Serum

The presence and relative effectiveness of antibody to XDH in the antiserum was determined by the ability of aliquots of the antiserum to inhibit XDH activity in extracts of Ore-R flies. Fly extracts were incubated with antiserum as described in Materials and Methods and the mixture was centrifuged to remove precipitated antigen-antibody complexes. The supernatants were then assayed for XDH activity by the TLC method. The results of the XDH assays are shown in Figure 3-A. XDH activity was inhibited by the antiserum, and the inhibition was dependent upon antiserum concentration. The XDH activity of a single fly equivalent was just completely inhibited by 0.0003 ml of undiluted antiserum. Controls for this experiment were incubated as described and chromatographed. The control mixtures consisted of:

1. 0.16 ml buffer incubated with Ore-R extract (uninhibited XDH activity); Figure 3-B (Ore-R).
2. 0.16 ml Norit-A-treated control serum (diluted 1:53) with Ore-R (test for non-specific inhibition of XDH); Figure 3-B (CS).
3. 0.16 ml of either antiserum or treated control serum (both diluted 1:53) with 0.05 ml buffer (tested by TLC for endogenous AHP and IXP); Figure 3-B (AS-END; CS-END).

4. 0.08 ml antiserum (1:53) plus 0.08 ml treated control serum (1:53) with Ore-R (tested by TLC for endogenous AHP and IXP); Figure 3-A (END).

The results of these controls are shown in Figure 3-B. The control serum did not inhibit XDH activity. There was no endogenous AHP or IXP in the Ore-R extract, the antiserum or the control serum.

B) XDH-CRM in Mutants Affecting XDH Activity

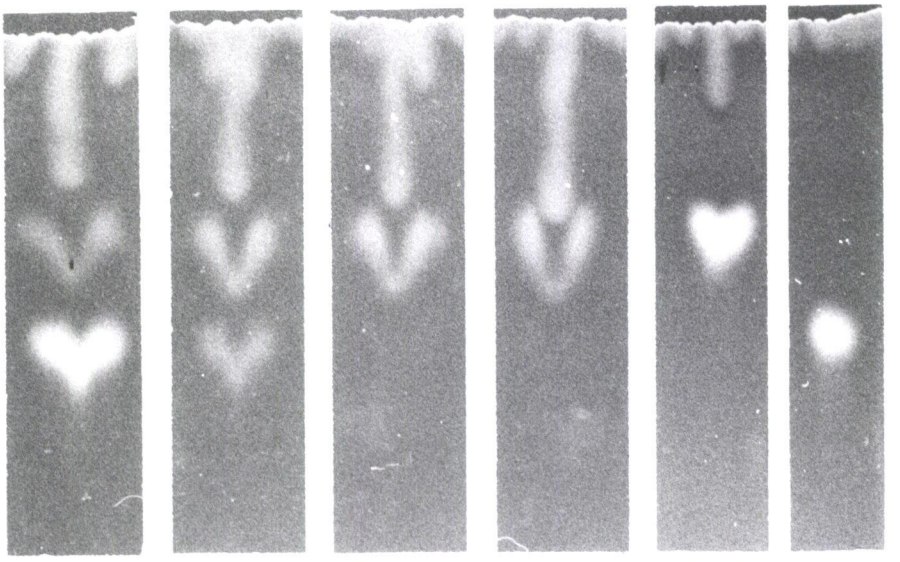
The presence or absence of XDH-CRM in Drosophila extracts was determined by a two-step immunoabsorption technique. If the extract contained XDH-CRM then precipitation of anti-XDH occurred in the first step and subsequently, Ore-R XDH activity could be detected after the second step by TLC Assay. The extent of immunoabsorption would be roughly proportional to the amount of IXP produced in the assay. The amount of XDH-CRM present in the test extracts relative to the amount of enzymatically active XDH in the Ore-R extract was determined by using various concentrations of test extract as described in Materials and Methods. Figure 4-A outlines results for a situation where the test extract contains the same amount of XDH-CRM as enzymatically active XDH in the Ore-R extract (wild-type levels). The amount of IXP detected increases as the concentration of test extract used increases. When a test extract contains the same number of fly equivalents as the

FIGURE 3

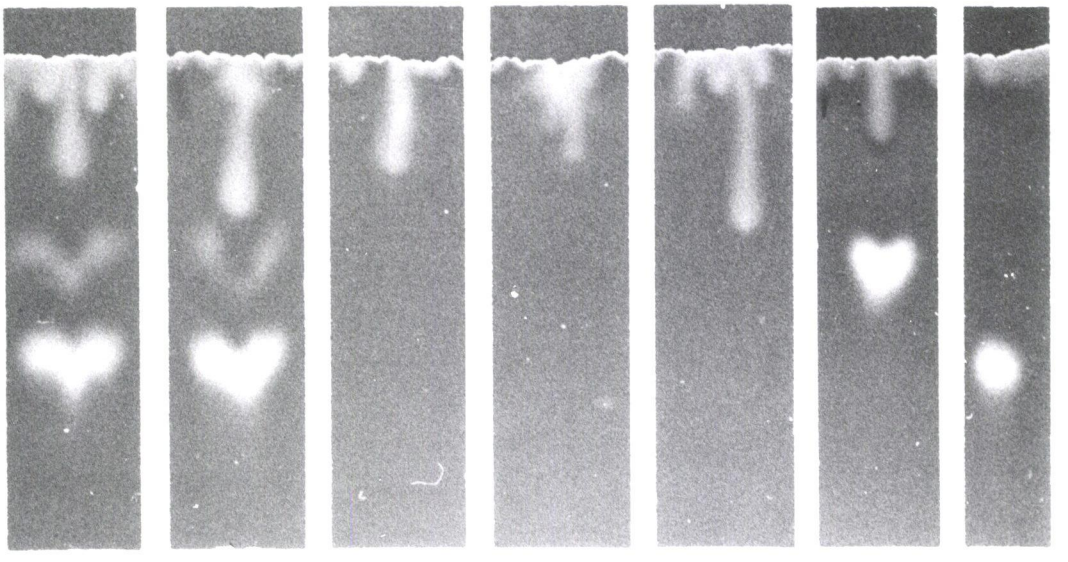
A. Titration of the anti-XDH content of the serum. The numbers refer to the volume of antiserum (ml x $10^{-3}$) used per Ore-R fly.

B. Controls performed: (Ore-R) maximal XDH activity; buffer substituted for antiserum. (CS) test for non-specific inhibition of XDH; control serum substituted for antiserum. (AS-END) antiserum tested for endogenous AHP and IXP. (CS-END) control serum tested for endogenous AHP and IXP. (END) test for endogenous AHP and IXP in the complete assay mixture; control serum, antiserum and Ore-R present. (AHP) (IXP) pure compounds chromatographed as markers.

3-A




3-B



Ore-R extract used, maximal levels of XDH activity are detected. Doubling the test extract concentration does not allow any additional XDH activity to be detected. If the test extract does not contain XDH-CRM then no immunoabsorption occurs and Ore-R XDH activity cannot be detected. Figure 4-B outlines results for such a situation. No Ore-R XDH activity is detected no matter what concentration of test extract is used. Figure 4 also outlines results for the controls used in the XDH-CRM determinations. When the antiserum is replaced with control serum, maximal Ore-R XDH activity is expected. The amount of IXP detected for this control is compared to the amount of IXP produced in the various determinations. Ideally, the test for endogenous AHP and IXP in the determination mixture should be negative as the presence of either compound may interfere with interpretation of the results. All test extracts of mutants affecting XDH activity with the exception of lxd should show no endogenous XDH activity. Pure AHP and IXP are run as markers identifying the spots.

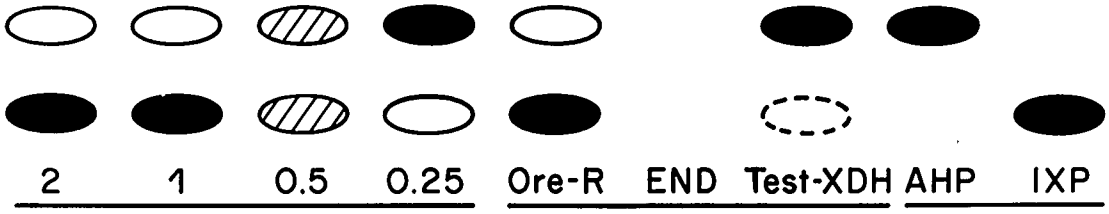
This technique for XDH-CRM determination was verified when extracts of mal, lxd and $ry^2$ flies were tested. Extracts of mal (Figure 5) and lxd (Figure 6) were found to contain wild-type amounts of XDH-CRM in accord with the observations of Glassman and Mitchell (1959) and Glassman (1965). In both cases, maximal Ore-R XDH activity was detected when test extract concentrations equivalent to the Ore-R extract were used. Only the lxd determination

FIGURE 4

TLC patterns for XDH-CRM determinations. The intensity of shading indicates the amount of AHP or IXP. The broken circle indicates presence only in certain instances depending upon the nature of the test extract used. The numbers refer to the number of test organisms used per Ore-R fly. The controls are: (Ore-R) maximal Ore-R XDH activity; control serum substituted for antiserum. (END) test for endogenous AHP or IXP in a determination mixture. (TEST-XDH) assay for XDH activity endogenous to the test extract. The markers are pure AHP and IXP. These symbols will be seen in subsequent figures.

# 4-A

TEST EXTRACTS CONTAINING XDH-CRM




# 4-B

TEST EXTRACTS NOT CONTAINING XDH-CRM

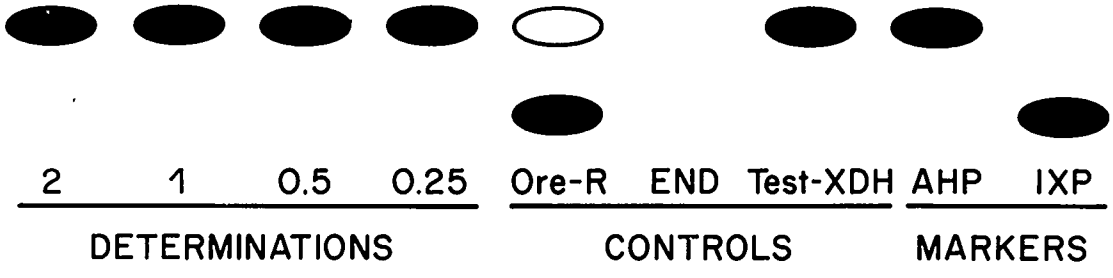


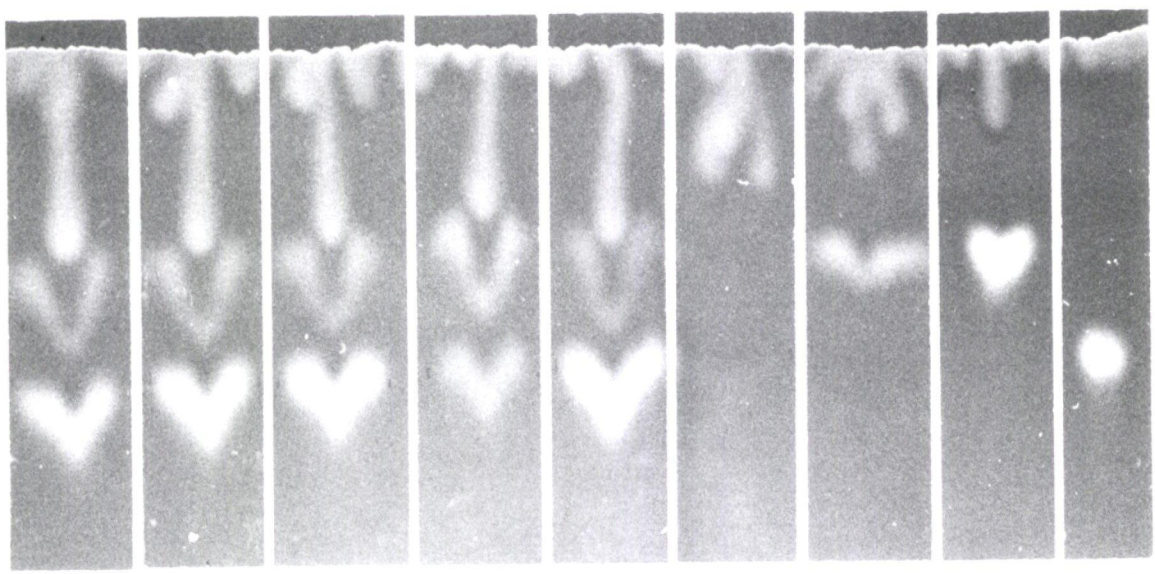
FIGURE 5

XDH-CRM determination for extracts of adult *mal*.

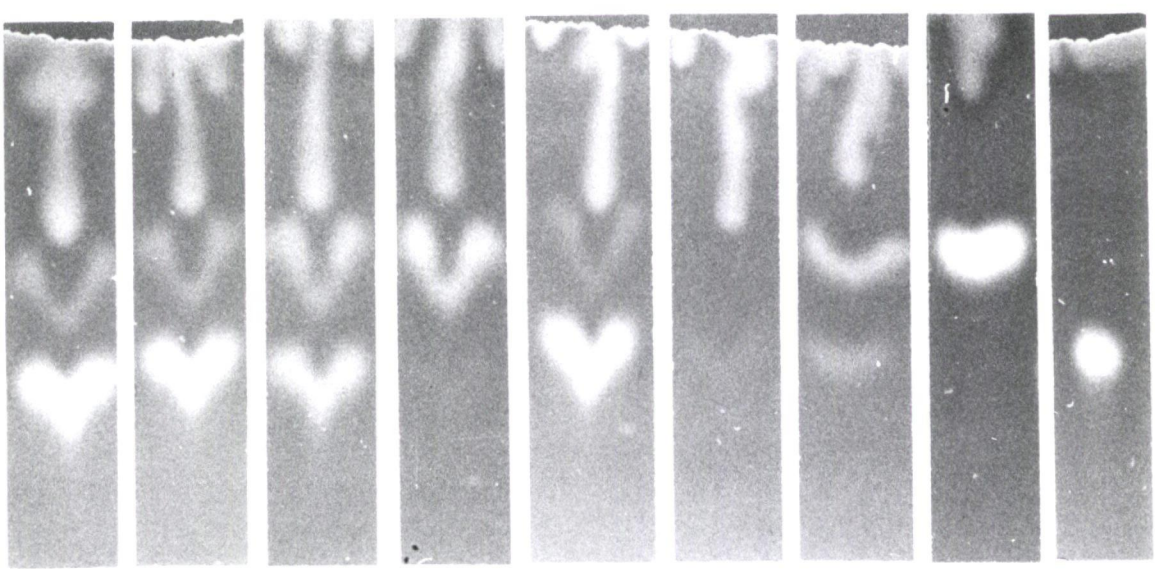
FIGURE 6

XDH-CRM determination for extracts of adult *lxd*.

5


2
1
0.5
0.25
Ore-R
END
Test-XDH
AHP
IXP

6


2
1
0.5
0.25
Ore-R
END
Test-XDH
AHP
IXP

showed any endogenous pteridines, this being a very small amount of IXP. The mal extract contained no endogenous XDH activity. The lxd extract, when assayed for XDH activity, produced a quantity of IXP considerably greater than the amount of endogenous IXP detected in the appropriate control. This indicates that lxd extracts contain enzymatically active XDH. This is in accord with the observation of Keller and Glassman (1964b) that lxd flies have 20 - 25% of wild-type levels of XDH activity. No XDH-CRM could be detected in extracts of $ry^2$ (Figure 7) and mal;$ry^2$ (Figure 8). Only traces of IXP could be seen in any of the determinations. However, this was probably endogenous IXP as the amounts detected were roughly equivalent to the amount of endogenous IXP shown in the controls. Neither test extract possessed any XDH activity of their own. These results are in accord with the findings of Glassman and Mitchell (1959).

It being desirable to know if the cin mutation affects XDH synthesis, maternally affected cin adults were tested. Such flies possess low levels of XDH activity upon eclosion; the activity dissappears as the flies age (Browder and Williamson, 1976). Since the presence of XDH activity in the test extract could affect the interpretation of results, these cin flies were aged prior to testing. In order to test cin flies which were not maternally affected, cin progeny of maternally affected cin mothers had to be obtained. Female progeny of a cross between $cin^1$ stock

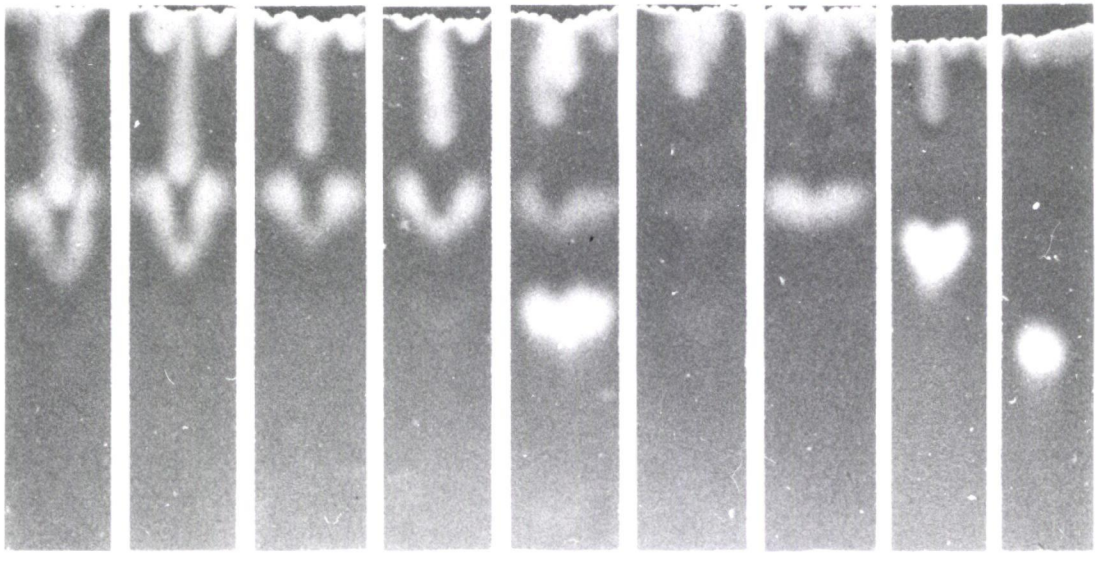
FIGURE 7

XDH-CRM determination for extracts of adult $\underline{ry}^2$.

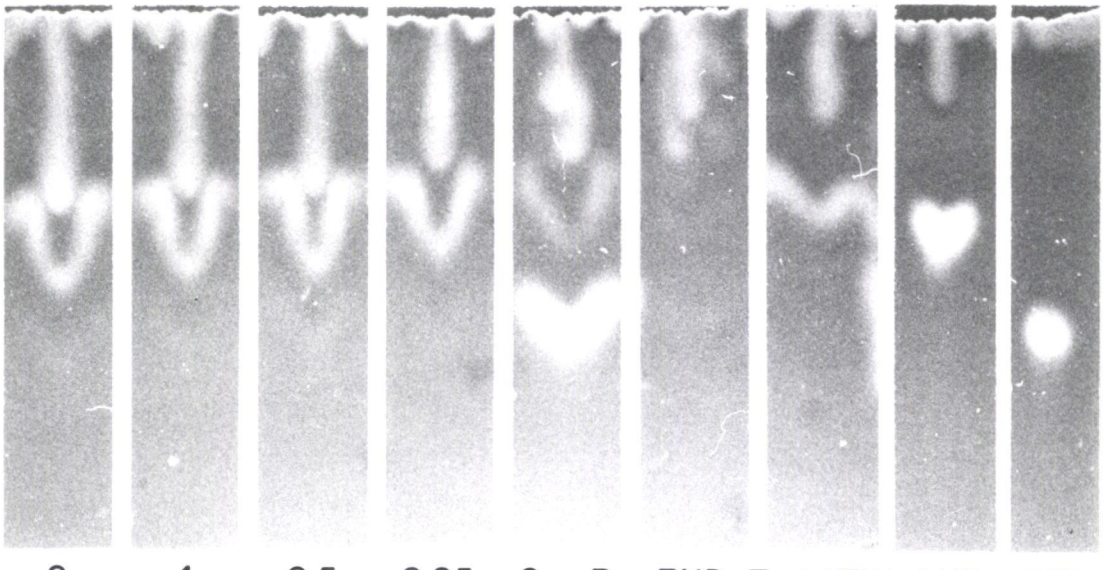
FIGURE 8

XDH-CRM determination for extracts of adult $\underline{mal};\underline{ry}^2$.

7


2
1
0.5
0.25
Ore-R
END
Test-XDH
AHP
IXP

8


2
1
0.5
0.25
Ore-R
END
Test-XDH
AHP
IXP

males and Ore-R females were back-crossed with *cin*$^{1}$ stock males. The resulting female progeny (maternally affected *cin*) were again crossed with *cin*$^{1}$ males. Since most *cin* progeny from *cin* mothers die during embryogenisis (Baker, 1973), few of the progeny of this cross were expected to survive to adults. Therefore, third instar larvae were first tested and compared to *mal* larval extracts, which represented wild-type levels of XDH-CRM for this developmental stage. A small number of *cin* progeny escaped the lethal effect and eclosed; they were also tested.

Maternally affected male adults of the *cin*$^{1}$ and *cin*$^{3}$ stocks as well as male *cin*$^{2}$ adults were found to contain wild-type amounts of XDH-CRM. The results of these determinations (Figure 9, 10, 11) were comparable to those obtained for *mal* and *lxd*. Although the amounts of detected IXP varied between the three determinations, maximal Ore-R XDH activity was found when the test extract used was equivalent to the Ore-R extract. The *cin*$^{1}$ determination mixture contained some endogenous AHP and IXP, the *cin*$^{2}$ mixture contained neither and the *cin*$^{3}$ mixture contained a trace of endogenous IXP. Only the *cin*$^{1}$ test extract showed any IXP when assayed for endogenous XDH activity. However, this IXP was probably not produced by the assay as it was roughly equivalent to the amount of endogenous IXP detected in the appropriate control. These results indicate that the aging of the maternally affected flies was sufficient to remove any XDH activity derived from the maternal effect.

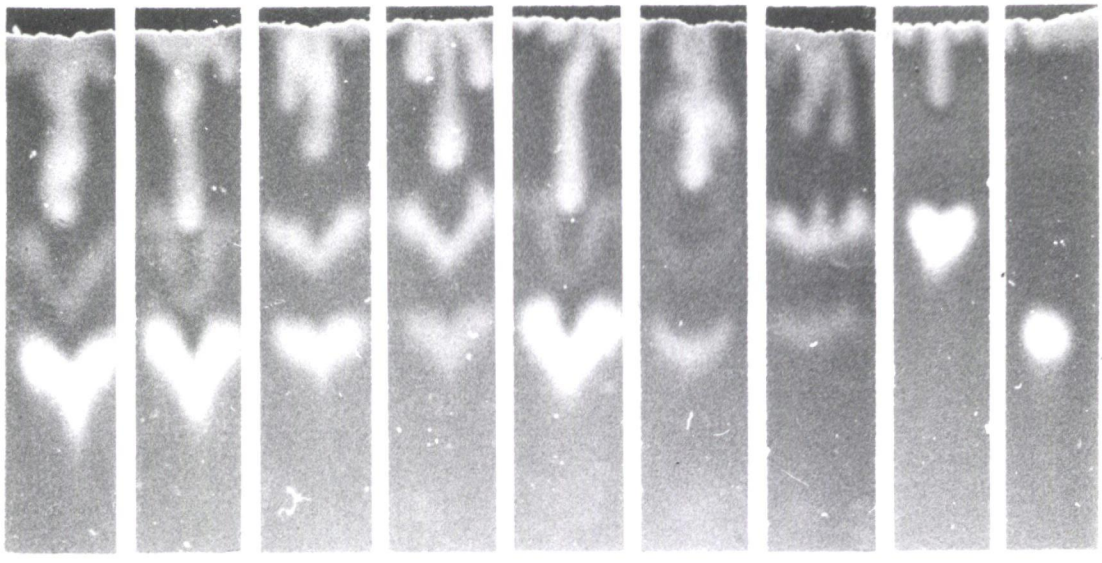
FIGURE 9

XDH-CRM determination for extracts of male adult *cin*[1].

FIGURE 10

XDH-CRM determination for extracts of male adult *cin*[2].

9


2
1
0.5
0.25
Ore-R
END
Test-XDH
AHP
IXP

10

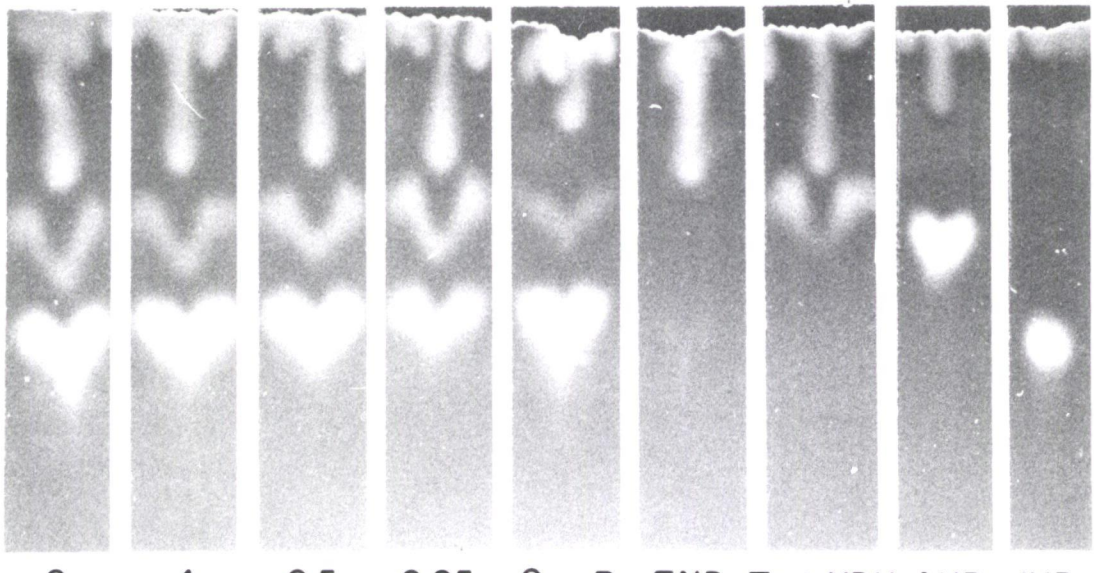
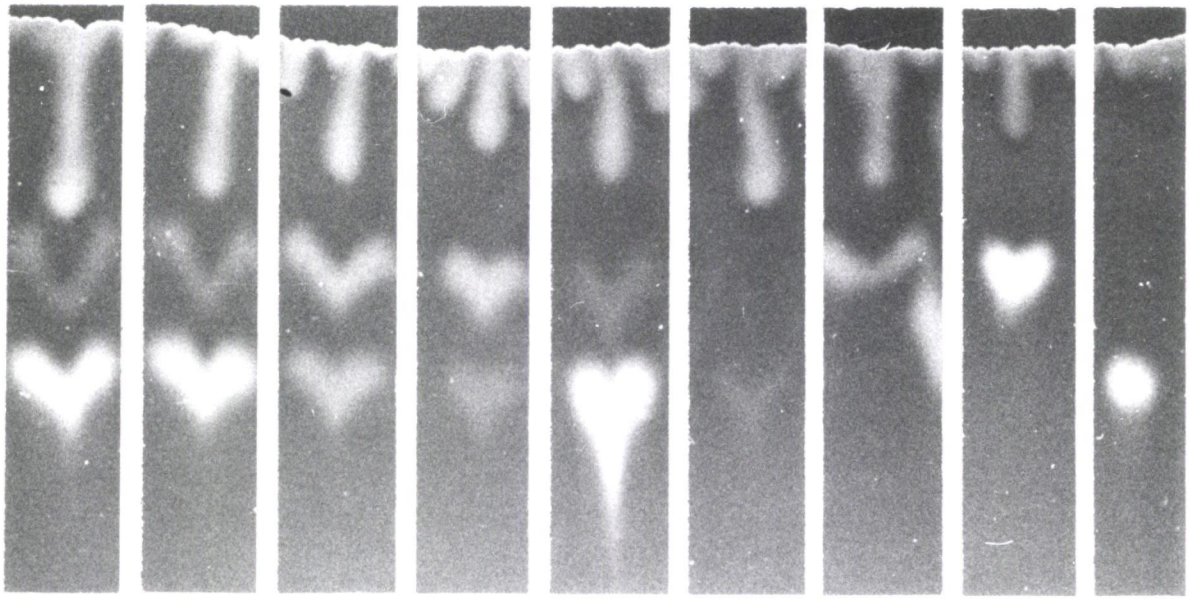

2
1
0.5
0.25
Ore-R
END
Test-XDH
AHP
IXP

FIGURE 11

XDH-CRM determination for extracts of male adult *cin.*[3]

11



Extracts of third instar *cin*$^{1}$ larvae from *cin*$^{1}$ mothers were also found to contain XDH-CRM (Figure 13). The results were comparable to those obtained using an extract of third instar *mal* larvae (Figure 12). This indicates that the *cin* larvae contain wild-type amounts of XDH-CRM. Neither the *cin* nor the *mal* determination mixtures contained any endogenous AHP or IXP nor did either test extract demonstrate XDH activity. From a collection of 760 embryos, only 13 female and 3 male adults were produced (2.1%). These had cinnamon eye colour, as the flies were not maternally affected. Extracts of these adults were shown to contain XDH-CRM (Figure 14). The results of this determination were comparable to those obtained for *mal* and *lxd*. When a test extract equivalent to the Ore-R extract was used, the amount of XDH activity detected compared well with the maximal activity shown by the uninhibited Ore-R control. This indicates that these flies contain wild-type levels of XDH-CRM. No endogenous AHP or IXP was found nor did the test extract possess any XDH activity of its own.

C) Synthesis of XDH-CRM in Embryos

Sayles *et al.* (1973) detected enzymatically active XDH of embryonic origin at the time of gastrulation in *ry*$^{+}$/*ry*$^{2}$ progeny of *ry*$^{2}$/*ry*$^{2}$ mothers. In order to verify this, extracts of *mal*/*mal*;*ry*$^{+}$/*ry*$^{2}$ embryos were tested for the appearance of XDH-CRM. The mothers were *ry*$^{2}$/*ry*$^{2}$, hence could contribute no XDH-CRM; both mothers and progeny were

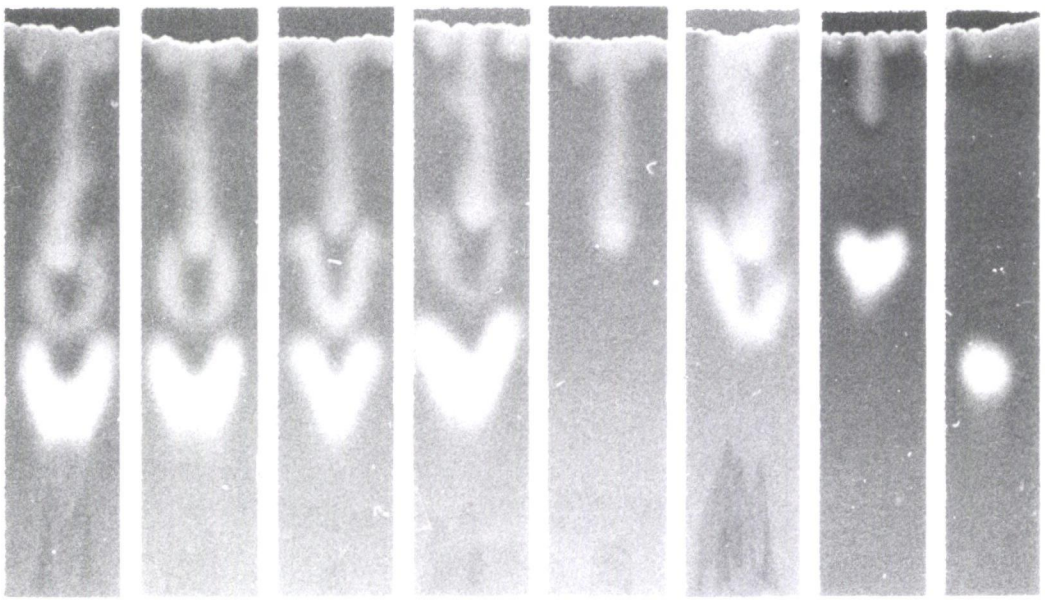
FIGURE 12

XDH-CRM determination for extracts of *mal* third instar larvae.

FIGURE 13

XDH-CRM determination for extracts of *cin*$^{1}$ third instar larvae from *cin*$^{1}$ mothers.

12




13

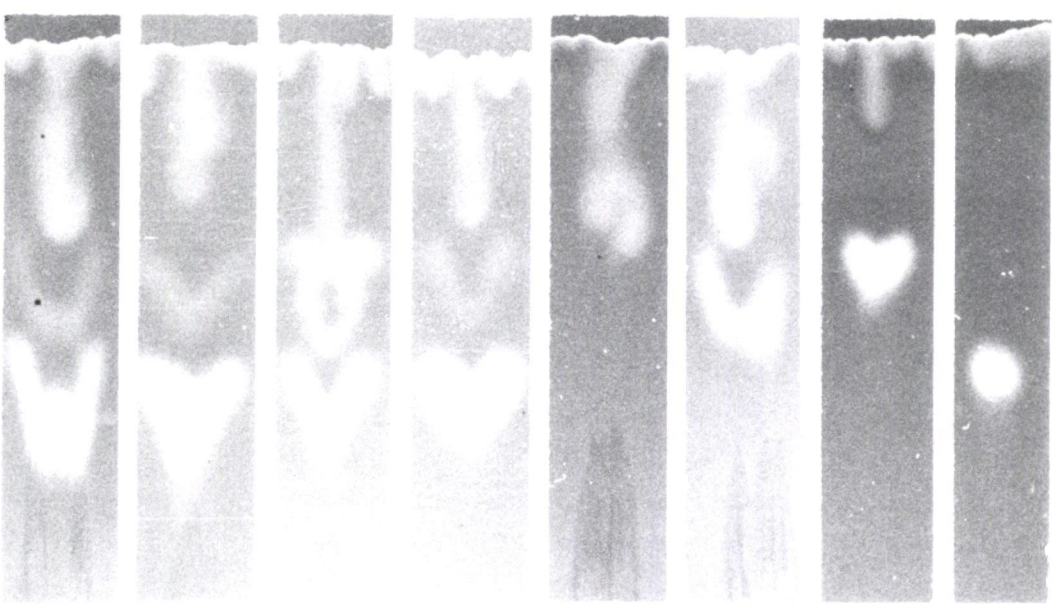
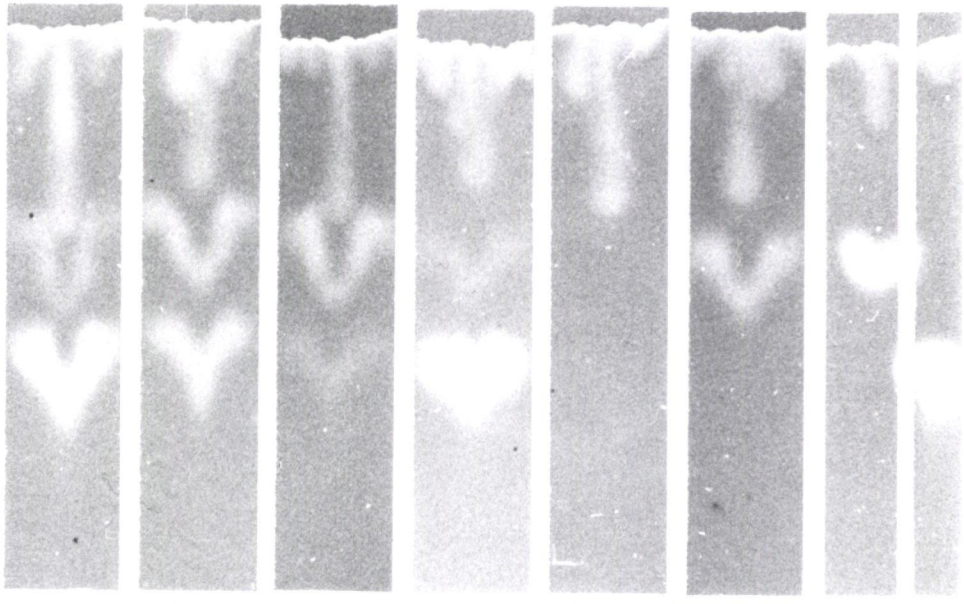


FIGURE 14

XDH-CRM determination for extracts of adult *cin*$^{1}$ from *cin*$^{1}$ mothers.

14



mal/mal. Thus no enzymatically active XDH would be present and possibly interfere with the determination. Extracts were made of embryos of both non-specific age and various staged collections.

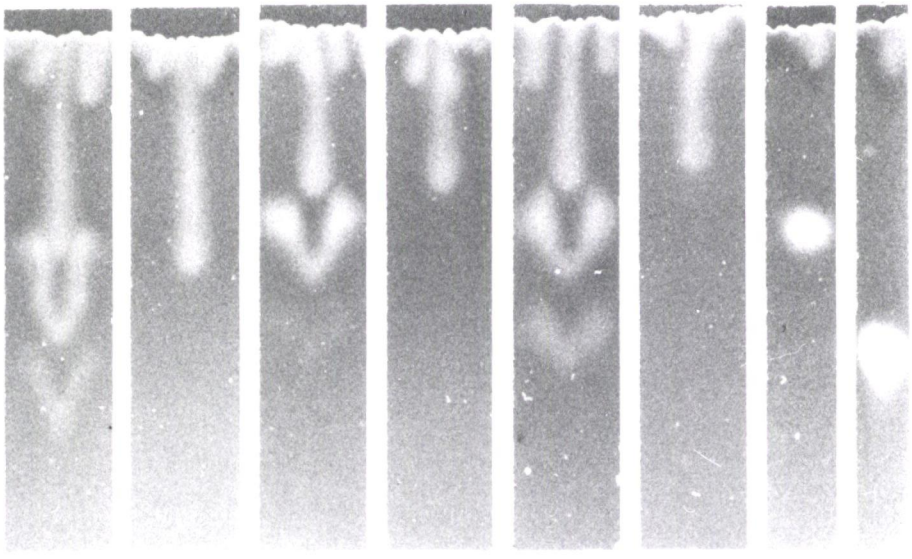
When an extract of embryos of non-specific age was tested, a small amount of XDH activity was detected (Figure 15). The determination mixture contained no endogenous AHP or IXP. A control representing full inhibition of the Ore-R XDH activity (no embryo extract added) failed to produce any IXP when assayed. These results indicate that XDH-CRM is synthesized sometime during the embryonic stage of development. Staged collections of embryos were tested for XDH-CRM (Figure 15):

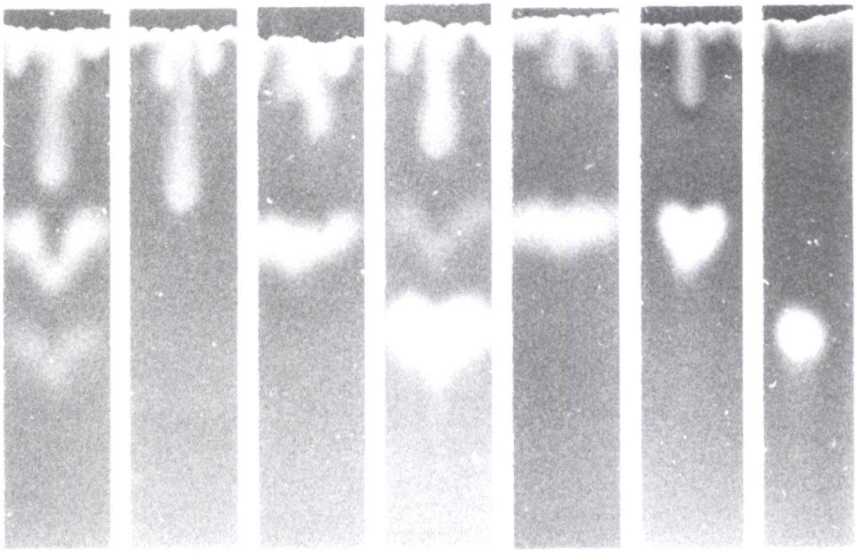
1. Stage 6, 7, 8 embryos: These stages encompass the process of gastrulation and are equivalent to 3 - 5.5 hours in the development of Ore-R embryos. By stage 8, the cephalic furrow and posterior midgut pocket can be seen (Bownes, 1975). An extract of these embryos contained a very small amount of XDH-CRM as the determination produced a trace of IXP (compared to none in the control). No endogenous AHP or IXP was detected.
2. Stage 9, 10 embryos: During these stages ($\simeq$ 5.5 - 9 hours in Ore-R development) ventral segments, especially the head segment, become distinct (Bownes, 1975). An extract of these embryos was found to contain XDH-CRM in excess of that detected in the previous extract. No endogenous AHP or IXP was found.

FIGURE 15

XDH-CRM determination for extracts of *mal*/*mal*;$ry^{+}$/$ry^{2}$ embryos. (NS) refers to embryos of non-specific age (100 embryos /Ore-R fly). Embryos of specific age (40/Ore-R): (A) stages 6 - 8. (B) stages 9, 10. (C) stages 12, 13. The controls: (END) test for endogenous AHP and IXP given for each assay mixture. (INB) full inhibition of Ore-R XDH activity; buffer substituted for test extract. (Ore-R) maximal Ore-R XDH activity. (TEST-XDH) test extract (C) assayed for XDH activity of its own.

15



3. Stages 12, 13 embryos: During these stages (≅ 11 - 17 hours in Ore-R development) the mouth becomes visible, the yolk divides, gut coils appear and active movements begin (Bownes, 1975). Approximately the same amount of XDH-CRM was found in this extract as was in the previous extract. No endogenous AHP or IXP was detected. A control, testing for XDH activity in this embryo extract was negative.

These results indicate that the $\underline{ry}^+$ gene becomes active and XDH protein is synthesized at or around the time of gastrulation (stage 6 - 8). Synthesis appears to intially increase then becomes stable during development of the embryo.

D) In vitro Complementation

Glassman (1962) reported that mixing of $\underline{ry}^2$ and $\underline{mal}$ extracts produces small amounts of enzymatically active XDH. These two extracts will complement each other presumably because the $\underline{mal}^+$ factor present in the $\underline{ry}^2$ extract will activate the $\underline{ry}^+$ factor (XDH protein) present in the $\underline{mal}$ extract. I have shown that the presence of the $\underline{cin}$ mutation does not affect synthesis of XDH protein. Hence, the $\underline{cin}^+$ factor must be involved in activation of XDH. In order to further characterize the role of the $\underline{cin}^+$ factor, the possibility of $\underline{in\ vitro}$ complementation between extracts of $\underline{cin}^1$ and $\underline{ry}^2$ or $\underline{mal}$ was investigated. All

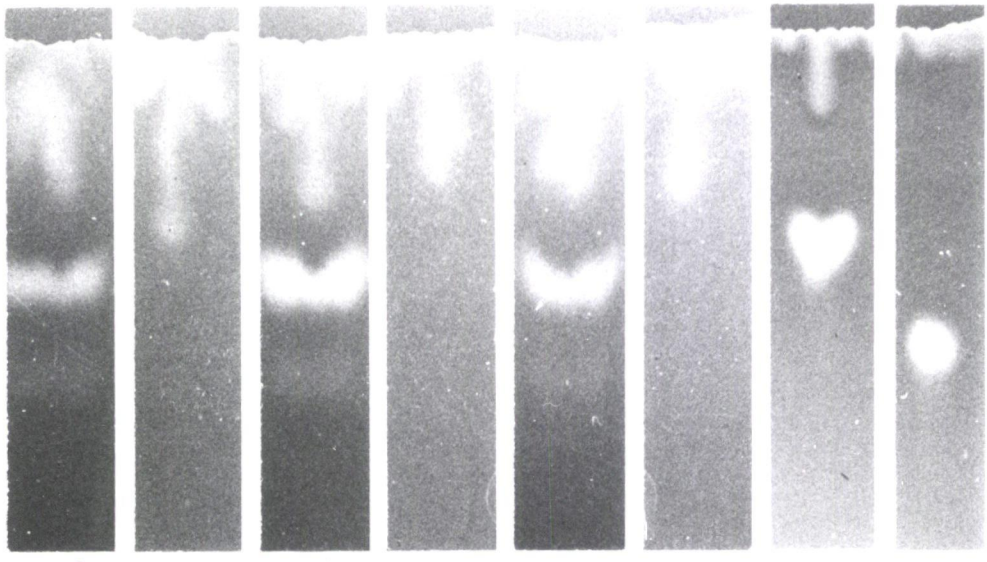
combinations of these extracts (*ry*$^2$ + *mal*; *ry*$^2$ + *cin*$^1$; *mal* + *cin*$^1$) were capable of converting AHP to IXP at approximately the same rate (Figure 16-A). No endogenous AHP or IXP could be found in any of these combinations. Therefore, the small amounts of IXP detected may have been produced as a result of complementation. However, lesser amounts of IXP were produced when controls consisting of extracts of a single genotype were assayed (Figure 16-B). In order to determine if the greater amount of IXP detected in the mixtures was produced at least in part as a result of complementation, a separate experiment was performed where different concentrations of extracts of a single genotype were incubated and assayed for XDH activity. It was demonstrated that the amount of IXP produced in single genotype assays was not dependent upon the concentration of extract used (Figure 17). Doubling the concentration of extract did not allow the production of any more IXP to occur. The amount of bacterial contamination of each assay mixture described in Figures 16 and 17 as determined on BHI plates could not be correlated with the amount of IXP produced (Table 4). This experimental approach does not lend itself to exact quantification of XDH activity nor, is it sensitive enough to clearly indicate if *in vitro* complementation did occur. Therefore, no definite conclusions can be made concerning these results.

FIGURE 16

A. In vitro complementation. Each mutant symbol represents an extract of 40 adults; the addition sign indicates a mixture of extracts. Endogenous AHP and IXP is determined for each assay mixture.

B. Controls performed. Each mutant symbol represents a single extract of 40 adults. Endogenous AHP and IXP is determined for each assay.

16-A


ry2+ mal
END
ry2+ cin1
END
mal+ cin1
END
AHP
IXP

16-B

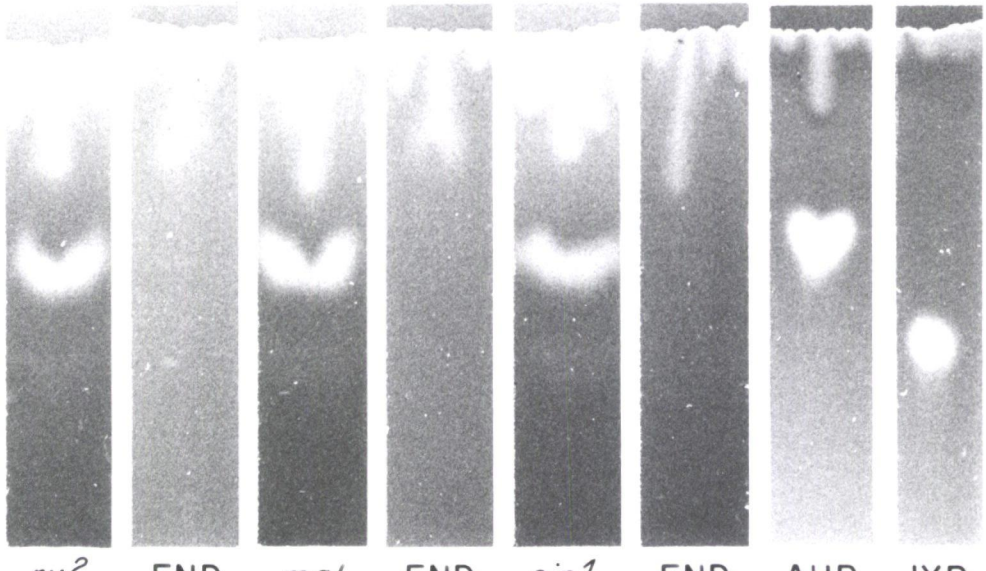

ry2
END
mal
END
cin1
END
AHP
IXP

FIGURE 17

Production of IXP in single genotype extracts. The mutant symbols identify extracts containing 40 adults. The addition sign indicates doubling of the amount of extract used.

17

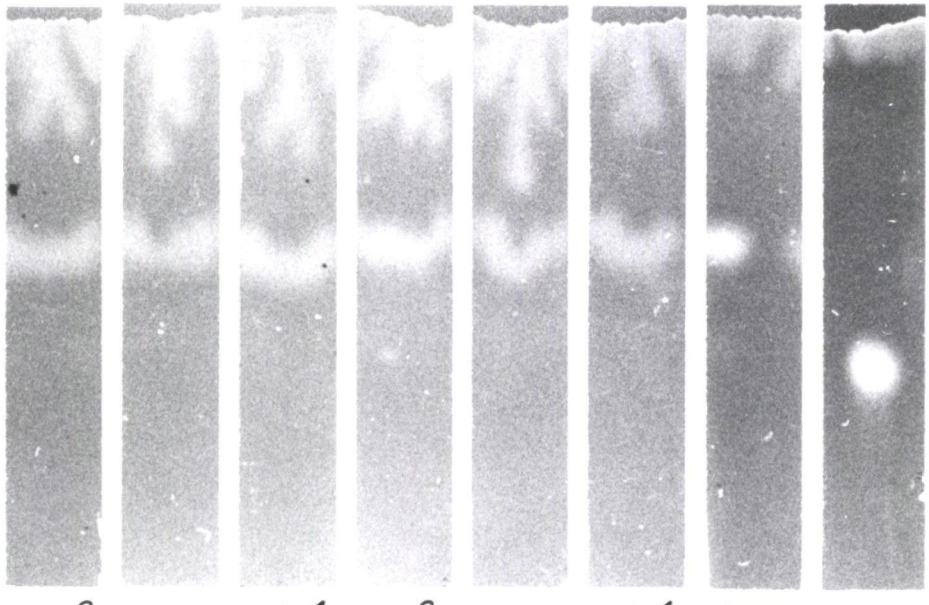


TABLE 4

Comparison of bacterial contamination and IXP production in extracts assayed for *in vitro* complementation. The symbols refer to estimated amounts of IXP and number of bacterial colonies present on BHI plates (-) no colonies; (+)< 50 colonies; (++) 50-100 colonies;

| Reference | Contents | IXP | Bacteria |
|---|---|---|---|
| Figure 16 | *ry*$^{2}$ + *mal* | + + | - |
| | *ry*$^{2}$ + *cin*$^{1}$ | + + | - |
| | *mal* + *cin*$^{1}$ | + + | + + |
| | *ry*$^{2}$ | + | - |
| | *mal* | + | + + |
| | *cin*$^{1}$ | + | - |
| Figure 17 | *ry*$^{2}$ | + | - |
| | *mal* | + | - |
| | *cin*$^{1}$ | + | + |
| | *ry*$^{2}$ + *ry*$^{2}$ | + | + |
| | *mal* + *mal* | + | + + |
| | *cin*$^{1}$ + *cin*$^{1}$ | + | + |

# DISCUSSION

Control of XDH activity can be exerted at two levels; regulation of the activity of the XDH protein or regulation of the synthesis of the protein. The two most described loci known to affect XDH activity (*mal* and *lxd*) appear to be involved at the former level since they are responsible for activation of the $ry^+$ gene product (XDH protein). It also seems reasonable that there would exist regulatory gene(s) involved in control of the synthesis of XDH protein by affecting transcription and/or translation of the $ry^+$ gene. Presumably it would be the influence of such a gene that would initiate the activity of the embryonic $ry^+$ gene during early development.

Antiserum against *Drosophila* XDH was prepared and used to detect XDH-CRM. The immunoabsorbtion technique used in the XDH-CRM determinations was verified experimentally by observing that *mal*, *lxd* and $ry^2$ flies contain the same quantities of this protein as reported in the literature (Glassman and Mitchell, 1959; Glassman, 1965).

Extracts of three *cin* alleles were tested for the presence of XDH-CRM. In the case of the $cin^1$ and $cin^3$ alleles, maternally affected males were used. These flies are rescued from the mutant phenotype presumably because some factor derived from the $cin^+$ gene of the mother is deposited in the unfertilized oocyte. This enables them to survive, possess XDH activity during development and hence,

wild-type eye colour as adults. The maternally affected *cin* flies were aged until all enzymatically active XDH derived from the maternal effect should have been eliminated. In the absence of XDH activity, wild-type amounts of XDH-CRM were still detected. Males of the $cin^2$ stock were also tested. These flies are heterozygous for the $cin^1$ and $cin^2$ alleles and are the progeny of mothers heterozygous for these alleles. They are not maternally affected, lack XDH activity and possess mutant eye colour yet they are viable. The $cin^2$ males were also found to possess wild-type amounts of XDH-CRM. In addition, extracts of surviving $cin^1$ progeny of $cin^1$ mothers were found to contain wild-type amounts of XDH-CRM. These flies also are not maternally affected and are never exposed to factors related to the $cin^+$ gene at any time of development. These results clearly indicate that the presence of the $cin^+$ gene is not necessary for synthesis of XDH protein. Therefore, its regulatory effect must be exerted at the post-translational level. As with *mal* and *lxd*, the $cin^+$ gene appears to be involved in activation of the XDH protein.

In order to further characterize the mode of action of the $cin^+$ factor, an attempt was made at producing *in vitro* complementation in extracts of $cin^1$ flies mixed with extracts of $ry^2$ and *mal* flies. Glassman (1962) reported that mixing extracts of $ry^2$ and *mal* flies produced low levels of XDH activity, presumably because of interaction of the $mal^+$ factor in the $ry^2$ fly extract with the

$ry^+$ factor (XDH protein) present in the mal extract. I detected low levels of conversion of AHP to IXP in extracts of $ry^2$, mal and aged $cin^1$ flies when incubated by themselves. This may have been due to spontaneous or photo-oxidation of the AHP during the lengthy incubation period. It was observed that after incubation of mixtures of $ry^2$ and mal, $ry^2$ and $cin^1$ as well as mal and $cin^1$ fly extracts, conversion of AHP to IXP occurred to a greater extent than in the single genotype assays. Complementation was expected at least in mixtures of $ry^2$ and mal fly extracts however, the results in all instances were equivocal. If complementation could be demonstrated in mixtures that included an extract of cin flies then it would appear that the mode of action of the $cin^+$ factor in activation of XDH is similar to that of the $mal^+$ factor. In particular, if complementation could be shown to occur between extracts of cin and mal flies, then the role of either the $cin^+$ or $mal^+$ factors would not be related to the synthesis of each other. Such a result would be supported by Courtright's observation (1975) that mal progeny of cin mothers and cin progeny of mal mothers are still maternally affected. The presence of maternal mal or cin genotypes does not prevent synthesis and transmission of the $cin^+$ or $mal^+$ factors to the oocytes of flies of the respective genotypes.

An important aspect in the study of the mechanisms of differentiation is determining the time at which specific embryonic genes become activated during development.

When this occurs, the zygote is seen to be capable of directing aspects of its own development. Sayles et al. (1973) found that enzymatically active XDH can be initially detected in $+/ry^2$ embryos at time of gastrulation. I have verified this result by detecting synthesis of XDH-CRM in precisely staged gastrulating $mal/mal;ry^+/ry^2$ embryos. The mothers of these embryos were homozygous for $ry^2$. Hence, the synthesis of XDH-CRM must have been directed by the embryonic $ry^+$ gene. This technique is an accurate means of determining if the $ry^+$ gene is active as it does not rely upon the activity of any other embryonic gene or maternally derived substance. However, while approximations of relative amounts of XDH-CRM can easily be made, this technique is not suitable for exact quantification. Nevertheless, it was apparent that the amount of XDH-CRM detectable in the embryo extracts increased after gastrulation. This may be due to an increase in synthesis of XDH protein or an increase in the number of embryos producing XDH-CRM in the collection. The amount of XDH-CRM in the older embryos appeared to remain constant. This observation is noteworthy as Sayles et al. found that XDH activity in Ore-R embryos initially increased and after 8 hours of development, remained relatively stable.

It has been determined that the $cin^+$ gene affects the activity of XDH rather than its synthesis. It would seem that neither the roles of the $cin^+$ or $mal^+$ genes fit into the Britten-Davidson model of gene regulation. Both

genes produce maternally transmitted products which are involved in the post-translational activation of the XDH structural gene product. It may be that the *cin*$^+$ and *mal*$^+$ gene products interact prior to activating XDH or they may function separately. In either case, they may act directly on the XDH protein or may first require interaction with some other factor. The role of the *lxd*$^+$ gene further complicates the system. With respect to XDH, it would appear that the role of the *lxd*$^+$ gene is that of activation. However, the *lxd*$^+$ gene may fit the Britten-Davidson model as it is possible that it regulates the activity of the *mal*$^+$ and/or *cin*$^+$ genes. If *lxd*$^+$ is a regulatory gene, it is possible that it responds to ambient levels of molybdenum. Flies which are *lxd*, raised on medium containing low concentrations of molybdenum show greatly increased levels of XDH activity (Duke *et al.*, 1975).

Courtright (1976) describes a model which is consistant with the information now known concerning the XDH gene-enzyme system. Accordingly, the *cin*$^+$ and *mal*$^+$ gene products interact to produce a factor necessary for the activity of XDH. This factor itself, is activated by the influence of the *lxd*$^+$ gene and molybdenum and is then equivalent to the *mal*$^+$ complementing factor described by Glassman (1962). The complementing factor is then capable of activating XDH protein by some mechanism not involving the addition of itself, another protein or FAD, molybdenum or iron cofactors (Andres, 1976).

A total understanding of genetic regulation requires the investigation of mechanisms affecting the transcription or translation of specific genes. Assuming that the activation of the $\underline{ry}^+$ gene during the time of gastrulation and continued synthesis of XDH protein is regulated by the genome, further study of the XDH gene-enzyme system may still provide insight as to the existence of such mechanisms.

# LITERATURE CITED

Andres, R. Y. 1976. Aldehyde oxidase and xanthine dehydrogenase from wild-type Drosophila melanogaster and immunologically cross-reacting material from mal mutants. Eur. J. Biochem. 62:591.

Baker, B. S. 1973. The maternal and zygotic control of development by cinnamon, a new mutant in Drosophila melanogaster. Develop. Biol. 33:429.

Bowness, M. 1975. A photographic study of development in the living embryo of Drosophila melanogaster. J. Embryol. exp. Morph. 33:789.

Britten, R. J. and E. H. Davidson. 1969. Gene regulation in higher cells: A theory. Science. 165:349.

Browder, L. W. and J. H. Williamson. 1976. The effects of cinnamon on xanthine dehydrogenase, aldehyde oxidase and pyridoxal oxidase activity during development of Drosophila melanogaster. Develop. Biol. 53:241.

Chovnick, A., A. Schalet, R. P. Kernaghan and M. Krauss. 1964. The rosy cistron in Drosophila melanogaster: Genetic fine structure analysis. Genetics. 50:1245.

Chovnick, A., G. H. Ballantyne and D. G. Holm. 1971. Studies on gene conversion and its relationship to linked exchange in Drosophila melanogaster. Genetics. 69:179.

Chovnick, A., W. Gelbart, M. McCarron, B. Osmond, E. P. M. Candido and D. L. Baillie. 1976. Organization of the rosy locus in Drosophila melanogaster: Evidence for a control element adjacent to the xanthine dehydrogenase structural element. Genetics. 84:233.

Courtright, J. B. 1967. Polygenic control of aldehyde oxidase in Drosophila melanogaster. Genetics. 57:25.

Courtright, J. B. 1975. Evidence for a new type of complementation among the cin, lxd and mal loci in Drosophila melanogaster. Molec. Gen. Genet. 142:231.

Courtright, J. B. 1976. Drosophila gene-enzyme systems. Advances in Genetics. 18:249. Academic Press. New York.

Davidson, E. H. 1968. "Gene Activity in Early Development." Academic Press, New York.

Davidson, E. H. and R. J. Britten. 1973. Organization, transcription and regulation in the animal genome. Quart. Rev. Biol. 48:565.

Dickinson, W. J. 1970. The genetics of aldehyde oxidase in Drosophila melanogaster. Genetics. 66:487.

Dickinson, W. J. 1971. Aldehyde oxidase in Drosophila melanogaster: A system for genetic studies on developmental regulation. Develop. Biol. 26:77.

Dickinson, W. J. and D. T. Sullivan. 1975. "Gene-enzyme systems in Drosophila." Springer-Verlag. New York.

Duke, E. J., D. R. Rushing and E. Glassman. 1975. Nutritional control of xanthine dehydrogenase. II. Effects on xanthine dehydrogenase and aldehyde oxidase of culturing wild-type and mutant Drosophila on different levels of molybdenum. Biochem. Genet. 13:53.

Ephrussi, B. and G. W. Beadle. 1936. A technique of transplantation of Drosophila. Am. Naturalist. 70:218.

Forrest, H. S., E. Glassman and H. K. Mithcell. 1956. The conversion of 2-amino-4-hydroxypteridine to isoxanthopterin in Drosophila melanogaster. Science. 124:725.

Forrest, H. S., E. W. Hanly and J. M. Lagowski. 1961. Biochemical differences between the mutants $rosy^2$ and maroon-like of Drosophila melanogaster. Genetics. 46:1455.

Gelbart, W. M., M. McCarron, J. Pandey and A. Chovnick. 1974. Genetic limits of the xanthine dehydrogenase structural element within the rosy locus in Drosophila melanogaster. Genetics. 78:869.

Gelbart, W., M. McCarron and A. Chovnick. 1976. Extension of the limits of the XDH structural element in Drosophila melanogaster. Genetics. 84:211.

Glassman, E. 1962. In vitro complementation between non-allelic Drosophila mutants deficient in xanthine dehydrogenase. Proc. Nat. Acad. Sci. U.S.A. 48:1491.

Glassman, E. 1965. Genetic regulation of xanthine dehydrogenase in Drosophila melanogaster. Federation Proc. 24:1243.

Glassman, E. and H. K. Mitchell. 1959. Mutants of Drosophila melanogaster deficient in xanthine dehydrogenase. Genetics. 44:153.

Glassman, E. and J. McLean. 1962. Maternal effect of $mal^+$ on xanthine dehydrogenase of Drosophila melanogaster. II. Xanthine dehydrogenase activity during development. Proc. Nat. Acad. Sci. U.S.A. 48:1712.

Glassman, E., E. C. Keller Jr., J. D. Karam, J. McLean and M. Cates. 1964. In vitro complementation between non-allelic mutants deficient in xanthine dehydrogenase. II. The absence of the $mal^+$ factor in lxd mutant flies. Biochem. Biophys. Res. Commun. 17:242.

Glassman, E., T. Shinoda, H. M. Moon and J. D. Karam. 1966. In vitro complementation between non-allelic Drosophila mutants deficient in xanthine dehydrogenase. IV. Molecular weights. J. Mol. Biol. 20:419.

Grell, E. H. 1962. The dose effect of $mal^+$ and $ry^+$ on xanthine dehydrogenase activity in Drosophila melanogaster. Genetics. 47:950.

Keller, E. C. and E. Glassman. 1964a. Xanthine dehydrogenase: Differences in activity among Drosophila strains. Science. 143:40.

Keller, E. C. Jr. and E. Glassman. 1964b. A third locus (lxd) affecting xanthine dehydrogenase in Drosophila melanogaster. Genetics. 49:663.

Kriegstein, H. J. and D. S. Hogness. 1974. Mechanism of DNA replication in Drosophila chromosomes: Structure of replication forks and evidence for bi-directionality. Proc. Nat. Acad. Sci. U.S.A. 71:135.

Lewin, B. 1974. "Gene Expression; Vol. 2: Eucaryote Chromosomes." John Wiley and Sons. London.

Lewis, E. B. 1960. A new standard culture medium. Drosophila Informat. Serv. 34:117.

Lindsley, D. L. and E. H. Grell. 1968. "The Genetic Variations of Drosophila melanogaster." Carnegie Institution. Washington. No. 627.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193:265.

McCarron, M., W. Gelbart and A. Chovnick. 1974. Intracistronic mapping of electrophoretic sites in Drosophila melanogaster: Fidelity of information transfer by gene conversion. Genetics. 76:289.

Mohler, S. D. 1977. Developmental genetics of the Drosophila egg. I. Identification of 59 sex-linked cistrons with maternal effects on embryonic development. Genetics. 85:259.

Monod, J. and F. Jacob. 1961. General conclusions: Teleonomic mechanisms in cellular metabolism, growth and differentiation. Cold Spring Harbour Symp. Quant. Biol. 26:389.

O'Brien, S. J. 1975. Biochemical Mutations in Drosophila melanogaster. Handbook of Genetics. 3:669.

Rechsteiner, M. C. 1970. Drosophila lactate dehydrogenase and ∝-glcerophosphate dehydrogenase: Distribution and change in activity during development. J. Insect Physiol. 16:1179.

Sayles, C. D., L. W. Browder and J. H. Williamson. 1973. Expression of xanthine dehydrogenase activity during embryonic development of Drosophila melanogaster. Develop. Biol. 33:213.

Schalet, A. 1963. Location of mal: maroon-like. Drosophila Informat. Serv. 38:82.

Seybold, W. D. 1974. Purification and partial characterization of xanthine dehydrogenase from Drosophila melanogaster. Biochem. Biophys. Acta. 334:266.

Yen, T. T. and E. Glassman. 1965. Electrophoretic variants of xanthine dehydrogenase in Drosophila melanogaster. Genetics. 52:977.

Yen, T. T. and E. Glassman. 1967. Electrophoretic variants of xanthine dehydrogenase in Drosophila melanogaster. II. Enzyme kinetics. Biochem. Biophys. Acta. 146:35.

Wright, D. A. and C. R. Shaw. 1969. Genetics and ontogony of ∝-glcerolphosphate dehydrogenase isozymes in Drosophila melanogaster. Biochem. Genet. 3:343.