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# AN IMMUNOLOGICAL APPROACH TO THE STUDY OF THE TUMOROUS-HEAD TRAIT IN *DROSOPHILA MELANOGASTER*

BY

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B.S., Florida Technological University, 1974

THESIS

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Special thanks are extended to Rita Neuhaus who provided the artwork for the figures in this thesis.

Last but not least, the most sincere thanks are offered to my husband and parents who have been an unending source of encouragement, support, and patience.

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# CHAPTER I

## INTRODUCTION

All strains of tumorous-head Drosophila melanogaster currently available are derived from a collection of wild flies found near the village of Acahuizolta, Mexico in 1941. During the Spring of 1945 abnormal structures were discovered in the head regions of the descendants of the Acahuizolta strain by investigators at the University of Texas. Those flies displaying the abnormal growths were selectively inbred and the strain was sent to the University of Utah where the name "tumorous-head" and the symbol "tu-h" were assigned to it. After years of intensive selection, the level of flies in that strain expressing the tumorous-head trait reached 80 - 90% (Gardner, 1970).

The distribution and morphology of the abnormal head growths were described by Newby in 1949. Usually unilateral, rarely bilateral, and never symmetrical, the tumorous head growths were not seen to cross the midline. They were, however, often seen to affect both sides of the head. Frequent involvement of the eyes, antennae, and other dorsal-lateral parts of the Drosophila head and the absence of growths in the mouth regions were observed

by Newby. Homoeosis was suspected by the replacement of antennae with leglike growths. The idea was further explored by Postlethwait and his coworkers in 1972 and, more recently by Kuhn and Dorgan (in press). Alterations from antennae to leg; from eye to abdominal, genital, and leg structures; and from rostralhaut to abdominal and genital structures have been reported.

In 1949, a semidominant third chromosome gene (symbolized tu-3) was shown by Gardner and Woolf (1949) to be responsible for the abnormal head growths. A sex-linked recessive gene (tu-1) causing a maternal effect was found to increase the penetrance of the tu-3 gene (Gardner and Woolf, 1949). The tu-3 gene was located by Gardner (1959) in the right arm of the third chromosome at 58.0. The tu-1 gene was deficiency mapped by Woolf to 65.3 in the X chromosome (Woolf personal communication). All tumorous-head flies are homozygous for these genes and all but one strain possess a third chromosome dimorphism, In(3L)P, known as the Payne inversion (Woolf and Phelps, 1960). Two types of third chromosomes are involved, 3A and 3B. Only 3A carries the tu-3 gene and only 3B carries the large paracentric inversion on its left arm (Woolf and Phelps, 1960). The Payne inversion being homozygous lethal, only two types of adult flies exist; those carrying 3A/3A and those carrying 3A/3B (Woolf and Phelps,

1960). Female homokaryotypes (3A/3A) are found to be less productive than female heterokaryotypes (Woolf, 1967). This phenomenon is due in part to a polygenic system involving the second chromosome and in part to a viability maternal effect controlled by the left arm of chromosome 3A (Woolf, 1967). Reduced fertility displayed by the homokaryotype female has been attributed to a second chromosome maternal effect. Reduced fecundity has been attributed to a controlling region near roughoid (0.0) in the left arm of chromosome 3A (Woolf, et al., 1964). Increased male and female heterokaryotype fitness has been attributed to an interaction between the second and third chromosomes (Knowles, 1967).

In 1968, Woolf described a genital disc defect displayed by the tumorous-head males and transformed females (XXY, tra/tra flies). In the presence of a maternal effect produced by a naturally occuring allele to tu-1, modifiers on the second chromosome, and tu-3, 0% to over 60% of the male flies lack seminal vesicles which derive from the genital disc. In the absence of these seminal vesicles, the testes cannot elongate and coil. Consequently, the affected males cannot reproduce.

Kuhn, in 1971 and 1973, observed that a maternal effect associated with the heterokaryotypic females affects sex ratios in favor of males. Genes in chromosome

3B, probably within In(3L)P, have been implicated.

In all, five maternal effects have been attributed to the tumorous-head flies. The first, affecting head tumors, is associated with tu-1. The second, affecting the viability of female homokaryotypes, is associated with the left arm of chromosome 3A. The third, affecting the fertility of female homokaryotypes, is associated with the second chromosome. The fourth, affecting testes development in male tumorous-head flies, is associated with a naturally occuring allele to tu-1, and the fifth, affecting sex ratios (in favor of males), is associated with the heterokaryotypic females.

Kuhn (in press) determined that extensive genetic relationships exist between the eclosion, sex ratios, karyotype, and penetrance of the tumorous-head trait. None of these characteristics is independent in its expression. Interactions are based on the fact that females are more likely to develop the tumorous-head trait than are males and that the heterozygous condition for chromosome 3B increases the probability of zygote survival to the adult stage despite abnormal development resulting in the expression of the tumorous-head trait (Woolf and Lott, 1965).

Recent investigations have drawn similarities between the tumorous-head trait and various enzyme systems

in Drosophila melanogaster. Certain locational similarities between the genes responsible for the expression of the tumorous-head trait and for the actions of three enzymes, xanthine dehydrogenase, aldehyde oxidase, and pryidoxal oxidase, have been suggested by Kuhn and Cunningham (personal communication).

In Drosophila melanogaster, three different gene loci produce and control xanthine dehydrogenase. No activity for this enzyme can be demonstrated in flies with an eye mutation maroon-like (ma-l) characterized by a brownish eye color. This gene is located at 64.8 on the X chromosome (Forrest et al., 1956) and functions in purine metabolism. Similarly, a second trait resulting from the recessive gene rosy (ry) located at 52.3 in the right arm of the third chromosome, shows no xanthine dehydrogenase activity and is characterized by a brownish eye pigment (Glassman and Mitchell, 1959). In 1965, Glassman suggested that the structural gene locus for xanthine dehydrogenase is positioned at ry. Keller and Glassman in 1964 described a third locus (3-33$^{+}_{-}$) affecting the quantity of enzyme produced. Those flies homozygous for the allele show only 25% of the normal wild-type activity for xanthine dehydrogenase. The allele, symbolized lxd (low xanthine dehydrogenase), is believed to serve a regulatory function.

The structural gene for a second enzyme, aldehyde oxidase, has been mapped by Dickinson (1970) to 56.6 ± 0.7 on the right arm of chromosome 3. Aldehyde oxidase is observed in reduced quantities in lxd mutants and is totally absent in ma-l flies (Dickinson, 1970).

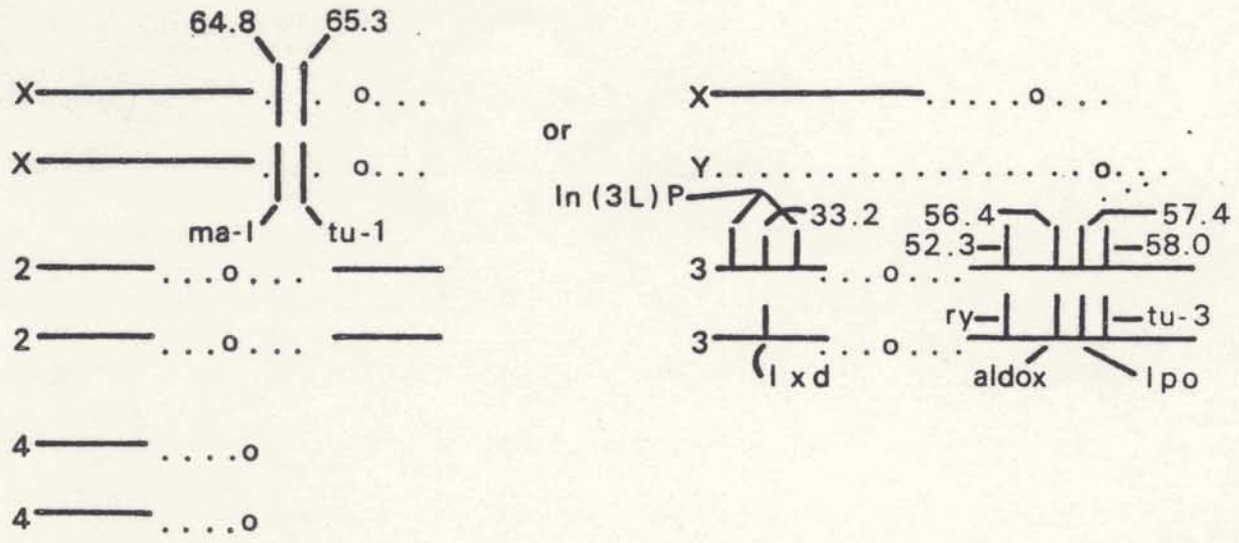
Like aldehyde oxidase a third enzyme, pyridoxal oxidase, is observed in reduced quantities (5% normal) in lxd mutants (Collins and Glassman, 1969) and is totally absent in ma-l flies (Forrest et al., 1961). Collins and Glassman (1969) suggest that the structural gene locus for pyridoxal oxidase is positioned at 57.0 ± on the third chromosome. Mutants at this locus produce low pyridoxal oxidase (lpo) showing only 2% of the wild-type activity.

Several theories have been forwarded to explain the similarities and relationships among these three enzymes. A cofactor theory suggested by Glassman (1965) is favored in explaining similarities between xanthine dehydrogenase and pryidoxal oxidase. Both these enzymes, having separate structural genes, rely on the products of $ma\text{-}l^+$ and $lxd^+$ for the formation of a cofactor necessary for their individual functioning (Collins and Glassman, 1969). Likewise, Courtright (1967) suggested that the functional aldehyde oxidase enzyme relies on the association of aldehyde oxidase and $ma\text{-}l^+$ and is controlled by the lxd locus.

Courtright (1967) suggested a second similarity between aldehyde oxidase and xanthine dehydrogenase by demonstrating maternal effect activity in maroon-like flies.

Kuhn and Cunningham (personal communication) have observed (Fig. 1) that the tumorous-head trait is produced by an interaction of tu-1 and tu-3 with the Payne inversion serving as an adaptive backbone having regulatory powers. Similarly, the structural gene loci for xanthine dehydrogenase, aldehyde oxidase, and pyridoxal oxidase are all located between 52.3 and 58.0 in the right arm of chromosome 3. All three enzymes require a gene product of $ma\text{-}l^{+}$ (next to tu-1) in order to function and all are regulated by lxd (within In(3L)P). Finally, both xanthine dehydrogenase and aldehyde oxidase demonstrate maternal effect activity as does the tumorous-head trait.

How these three enzyme systems relate to the tumorous-head trait is currently under investigation by Kuhn and Cunningham. They have already demonstrated that the Arizona State University (ASU) strain of tumorous-head Drosophila shows severely depressed levels of xanthine dehydrogenase and increased levels of aldehyde oxidase. The difference in activity of the latter enzyme when compared to wild-type laboratory strains is greatest during embryogenesis and metamorphosis. These developmental




| | | |
|---|---|---|
| ——— | = | euchromatin |
| . . .o. . . | = | heterochromatin with centromere |
| X, Y, 2, 3, 4 | = | chromosomes of Drosophila melanogaster |
| ma- l | = | maroon-like mutant lacking xanthine dehydrogenase |
| tu-1 | = | maternal effect genes for head tumors or sac testes |
| l x d | = | mutant causing low xanthine dehydrogenase |
| In (3L) P | = | Payne Inversion |
| ry | = | rosy eye mutant lacking xanthine dehydrogenase |
| aldox | = | aldehyde oxidase structural gene locus |
| lpo | = | mutant causing low pyridoxal oxidase |
| tu- 3 | = | semidominant gene causing head tumors |

Fig. 1. Gene locations for the major genes controlling the tumorous-head trait, xanthine dehydrogenase, aldehyde oxidase, and pyridoxal oxidase.

stages correspond to the tissue determination and differentiation (Kuhn and Cunningham, submitted).

Undoubtly the genetics, regulation, and expression of the tumorous-head trait in Drosophila melanogaster is an interesting study having much area open for investigation. An understanding at the molecular level, as well as at the genetic level, would greatly add to the understanding of abnormal growth and development in general.

Many different approaches to the study of the tumorous-head trait have been used. It was the purpose of the present investigation to utilize yet another approach. If, indeed, there is some product (or products) released by the tumorous-head system that is unique to that system and that is of large enough molecular weight to be antigenic, its presence should be detectable by immunological means. Immunology is a relatively young and rapidly changing science. Until recently it has not been used in those areas outside of the strictly medical fields (Gordon, 1974). Immunology provides a powerful means of detecting very minor differences among substances that otherwise appear similar. It was the intent, therefore, of this investigation to use immunological techniques in the study of the tumorous-head trait.

# CHAPTER II

## MATERIALS AND METHODS

### Immunization of Rabbits

The tumorous-head fly preparations (tuh antigens) utilized in the initial immunizations of two male New Zealand white rabbits were obtained by emulsifying in a tissue grinder 200 male and 200 female tumorous-head flies (ASU strain) in 4 ml of distilled water. A dosage, therefore, of 100 male and 100 female flies per rabbit per immunization was used.

Similarly, the wild-type fly preparations (wild-type antigens) utilized in the immunizations of two other male New Zealand white rabbits were obtained by emulsifying 50 male and 50 female flies each of four wild-type laboratory strains in 4 ml of distilled water. Swedish-C, Urbana-S, Oregon-R-C, and Canton-S strains were used. The dosage, therefore, per immunization per rabbit was 25 male and 25 female flies each of the four laboratory strains mentioned.

Prior to each immunization, a small portion of each preparation was plated out on Tryptic-soy-agar (TSA) and incubated at 37°C for 48 hours. Subsequently, the cultures

were transferred to TSA slants, reincubated at 37°C for 48 hours, and then stored at 4°C for later use in the immunoabsorption studies.

An immunization schedule of 5 intramuscular injections administered at 10 day intervals was set. A six week "resting period" was then allowed the rabbits. Ten days prior to serum collection, booster injections were administered. To prevent paralysis of the immune system, a smaller dose of the antigen preparation was used (Gordon, 1974): in this case, 0.4 of the previous dosage was used.

## Serum Collection

Blood was obtained from the immunized rabbits 10 days and 13 days following the booster injections. Approximately 15 ml of blood was collected each time by ear vein laceration. Samples were refridgerated overnight to allow for maximum shrinkage of the clot which was subsequently removed by centrifugation at 3000 x g for 15 minutes. Those serum samples not immediately used were stored in small volumes at -40°C.

## Serum Titer

Individual serum samples were titered against the antigen responsible for their synthesis. A slide dif-

fusion (Ouchterlony double diffusion) technique employing a standard 7-well pattern was utilized. Serum dilutions of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and 1:1024 were used (Burrell and Mascoli, 1970).

## Globulin Precipitation

Serum samples from each set of rabbits were pooled prior to the ammonium sulfate precipitation of the globulins. Each pooled sample was treated as described below:

Saturated ammonium sulfate, pH 7.0, was added dropwise to the serum sample to a final ammonium sulfate concentration of 40%. After stirring constantly at room temperature for 30 minutes, the mixture was centrifuged at 1000 x g, room temperature for 30 minutes. The supernate was discarded and the pellet was resuspended in one volume of 40% w/v $(NH_4)_2SO_4$. The mixture was centrifuged as above and the precipitate was restored to the original serum volume with 0.01 M sodium phosphate buffered saline pH 7.2 (PBS). Excess ammonium sulfate was removed by passage through a sephadex G-25 column (49.5 x 4 cm, Ace Glass, Inc. Vineland, New Jersey). Elution was with PBS. Protein peaks measured at 280 nm were collected and the eluate was concentrated to the original serum volume by Amicon Ultrafiltration. A 50 UM filter and 18 $lb/in^2$ nitrogen were used. The filtration was performed at 4°C.

The globulin concentration of the sample was estimated by absorbance at 280 nm: a reading of 1.24 U equaling 1 mg of globulin per ml (Calcagno, et al., 1973).

## Drosophila Strain Synthesis

Six strains of Drosophila melanogaster were synthesized according to the flow diagram appearing in the Appendix. It was the intent of this synthesis to locate at the chromosome level genes responsible for the unique tumorous-head antigens that may be involved with the amorphous growths appearing in the head regions of the tumorous-head flies. All flies were maintained by Kuhn at 25°C in ½-pint milk bottles containing standard Drosophila medium.

## Developmental Studies

For immunodiffusion and absorption studies, flies in various developmental stages were collected in accordance with Paulson's (1950) and Bodenstein's (1950) description and timing of metamorphological events in Drosophila melanogaster.

Unetherized male and female flies of the selected strain were placed in empty ½-pint milk bottles and inverted over a stender dish containing standard Drosophila medium. Eggs were collected 16 - 18 hours after laying;

first instar larvae, 40 - 42 hours after laying; second instar larvae, 64 - 66 hours after laying; early third instar larvae, 72 - 74 hours after laying; middle third instar larvae, 80 - 82 hours after laying; and late middle third instar larvae, 92 - 94 hours after laying. Both male and female late third instar larvae (having an average age of 100 - 110 hours) were collected as they crawled up the sides of the culture bottles prior to pupation. Pupae were collected from vials containing standard Drosophila medium in which previously sexed late third instar larvae had been placed and allowed to pupate. Pupae having an average age of 150 - 170 hours (medium pupae) were collected.

## Immunodiffusion Studies

A double diffusion slide technique as devised by Orjan Ouchterlony was used throughout the investigation (Gordon, 1974).

Standard size microscope slides were coated with a 1% Nobel Agar solution prior to the actual pouring of the gel diffusion medium (a 1½% Nobel Agar solution prepared with 0.01 M potassium phosphate buffer, pH 8.0). Approximately 3 ml of the gel diffusion medium were used per slide. Standard gel diffusion equipment (Gelman Instrument Co. Ann Arbor, Michigan) was used throughout the

investigation in the pouring and cutting of the diffusion slides. Seven- and nine-well patterns were utilized. In most studies, the center wells were chosen for the antibody solutions and the outer wells for the various antigen preparations.

Antigen samples used in the diffusion studies were prepared on the day of use by emulsifying 20 male and 20 female flies of the selected strain in 1 ml of 0.01 M potassium phosphate buffer, pH 8.0. Wild-type antigen preparations were obtained by emulsifying 5 male and 5 female flies each of the four previously mentioned strains in 1 ml of the phosphate buffer. Samples for the developmental studies were similarly obtained on the day of use by emulsifying either 400 eggs, 200 first instar larvae, 100 second instar larvae, 40 early, middle, or late-middle third instar larvae, 20 male and 20 female late third instar larvae, or 20 male and 20 female pupae in 1 ml of the phosphate buffer.

Antigen preparations were allowed to diffuse 24 hours at room temperature in a moist chamber prior to the addition of antibody to the center well. Following another 24 hour incubation period at room temperature, the slides were stored a minimum of 24 hours at $4^{o}$ C before staining.

Slides to be stained were placed in a 0.01 M potas-

sium phosphate buffer (pH 8.0) bath for 2 hours. They were removed to a distilled water bath for 10 minutes; stained subsequently in a 0.3% w/v Thiazine Red solution (prepared in 1% v/v acetic acid) for 10 minutes; washed in two changes of 1% acetic acid for 10 minutes each; and finally preserved in a 1% acetic acid - 1% v/v glycerol solution for 10 minutes. The slides were then dried at room temperature.

All slides were examined and interpreted by means of a high intensity light source and a hand lens.

## Immunoabsorption Studies

Absorption of bacterial cultures was accomplished by incubating undiluted globulin and a heavy bacterial suspension (prepared by suspending the bacterial growth of each slant in 2 ml of sterile distilled water) in a ratio of 1:1 at 37°C for 4 hours. The solution was stored at 4°C for 12 - 18 hours and subsequently centrifuged at 1000 x g for 5 minutes to pellet the reacted materials.

Absorption of *Drosophila* antigens was performed as above utilizing a *Drosophila* - antibody suspension prepared by emulsifying 20 male and 20 female flies of the selected strain or 5 male and 5 female flies *each* of the four laboratory wild-type strains in 1 ml of undiluted

globulin. Similarly, for the developmental studies either 400 eggs, 200 first instar larvae, 100 second instar larvae, 40 early, middle, or late-middle third instar larvae, 20 male and 20 female late third instar larvae, or 20 male and 20 female pupae were suspended in 1 ml of the undiluted globulin. Centrifugation as above allowed for the pelleting of reacted materials. The resulting supernatants were used exclusively as sources of unreacted antigen (Burrell and Mascoli, 1970).

## FITC Conjugation

An aliquot of the serum globulins from the rabbits immunized with tuh antigens was set aside for fluorescent labeling. To the globulins were added a 0.1 volume of 1 M carbonate-bicarbonate buffer pH 9.5 and 16 mg of fluorescein isothiocyanate (FITC) per 1000 mg of globulin. The FITC had been previously dissolved in 0.1 ml of acetone. The solution was stirred 20 hours at $4^{o}C$ in a foil lined container. To remove unbound FITC, the mixture was passed through a sephadex G-25 column (as described) and eluted with PBS. Protein peaks at 280 nm were collected and the eluates were concentrated to the original aliquot volume by Amicon Ultrafiltration. A 50 UM filter and 18 $lb/in^2$ nitrogen were used. The filtration was performed at $4^{o}C$.

To the concentrated FITC-globulin solution was added

Whatman diethylaminoethyl (DEAE) cellulose (DE 32 microgranular) pre-equilibrated in 0.01 M sodium phosphate buffer, pH 7.5. Five grams wet weight DEAE were added per ml of globulin solution. The slurry was stirred for 10 minutes and then centrifuged for 10 minutes at 4°C and 1500 x g. The supernatant was collected and stored at 4°C for subsequent concentration.

The pellet was resuspended in 0.01 M sodium phosphate buffer pH 7.5 containing 0.1 M sodium chloride (NaCl) using 1 ml of buffer per gram of DEAE. The mixture was stirred 10 minutes on a magnetic stirrer and then was centrifuged 10 minutes at 4°C and 1500 x g. The supernatant was collected and stored at 4°C for subsequent concentration.

The above elution procedure was repeated once more with the 0.01 M sodium phosphate buffer pH 7.5 containing 0.1 M NaCl and twice more with 0.01 M sodium phosphate buffer pH 7.5 containing 0.2 M NaCl.

The supernatants collected from each elution were pooled and concentrated to the original aliquot volume by Amicon Ultrafiltration. PBS was used as a final wash in five-fold volume and the tagged globulin solution was restored again to its original aliquot volume by ultrafiltration. A 50 UM filter and 18 $lb/in^2$ nitrogen at 4°C were used. The resulting solution was stored in small

volumes at -40°C.

## Fluorescent Staining

Adult, egg (embryo), first instar, second instar, third instar, and Drosophila pupae were collected and frozen prior to squashing. In selected studies, various parts of the adult fly were dissected. Only precleaned standard size microscope slides were used.

The Drosophila squashes were alcohol fixed (95% ethanol); 2 - 3 drops of the undiluted FITC-labeled antibody (anti-tuh antigens) were added; and, the slides were incubated in a moist dark chamber for 30 minutes at 37°C. Following incubation, the slides were washed in two changes of PBS and dipped in distilled water. After a brief drying period, they were examined for areas of fluorescence. A standard Zeiss RA fluorescent microscope with a BG 12 exciter filter, an OG 1 barrier filter, and an HBO 200 W/4 super pressure mercury lamp ultra-violet light source was used under dark field observation at 63x magnification.

In order to determine the specificity of fluorescence a fluorescent inhibition test was performed. Four male and 4 female tumorous-head flies were emulsified in 0.2 ml of the undiluted FITC-labeled antibody. Following incubation for 30 minutes at 37°C, the antibody was

centrifuged 2 minutes at 1500 x g. The supernatant was then added to previously fixed tumorous-head Drosophila squashes. The slides were incubated, washed, and examined as above.

Likewise, in order to determine the specificty of tumorous-head antigens for the fluorescent antibody, 1 male and 1 female from each of the four laboratory wild-type strains were emulsified in 0.2 ml of undiluted FITC-labeled antibody. Following incubation of 30 minutes at 37°C, the antibody was centrifuged 2 minutes at 1500 x g. The supernatant was then added to various strains of previously fixed Drosophila squashes. The slides were incubated, washed, and examined as above.

# CHAPTER III

# RESULTS

## Immunization of Rabbits

No adverse reactions to the immunization or boostering procedures were observed either in the rabbits immunized against antigens from the tumorous-head flies or in those rabbits immunized against antigens from wild-type flies. All animals appeared healthy, ate well, and drank sufficient quantities of water. At the conclusion of the project, autopsy of the rabbits revealed a gross difference in the type of inflamation response at the immunization site.

Areas of necrotic tissue were observed at the sites of injection in both sets of rabbits. In those rabbits immunized against the wild-type antigens, these areas were diffuse and were filled with a viscous pus-like substance. In those rabbits immunized against the tumorous-head antigens, the necrotic areas were localized and were indurated. Subsequent biopsy of the tissue revealed no apparent abnormal cells in either set of rabbits.

During blood collection, it was noted that the rabbits immunized with tumorous-head flies showed

decreased clotting time and marked hemolysis in the serum. Rabbits immunized with wild-type flies showed normal clotting time and little hemolysis in the serum.

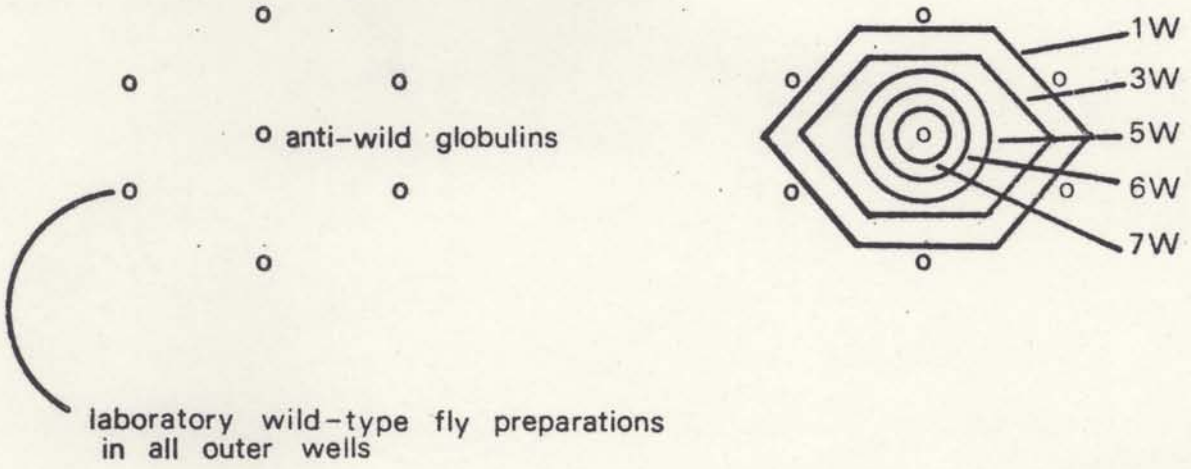
Initially, a greater antibody titer was observed in the pooled serum of the rabbits immunized with the wild-type flies. A titer of 1:256 was noted. The pooled serum of the rabbits immunized with tumorous-head flies showed an antibody titer of 1:64.

All animals were boostered prior to the termination of the project in an effort to obtain sufficient quantities of high titered serum for future experimentation. At that time, the pooled serum from each set of rabbits showed an antibody titer of 1:1024.

## Diffusion and Absorption Studies

All studies were performed not less than three times, each on separate days, utilizing fresh globulin solutions and flies, eggs, larvae, or pupae collected on the day of the study. All results were reproducible with no variation.

Ouchterlony double diffusion of emulsified unabsorbed wild-type fly antigen preparations with antibody directed against wild-type flies (anti-wild globulins) revealed five distinct bands, 1W, 3W, 5W, 6W, and 7W (Fig. 2). Diffusion of the same antigen preparations with antibody



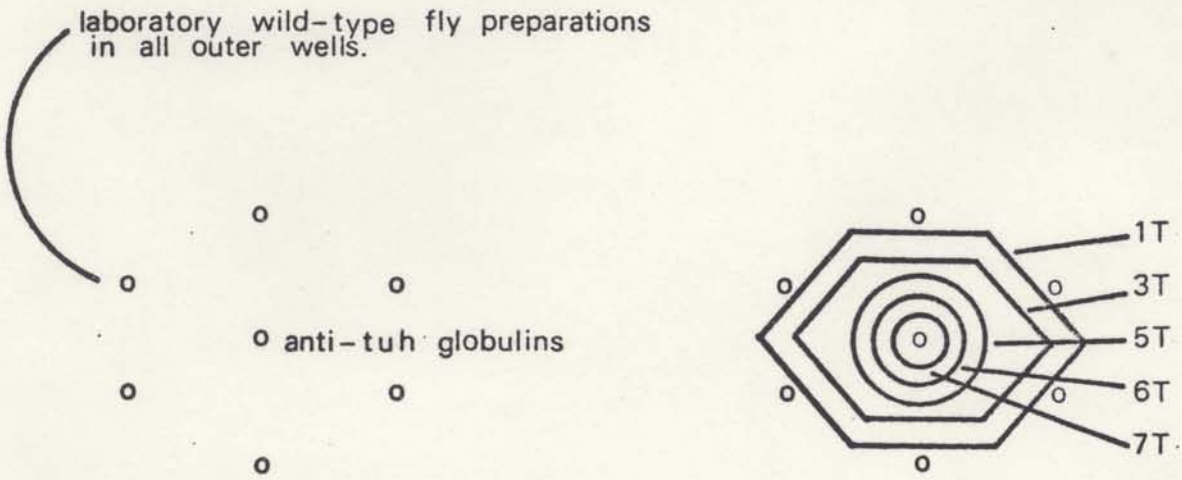




Fig. 2. Ouchterlony slides showing banding patterns resulting from the diffusion of unabsorbed wild-type fly preparations with anti-wild and anti-tuh globulins.

directed against tumorous-head flies (anti-tuh globulins) similarly revealed five distinct bands, 1T, 3T, 5T, 6T, and 7T[1] (Fig. 2).

Diffusion of emulsified unabsorbed tumorous-head fly antigen preparations with anti-wild globulins showed five distinct bands, 1W, 3W, 5W, 6W, and 7W (Fig. 3). Diffusion of the same antigen preparations with anti-tuh globulins revealed seven distinct bands, 1T, 2T, 3T, 4T, 5T, 6T, and 7T (Fig. 3).

Simultaneous diffusion of unabsorbed wild-type fly preparations in the upper three wells and of unabsorbed tumorous-head fly preparations in the lower three wells with anti-wild globulins showed that the five bands present displayed identity patterns (Fig. 4). Similar diffusion of both preparations with anti-tuh globulins showed that five of the seven bands present displayed identity patterns and that two bands, 2T and 4T, were unique to the tumorous-head preparations (Fig. 4).

Bacterial absorption studies revealed no differences in the banding patterns already mentioned. Drosophila antigen absorption studies did however reveal variations in these banding patterns.

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[1] The notation nW or nT was used in labeling the bands by indicating both the band number (n) and the diffusing antibody, anti-wild or anti-tuh globulins respectively.

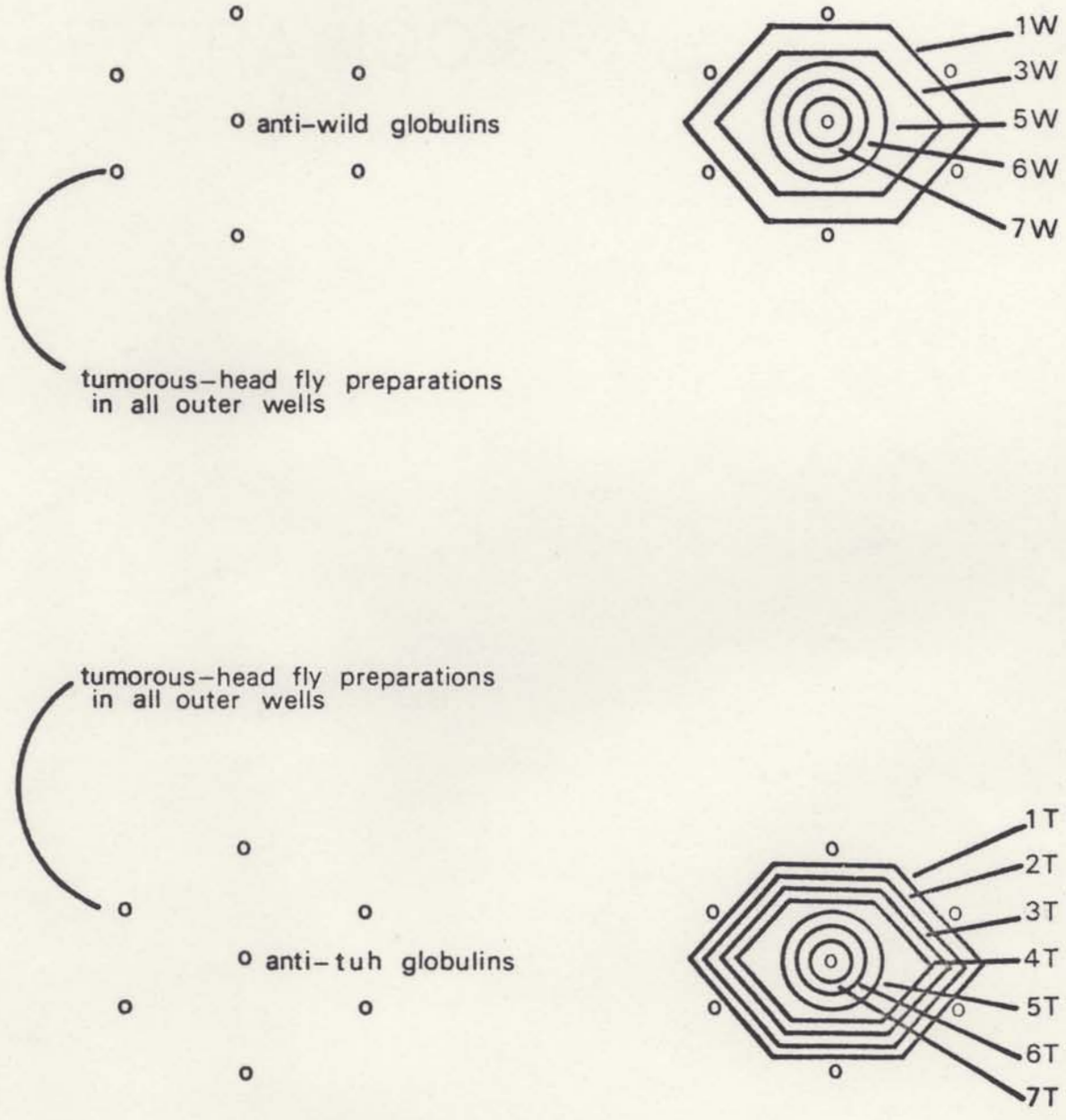



Fig. 3. Ouchterlony slides showing banding patterns resulting from the diffusion of unabsorbed tuh antigens with anti-wild and anti-tuh globulins

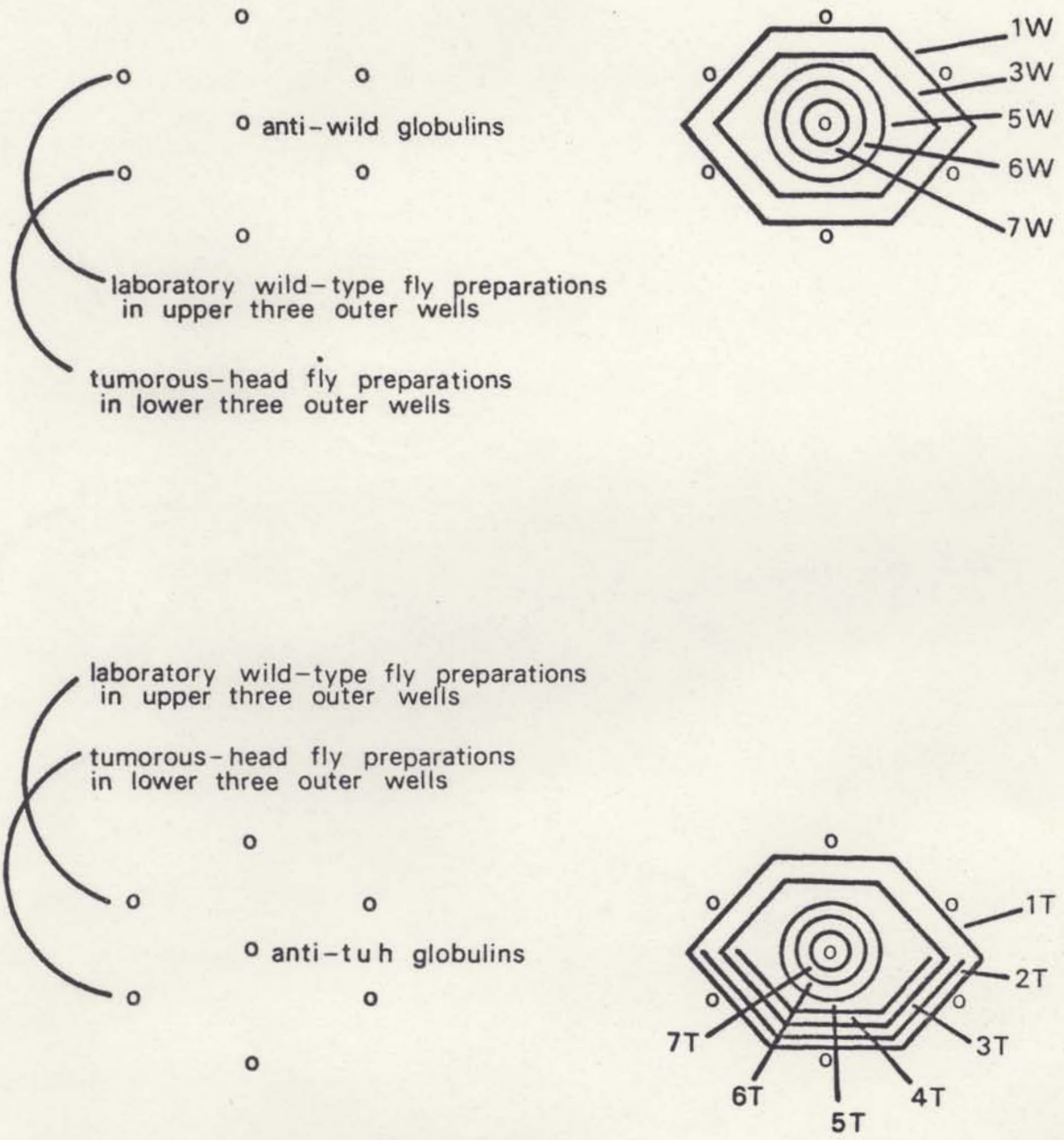



Fig. 4. Ouchterlony slides showing the identity and non-identity patterns resulting from the diffusion of both unabsorbed laboratory fly preparations and tumorous-head fly preparations with anti-wild and anti-tuh globulins.

Absorption of either wild-type or tumorous-head fly preparations with anti-tuh globulins and diffusion with anti-wild globulins revealed only bands 5W, 6W, and 7W. Diffusion of these similarly absorbed antigens with anti-tuh globulins revealed only bands 5T, 6T, and 7T. Absorption of wild-type fly preparations with anti-wild globulins and diffusion with anti-wild or anti-tuh globulins revealed bands 5W, 6W, and 7W or 5T, 6T, and 7T respectively. Absorption of tumorous-head fly preparations with anti-wild globulins and diffusion with anti-wild or anti-tuh globulins revealed bands 5W, 6W, and 7W or 2T, 4T, 5T, 6T, and 7T respectively.

When absorbed with anti-wild globulins and diffused with anti-tuh globulins, preparations of six strains of Drosophila melanogaster synthesized by Kuhn showed the banding patterns given in Table 1. Banding patterns characteristic of wild-type adult flies, tumorous-head adults, and M5; Cy/Pm; Sb/Ubx adults are also given in Table 1. Regardless of genotype, all absorbed preparations showed bands 5T, 6T, and 7T. Only those preparations having a tumorous-head X chromosome showed band 4T and only those preparations having both a tumorous-head X and tumorous-head 3rd chromosome showed band 2T.

Preparations of various developmental stages from both the tumorous-head (ASU) and pooled wild-type

TABLE 1

Ouchterlony diffusion patterns
observed in preparations
of several Drosophila melanogaster strains
absorbed with anti-wild globulins
and diffused with anti-tuh globulins

| Strain | Bands | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1T | 2T | 3T | 4T | 5T | 6T | 7T |
| wild* | - | - | - | - | + | + | + |
| tuh(ASU) | - | + | - | + | + | + | + |
| M5; Cy/Pm; Sb/Ubx | - | - | - | - | + | + | + |
| M5; Cy/Pm; 3** | - | - | - | - | + | + | + |
| M5; 2; Sb/Ubx | - | - | - | - | + | + | + |
| M5; 2; 3 | - | - | - | - | + | + | + |
| 1; Cy/Pm; Sb/Ubx | - | - | - | + | + | + | + |
| 1; Cy/Pm; 3 | - | + | - | + | + | + | + |
| 1; 2; Sb/Ubx | - | - | - | + | + | + | + |

*Pooled antigen preparations from Swedish-C, Urbana-S, Oregon-R-C, and Canton-S strains were used.

**See Appendix.

laboratory strains of Drosophila when absorbed with anti-wild globulins and diffused with anti-tuh globulins showed the banding patterns presented in Table 2 and Table 3. Banding patterns characteristic of preparations of late third instar larvae from three of the Drosophila strains synthesized by Kuhn absorbed with anti-wild globulins and diffused with anti-tuh globulins are presented in Table 4. Bands 5T, 6T, and 7T appear first developmentally with the second instar larvae preparations. Bands 2T and 4T appear first developmentally with late third instar larvae preparations.

## Fluorescent Staining

Fluorescein isothiocyanate labeling of anti-tuh globulins, as previously described, produced a fluorescent antiserum which showed non-specific fluorescence with several Drosophila melanogaster strains tested. The antiserum showed non-specific fluorescence with dissected adult tumorous-head (ASU) flies as well as with tumorous-head (ASU) eggs.

TABLE 2

Ouchterlony diffusion patterns
observed in preparations of various developmental stages
from wild-type *Drosophila* laboratory strains
absorbed with anti-wild globulins
and diffused with anti-tuh globulins

| Stage | Bands | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1T | 2T | 3T | 4T | 5T | 6T | 7T |
| adult* | - | - | - | - | + | + | + |
| pupae | - | - | - | - | + | + | + |
| late 3rd instar | - | - | - | - | + | + | + |
| late mid 3rd instar | - | - | - | - | + | + | + |
| mid 3rd instar | - | - | - | - | + | + | + |
| early 3rd instar | - | - | - | - | + | + | + |
| 2nd instar | - | - | - | - | + | + | + |
| 1st instar | - | - | - | - | - | - | - |
| egg (embryo) | - | - | - | - | - | - | - |

*Pooled antigen preparations from Swedish-C, Urbana-S, Oregon-R-C, and Canton-S strains were used.

TABLE 3

Ouchterlony diffusion patterns
observed in preparations of various developmental stages
from tumorous-head Drosophila melanogaster
absorbed with anti-wild globulins
and diffused with anti-tuh globulins

| Stage | Bands | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1T | 2T | 3T | 4T | 5T | 6T | 7T |
| adult | - | + | - | + | + | + | + |
| pupae | - | + | - | + | + | + | + |
| late 3rd instar | - | + | - | + | + | + | + |
| late mid 3rd instar | - | - | - | - | + | + | + |
| mid 3rd instar | - | - | - | - | + | + | + |
| early 3rd instar | - | - | - | - | + | + | + |
| 2nd instar | - | - | - | - | + | + | + |
| 1st instar | - | - | - | - | - | - | - |
| egg (embryo) | - | - | - | - | - | - | - |

TABLE 4

Ouchterlony diffusion patterns
observed in preparations of late 3rd instar larvae
from three synthesized Drosophila strains
absorbed with anti-wild globulins
and diffused with anti-tuh globulins

| Strain | Bands | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1T | 2T | 3T | 4T | 5T | 6T | 7T |
| 1; Cy/Pm; Sb/Ubx* | - | - | - | + | + | + | + |
| 1; Cy/Pm; 3 | - | + | - | + | + | + | + |
| 1; 2; Sb/Ubx | - | - | - | + | + | + | + |

*See Appendix.

# CHAPTER IV

## DISCUSSION

In the Fall of 1973, experiments employing an immunological approach to the study of tumorous-head *Drosophila melanogaster* were initiated at Florida Technological University. It was hoped that antibody produced in rabbits against the tumorous-head (ASU) strain could be fluorescently labeled and used to tag specifically the head tumors. It was also hoped that this fluorescent antibody would label the eyes, antennae, and other dorsal-lateral parts of the *Drosophila* head in those tumorous-head flies not displaying head tumors. In short, it was believed that a fluorescent antibody technique could be developed for detecting abnormal tissue unique to the tumorous-head strain.

A set back was met when the rabbit being immunized died suddenly. Autopsy of the animal revealed massive areas of necrotic tissue and abnormal growth especially in the gut region. It was immediately assumed that the death of the rabbit was due to the immunization. Within a few weeks, three more rabbits were immunized with the tumorous-head flies in the hope of repeating the previous

results. However, the new set of rabbits showed little more than small areas of induration at the immunization site. These, on biopsy, displayed no apparently abnormal cells. Interests returned, therefore, to the goals of the original project.

The immunized rabbits were bled; their serum pooled, and the globulins precipitated. Before fluorescently labeling the globulins, an attempt was made to demonstrate the action of antibody by means of gel diffusion. Well defined banding patterns resulted and once again, the goals of the original project were temporarily abandoned. A number of Drosophila strains were subsequently tested for banding reactions. Little conclusive data was obtained, however, and as the need for controlled experimentation became evident, the goals and guidelines for the present investigation were formulated.

The concepts of the original project and the investigation reported here are similar and will be discussed together.

A major criticism with the original project was that it lacked control. Without control, any experimentation is virtually useless. The rabbit, which met its untimely death at the onset of the project, was a female New Zealand white rabbit previously used in diethylstilbestrol (DES) studies. The original immunization had been

administered intravenously thereby introducing huge amounts of particulate matter into the bloodstream. It is quite difficult to say, therefore, whether or not the death of the rabbit was due solely to the tumorous-head fly immunization. The weakened system of the rabbit from previous unrelated experimentation, as well as, the route of innoculation certainly played a major role. The subsequent immunization of the three rabbits did not produce the same type of results. Again all three rabbits ( 1 female and 2 male New Zealand whites) had been previously used in unrelated experiments. More specifically, one of the males had been used in DES studies and the others had been used for producing antibody to unreported antigens. All three rabbits were immunized with the tumorous-head flies intramuscularly and all survived to give high titered serum.

In the present investigation, all rabbits were of the same sex, age, and weight. None had been used for experimental purposes prior to the study and all were immunized intramuscularly. None died during the project and all produced serum of high titer. It is interesting to note that the three original rabbits, as well as the two rabbits of the present study, immunized with the tumorous-head flies showed a decreased clotting time and marked hemolysis in the serum. Quite possibly, some

substance present in the tumorous-head fly preparations directly caused the decreased clotting time, which in turn, may have caused the hemolysis of red blood cells during collection. Equally possible is that a substance present in the preparations indirectly caused the observed effects by eliciting the formation of some product by the immune system which acted to decrease the clotting time and increase the amount of hemolysis. That the immune system responded differently to the tumorous-head fly preparations and the wild-type fly preparations is evident in the types of local reactions seen. Without further experimentation geared solely at observing the types of immune responces elicited, it is difficult to speculate on other possible causes.

The immunodiffusion and absorption studies gave reproducible, clear-cut results. The original studies showed the two unique bands, 2T and 4T, for the tumorous-head preparations, as well as, bands 1T, 3T, 5T, 6T, and 7T. A number of laboratory wild-type Drosophila strains were diffused with the original globulin solution and showed the banding patterns seen in the present investigation. As a need for a control serum prepared against wild-type flies became evident, no further experimentation was made.

The current study offers many conclusions. First,

looking solely at the diffusion and absorption studies of only the laboratory wild-type and tumorous-head strains, one very evident conclusion is that band 1T is the same as band 1W (i.e., it is formed by the same antigen-antibody complex) and that band 3T is the same as band 3W. Band 1T was located proportionally the same distance from the center well as was band 1W. Likewise, band 3T was located proportionally the same distance from the center well as was band 3W. Both sets of bands were removed by absorption regardless of the absorbing or diffusing antibody.

Less evident is the relation of band 5T to 5W, of band 6T to 6W, and of band 7T to 7W. Each band was located proportionally the same distance from the center well as its numerical counterpart. None of the sets of bands was removed by absorption despite the absorbing or diffusing antibody, however. As implied by the numbering system chosen, these bands are probably equal. Possibly, the concentration of absorbing antibody was not great enough to completely tie up the responsible antigens and thus, on subsequent diffusion, bands formed. Alternately, any or all of the three bands may have been residual matter. Specifically the 7th band might have been a "halo" or "shadow" created by the antibody as it diffused. Further experimentation is needed to determine the relationships of these particular band sets. Concentration of

the absorbing antibody solution is suggested as a first step.

The most evident conclusion from these studies is that bands 2T and 4T are unique to the tumorous-head fly preparations. There was neither a 2W nor 4W band and absorbing with anti-wild globulins did not remove either band. Although band 2T was positioned very close to band 1T, it was distinct. Band 4T had no close associate and consequently was distinct.

Absorption studies utilizing the synthesized strains of Drosophila melanogaster indicate that the tumorous-head X and 3rd chromosomes are responsible for the production of the antigens which when bound with antibody result in the formation of the 2T and 4T bands. More specifically, the tumorous-head X chromosome is necessary for the 4T band and both the tumorous-head X and 3rd chromosomes are necessary for band 2T. The tumorous-head 2nd and 4th chromosomes play no apparent role in band formation.

As previously stated, the tumorous-head trait has been genetically shown to be produced by an interaction of tu-1 and tu-3 with the Payne inversion serving as an adaptive backbone having regulatory powers (Gardner, 1970). It is possible that a product of the tu-1 locus (when homozygous) is the antigen responsible for forming band 4T. Post-thesis data does not support this possibility,

however. Rather a product of some naturally occuring allele to tu-1 is implied.

Similarly, it is possible that some product of the tumorous-head third chromosome functional only in the presence of another product of the tumorous-head X chromosome (or vice versa) is the antigen responsible for forming band 2T. An interaction of the two chromosomes is evidently necessary for the formation of this band. By itself, the tumorous-head 3rd chromosome does not play a role in band formation; although, it may produce a hapten (incomplete antigen) which when bound to a product of the tumorous-head X chromosome results in the antigen responsible for the 2T band. Post-thesis data does support this possibility.

An interesting consideration in explaining this phenomenon may lie in related enzyme studies. As already stated, the structural gene loci for xanthine dehydrogenase, aldehyde oxidase, and pyridoxal oxidase are all located in the right arm of the third chromosome near the tu-3 locus. All three enzymes require a product of $ma\text{-}1^{+}$ (next to tu-1) in order to function and all are regulated by lxd within the Payne inversion. An interaction of the X and 3rd chromosomes is evident here as it is in the tumorous-head system. Further support is given to the relationship between these three enzyme systems and

tumorous-head by the demonstration of depressed xanthine dehydrogenase (Kuhn and Cunningham, personal communication) and increased aldehyde oxidase (Kuhn and Cunningham, submitted) levels in tumorous-head flies.

Future experimentation along these lines must show that the tumorous-head X and 3rd chromosomes are responsible for the 2T and 4T bands regardless of other genes present. All possible combinations of the two chromosomes must be exhausted (i.e., homozygosity for one or both chromosomes; heterozygosity for one or both; the absence or presence of tu-1, tu-3, and the Payne inversion; and all combinations thereof). Once the responsible chromosomes are indisputably determined, strain syntheses geared at pinpointing the responsible genes should be initiated to demonstrate at a gene level the loci responsible for band formation. Finally, future experimentation must demonstrate whether or not the three enzyme systems are indeed related to the tumorous-head trait and, in the same light, to the banding patterns seen.

Developmentally it was shown that the 5th, 6th, and 7th bands first appear with 2nd instar larval preparations. Both the 2T and 4T bands first appear with late-3rd instar larval preparations. No attempt was made to determine the developmental appearance of the 1st and 3rd bands (only absorbed preparations being used). However, it can be

safely assumed that these bands would appear no earlier than 2nd instar and no later than late-3rd instar. It is intersting to note that the appearance of the 2T and 4T bands corresponds closely with the appearance of -ecdysone, the molting hormone responsible for metamorphosis in Drosophila (Borst, et al., 1974). This suggests that adult proteins are involved in the 2T and 4T band formation. Post-thesis data further demonstrates that the formation of these particular bands is associated with the imaginal discs. Also interesting to note is that the appearance of these bands relates to the point in development during which the difference in activity of aldehyde oxidase between tumorous-head and wild-type laboratory strains is the greatest (Kuhn and Cunningham, submitted). The latter observation adds credence to the possible relationship between the enzyme systems mentioned and the banding patterns.

The initial goal of utilizing a fluorescent antibody to detect abnormal tissue in tumorous-head Drosophila bore the least light on the immunological study of that particular strain. A fluorescent antibody was developed but tests showed it to be non-specific, labeling the wing of one fly, the rostralhaut of another, the eyes, antennae, legs, and abdomen of still others. Many flies completely fluoresced while others showed no fluorescence at

all. The only conclusive data came from making thinner and thinner squashes of the fly specimens. Fluorescence became more defined but the integrity of the structure examined was destroyed in the process. Freeze- or paraffin-sectioning of the fly specimen would offer a solution to preserving structure integrity but would possibly destroy the effectiveness of the FITC label. In answer to this, ferritin is a possible solution. It is obvious that FITC-conjugation is not the method of choice in labeling antibodies directed against anything so large as an insect. The thickness of a usable specimen simply prevents the proper excitation of the fluorescein isothiocyanate by the ultra-violet light source.

In all, immunology has proved to be an extremely useful means for studying the tumorous-head trait in Drosophila melanogaster. Especially by means of immunodiffusion and absorption techniques, reproducible, well defined, and rapid methods for investigating the tumorous-head system have developed. More importantly, however, these techniques can be applied to other systems in Drosophila which merit investigation. Traits can be pinpointed to their responsible chromosomes and possibly even to their responsible genes. Relations between various genetic and biochemical systems can be demonstrated and finally, the points during development when various traits first appear can be determined.

# CHAPTER V

## SUMMARY

A rapid reproducible method for studying eukaryotic systems as a means of further investigating abnormal growth and development is presented.

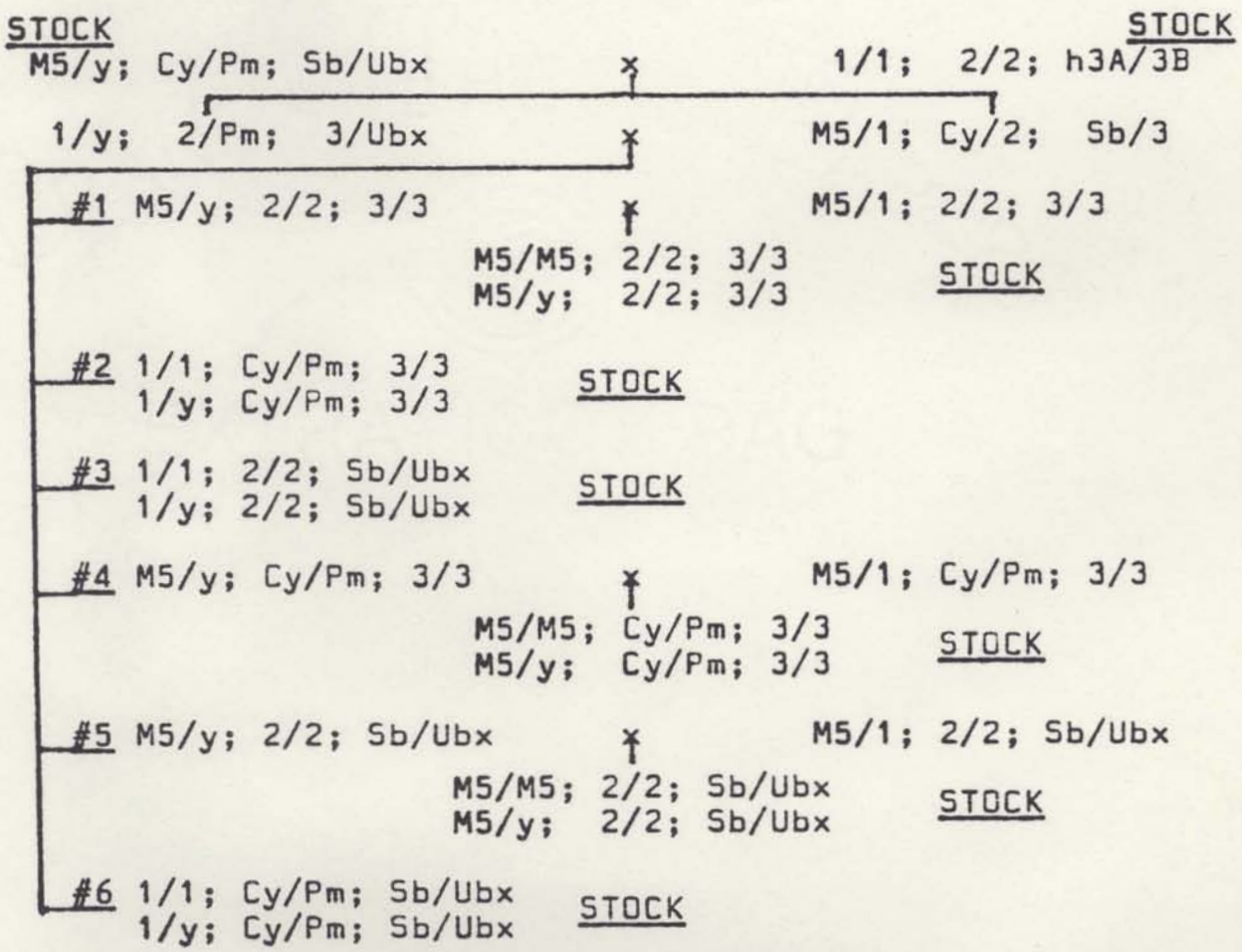
Utilizing immunological techniques, the tumorous-head strain of Drosophila melanogaster was studied.

Antisera was produced in rabbits against both laboratory wild-type and tumorous-head (ASU) fly preparations. Banding patterns common to all strains tested, as well as, patterns unique to the tumorous-head strain were demonstrated through immunodiffusion studies. The action of the tumorous-head X chromosome and the interaction of the tumorous-head X and 3rd chromosomes were shown by immunoabsorption studies to be responsible for apparently unique tumorous-head bands. The developmental appearance of bands common to all strains tested were demonstrated in 2nd instar larval preparations; whereas, the developmental appearance of bands unique to tumorous-head were demonstrated in late-3rd instar preparations. Fluorescent antiserum to tumorous-head fly preparations was shown to non-specifically label all strains tested, probably due to the presence of the three unabsorbed common antigens.

# APPENDIX

## Drosophila Strain Synthesis

Six strains of Drosophila melanogaster were synthesized by Kuhn with the intent of locating at a chromosome level, genes responsible for the unique antigens involved with the amorphous head growths.



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