A STUDY OF THE ENZYME XANTHINE DEHYDROGNASE FROM DROSOPHILA MELANOGASTER

M. S.

MICHIGAN STATE UNIVERSITY

SHELDON D. PARZEN

1963

ABSTRACT

A STUDY OF THE ENZYME XANTHINE DEHYDROGENASE
FROM DROSOPHILA MELANOGASTER

by Sheldon D. Parzen

The purpose of the work reported was to study the biochemical characteristics of xanthine dehydrogenase from Drosophila melanogaster. This involved the development of an assay of enzymatic activity which was linear in relation to enzyme concentration and was sensitive enough to detect activity in single flies.

A method of purification was devised, this resulting in a 528 fold purification of the enzyme. The enzyme was found to have a pH optimum of 8.0. K_m's were determined for various substrate and electron acceptors of the enzyme. A study of the stoichiometry of the reaction using purified preparations indicate that 1 mole of NAD is reduced for each mole of hypoxanthine converted to xanthine and another mole of NAD is reduced for each mole of xanthine converted to uric acid.

A complementation experiment was performed resulting in the production of active xanthine dehydrogenase from extracts of two mutants of D. melanogaster deficient with regard to that enzyme.

A STUDY OF THE ENZYME XANTHINE DEHYDROGENASE FROM DROSOPHILA MELANOGASTER

By

Sheldon D. Parzen

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1963

PREFACE

The author wishes to gratefully acknowledge his indebtedness to Dr. A. S. Fox for his understanding and patience, and under whose direction this work was performed; to Dr. James Kan for his aid in the statistical analysis of some of the data contained in this work; and to his wife for her aid in the preparation of this manuscript and her patience and understanding during the final months of the completion of this work.

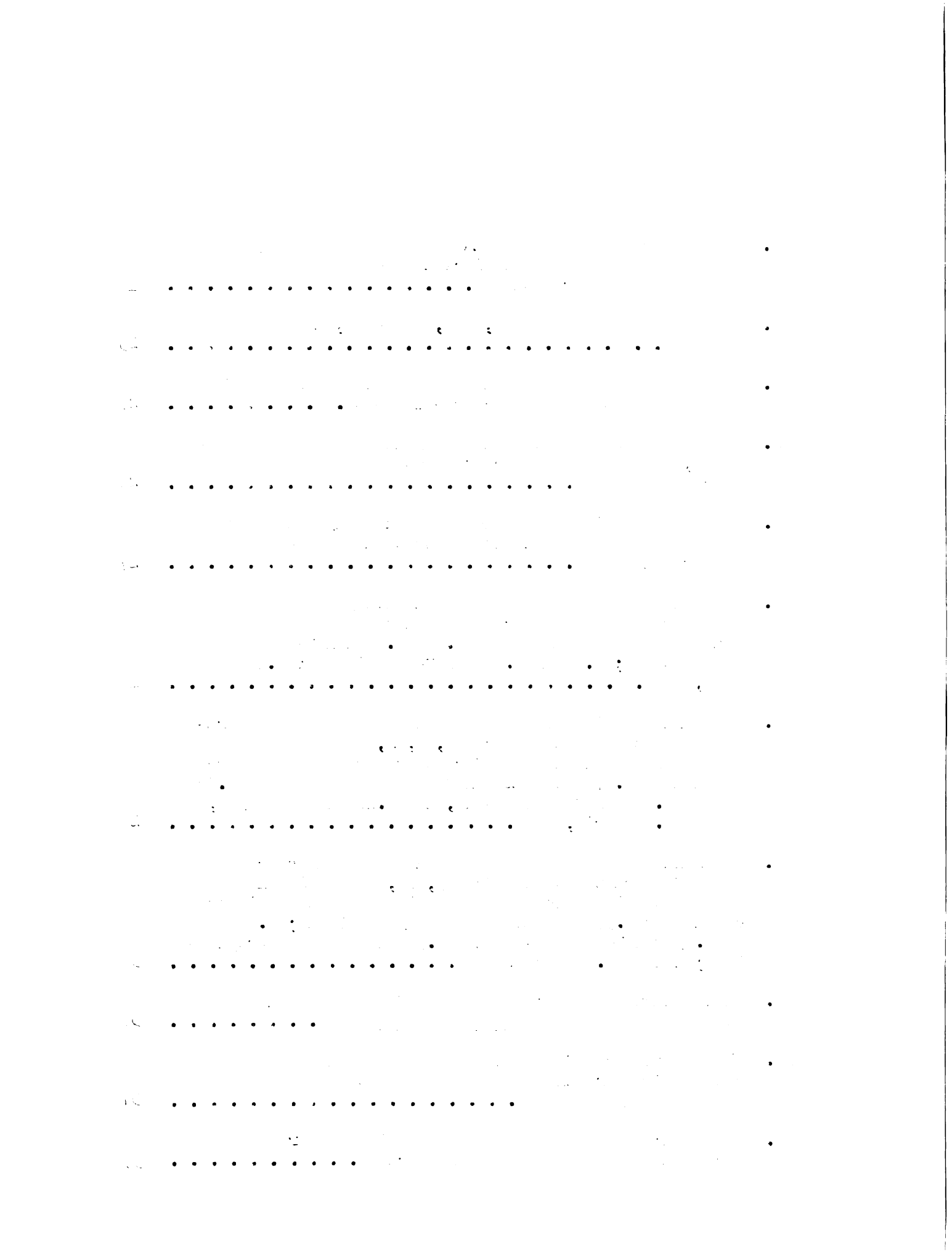
Sheldon D. Parzen

August, 1963

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES



I. INTRODUCTION

In recent years several problems have arisen which are of great interest to the biochemist who is genetically oriented. These are the problems of the genetic control of protein structure; the synthesis of specific proteins in cell free systems; and the general question of the nature of the genetically controlled biochemical mechanisms involved in the differentiation of multicellular organisms.

The first problem facing one who is interested in these questions is that of finding an organism which is suitable for study with regard to these problems. The organism must, by necessity, be multicellular if the problem of differentiation is to be studied. Secondly the mechanism of genetic control, that is the genetics of the organism, must be well understood if the problem of determination of protein structure is to be studied. Finally, knowledge of the biochemical make-up of the organism should be known if cell free systems are to be isolated in which specific proteins can be synthesized. Meeting these criteria, perhaps better than any other organism, is the common fruit fly Drosophila melanogaster, which for years has been under the study of geneticists and is now under the analytical tools of the biochemist.

The enzyme xanthine dehydrogenase, isolable from Drosophila, has several advantages which make it ideal as a subject of study for the aforementioned areas of interest. Initially it is known that wild type Drosophila possess the enzyme and that certain well defined eye color mutants of the organism lack it; that is, there is a strong

correlation between the phenotype of the fly and the occurrence of the enzyme. Secondly, the mutants which lack the enzyme have been well studied on a genetic basis and thirdly, those mutants have been studied on a biochemical basis, not only as regards the presence or absence of the enzyme, but also as regards their general biochemical make-up both as adult and larval forms.

Hence it was the purpose of this work to initiate a study of the biochemistry of the enzyme xanthine dehydrogenase. This work involved the design of a qualitatively and quantitatively sensitive and reliable assay for the enzyme, purification and characterization of the enzyme, and a study of the nature of the biochemical bases for lack of activity of the enzyme in the mutant forms of the organism.

II. REVIEW OF THE LITERATURE

A. GENETICS OF ROSY AND MAROON-LIKE

Rosy (ry) is a recessive mutant which was first isolated as a spontaneous mutant in a Canton (Ohio) wild stock by Bridges in 1938 (Bridges and Brehme, 1944). It was located at 3-51± and described as having a phenotype of a deep ruby eye color, ocelli slightly diluted, and larval malpighian tubes considerably lighter than wild type (Brehme and Demerec, 1942).

In 1956 Hadorn and Schwink reported the isolation of an allele of rosy, $rosy^2$ (ry^2), from other stocks and also located at 3-51. The mutant lacked isoxanthopterin and was non-autonomous for the red eye pigments (Hadorn and Schwinck, 1956a, 1956b; Hadorn and Graf, 1958). Thus $rosy^2$ eye anlagen implanted into wild type hosts develop a drosopterin phenotype identical to wild type. Moreover, implants of wild type malpighian tubes into $rosy^2$ hosts caused the drosopterin content in the hosts' head to approximate that of wild type. It was also reported by these workers that wild type eye discs implanted into $rosy^2$ hosts develop non-autonomously, i.e. resemble $rosy^2$ rather than the wild phenotype.

Eye color in this mutant was described as being dark reddish brown due to the partial reduction of the red eye pigments with the color of the ocelli and testes approximating wild type, but the malpighian tubes were shortened and malformed containing in the lumen

yellow to orange colored globular inclusions. Aside from the lack of isoxanthopterin, there were increased amounts of other pterins. The viability of the mutant was normal at 18°C, but subvital to semilethal in late pupae and early adults at 25°C. Schwink (1960) also noted that rosy and $rosy^2$ were both lacking in xanthine dehydrogenase activity.

Further elucidation of the rosy locus came from the laboratory of Chovnick (Chovnick, Schalet and Kernaghan, 1961a; Chovnick, Schalet, Kernaghan and Talsma, 1962) in their study of recombination at the rosy locus. Using a series of spontaneous and x-ray induced mutants at the rosy locus, they applied a recombinational analysis utilizing schemes which are modifications of systems designed for the study of induced crossing over in Drosophila males which selects for crossovers (Whittinghill, 1950) and which had been applied by Chovnick to other work (Schalet and Chovnick, 1960; Chovnick, Schalet and Kernaghan, 1961b).

The scheme is based on the utilization of various lethal markers adjacent to the area to be mapped, and used in combinations such that all non-crossovers die and only a fraction of the crossovers survive. The selective efficiency of such a system will be a function of the distance between the lethal markers, this system then making possible investigation of genetic fine structure in a higher organism which previously had been attempted only in lower organisms as exemplified by the work of Benzer (1959, 1961).

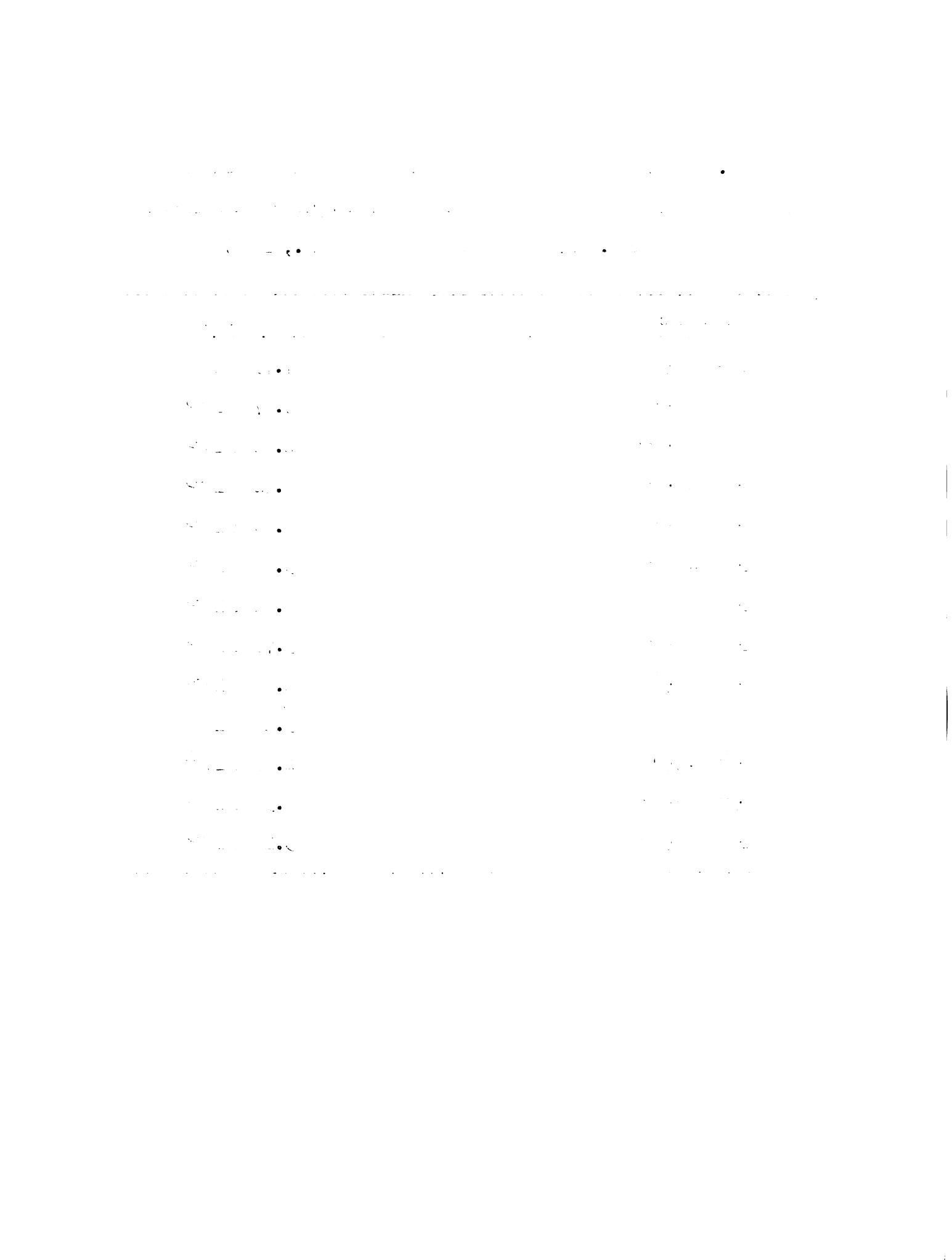
Using the markers curled (3-50.0) and karmoisin (3-52.0) which are recessive visibles; Minute-34 (3-44.4) and lethal-26 (3-52.5)

which are recessive lethals; Deformed (3-47.5), Stubble (3-58.2), and Ultabithorax (3-58.6) which are dominant visibles with recessive lethal effects, Chovnick and co-workers were able to map 14 x-ray induced rosy mutants with map distances ranging from 7.73×10^{-4} for the distance between $rosy^{1}$ and $rosy^{26}$ to 5.87×10^{-3} for the distance between $rosy^{3a}$ and $rosy^{26}$. Table I shows a summary of the recombination data of an unselected sample of 13 independent mutations of rosy tested against $rosy^{26}$ acquired in these selective recombination tests.

Chovnick et al (1962) attempted a conversion of map distance in terms of percent of recombination to distance in terms of nucleotide pairs in a single double helix molecule of DNA. Assuming that recombination is uniform throughout the third chromosome of D. melanogaster, Rudkin (1962) has provided a maximum estimate of the number of nucleotide pairs per map unit of 1.3×10^{6}. Using the minimum estimate of the smallest distance thus far resolved, one emerges with an estimate of 40 nucleotide pairs as the distance separating $rosy^{26}$ and $rosy^{2}$. Estimate of the total length of the rosy cistron, using the maximum distances thus far obtained for $rosy^{3a}$-$rosy^{26}$-$rosy^{41}$, indicates a value of 11.8×10^{3} nucleotide pairs. If one assumes that this structure completely determines the amino acid sequence of that part of xanthine dehydrogenase controlled by the rosy locus, that the genetic code is a three-letter, non-overlapping, commaless code with no "nonsense" information (Crick et al., 1961), and that the average molecular weight of an amino acid in this protein is 100, then the molecular weight of the rosy contribution to xanthine dehydrogenase is estimated to be 390,000. Of

Table 1. Summary of recombination data of an unselected sample of 13 independent mutations of *rosy* tested against *rosy*^{26} in selective recombination tests. (Adapted from Chovnick *et al.*, 1962)

Mutants tested	map distance
$ry^{1} - ry^{26}$	7.73×10^{-4}
$ry^{3a} - ry^{26}$	5.87×10^{-3}
$ry^{4} - ry^{26}$	2.32×10^{-3}
$ry^{5} - ry^{26}$	4.42×10^{-3}
$ry^{8} - ry^{26}$	2.80×10^{-3}
$ry^{9} - ry^{26}$	3.48×10^{-3}
$ry^{23} - ry^{26}$	4.51×10^{-3}
$ry^{24} - ry^{26}$	3.75×10^{-3}
$ry^{26} - ry^{2}$	2.60×10^{-4}
$ry^{26} - ry^{6}$	3.16×10^{-4}
$ry^{26} - ry^{7}$	2.85×10^{-4}
$ry^{26} - ry^{25}$	8.38×10^{-4}
$ry^{26} - ry^{41}$	3.18×10^{-3}

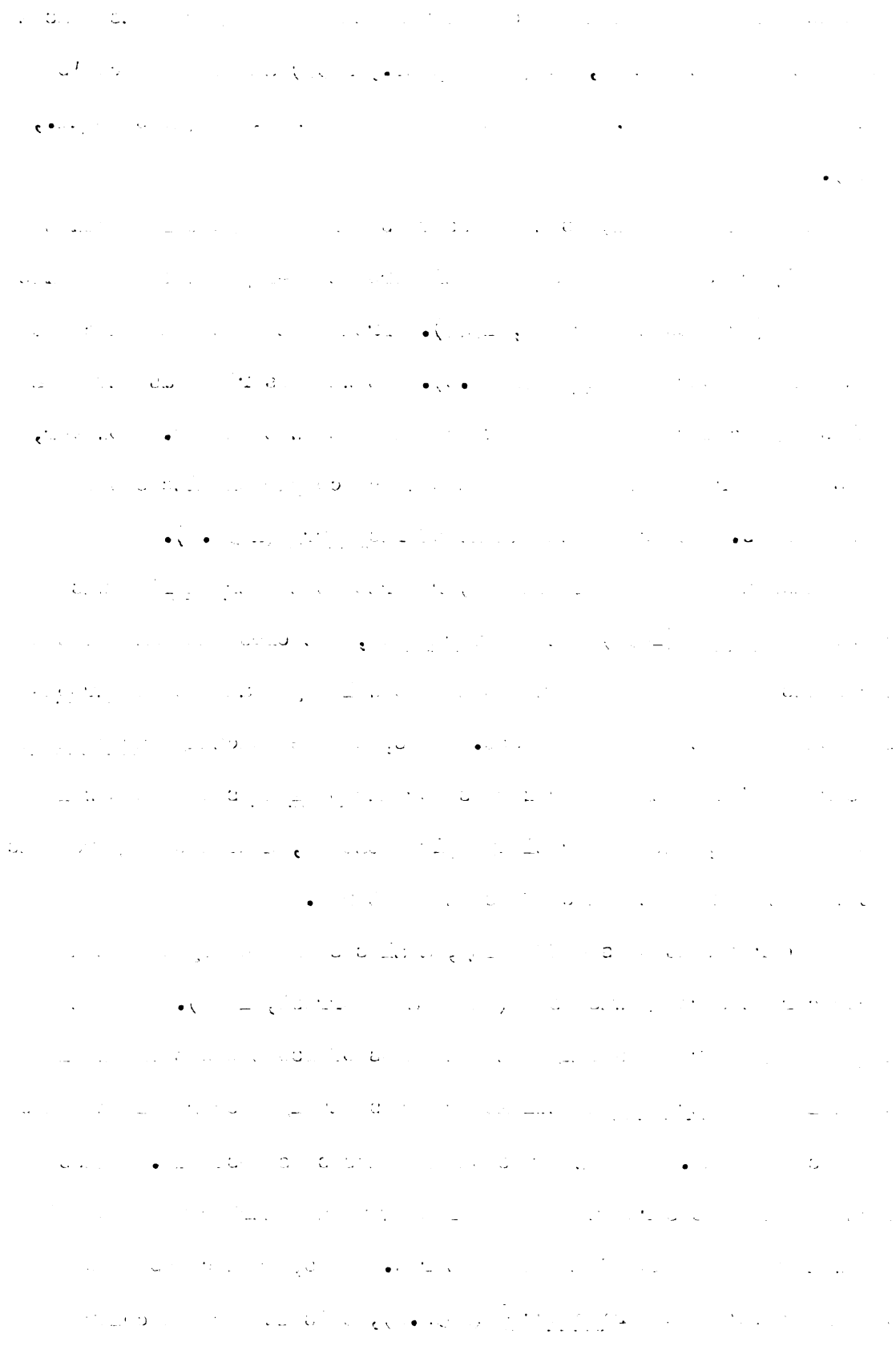


interest is the fact that chicken liver xanthine dehydrogenase has a molecular weight of 480,000 (Remy et al., 1955) and that of cow's milk xanthine oxidase, an estimated weight of 290,000 (Avis et al., 1956).

Maroon-like (ma-1) is a recessive sex-linked eye color mutant originally recovered in a single male from an x-rayed wild type male by Oliver (Bridges and Brehme, 1944). Preliminary mapping placed the mutant locus near vermilion (1-33.0). It was described as having a dullish eye color on emergence which darkened with aging. However, it is brighter than the brown mutant which completely lacks the drosopterins. It is not an allele of raspberry (1-32.8).

Glassman and Mitchell (1959b) reported that maroon-like was closer to Beadex (1-57) than to vermilion, but close mapping of the locus was hindered by the fact that the wild type allele of maroon-like exhibited a maternal effect. Thus, the genetically maroon-like offspring of a female heterozygous for maroon-like showed the wild type phenotype, having normal eye pigmentation, xanthine dehydrogenase activity and trace amounts of isoxanthopterin.

To circumvent this difficulty, analysis of chemotype obtained by paper chromatography was used (Hubby and Forrest, 1960). The maroon-like genotype produces only trace amounts of isoxanthopterin while the wild type maroon-like allele produces easily discernible amounts of this compound. Thus a division of classes is possible. Classification of this sort may be performed with any marker that contains the wild type amount of isoxanthopterin. Thus, in a cross involving the eye color mutant raspberry^2 (1-32.8), this latter eye color



mutant was phenotypically indistinguishable from the double mutant raspberry^2, maroon-like; but chemotypically this double mutant lacked the wild type amount of isoxanthopterin that is characteristic of raspberry^2.

Consequently, using this technique Hubby and Forrest (1960) made a preliminary cross involving the markers yellow (1-0.0), cut^6 (1-36.1), raspberry^2 (1-32.8), forked^5 (1-56.7), and miniature (1-36.1). The results of this cross indicated that maroon-like was situated 12.7$\pm$2 crossover units to the right of forked^5. A more exhaustive analysis was then made using Beadex^3 (1-59.4) as the most distal, well located marker. From this cross maroon-like was located at 67.2 $\pm$ 0.7 on the X chromosome.

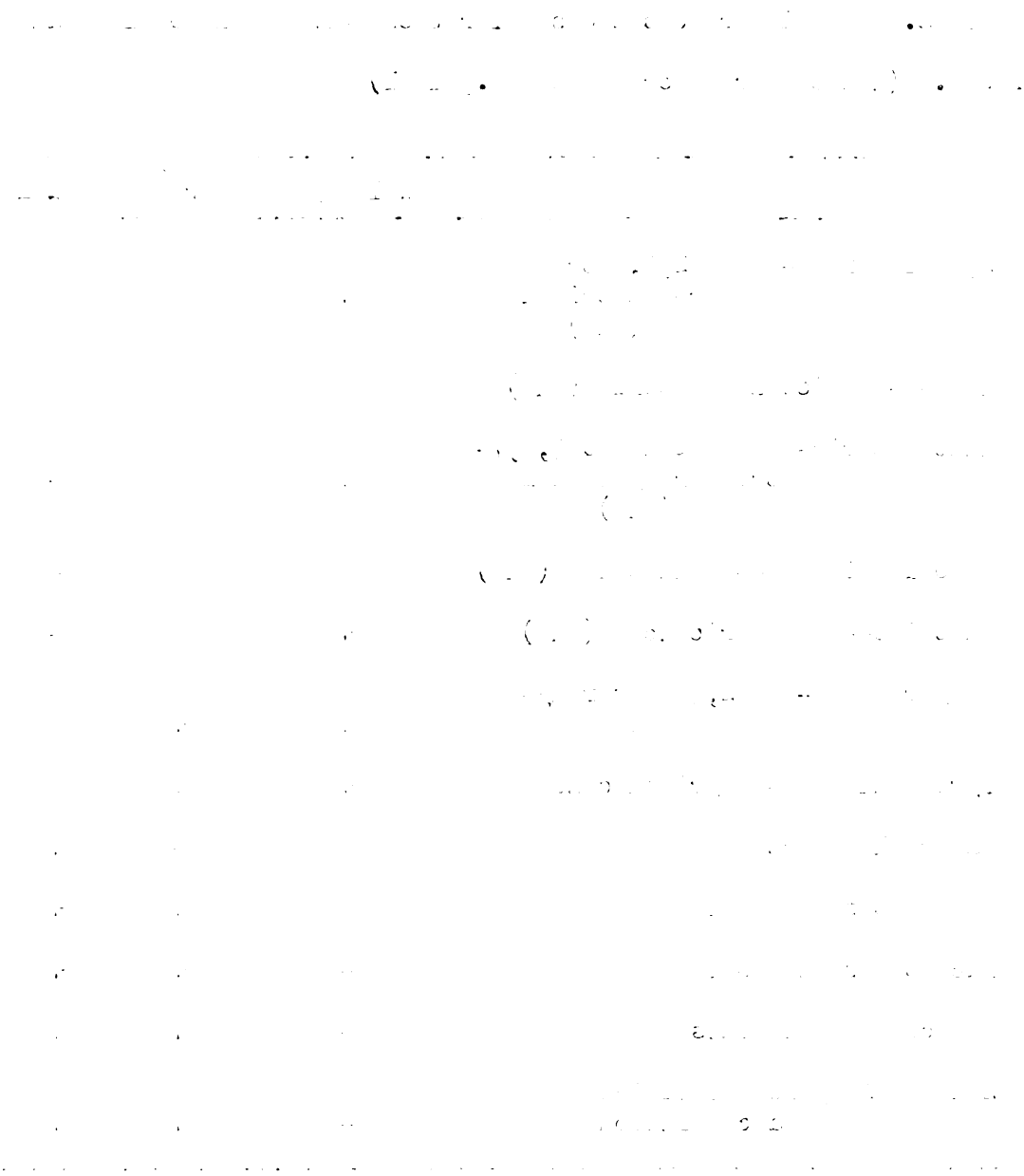
B. BIOCHEMISTRY

From a biochemical point of view, the deficiencies exhibited by the rosy mutants are quite similar to those shown by the maroon-like mutants in that neither can carry out those reactions catalyzed by xanthine dehydrogenase, with the exception of certain reactions not requiring NAD which can be catalyzed by the rosy mutants but not by maroon-like. In addition, rosy exhibits no maternal effect and has no effect on that shown by maroon-like (Glassman and Mitchell, 1959b). The differences and similarities between maroon-like and rosy are shown in Table 2.

The eye pigments in Drosophila are a complex of at least three compounds (Viscontini, Hadorn, and Karer, 1957; Viscontini, 1958).

Table 2. The differences and similarities between maroon-like and rosy^2. (Adapted from Forrest et al., 1961)

Reaction	wild type	ry^2	ma-1
2,4-dihydroxy-----→ 2,4,7-trihydroxypteridine (NAD)	+	-	-
AHP------→ isoxanthopterin (NAD)	+	-	-
Xanthopterin------→ 2-amino-4,6,7-trihydroxypteridine (NAD)	+	-	-
Hypoxanthine------→ Xanthine (NAD)	+	-	-
Xanthine------→ Uric Acid (NAD)	+	-	-
4-hydroxy------→ 2,4-dihydroxypteridine	+	+	-
Pyridoxal------→ pyridoxic acid	+	+	-
Maternal effect	-	-	+
Endogenous hypoxanthine	-	+	+
Lack of isoxanthopterin	-	+	+
Reduced red pigments	-	+	+
2-amino-4-hydroxypteridine accumulation	-	+	+



These pigments have been designated drosopterin, isodrosopterin, and neodrosopterin by Viscontini et al. (1957). The three compounds are orange in visible light and fluoresce orange under ultra-violet light. A fourth pigment which is red under both sources is demonstrable upon electrophoretic separation. The chemical constitution of these compounds is unknown, though their pteridine nature has been demonstrated by Forrest and Mitchell (1955) and confirmed by Viscontini et al. (1957).

Several attempts to establish the chemical nature of these pigment components have been made (Wald and Allen, 1946; Maas, 1948; Heymann, Chan, and Clancy, 1950; Chan, Heymann, and Clancy, 1951). Lederer (1940) first suggested that the pigments were pteridines. This was denied by Maas on the basis of a low nitrogen elementary analysis. Later Forrest and Mitchell (1955) determined that these pigments were pteridines and gave rise by photo-oxidation to 2-amino-4-hydroxy-6-carboxypteridine. The pteridine nature of the photolysis product of the red pigments has also been confirmed (deLerma and Vincentiis, 1955; Viscontini, Hadorn, and Karrer, 1957).

In a series of papers by Forrest and Mitchell (1954a, 1954b, and 1955) five other pteridines have been identified and characterized. This work was stimulated by the discovery of a paper chromatographic technique for the separation of fluorescent compounds and other pigments in Drosophila (Hadorn and Mitchell, 1951). This technique was particularly fruitful in the mutant sepia in which Hadorn and Mitchell demonstrated the lack of drosopterin and the occurrence of large amounts of a yellow fluorescent compound. This

compound is present in trace amounts in wild type strains. Forrest and Mitchell (1954a, 1954b) isolated this pigment in crystalline form from the mutant sepia and characterized it as 2-amino-6, 7-dihydro-4, 6-dihydroxy-6-lactylpteridine.

Shortly after this Forrest, Glassman, and Mitchell (1956) described the absence of isoxanthopterin in the mutant maroon (ma) and maroon-like. Simultaneously, Wadorn and Schwinck (1956a) reported that rosy^2 lacked isoxanthopterin. In addition the former work described the enzymatic conversion of 2-amino-4-hydroxypteridine to isoxanthopterin by extracts of wild type and numerous mutant flies, as well as the absence of this enzyme activity in maroon-like. Rosy^2 was also reported to lack enzyme activity (Glassman, Forrest, and Mitchell, 1957).

This was the first report of a lack of enzyme activity associated with a mutant in Drosophila melanogaster. Moreover, the mutants involved affected a number of well defined and easily identifiable compounds.

Forrest, Glassman, and Mitchell (1956) demonstrated that extracts of wild type stocks of Drosophila melanogaster contain enzyme activity for the conversion of 2-amino-4-hydroxypteridine to isoxanthopterin, hypoxanthine to xanthine to uric acid, xanthopterin to leucopterin, and benzaldehyde to benzoic acid. Later, Glassman and Mitchell (1959a) showed that this activity could be ascribed to a xanthine dehydrogenase rather than to a xanthine oxidase because well dialyzed preparations require methylene blue or NAD for activity. Nawa, Taira, and Sakaguchi (1958) derive the same conclusion. In this latter report

it was found that extracts treated with sufficient activated charcoal to remove the last traces of fluorescent materials are essentially devoid of activity without adding an electron acceptor such as NAD.

Hubby and Forrest (1960), using an assay based on the reduction of NAD with activity expressed as positive change in optical density at 340 mu/minute, found a pH optimum for the enzyme at 8.0 with an optimum molar concentration for NAD being unity with respect to hypoxanthine. Glassman and Mitchell (1959a) reported the oxidation of purines, pteridines, and aldehydes by the enzyme. They found the K_m for 2-amino-4-hydroxypteridine to be 6.7×10^{-6} M; for xanthine and hypoxanthine, 2.5×10^{-5} M and 2.1×10^{-5} M, respectively; the latter compound was oxidized 2.5 times faster than 2-amino-4-hydroxypteridine with NAD as the electron acceptor. However, this assay utilizing NAD was found to be undesirable in the measurement of the conversion of 2-amino-4-hydroxypteridine to isoxanthopterin. This is due to the overlapping of the 340 mu peak of reduced NAD with an absorbance peak of 2-amino-4-hydroxypteridine, thus making the assay unsuitable for quantitative studies of the reaction (Parzen and Fox, unpublished data).

A purification scheme for the enzyme was reported by Glassman and Mitchell (1959a) which utilized ammonium sulfate fractionations and chromatography on calcium phosphate gel. However, this scheme resulted in a purification of only 10 to 50 fold with a recovery of enzyme activity between 50 to 75 percent. The assay devised by these workers for this work was based on the change in fluorescence when

2-amino-4-hydroxypteridine is converted to isoxanthopterin. Parzen and Fox (unpublished data), however, found this assay undesirable because of secondary interactions between the fluorescent emissions of isoxanthopterin and 2-amino-4-hydroxypteridine when together in reaction mixtures. Consequently, this assay was also non-usable for quantitative work with the enzyme.

The maternal effect exhibited by the maroon-like genotype has the result of causing maroon-like progeny from females with a wild allele of maroon-like to exhibit a wild type phenotype. Hence, the male progeny from a cross of an attached-X female (homozygous for the wild allele of maroon-like) with maroon-like males unexpectedly showed a wild type eye color although genetically they were maroon-like. Similarly all progeny of a cross of heterozygous maroon-like females with maroon-like males were phenotypically wild type, even though a 1:1 ratio of wild type to maroon-like was expected. These effects were noted and studied by Glassman and Mitchell (1959b), Hubby and Forrest (1960), and Glassman and McLean (1962).

The maternal effect involved not only eye color, but also morphology and function of the malpighian tubes. In maroon-like flies not exhibiting the maternal effect there is aberrant morphology and function of the malpighian tubes (shorter, irregularly shaped, puffed up, containing yellow to orange globules). However, in maternally affected flies these abnormalities are not exhibited (Schwinck, 1960).

This maternal effect exhibited itself only in maroon-like flies which emerged in the first six to eight days after the first appeared;

after this a short period occurred during which flies emerging had eye colors intermediate between maroon-like and wild, but later none were maternally affected. However, when the egg-laying female was transferred to new food, the maternal effect again appeared (Glassman and Mitchell, 1959b). This would indicate that the previously mentioned change was not due to depletion of the maternal substance in the aging female, but to some environmental cause. Glassman and Mitchell also pointed out that the maternal substance was probably not xanthine dehydrogenase, since females homozygous for rosy, and hence lacking the enzyme, can still have maternally affected maroon-like progeny.

Glassman and McLean (1962) also found xanthine dehydrogenase activity in maternally affected larvae, but none in eggs, the amount of enzyme slowly declining during development. This observation indicates either activation, complementation, or synthesis de nova of xanthine dehydrogenase during early development of maternally affected maroon-like flies. These workers also found xanthine dehydrogenase activity in maroon-like larvae dericed from doubly attached-X, scarlet, rosy^{1} females which indicated to them a type of complementation in vivo in which the product of the wild type allele of maroon-like in the maternal parent reacts with the product of the wild type rosy gene in the progeny to produce active xanthine dehydrogenase.

An example of this type of maternal influence is that of some egg color mutants in Bombyx (Kikkawa, 1957) in which the pigmentation of the egg is passively passed from the female parent into the egg, but is diluted out during development. In contrast to this observation

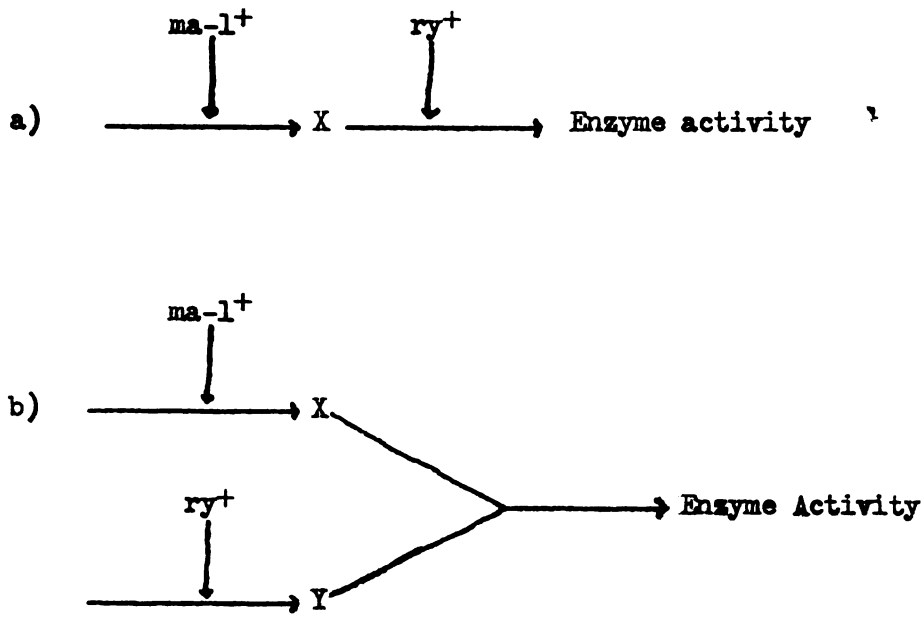
is the finding of Fox (1958, 1959) and Fox, Yoon, and Mead (1962) wherein they report that a segment of the Y chromosome in D. melanogaster, when present in the oocyte of a female, has a structural effect on a protein in her offspring even though the latter lack the chromosome segment in question. Since the effect persists through ontogeny, it implies the existence of a self-perpetuating information transfer mechanism in protein synthesis. This is quite dissimilar to the maternal effect of maroon-like in which there is a progressive dilution and eventual disappearance of the characteristic passed from parent to offspring.

The presence of xanthine dehydrogenase activity in maroon-like larvae derived from attached-X females having the $rosy^1$ gene indicates a type of complementation in vivo, in which the product of the wild type allele of maroon-like in the maternal parent reacts with the product of the wild type rosy gene in the progeny to produce active xanthine dehydrogenase (Glassman and McLean, 1962).

Thus, there are at least two loci which control xanthine dehydrogenase in Drosophila melanogaster. The fact that either locus can cause the deficiency of the enzyme while the other is normal indicates that each locus has a different function in the genetic control of this enzyme. This is further indicated by the fact that the maroon-like and rosy mutants can complement each other in vivo.

Glassman and Mitchell (1959a) have proposed two possible schemes for the action of the two genes in the production of xanthine dehydrogenase (figure 1). Xanthine dehydrogenase could have two sites of enzyme activity each controlled by a different gene. (Dual

Figure 1. Schemes proposed by Glassman and Mitchell (1959a) for the action of the two loci in the production of xanthine dehydrogenase.



functions for the classical xanthine oxidase and chicken liver dehydrogenase, where purines, pteridines, and aldehydes are oxidized at one site and reduced NAD is oxidized at another site, have been suggested by other data (De Renzo, 1956)). Thus the mutant rosy^2 would affect the dehydrogenase site (perhaps the site where the co-factor is bound), leaving the site of oxidation unaffected but probably with a markedly reduced efficiency. On the other hand, the mutant maroon-like would necessarily affect the site of substrate binding, consequently interrupting both the oxidase and dehydrogenase activities. Alteration of the substrate site would be assumed to have little or no effect on the protein's ability to react with specific antibodies. Hence, cross reacting ability was found by Forrest, Hanley, and Lagowski (1961) with maroon-like and wild type extracts to antibodies formed to the enzyme itself.

This explanation would be more in accord with the second of Glassman and Mitchell's schemes for the action of the two genes on the activity of the enzyme (figure 1b). Thus, maroon-like flies would contain the product of the wild type rosy allele, Y, having all the cross-reacting ability but no enzyme activity. In contrast, rosy flies would contain the product of the wild type maroon-like allele, X, having no cross-reacting ability, but which would be enzymatically active with an efficiency, however, less than that of wild type flies. In fact, by an extension of this scheme, it becomes somewhat analogous to the system in Escherichia coli controlling tryptophan synthetase (Crawford and Yanofsky, 1958), where two proteins, A and B, are necessary to make a fully functional enzyme, although each, by itself,

has some enzyme activity in the half reactions involved in the production of tryptophan. Cross-reacting activity, in contrast to this, is confined to one of the proteins (Lerner and Yanofsky, 1957).

On this basis, extracts of the two mutants when incubated together in specific fashions should result in xanthine dehydrogenase activity if this incubation allows for assumption of proper tertiary and quaternary structure necessary for enzymatic activity. Glassman (1962) reported just such an experiment, utilizing a fluorometric assay supposedly sensitive enough to detect activity of the enzyme in single flies (Glassman, 1962). Results of this experiment indicated that although the mutant extracts incubated alone exhibited no xanthine dehydrogenase activity, incubation of mixtures prepared by combining extracts from the mutants of each locus results in the production of enzyme activity.

III. MATERIALS AND METHODS

A. STOCKS

1. Oregon-R (Ore-R). A wild type stock maintained by Dr. A. S. Fox. The stock contains a slight ebony allele, a slight branching of the posterior crossvein and an occasional scooped wing. It is homozygous for Df (2) Ore-R at the tip of 2R.

2. Oregon-R-isogenic (Ore-R-I). A wild type stock originally isogenized by J. Schultz and subsequently maintained in this laboratory by single pair, brother-sister matings for 168 generations at the beginning of this work.

3. Maroon-like (ma-l). A recessive sex-linked eye color mutant. It has a dullish eye color which darkens with aging but is brighter than brown which lacks all the drosopterins. This stock was obtained from Dr. Arthur Chovnick.

4. Rosy (ry), Rosy^2 (ry^2). Recessive eye color mutants having deep ruby eye color. These stocks were also obtained from Dr. Chovnick.

5. White-apricot no. 2 (w^{a2}), white-apricot-Sydney (w^a-Syd). Two eye color mutants in the white region typified by orange pink eye color which is darker in the males.

6. Swedish and Samarkand and Oregon-R-Sydney. Three additional wild type stocks.

Oregon-R-Sydney, Oregon-R-I, and white-apricot-Sydney are all inbred isogenic stocks. Samarkand, Swedish, and white-apricot no. 2 are non-inbred isogenic stocks.

B. GROWTH AND COLLECTION OF FLIES

All stocks were grown in half-pint milk bottles at 25°C. on a standard corn meal-molasses-agar medium enriched with brewer's yeast and seeded with living yeast. Flies were collected by light etherization after a period of three to four weeks, and either used immediately or stored in a freezer at -20°C.

C. PREPARATION OF EXTRACTS

Extracts were prepared by homogenizing the flies in a 2.5 w/v ratio of 0.1 M Tris (hydroxymethyl) amino-methane ("Tris") buffer, pH 8.0 which was 5 mg/ml with respect to crystalline serum bovine albumin in an all-glass, conical homogenizer at 5°C. The homogenate was then centrifuged at 30,000 x g for 30 minutes at 0°C. To the resulting supernatant was added Norite-A to give a concentration of 100 mg/ml. This was allowed to stand in the cold for one hour with occasional stirring, immediately after which the mixture was centrifuged at 30,000 x g for 30 minutes at 0°C., and the resulting supernatant was poured through a coarse sintered glass filter to remove any remaining charcoal. The resulting filtrate, designated extract, was then assayed for enzymatic activity.

The preparation of extracts of single flies for enzymatic assay duplicated the foregoing assay with the exceptions that the fly was homogenized in 1 ml. of the buffer, 10 to 20 mg. of Norite-A then being added to this homogenate. This was then allowed to stand for one hour at 5°C., and was centrifuged.

D. ASSAY METHODS

Assay of the enzyme is based on the conversion of nicotinamide-adenine-dinucleotide (NAD) to reduced NAD, or thionicotinamide-adenine-dinucleotide (thio-NAD) to reduced thio-NAD, with the concommitant increase in absorbance at 340 mu in the former case and at 395 mu in the latter case.

Thio-NAD was purchased from Pabst Laboratories. 2-amino-4-hydroxypteridine (AHP) was purchased from General Biochemicals. Other samples of AHP were gifts from Lederle Laboratories and from Dr. Arthur Chovnick. 2-amino-4, 7-dihydroxypteridine (isoxanthopterin) was also a gift of Dr. Chovnick. Tris (hydroxylmethyl) aminomethane, "Tris," was purchased from Sigma Chemical Co.

All solutions were made up in 0.1 M Tris buffer, pH 8.0. Hypoxanthine was prepared in concentrations of 5.1×10^{-3} M and 2×10^{-4} M; Thio-NAD, 3.43×10^{-3} M; NAD, 10^{-2} M; xanthine, 3.3×10^{-3} M; NADH, 10^{-1} M; uric acid, 7×10^{-5} M; AHP, 2×10^{-4} M.

Reaction mixtures contained either hypoxanthine or xanthine and a suitable electron acceptor in the form of NAD or thio-NAD to follow the reaction in the forward direction. Increase in absorbance at 340 mu or 395 mu was then followed on a Beckman DU spectrophotometer with a Gilford Recording attachment at 25°C. Thio-NAD was used at these times when increased sensitivity was desired. The increased sensitivity is due to the fact that reduced thio-NAD has an a_m of 11.3×10^3 in comparison to the a_m of reduced NAD of 6.22×10^3.

Conversion of AHP to isoxanthopterin was followed by using thio-NAD as the electron acceptor and recording the increase in absorbance at 395 mu as the thio-NAD was reduced. In this case the use of thio-NAD was mandatory due to the overlapping of absorbance curves of $NADH_2$ and AHP (figure 2).

One unit of enzyme activity is defined as equal to an increase in optical density at 340 or 395 mu (depending on electron acceptor) of 0.001 per minute per ml. of enzyme preparation.

Study of the reverse reaction, that is, the conversion of uric acid to xanthine to hypoxanthine, with the concommitant oxidation of reduced NAD, was attempted by using an assay mixture containing uric acid and reduced NAD and observing the decrease in absorbance at 340 mu.

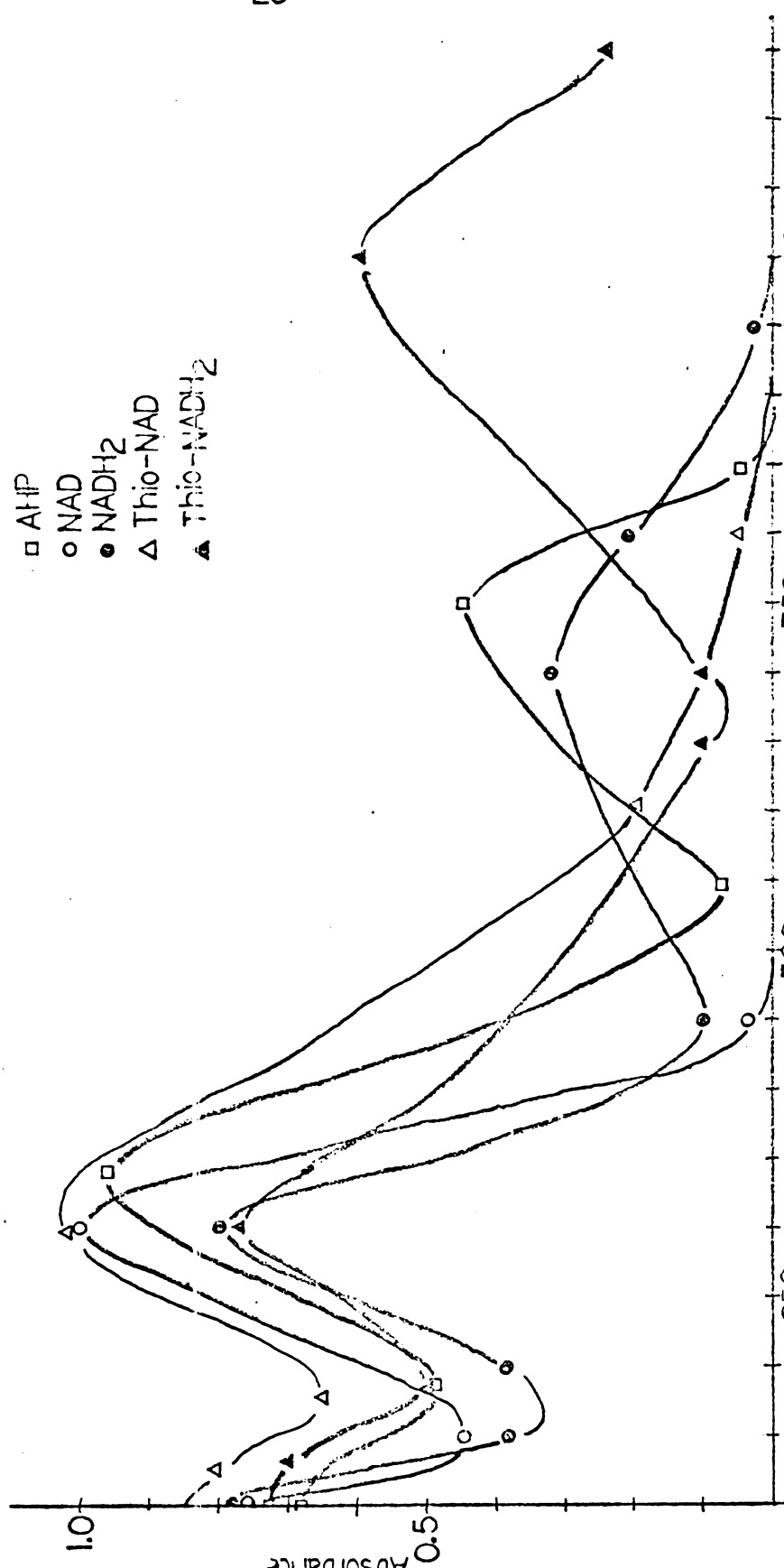


Figure 2. Absorption spectra of AHP, NAD, thio-NAD, $NADH_2$, and thio-$NADH_2$. Past Laboratories, Circular No. OR-18.

IV. RESULTS

RELIABILITY OF ASSAY: The dependance of measurable enzyme activity on suitable substrate and electron acceptor is demonstrated in figure 3. In this experiment, using hypoxanthine and NAD, optical density at 340 mu increased linearly for about 2 to 3 minutes and reached a maximum after 15 minutes of incubation. No increase occurred in the absence of NAD or enzyme. A slow increase was observed in the absence of hypoxanthine. This was probably attributable to a small amount of endogenous substrate in the crude extract, and other experiments have demonstrated that complete dependence on added substrate occurs after an additional passage of the extract over Norite.

Figure 4 shows the relationship between measured enzyme activity and the concentration of crude extract using hypoxanthine as substrate and NAD as electron acceptor. Figure 5 shows the same relationship using hypoxanthine and thio-NAD. In both cases the relationship is linear over the range studied. The same linearity is exhibited with extracts from single flies (figure 6).

It can also be demonstrated that the amount of measured activity is linearly proportional to the number of flies homogenized in a given volume of buffer. Figure 7 demonstrates this for one to eight flies homogenized in 1 ml. of buffer, while figure 8 shows such a relationship for 5 to 40 flies homogenized in 5 ml. of buffer.

Figure 3. The dependance of enzyme activity on the presence of suitable substrate and electron acceptor. Complete reaction of 3 ml contains: 3.00 ml, 2×10^{-4} M hypoxanthine; 0.2 ml, 10^{-2} M NAD; 0.3 ml, of enzyme in 0.1 M Tris, pH 8.0. (o—o, Complete rx mixture; □—□, less hypoxanthine; △—△, less NAD or enzyme.)

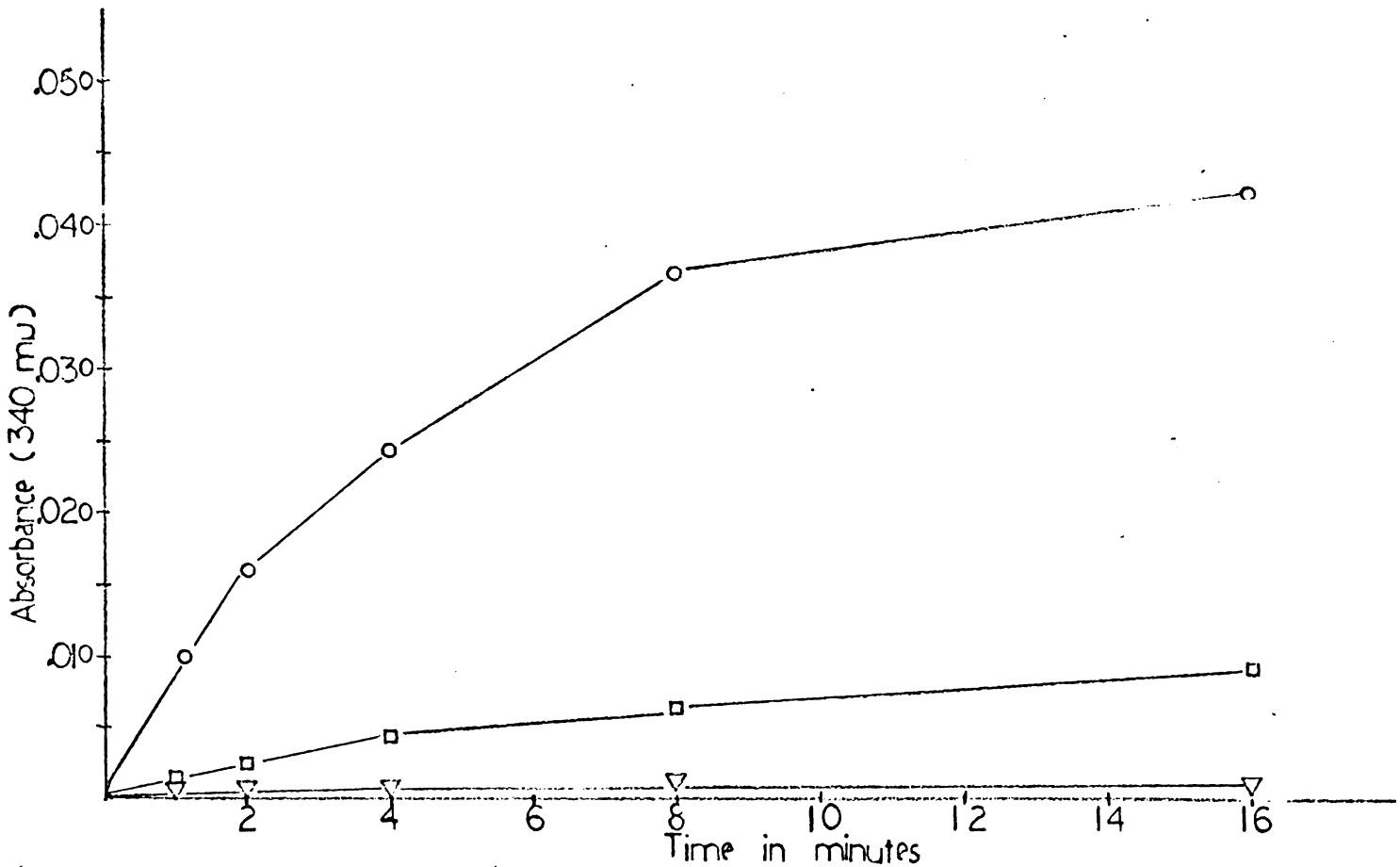


Figure 4. The relationship between activity and enzyme concentration using hypoxanthine as substrate and NAD as electron acceptor. Reaction mixture contains 2.7ml of 2×10^{-4}M hypoxanthine; 0.3ml of 10^{-2}M NAD and enzyme varying in volume from 0.0ml to 0.5ml; 0.1M Tris, pH 8.0.

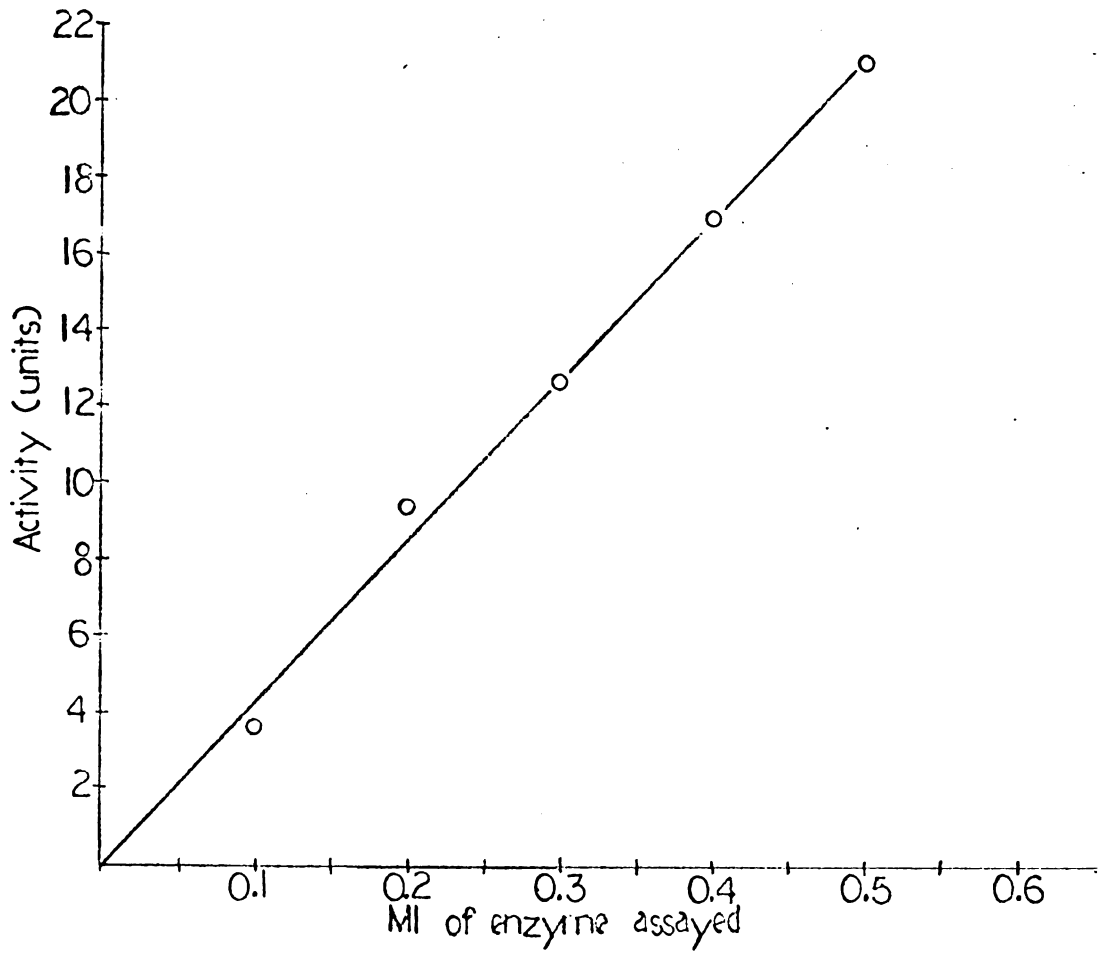


Figure 5. The relationship between activity and enzyme concentration using hypoxanthine as substrate and Thio-NAD as electron acceptor. Reaction mixture contains 2.7 ml of 2×10^{-4} M hypoxanthine; 0.3 ml of 3.43×10^{-3} M Thio-NAD and enzyme varying in volume from 0.0 ml to 0.5 ml; 0.1 M Tris, pH 8.0.

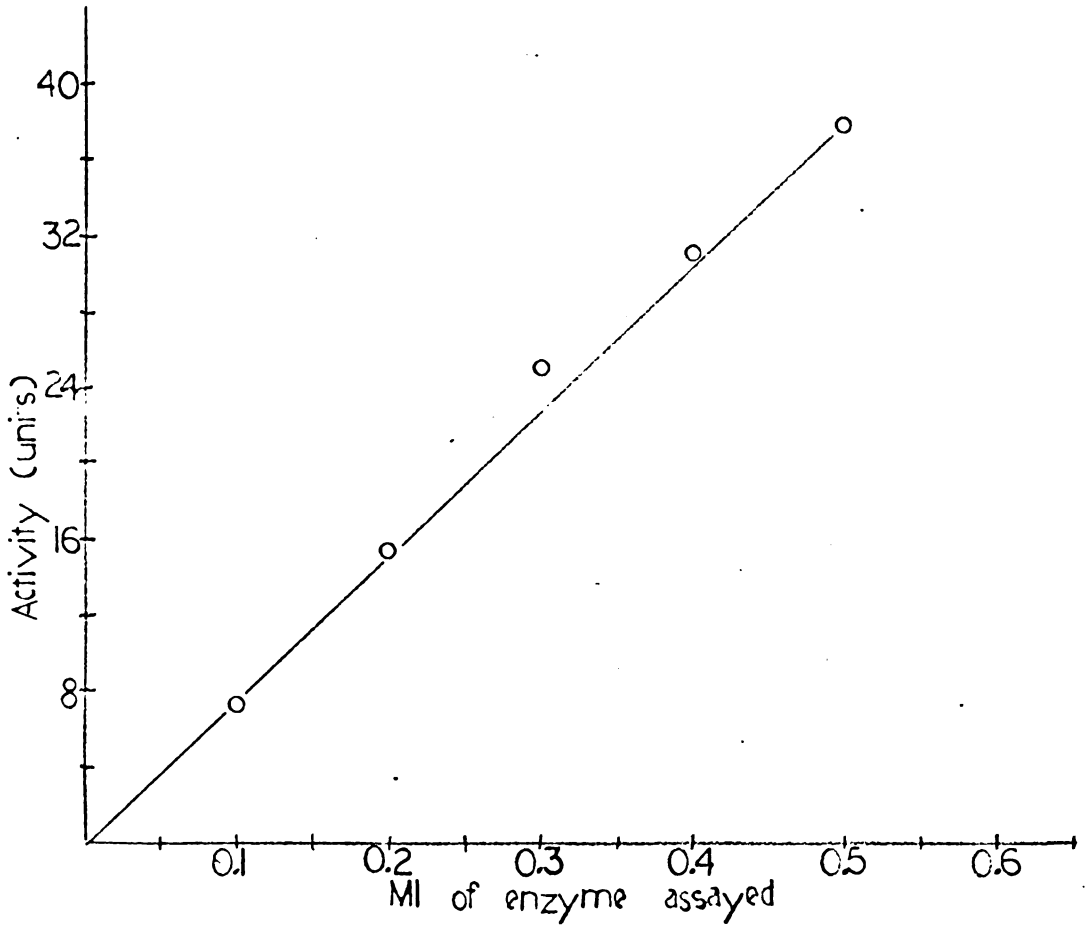


Figure 6. The relationship between enzyme activity and the concentration of an extract from a single fly in a reaction containing 0.57ml of 2×10^{-4}M hypoxanthine; 0.39ml of 3.43×10^{-4}M Thio-NAD; 0.1M Tris, pH 8.0.

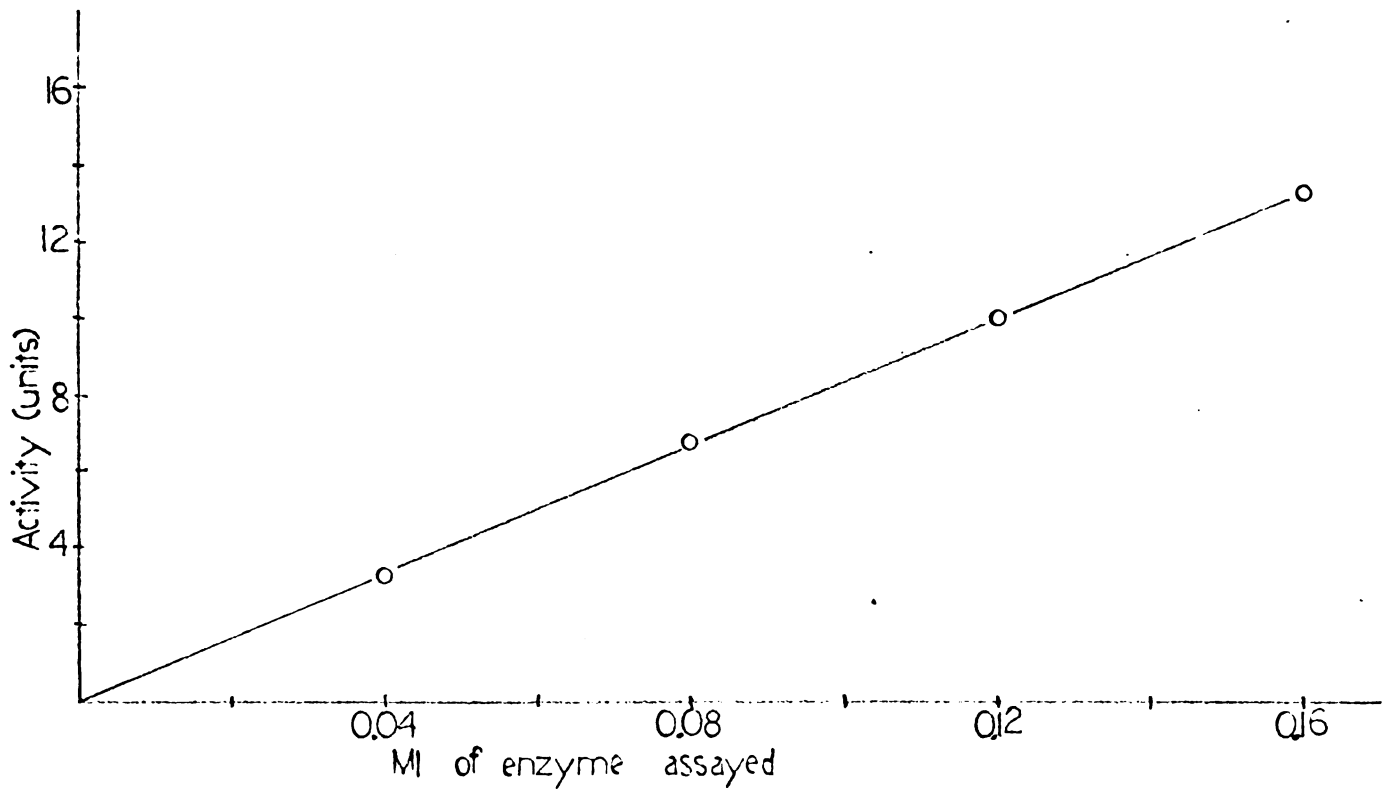


Figure 7. The relationship between enzyme activity and concentration of enzyme obtained from 1, 2, 4, and 8 flies homogenized in 1 ml of buffer using a reaction mixture containing 0.57ml of 2×10^{-4} M hypoxanthine and 0.39ml of 3.43×10^{-3} M Thio-NAD, 0.16ml enzyme in 0.1 M Tris, pH 8.0.

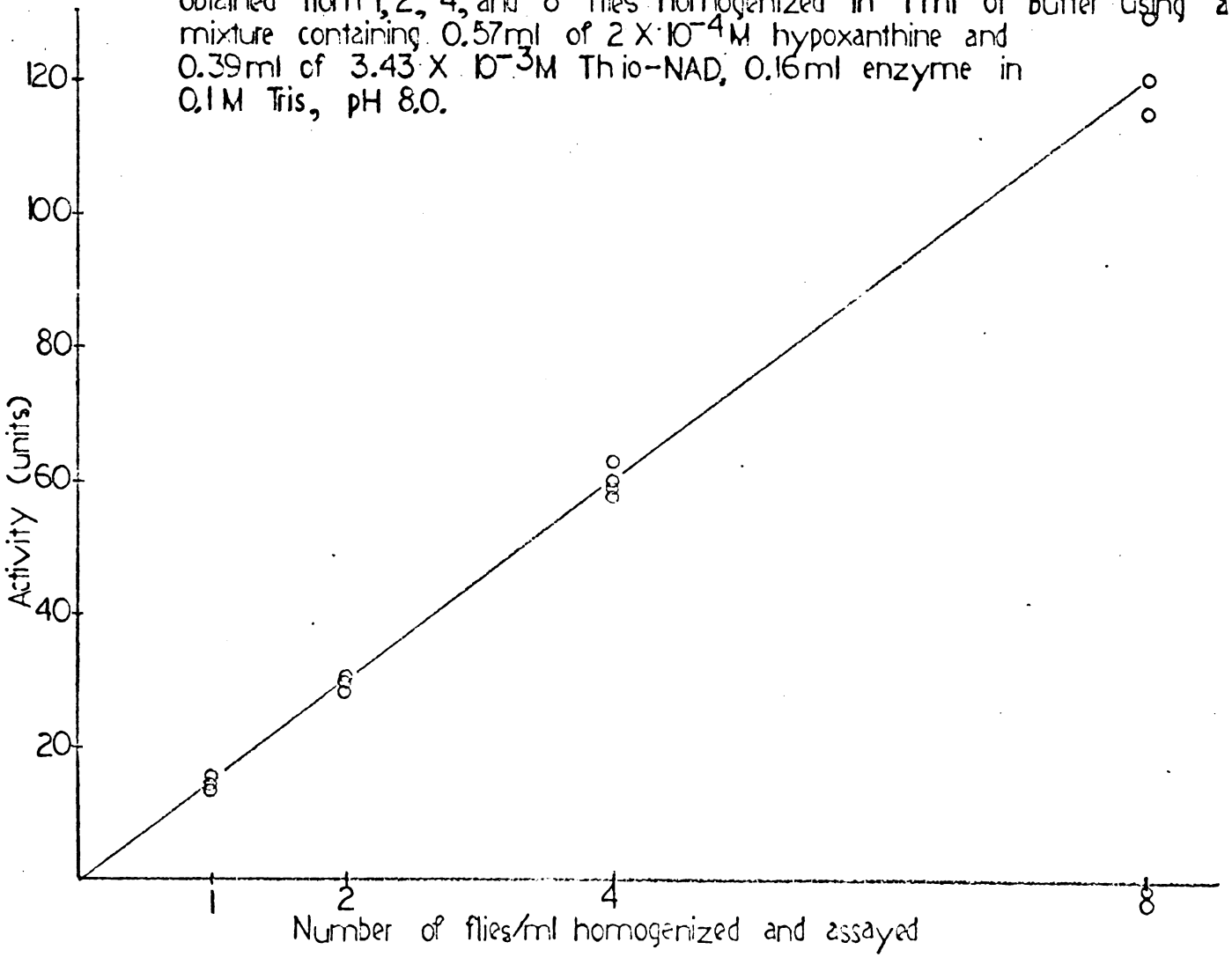
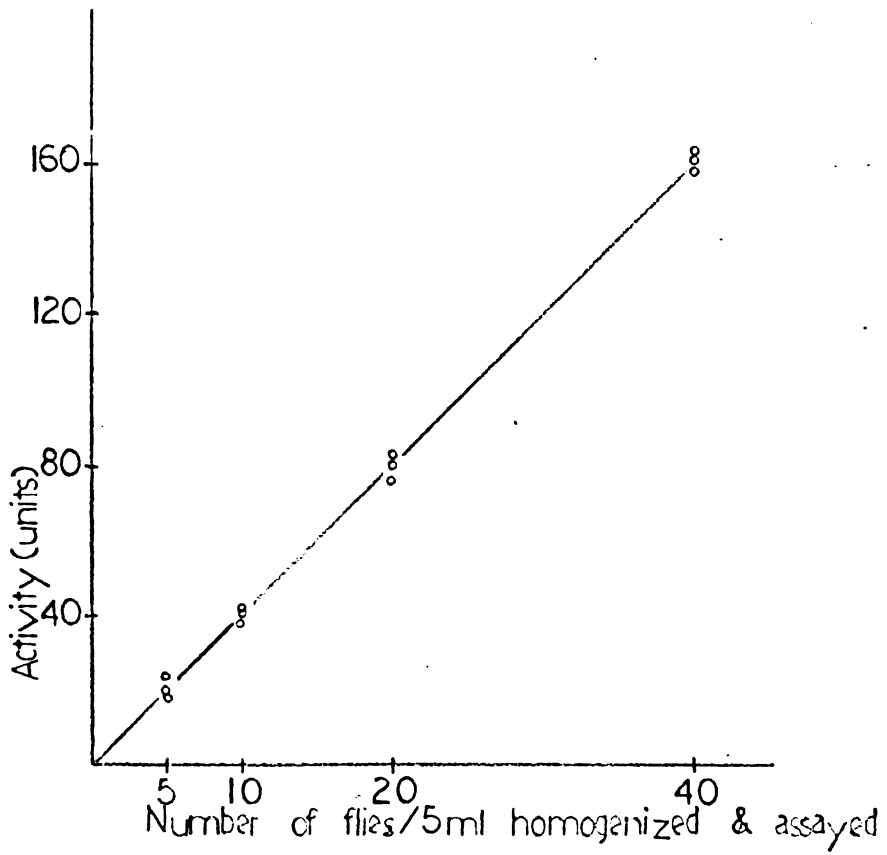


Figure 8. The relationship between enzyme activity and concentration of extract obtained from 5, 10, 20, and 40 flies homogenized in 5ml of buffer using a reaction mixture containing 0.57 ml of 2×10^{-4}M hypoxanthine; 0.39ml of 3.43×10^{-3}M Thio-NAD and 0.39ml of enzyme preparation; all in 0.1M Tris buffer, pH 8.0.



Test of the reliability of single fly assays were carried out using several inbred and several non-inbred stocks. Flies used for these assays were all one day old, each being collected and immediately frozen until homogenization. The results are given in Table 3.

An analysis of variance of these data has been performed by Dr. James Kan and is given in Table 4. To be significant at the 1% level, an F value must equal or exceed 3.34. This is true only in the case of variation between stocks; i.e., there are significant differences in the enzyme activities exhibited between stocks, but not between sexes in general or between sexes within each stock.

Enzyme Purification: Due to the extreme lability of the enzyme, a method of purification had to be designed which would treat the enzyme in the most gentle way. Accordingly the following scheme was evolved which resulted in a 528 fold purification of the enzyme (figure 9).

Flies were homogenized in a 2.5 (w/v) ratio of 0.1 M Tris buffer, pH 8.0, which was 5 mg/ml with respect to crystalline serum bovine albumin. All procedures were carried out at below 5° C unless otherwise specified. The resulting homogenate was centrifuged for 30 minutes at 30,000 x g in an International Model HR-1 centrifuge. The precipitate was discarded and Norite-A was added to the supernatant at a concentration of 100 mg/ml. This was allowed to stand with occasional stirring for 60 minutes at the end of which time the solution was recentrifuged at 30,000 x g for 20 minutes. The resulting supernatant was poured through a coarse sintered glass filter to

Table 3. Test of the reliability of single fly assays using several inbred and non-inbred stocks.

Stocks and Sex	Activity of single flies	Mean and Standard Error
Samarkand male	28.4 27.3 27.5 28.4 26.0 25.0	$\bar{X}$ = 27.100 $S_{\bar{X}}$ = 0.56
Samarkand female	24.0 28.0 23.9 27.8 28.0 28.0	$\bar{X}$ = 26.617 $S_{\bar{X}}$ = 0.85
Swedish male	27.6 27.6 25.4 28.2 26.6 28.6	$\bar{X}$ = 27.333 $S_{\bar{X}}$ = 0.47
Swedish female	28.6 28.6 29.3 24.3 28.7 28.7	$\bar{X}$ = 28.033 $S_{\bar{X}}$ = 0.75
w^{a2} male	15.4 15.8 17.8 16.3 17.5 18.0	$\bar{X}$ = 16.800 $S_{\bar{X}}$ = 0.45
w^{a2} female	19.0 17.0 19.3 16.0 15.0 18.8	$\bar{X}$ = 17.517 $S_{\bar{X}}$ = 0.73

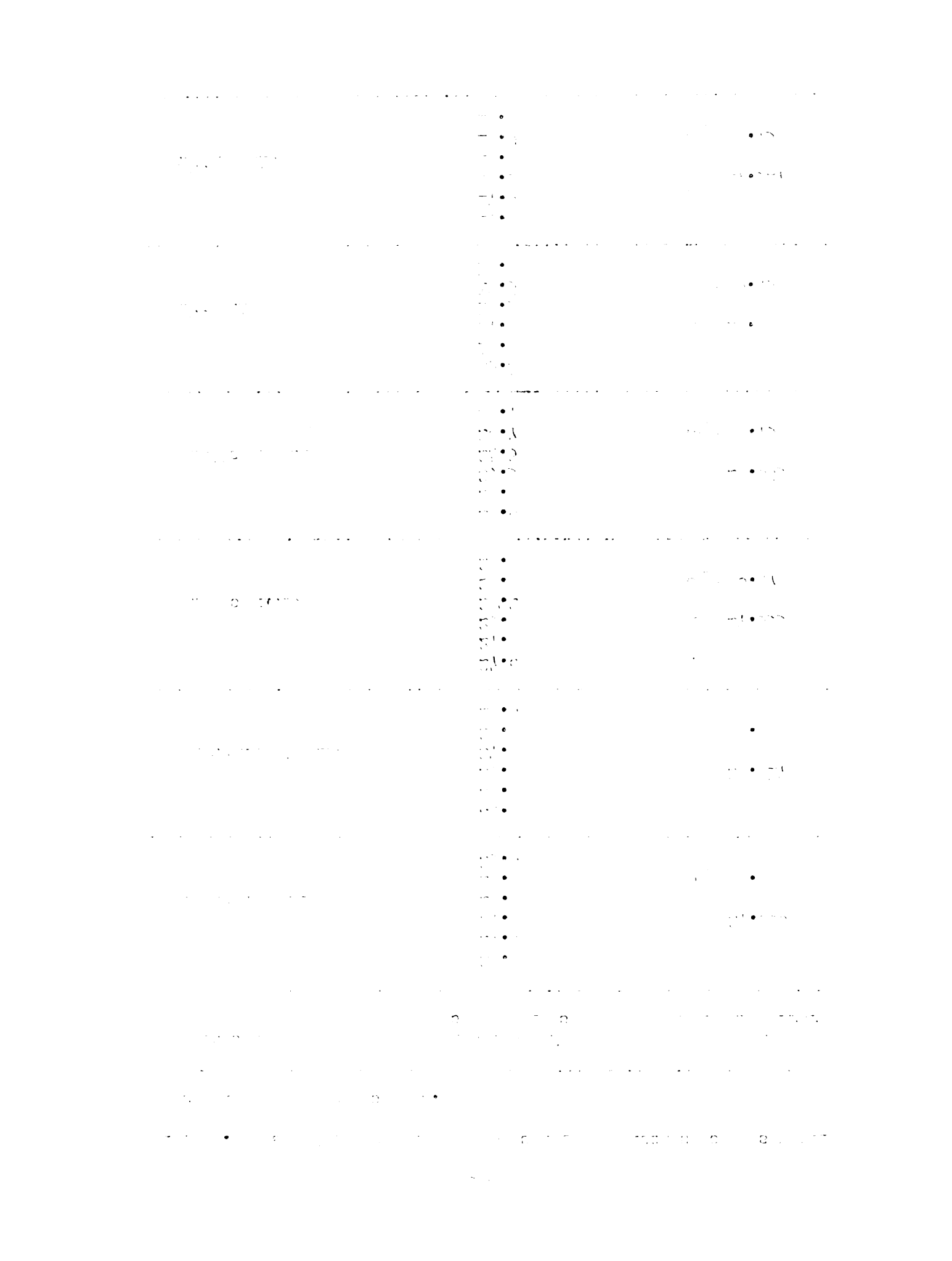


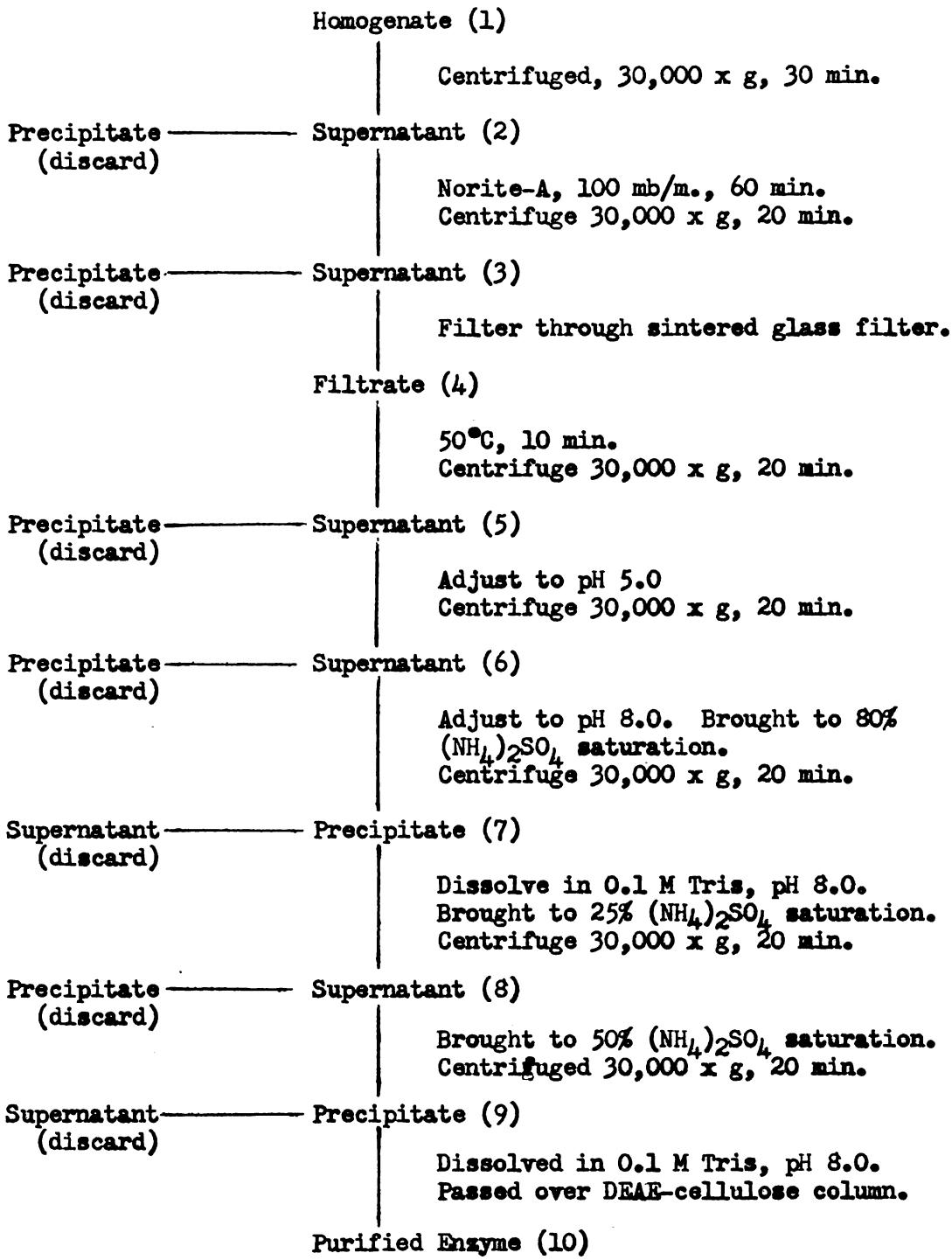
Table 3.-- continued

Stocks and Sex	Activity of single flies	Mean and Standard Error
Oregon-R-Sydney male	27.6 27.4 27.4 27.7 27.8 28.0	$\bar{X} = 27.65$ $S_{\bar{X}} = 0.096$
Oregon-R-Sydney female	28.0 28.1 27.6 27.6 27.4 28.0	$\bar{X} = 27.783$ $S_{\bar{X}} = 0.12$
Oregon-R-I male	24.5 23.7 25.8 28.8 24.3 24.5	$\bar{X} = 25.267$ $S_{\bar{X}} = 0.75$
Oregon-R-I female	24.3 24.7 26.9 28.3 20.0 22.0	$\bar{X} = 24.367$ $S_{\bar{X}} = 1.18$
w^a- Sydney male	18.0 23.0 28.6 27.6 28.0 29.0	$\bar{X} = 25.700$ $S_{\bar{X}} = 1.78$
w^a- Sydney female	17.0 18.3 25.4 27.6 28.0 23.0	$\bar{X} = 23.217$ $S_{\bar{X}} = 1.92$

Table 4. Analysis of variance of enzyme activity in single fly assays among six stocks of Drosophila melanogaster.

Source of Variation	Degrees of Freedom	Mean Square	F
Between sexes	1	2.68	-
Between stocks	5	190.96	33.2
Sex and Stock	5	4.41	-
Error	60	5.76	-

Figure 9. Flow diagram of the scheme of purification of xanthine dehydrogenase from Drosophila melanogaster.

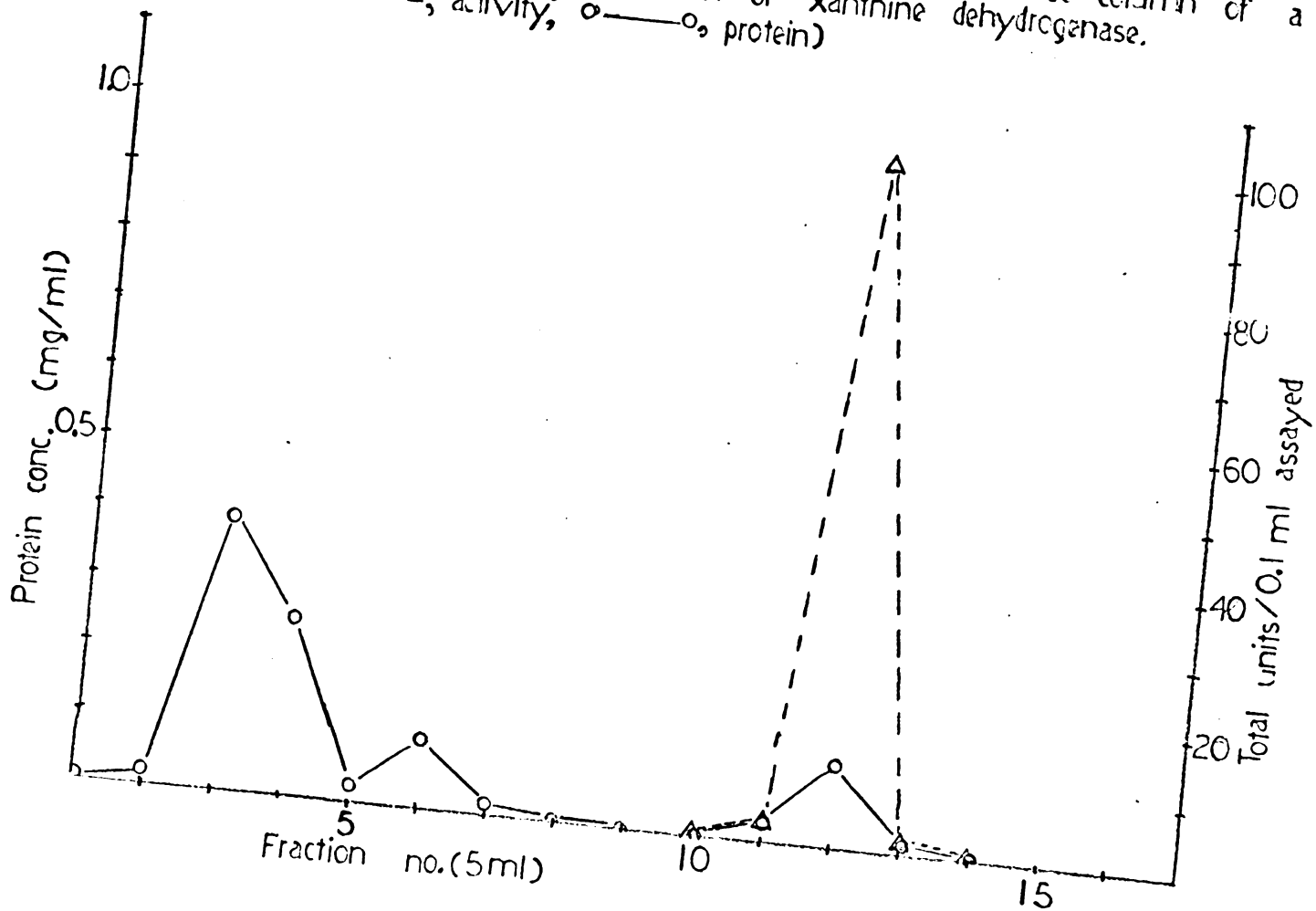


remove any remaining charcoal. An increase in total enzyme activity was characteristically observed after passage over Norite.

The filtrate was then heated to 50° C for 10 minutes, cooled immediately, centrifuged as above, and the precipitate discarded. The supernatant was then adjusted to pH 5.0 with 1 M acetic acid. This was immediately centrifuged, the precipitate being discarded and the supernatant being adjusted to pH 8.0 with 0.1 M NaOH. A saturated solution of ammonium sulfate was then added to give a final concentration which was 80% saturated. This was allowed to stand for 60 minutes at the end of which time the solution was centrifuged, the supernatant discarded, and the precipitate redissolved in 0.1 M Tris buffer, pH 8.0. To this solution was added a saturated solution of ammonium sulfate to give a final concentration of 25% saturation. After 1 hour the solution was centrifuged, the precipitate discarded, and the supernatant brought to 50% saturation with ammonium sulfate. This was allowed to stand for 1 hour at the end of which time the solution was centrifuged, the supernatant discarded, and the precipitate redissolved in 0.1 M Tris buffer, pH 8.0.

Samples of this solution were then added to a DEAE-cellulose column (1.5 cm x 24 cm) previously equilibrated with 0.1 M Tris buffer, pH 8.0. The column was then eluted with 50 ml of 0.1 M NaCl in Tris, and then with 50 ml of 0.15 M NaCl in the same Tris buffer. 5 ml samples were collected, protein being determined by the method of Warburg and Christian (1942). Those samples with protein were assayed for enzymatic activity. Figure 10 shows the elution pattern of the column chromatography. Fractions 11, 12, and 13 were pooled

Figure 10. Elution pattern on a DEAE-cellulose column of a partially purified preparation of xanthine dehydrogenase. (△---△, activity; o——o, protein)



to give the final purified enzyme preparation. Table 5 indicates the steps in the purification and the assay of each stage of purification of the enzyme. Specific activity is in terms of units of activity per mg of protein.

Kinetic characteristics of the purified enzyme: Using samples of this purified preparation, Michaelis-Menten constants were determined for hypoxanthine, xanthine, NAD, and thio-NAD. The K_m for NAD was found to be 2.5×10^{-4} M (figure 11); for thio-NAD, 2.8×10^{-5} M (figure 12); for hypoxanthine, 2.0×10^{-5} M (figure 13); for xanthine, 2.36×10^{-5} M (figure 14). By way of comparison, the K_m's exhibited by crude extracts were as follows: NAD, 3.25×10^{-4} M; hypoxanthine, 2.032×10^{-5} M.

The maximum velocity of reaction attained using xanthine as substrate was found to be 40% of that with hypoxanthine as substrate. The pH optimum for the enzyme was found at 8.0, decreasing in activity above and below that pH (figure 15).

Studies involving the stochiometry of the conversion of hypoxanthine to uric acid were also attempted. In this instance a known concentration of hypoxanthine was added to a reaction mixture containing a known excess of thio-NAD and the reaction, after addition of enzyme, was allowed to run to completion. From the change in absorbance at 395 mu, the amount of reduced thio-NAD formed could be calculated. In the specific case, 1.0×10^{-7} moles of hypoxanthine and 3.43×10^{-6} moles of thio-NAD were allowed to incubate together in a reaction mixture of 3 ml with excess enzyme. The progress of the

Figure 11. The determination of the K_m of xanthine dehydrogenase for NAD with hypoxanthine as substrate.

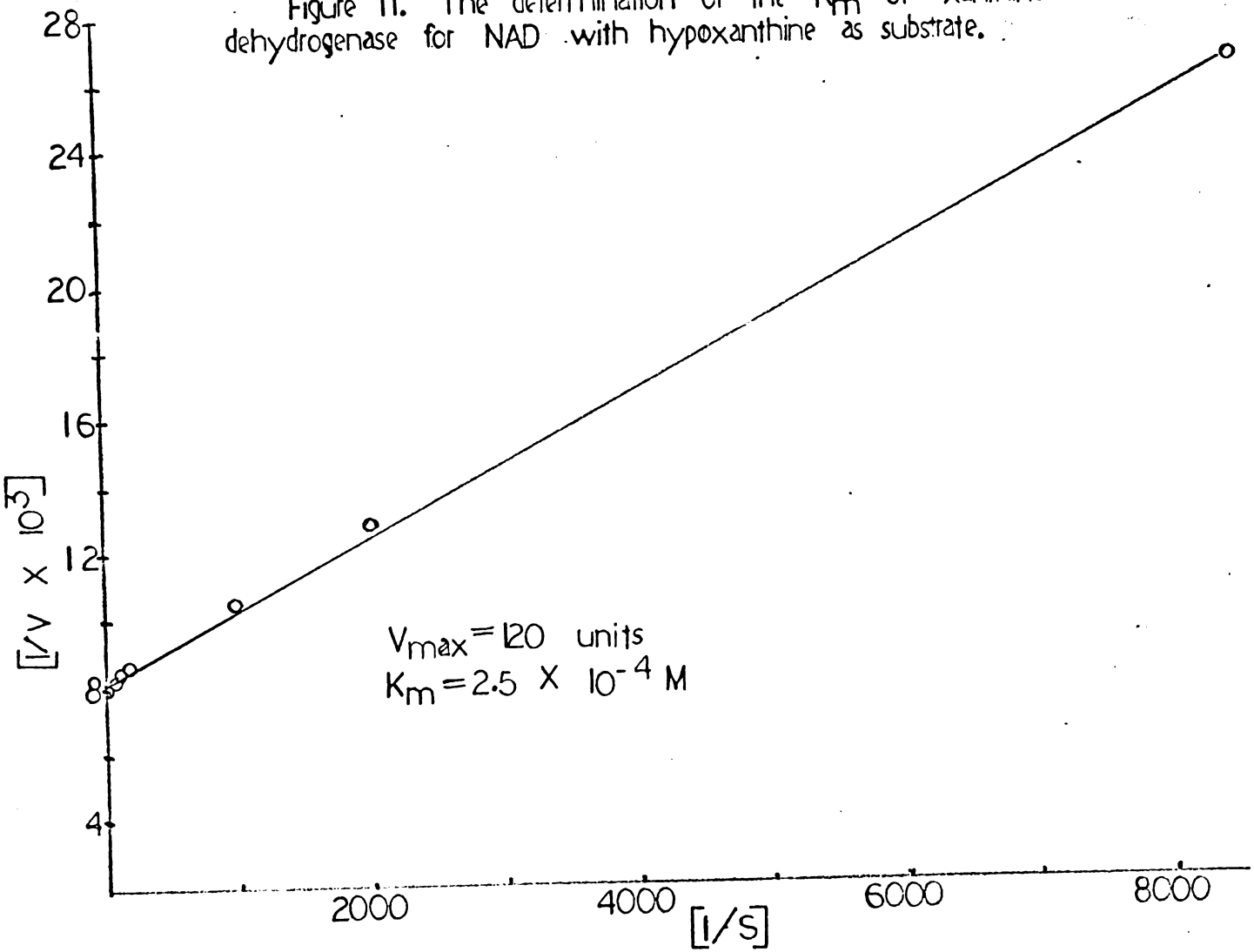


Figure 12. The determination of the K_m for xanthine dehydrogenase of thio-NAD with hypoxanthine as substrate. Best straight fitted by least squares method.

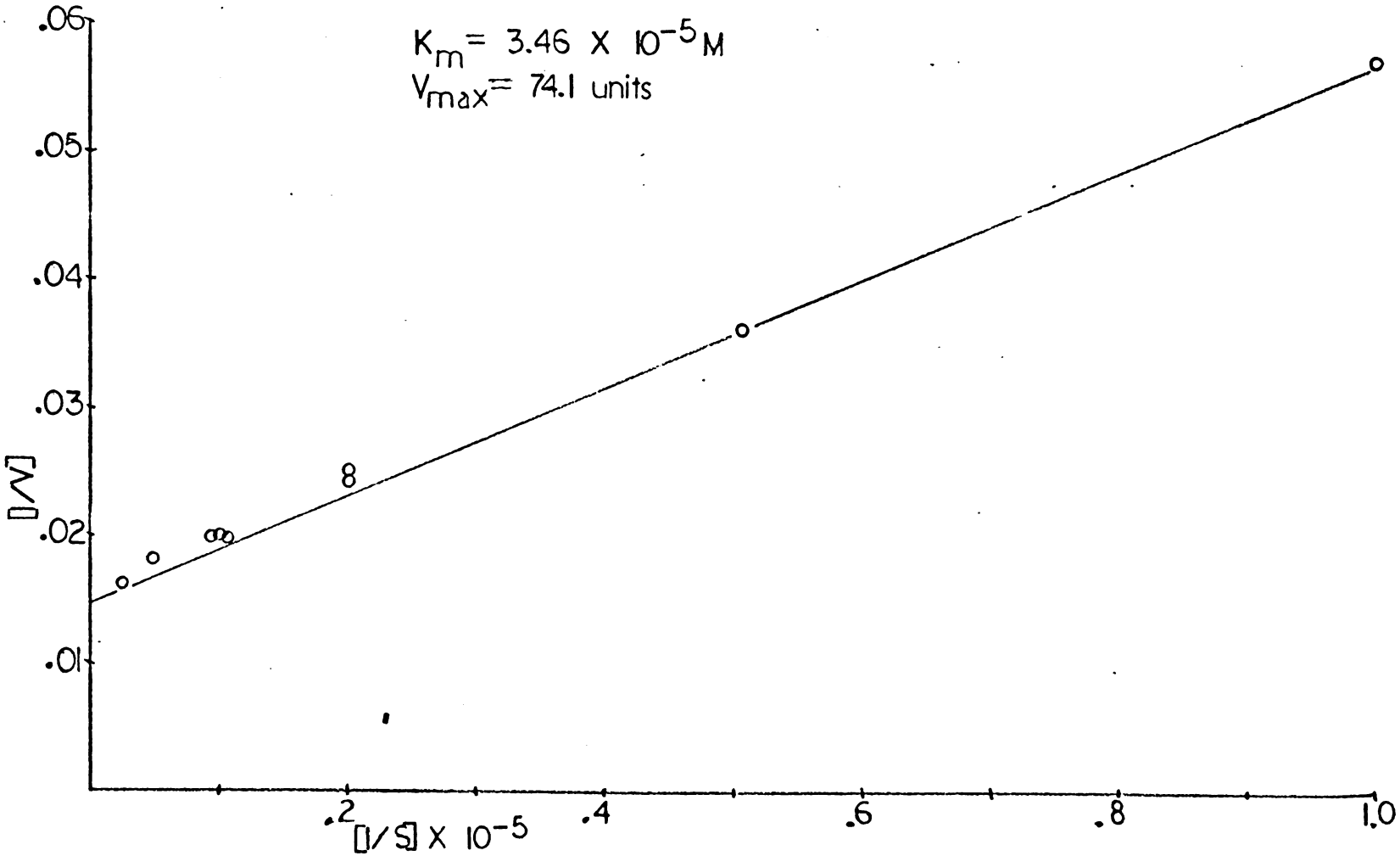


Figure 13. The determination of the K_m of xanthine dehydrogenase for hypoxanthine with NAD as electron acceptor.

$V_{max} = 80$ units
$K_m = 2.0 \times 10^{-5} M$

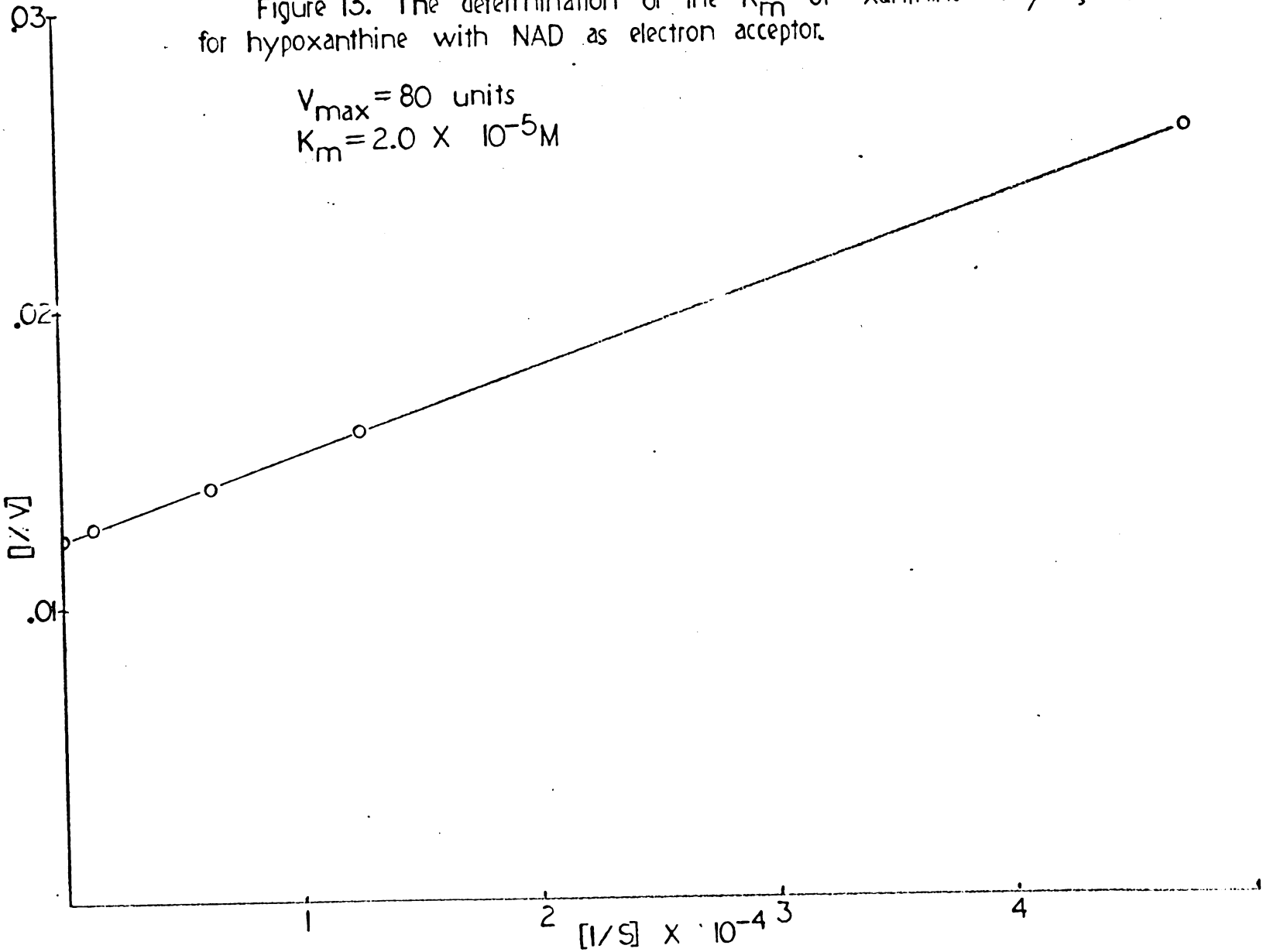


Figure 14. The determination of the K_m of xanthine dehydrogenase for xanthine with NAD as electron acceptor.

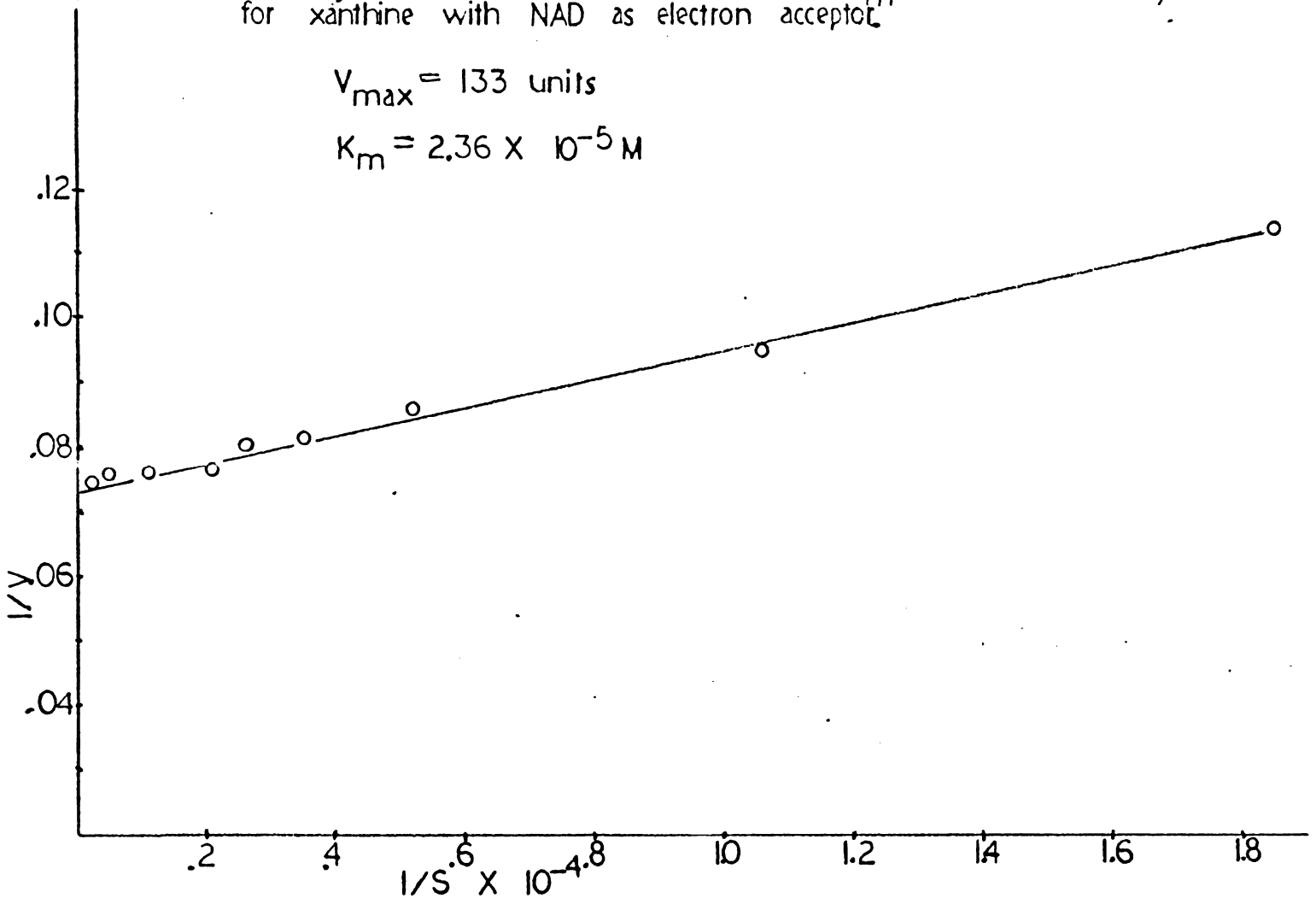


Table 5. Purification of xanthine dehydrogenase

Step*	Volume (ml)	Activity (units/ml)	Total units	Protein (mg/ml)	Specific Activity	Yield %	Fold purified
1	20	10	200	48.87	0.202	-	-
3	17	618	10,506	39.68	15.6	100	1
5	14	571	7,994	20.43	23.0	76	1.47
6	13	577	7,501	15.29	27.7	71	2.42
7	5	1400	7,000	8.88	157.5	66.5	10.1
8	6.5	1000	6,500	5.43	184.5	61.8	11.8
9	5	1000	5,000	1.44	695	47.5	44.5
10	5	990	4,950	0.12	8,250	47.0	528

*Refer to Figure 9.

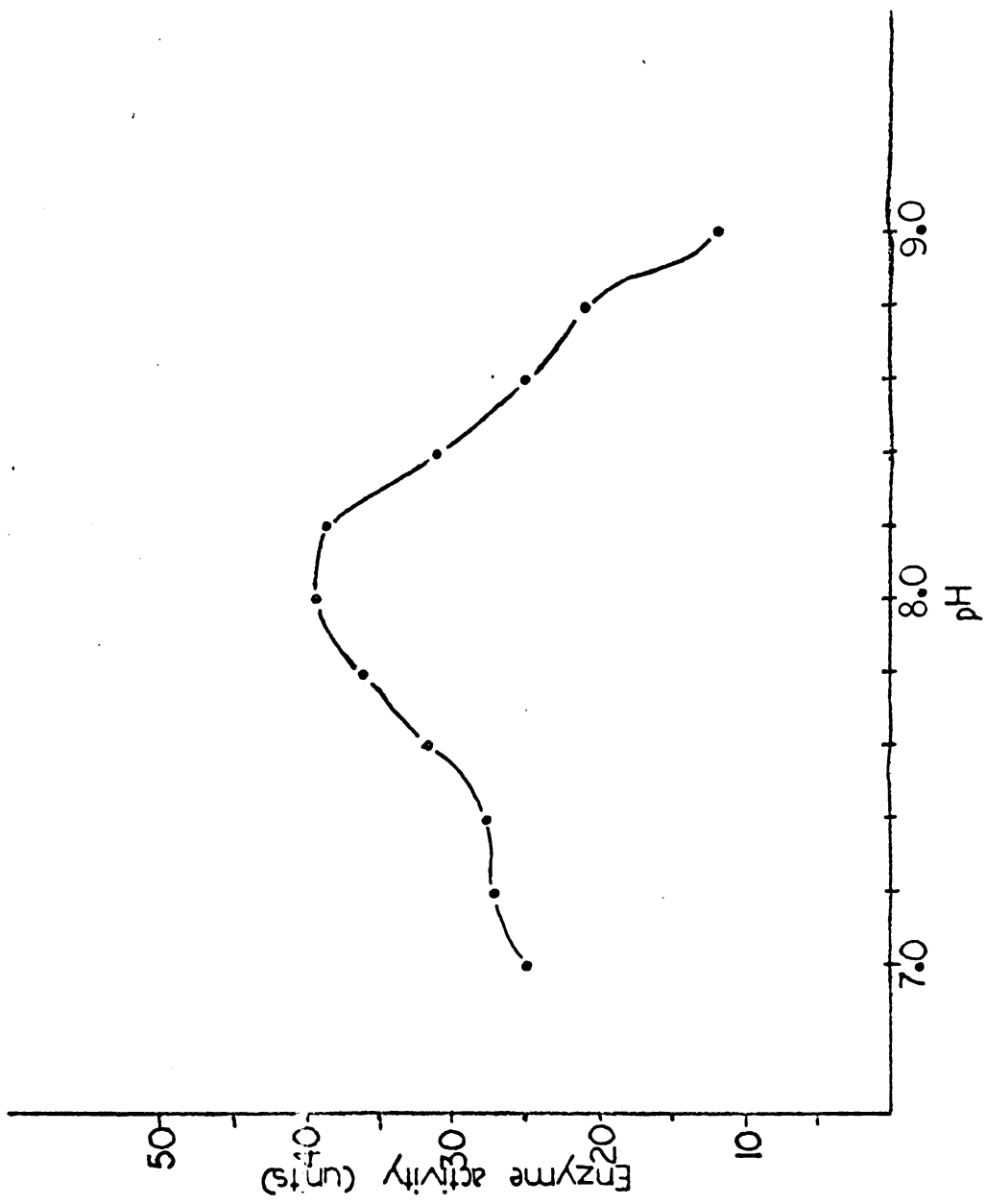


Figure 15. Enzyme activity in relation to pH.

reaction was followed at 395 mu. When optical density reached a maximum the change in absorbance was noted, and from the known molar absorbancy of reduced thio-NAD the amount of this compound formed was calculated. The molar absorbancy of reduced thio-NAD is 11.3 x 10^3. The change in absorbance at 395 mu was found to be 0.710. This is equivalent to 1.89 x 10^{-7} moles of reduced thio-NAD formed. On the basis that 1.0 x 10^{-7} moles of hypoxanthine were initially added, this would imply that 2 moles of NAD are required for the conversion of 1 mole of hypoxanthine to uric acid (Table 6).

Similar experiments were carried out with xanthine as substrate and NAD as electron acceptor (Table 6). The results of this experiment indicated that the conversion of 1 mole of xanthine to uric acid involved a concommitant conversion of 1 mole of NAD to reduced NAD.

The reverse reaction, that is the conversion of uric acid to hypoxanthine with the concommitant oxidation of reduced NAD was attempted using reaction mixtures containing various concentration of uric acid and reduced NAD. Activity in the reverse reaction would be indicated by a decrease in optical density at 340 mu. In no case was this observed. It is interesting, however, to note that this phenomenon did take place in certain extracts prior to treatment with Norite-A. This finding could be indicative of a system capable of oxidizing reduced NAD in the extracts. Nagelein and Schon (1957) have previously described such a system in D. melanogaster.

Reaction mixtures containing uric acid in concentrations as high as 7 x 10^{-5} M were also used to test for the possible inhibition of the forward reaction by that compound. Such inhibition would be

Table 6. The stoichiometry of the conversion of hypoxanthine to uric acid by xanthine dehydrogenase.

Experiment	Substrate Added	Electron acceptor added	Reduced electron acceptor formed	Mole of Electron Acceptor reduce per mole of substrate
1	Hypoxanthine 1×10^{-7} moles	thio-NAD 3.43×10^{-7} moles	1.89×10^{-7} moles	1.89
2	Xanthine, 0.3×10^{-7} moles	NAD, 0.33×10^{-5} moles	0.33×10^{-7} moles	1.1

suggested by a lessened increase in absorbance at 340 mu in comparison to assay mixtures having the identical concentration of substrate, electron acceptor, and enzyme, but lacking uric acid. No such inhibition was indicated in any case.

In vitro complementation: An in vitro complementation experiment involving extracts of the two non-allelic mutants deficient in xanthine dehydrogenase was also attempted. Extracts were prepared from maroon-like and $rosy^2$ mutants on the presumption that maroon-like should contain the normal $rosy^2$ substance and $rosy^2$ extracts would contain the normal maroon-like substance. By incubating the two extracts together under specified conditions, it was hoped that the two pieces would come together in the structure necessary for xanthine dehydrogenase activity.

Flies of both mutant types were homogenized separately at 5° C in 0.1 M Tris buffer, pH 8.0 in the usual fashion. The homogenates were then centrifuged at 30,000 x g for 30 minutes and the resulting supernatant was adjusted to pH 5.0 with 1 M acetic acid. They were centrifuged immediately, and the resulting supernatant was readjusted to pH 8.0 with 0.1 M NaOH.

This preparation was modified in some cases by treatment with Norite-A prior to the pH 5.0 fractionation. This involved adding 100 mg of activated charcoal per ml of homogenate. The mixture were allowed to stand with occasional stirring for 1 hour and then centrifuged to remove all charcoal. The charcoal-free supernatants were then adjusted to pH 5.0, centrifuged, and readjusted to pH 8.0.

In each case, the extracts were adjusted to approximately equal protein concentration. 0.5 ml of each extract alone was then assayed to test for enzymatic activity. Complementation experiments were performed by mixing equal volumes of $\underline{ry^2}$ and $\underline{ma\text{-}1}$ extracts, incubating for 1 hour at 30° C, and assaying 0.5 ml of the mixture. In addition to 0.5 ml of extract (single or mixed), the complete assay mixture contained 0.4 ml of 5.1×10^{-3} M hypoxanthine and 0.1 ml of 1.37×10^{-3} M thio-NAD. Enzyme activity was measured by rate of increase in optical density at 395 mu.

The results obtained with the extracts that were not treated with Norite-A are given in Table 7. As may be noted, a slow increase in optical density was observed in the presence of enzyme and thio-NAD even without added substrate. Such an increase was remarked above in discussion of the dependence of enzyme activity on substrate and electron acceptor, and probably reflects the presence of endogenous substrate in extracts prior to Norite treatment. There occurs, however, a marked increase in enzyme activity when the mixture of $\underline{ry^2}$ and $\underline{ma\text{-}1}$ extracts is preincubated prior to assay.

This increase is even more marked when Norite treated extracts are used (Table 8). In this case no endogenous reduction of thio-NAD occurs, and activity is observed only with preincubated $\underline{ry^2}$ and $\underline{ma\text{-}1}$ mixtures in the presence of both hypoxanthine and thio-NAD.

Table 7. Results of the complementation experiment with extracts not treated with Norite-A. All reaction mixtures contain 20.2 mg of protein.

	Enzyme activity (units)		
	ry^2	ma-1	Mixture
Complete	3.0	4.0	16.25
- Hypoxanthine	5.3	4.0	4.25
- Thio-NAD	0	0	0
- Hypoxanthine and Thio-NAD	0	0	0

Table 8. Results of the complementation experiment with Norite-A treat extracts. All reaction mixtures contain 18.8 mg of protein.

	Enzyme activity (units)		
	ry^2	ma-1	Mixture
Complete	0	0	22.0
- Hypoxanthine	0	0	0
- Thio-NAD	0	0	0
- Hypoxanthine and Thio-NAD	0	0	0

V. DISCUSSION

The results indicate the successful development of an assay method for the enzyme xanthine dehydrogenase from Drosophila melanogaster. This assay is sensitive enough to detect enzymatic activity in a single fly; the measured activity is linear with respect to enzyme concentration. Furthermore, the assay has none of the shortcomings of the fluorometric assay previously described.

The assay was utilized in the testing of enzymatic activity of single flies from several different stocks. Statistical analysis of the results of these assays indicate no significant differences in enzyme activity between males and females of any stock, but a significant difference between stocks. The causes for this variability are probably genetic, but do not involve the ry or ma-l loci.

A 500 fold purification of the enzyme was accomplished and determinations, using this purified preparation, were made of the Michaelis-Menten constants of various substrates and electron acceptors. Using NAD as the electron acceptor a K_m of 2.0×10^{-5} M was found for hypoxanthine; 2.36×10^{-5} M for xanthine. This is in close agreement with Glassman (1959) who reported a K_m of 2.1×10^{-5} M for hypoxanthine and 2.5×10^{-5} M for xanthine.

Using hypoxanthine as substrate, K_m's were also determined for NAD and thio-NAD. These were found to be 2.5×10^{-4} M and 3.46×10^{-5} M respectively. The difference in K_m's suggests that the enzyme has a higher affinity for thio-NAD than for NAD. The explicit causes

for the differences observed are unknown.

The stiochiometry of the reaction catalyzed by xanthine dehydrogenase was also studied using the purified enzyme. The results indicate that conversion of a mole of hypoxanthine to a mole of uric acid requires the reduction of 2 moles of NAD. Similarly, the conversion of a mole of xanthine to a mole of uric acid requires the reduction of only 1 mole of NAD. The inability to run the reaction in the reverse direction suggests that the equilibrium for the reaction lies far to the side of uric acid. This is perhaps also indicated by the fact that relatively high concentrations of uric acid in reaction mixtures do not inhibit the forward reaction.

Results of the complementation experiment involving extract of rosy^2 and maroon-like, each of which is deficient in regard to xanthine dehydrogenase, suggests that each locus in wild type form synthesizes a necessary part of the enzyme. This is also in agreement with the findings of Glassman (1962), though it in no way indicates the exact composition of each part. The data could be interpreted in terms of a "combining-subunits" hypothesis in which each locus produces a protein, both proteins being necessary for enzymatic activity; or in terms of a "catalytic-activator" hypothesis in which the product of one locus is activated by some product or action of the other locus. Unfortunately, no method has been devised to test either of these hypotheses.

Forrest, Hanley, and Lagowski (1961) have reported enzymatic activities in extracts of rosy^2 which are not present in extracts of maroon-like, e.g., the conversion of pyridoxal to pyridoxic acid and

4-hydroxypteridine to 2, 4-dihydroxy pteridine; neither of these reactions require NAD. These reactions may be able to serve as an assay for the product of the wild type maroon-like locus. However, no reaction has been found which is restricted to maroon-like extracts.

It is known that at least 2 different loci control the formation and activity of xanthine dehydrogenase in Drosophila. However, the situation may be much more complex. Glassman (unpublished data) has reported the existence of strains of wild type D. melanogaster which exhibit different levels of enzyme activity. This perhaps suggests some form of quantitative inheritance; that is, quantitative genes controlling xanthine dehydrogenase levels in the flies. The results of the test of reliability of the single fly assay reported in this work may also indicate the same situation.

Schepers (1962) has also reported an interaction in pteridine metabolism involving alleles of the mutants garnet and brown in Drosophila. He reports that 2-amino-4-hydroxypteridine is present in both single mutants, but in lower concentration than in wild type forms. The activity of xanthine dehydrogenase in those mutants is the same as in wild type. However, flies which are mutant with respect to both of these alleles completely lack 2-amino-4-hydroxypteridine and exhibit a reduction in xanthine dehydrogenase activity as compared to wild type.

This observation suggests some type of mechanism controlling xanthine dehydrogenase activity. If such is the case, it will be the first found in D. melanogaster and will be invaluable in the study of genetic control mechanisms.

VI. SUMMARY

1. The purpose of the work reported was to study the biochemical characteristics of xanthine dehydrogenase from Drosophila melanogaster. This involved the development of an assay of enzymatic activity which was linear in relation to enzyme concentration and was sensitive enough to detect activity in single flies. The assay is based on the change in optical density at 340 mu as NAD is reduced or at 395 mu as thio-NAD is reduced, with either xanthine or hypoxanthine as substrate. Tests of reliability of the assay were performed on single flies from various inbred and non-inbred stocks.

2. A method of purification was devised resulting in a 528 fold purification of the enzyme, the purified enzyme having a pH optimum of 8.0.

3. Using this purified preparation of the enzyme, K_m's were determined for hypoxanthine, xanthine, NAD, and thio-NAD and found to be 2.0×10^{-5} M, 2.36×10^{-5} M, 2.5×10^{-4} M, and 2.0×10^{-5} M respectively.

4. Stoichiometry of the reaction was studied using purified preparations and the results indicate the reduction of 1 mole of NAD for each mole of xanthine converted to uric acid and the reduction of 2 moles of NAD for each mole of hypoxanthine converted to uric acid.

5. A complementation experiment was carried out using extracts of maroon-like and rosy^{2}. Prior to incubation together, neither of the extracts exhibited any xanthine dehydrogenase activity. After incubating the two extracts together for one hour at 30° C., the mixture was assayed for enzymatic activity. Results show the presence of activity in the incubated mixture, but none in the separate extracts.

BIBLIOGRAPHY

Avis, P. G., C. Bergel, R. C. Bray, D. W. F. James and K. V. Shooter, 1956, The chemistry of xanthine dehydrogenase. II. The homogeneity of crystalline metallofloavoprotein fractions. J. Chem. Soc. 1212-1219.

Benzer, Seymour, 1959, On the topology of the genetic fine structure. Proc. Natl. Acad. Sci. U.S. 45:1607.

1961, On the topology of the genetic fine structure. Proc. Natl. Acad. Sci. U.S. 47:403.

Brehme, K. S., and M. Demerec, 1942, Growth 6:351.

Bridges, C. B., and K. S. Brehme, 1944, The mutants of Drosophila melanogaster. Carnegie Inst. Wash. Publ. No. 552, Washington, D.C.

Chan, F. L., H. Heymann and C. W. Clancy, 1951, Chemical composition of the red eye pigments in Drosophila. J. Amer. Chem. Soc. 73:5448.

Chovnick, A., A. Schalet and R. P. Kernaghan, 1961a, Recombination at the rosy locus in Drosophila melanogaster. Genetics 46:858.

1961b, Recombination at the rosy locus in Drosophila melanogaster. Rec. Gen. Soc. Amer. Abstracts 30:68.

Chovnick, A., A. Schalet, R. Peter Kernaghan, and Joy Talsma, 1962, The resolving power of genetic fine structure analysis in higher organisms as exemplified by Drosophila. Amer. Naturalist 96:281.

Crawford, I. P. and Charles Yanofsky, 1958, On the separation of the tryptophan synthetase of Escherichia coli into two protein components. Proc. Natl. Acad. Sci. U.S. 44:161.

Crick, F. H. C., L. Barnet, S. Brenner and R. J. Watts-Tobin, 1961, General nature of the genetic code for proteins. Nature 192: 1227.

DeRenzo, E. C., 1956, Chemistry and biochemistry of xanthine oxidase. Adv. in Enzymol. 17:293.

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

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

Forrest, H. S., and H. K. Mitchell, 1954a, Pteridines from Drosophila. I. Isolation of a yellow pigment. J. Amer. Chem. Soc. 76:5656.

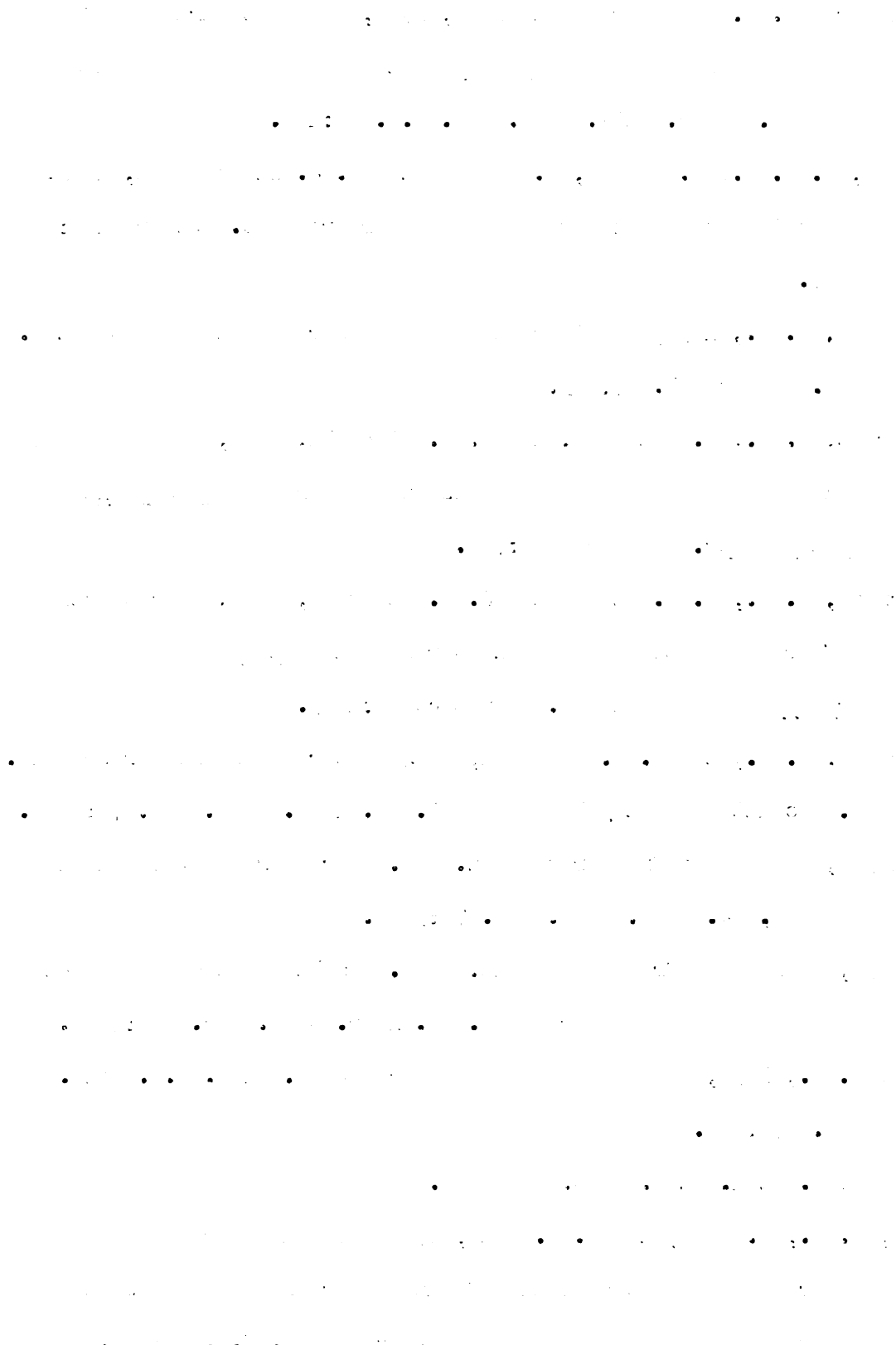
1954b, Pteridines from Drosophila. II. Structure of the yellow pigment, J. Amer. Chem. Soc. 76:5658.

1955, Pteridines from Drosophila. III. Isolation and identification of three more pteridines. J. Amer. Chem. Soc. 77:4856.

Fox, A. S., 1958, Genetics of tissue specificity. Ann. N.Y. Acad. Sci. 73:611.

1959, J. Natl. Canc. Inst. 23:1297.

Fox, A. S., S. Yoon, and C. G. Mead, 1962, Evidence for the persistence in protein synthesis of an information transfer mechanism after the removal of genes. Proc. Natl. Acad. Sci. U.S. 48:546.



Glassman, E., 1962, In-vitro complementation between nonallelic Drosophila mutants deficient in xanthine dehydrogenase. Proc. Natl. Acad. Sci. U.S. 48:149.

Glassman, E., H. S. Forrest and H. K. Mitchell, 1957, Genetic control of xanthine dehydrogenase in Drosophila melanogaster. Genetic 42:372 (Abstr.).

Glassman, E., and J. McLean, 1962, Maternal effect of ma-l^+ on xanthine dehydrogenase of Drosophila melanogaster. II. Xanthine dehydrogenase activity during development. Proc. Natl. Acad. Sci. U.S. 48:1712.

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

1959b, Maternal effect of ma-l^+ on xanthine dehydrogenase of Drosophila melanogaster. Genetics 44:547.

Hadorn, E., and G. E. Graf, 1958, Weiter Untersuchungen uber den nicht-autonomen Pterinstoffwechsel der Mutant rosy von Drosophila melanogaster. X. Anzeiger 160:231.

Hadorn, E., and H. K. Mitchell, 1951, Properties of mutants of Drosophila melanogaster and changes during development as revealed by paper chromatography. Proc. Natl. Acad. Sci. U.S. 37:650.

Hadorn, E. and I. Schwinck, 1956a, A mutant of Drosophila without isoxanthopterin which is non-autonomous for the red eye pigments. Nature 177:940.

1956b, Fehlen von Isoxanthopterin and Nicht-Autonomie in der Bildung

der roten Augenpigmente bei einer Mutant (rosy^2) von Drosophila melanogaster. Z. Vererbungslehre 87:528.

Heymann, H., F. L. Chan, and C. W. Clancy, 1950, Partition chromatography of red eye pigment of Drosophila melanogaster. J. Amer. Chem. Soc. 72:1112.

Hubby, J., and H. S. Forrest, 1960, Studies on the mutant maroon-like in Drosophila melanogaster. Genetic 45:211.

Kikkawa, H., 1941, Mechanism of pigment formation in Bombyx and Drosophila. Genetics 34:564.

Lederer, E., 1940, Les pigments des invertebres. Biol. Rev. Cambridge Phil. Soc. 15:273.

deLerma, B., and M. deVincentiis, 1955, On the pteridine nature of an ultraviolet radiation photolysis product of the red eye pigment from the eyes of Drosophila melanogaster. B. Soc. Ital. di Biol. Sper. 31:1606.

Lerner, P., and C. Yanofsky, 1957, An immunological study of mutants of Escherichia coli lacking the enzyme tryptophan synthetase. J. Bacteriol. 74:494.

Mass, W., 1948, Spectrophotometric and chromatographic adsorption analysis of the red eye pigment of Drosophila melanogaster. Genetics 33:177.

Nawa, S. T. Taira, and B. Sakaguchi, 1958, Pterin dehydrogenase found in D. melanogaster. Proc. Jap. Acad. 34:115.

Negelein, E., and R. Schon, 1957, Reduced diphosphopyridine nucleotide dehydrogenase enzyme system in Drosophila melanogaster.

Biokhimiya 22:191.

Remy, C. N., D. A. Richert, R. J. Doisy, I. C. Wells and W. W. Westerfield, 1955, Purification and characterization of chicken liver xanthine dehydrogenase. J. Biol. Chem. 217:293.

Schalet, A. and A. Chovnick, 1960, A crossover selecter system for the study of pseudoallelic recombination in Drosophila melanogaster. D.I.S. 34:104.

Schepers, A. M., 1962, An interaction in pteridine metabolism between garnet and brown genes in D. melanogaster, D.I.S. 36:114.

Schwinck, I., 1960, Studies on maroon-like and rosy. D.I.S. 34:105.

Viscontini, M., 1958, Fluorescent substances from Drosophila melanogaster. Part 10. Constitution of drosopterin. Helv. Chim. Acta 41:1299.

Viscontini, M., E. Hadorn, and P. Karrer, 1957, Fluorescent material from Drosophila melanogaster. Part 5. The red eye coloring matter. Helv. Chim. Acta 40:579.

Wald, G., and G. Allen, 1946, Fractionation of the eye pigments of Drosophila melanogaster. J. Gen. Physiol. 30:41.

Warburg, O., and S. Christian, 1942, Biochem. Z. 310:384.

Whittinghill, Maurice, 1950, Two crossover-selector systems: New tools in genetics. Science 111:377.