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THE IDENTIFICATION OF PUTATIVE NONSENSE MUTANTS OF THE *ROSY* LOCUS IN *DROSOPHILA MELANOGASTER*

by

© LOIS E. GIRTON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "The Identification of Putative Nonsense Mutants of the *rosy* Locus in *Drosophila melanogaster*" submitted by Lois E. Girton in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Of the three types of conditional mutations widely studied in micro-organisms, namely: auxotrophic, temperature-sensitive, and nonsense mutations, only the first two types have been identified in *Drosophila melanogaster*. The goal of the present study then has been to identify putative nonsense mutations in *D. melanogaster*. X-ray and ethyl methane sulfonate (EMS) induced mutants at the *rosy* (*ry* 3-52) locus which codes for the enzyme xanthine dehydrogenase (XDH) have been examined for the presence of XDH protein. Three newly induced mutants were found to have substantial XDH activity in the fluorometric assay and were not analyzed further. One EMS induced mutant had low activity (<2%) as detected by gel electrophoresis and staining for XDH activity, but it retained the *ry* mutant eye color. This mutant and five other EMS induced mutatnts exhibited interallelic complementation as detected by the restoration of wild type or intermediate eye color in certain heterozygous combinations. All of the mutants were also tested for cross-reacting material (CRM) to anti-XDH serum by Laurell rocket gel electrophoresis. All but one of the complemnting mutants and two mutants which did not exhibit complementation were found to have inactive XDH protein, that is, were CRM^+ by this test. Those mutants which were induced by EMS and appeared negative for activity, complementation, and CRM may be nonsense mutants, or possible, regulatory mutants. These can be tested for suppressibility by an EMS induced second site mutation through the use of the purine lethal screen which allows only ry^+ flies to survive.

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I. Description of the purpose of research

(1) Conditional mutants in *Drosophila melanogaster*

In prokaryotes and lower eukaryotes, several classes of conditional mutants have been described, namely, temperature-sensitive, auxotrophic, and nonsense mutants. Temperature-sensitive mutations are those whose mutant phenotype is expressed only at certain temperatures (restrictive) usually either colder or warmer than the optimum temperature for that particular organism. In the past ten years, many such mutations have been produced in *Drosophila* as well, and have been particularly useful in the study of development. Auxotrophic or nutritionally conditional mutants have also been described, mainly in the purine and pyrimidine biosynthetic and salvage pathways. The third class of mutants, nonsense mutants, have not yet been identified in *Drosophila*. (For a review of conditional mutants, see Suzuki *et al.*, 1976.)

A nonsense mutation is produced by a base substitution which results in a chain termination codon (UAA, UAG, or UGA) replacing a sense codon at any point preceding the normal termination site of a structural gene. (Garen, 1968) The result, upon translation of the mRNA, is the release of an incomplete polypeptide chain. This type of mutation can however be "suppressed" by a corresponding mutation in a transfer RNA anticodon such that the nonsense codon is read by that tRNA and an amino acid added to the polypeptide chain allowing translation to proceed.

There have been many genes identified in *Drosophila* which suppress a mutation at another locus (Lindsley and Grell, 1968). Of these, most

are spontaneous mutations which mask the phenotype of particular alleles, almost all of spontaneous origin, at specific loci. Many of the same phenotypes which can be suppressed can also be enhanced by mutations at other loci. Some mutations in fact both suppress and enhance, at different loci, simultaneously, for example, *suppressor of forked su(f)* enhances *white-apricot* w^a. None of the known suppressors appears to be acting as an informational suppressor, that is, translating a nonsense codon.

The *suppressor of sable* (*su(s)* 1-0.0) gene has been investigated more thoroughly than all other suppressor genes in *Drosophila*. Mutants at this locus suppress some alleles at the *sable*, *speck*, *purple*, and *vermilion* loci. The *vermilion* (*v*) locus codes for the enzyme tryptophan pyrrolase (Baglioni, 1959) and wild type and suppressed flies produce similar enzyme (Baillie and Chovnick, 1971). Tartof (1969) suggested that the suppression was probably not nonsense suppression because: *su(s)* alleles are recessive, not dominant as expected for nonsense suppressors and the restored level of enzyme activity was too high (10-25%). It was originally thought that *su(s)* flies lacked the second isoaccepting form of tyrosine transfer RNA ($tRNA^{tyr}_2$) (Twardzik *et al.*, 1971), that *v* tryptophan pyrrolase was inhibited by this tRNA, and that, in the absence of this tRNA, *v* mutant enzyme was functional. However, it has recently been shown that this form of tyrosine tRNA is not missing in *su(s)* mutants, only altered such that under certain conditions of preparation it can be detected (Jacobson, 1975). Thus, tRNA mutants have not been implicated in suppression in *Drosophila* at this time.

(2) Definition of the goal of the research

In view of the present status of conditional mutants in *Drosophila*, it would be interesting to identify mutants of the nonsense type and suppressors of these. These would add to the knowledge of translational processes in *Drosophila* as well as provide another tool for the maintenance of conditionally mutant or lethal stocks. The ultimate definition of a nonsense mutant lies in its ability to be suppressed by a tRNA with an altered anticodon capable of reading a UAA, UAG, or UGA codon. This, however, is difficult to attain since it involves the induction of two types of mutations both requiring very specific base substitutions. It is therefore of great importance to characterize a mutants as fully as possible before attempting to suppress the mutant.

Logically, one must start with a gene whose protein product is clearly established and available for purification, thus a gene coding for an enzyme is a good choice. Secondly, it is helpful if mutations in the gene can be easily identified on the basis of a visible phenotype. Thirdly, it is important to induce mutations of the base substitution type so that a nonsense codon may be produced.

Having induced an array of mutations, it is then necessary to characterize them to find which would be likely candidates for chain termination mutations. The studies in prokaryotes (Hartman *et al.*, 1971) have indicated that nonsense mutants are usually: null activity mutants, incapable of intragenic complementation, do not produce cross-reacting material (CRM) to antibodies against the wild type protein, and do not affect dimerization of wild type protein in heterozygotes as detected by altered mobility in gels or by negative complementation. It should

be noted that all of these characteristics are not sufficient to identify a nonsense mutant, and indeed, may equally describe a mutant of a regulatory nature.

Using the above criteria as the best available, the purpose of the following work has been to identify putative nonsense mutants in *Drosophila*.

(3) The approach to the problem

There are few gene-enzyme systems in *Drosophila* which would be suitable for such a study. For example, the *vermilion* locus (*v* 1-33.0) codes for tryptophan pyrrolase and mutants have a visible eye phenotype; this enzyme has been purified (Baillie and Chovnick, 1971) but no lethal screen exists for reversions or second site mutations. The enzyme alcohol dehydrogenase is coded for by the *Adh* gene (2-50±0.1) and mutants at this locus can be selected by addition of pentenol to the media; pentenol is metabolized by Adh^+ flies to a toxic substance, such that only Adh^- flies survive. Furthermore, ethanol is toxic to Adh^- flies giving a lethal screen for revertants or suppressors. Thus, this enzyme would be a good choice of study.

The system which was selected for study is the *rosy* locus (*ry* 3-52.0) coding for xanthine dehydrogenase (XDH), the earliest gene-enzyme system identified in *Drosophila*. Null mutants at this locus have a visible phenotype, dull red eyes, and a lethal screen exists for picking up reversions or second site suppression.

The gene product, XDH, has been purified and several workers have successfully raised antibodies to XDH in rabbits, allowing for studies

of CRM. The active XDH enzyme is a homodimer; this means that intragenic complementation of mutants is a possibility. In addition, inactive polypeptides may affect dimerization of the wild type XDH and/or may show a protein band in sodium dodecyl sulfate gels in the region of the wild type purified monomer (Schwartz and Sofer,1976). The *rosy* locus and its protein product, XDH, have been fully described in the following parts of the Introduction.

The mutagen of choice in attempting to produce base substitutions in *Drosophila* is probably the monofunctional alkylating agent ethyl methane sulfonate (EMS). Lim and Snyder (1968) suggested that EMS is unable to produce even small chromosomal deletions in *D. melanogaster*. This work was questioned by Lifschytz and Falk (1969) who found that 20% of high dosage EMS-induced recessive lethals were apparent deletions. Lim and Snyder (1974) re-examined this question using the EMS-feeding method of Lewis and Bacher (1968) to induce 82 recessive lethals and found all of them to be mutations involving only one cistron with no apparent deletions. Support for their hypothesis also comes from Hochman (1971), in an analysis of EMS induced mutations on the fourth chromosome; he found 81 point mutations, one deficiency (suspected on genetic grounds only) and one translocation; whereas X-rays produced 30 point mutations, 7 deficiencies (4 confirmed by cytological analysis), and 4 translocations. Additional confirmation comes from Hilliker (1976); of 113 EMS induced lethals in the heterochromatic region of chromosome 2, none behaved as a deficiency. EMS was therefore used in all mutagenesis experiments in this laboratory.

It should be noted, however, that several X-ray induced *ry* mutants

obtained from A. Chovnick were included in the studies. These mutants map to specific sites in the *ry* locus and it is the belief of Dr. Chovnick that most X-ray induced mutations restricted to one gene are single-site mutations (Chovnick *et al.*, 1977). However, it is possible that many of the single site mutations are base-pair insertions or deletions rather than substitutions. Malling and de Serres (1973) studied 101 X-ray induced mutations at the *ad-3B* locus in *Neurospora crassa* for ability to be reverted by specific mutagens. They interpreted 42% of the X-ray induced lesions to be base substitutions, 37% insertions or deletions, 6% non-revertible, and the remaining 15% were revertible spontaneously. Thus, X-ray induced mutants should probably not be included as putative nonsense mutants even if they fulfil other criteria applied in the study.

Predating the following work on characterization of mutants at the *ry* locus for the presence of an inactive protein product are comparable studies on at least two other loci, *Acid phosphatase-1* (*Acph-1* 3-101.1) and *Adh*. Bell *et al.* (1972) and Bell and MacIntyre (1973) analyzed 15 *Acph-1* null alleles; 6 of these proved to be leaky mutants and a further 6 exhibited intragenic complementation. The three remaining mutants were tested for CRM and found to have levels less than 5% of wild type; these levels are so low as to be indistinguishable from 0% or CRM^-. These mutants were thus interpreted as putative nonsense mutants.

Schwartz and Sofer (1976) used similar methods to analyze mutants at the *Adh* locus in order to distinguish mutants of the structural gene from mutants of a regulatory gene. They examined 16 EMS induced mutants for hybrid enzyme formation, intracistronic complementation, CRM (by

antibody precipitation in Ouchterlony tests), and migration in sodium dodecyl sulfate (SDS) gels of a protein from a mutant to a position occupied by purified wild type ADH. They concluded by these methods that 11 of the 16 mutants appeared to be structural gene mutations. Of these tests, hybrid enzyme formation appeared to be the most stringent, whereas all of the mutants produced at least faint staining in SDS gels. The antibody precipitation test was fairly adequate in identifying protein -- only one CRM^- mutant produced a distinct band in the SDS gels and one other CRM^- mutant showed intracistronic complementation.

The *ry* locus has been studied extensively by Chovnick and his co-workers who have mapped null alleles, complementing alleles, and electrophoretic sites within the locus. However, no information on CRM levels of any of the mutants with the exception of ry^2 (Karam, 1965) has been made available. The present study is intended to complete this information for a group of alleles at this locus.

II. The *rosy* system

(1) Identification of mutants

Two mutants of *Drosophila melanogaster* with a reddish-brown eye color were originally observed as reported in Bridges and Brehme (1944). One of these, a spontaneous mutant, mapped to the third chromosome and was designated *rosy* (*ry*); the other, *maroon-like* (*ma-l*), was X-ray induced by Oliver and mapped to the first chromosome. A second mutant at the *ry* locus, ry^2, was discovered by Hadorn and Schwink (1956a). In addition to these, many other *ry* mutants have been induced by X-ray and EMS mutagenesis by screening for the eye color obtained in combination

with either ry^2 or a deletion of *ry* (Schalet *et al.*, 1964 and later papers by Chovnick and co-workers). The dull red eye color has been shown to be due to reduced amounts of drosopterins and increased amounts of biopterine and sepiapteridine (Hadorn and Schwink, 1956 a,b; Hubby and Forrest, 1960).

(2) Identification of the lack of xanthine dehydrogenase in *ry* and *ma-1*

A paper chromatographic technique to detect fluorescent substances in fly extracts, particularly eye pigments, was developed by Hadorn and Mitchell (1951) and used to test several eye and body color mutants, *ry* and *ma-1* not being among those tested. The fluorescent substances were later purified and compared to synthetic specimens by Forrest and Mitchell (1955) and five pteridines were thus identified. Two of these were found to be 2-amino-4-hydroxypteridine (AHP) and isoxanthopterin (IXP).

A new spontaneous reddish-brown eye color mutant was isolated and found not to contain the fluorescent spot, isoxanthopterin, at any stage during development (Hadorn and Schwink, 1956 a,b). The mutant mapped to 3-51±1 and failed to complement Bridges' *ry* allele, thus it was designated ry^2. The eye phenotype was observed to be non-autonomous, that is, *ry* eye discs tranplanted into wild type hosts produced a red pigmented eye.

Since pteridines had been implicated in eye pigments and these greatly increase in pupal life, a preparation of young pupae was made and found to readily oxidize 2-amino-4-hydroxypteridine to isoxanthopterin by following the increase in optical density at 340 nm. in a spectrophotometer (Forrest and Mitchell, 1955). More satisfactory assays were

obtained when the homogenate was pretreated with charcoal (Norite-A) and heat (50°C) for 10 minutes. The same extract also oxidized xanthopterin to leucopterin and xanthine to uric acid, as did a preparation of xanthine oxidase from fresh cream. Thus, xanthine oxidase was thought to be the enzyme being assayed. In another study (Forrest *et al.*,1956), late third instar larvae of 44 eye color mutants were tested qualitatively for this enzyme and only two, *ma-l* and *maroon* (*ma*), contained very little activity. It should be noted that *ry* was not tested by these workers. Glassman and Mitchell (1959) later ascribed the activity to a xanthine dehydrogenase rather than xanthine oxidase as methylene blue or DPN (NAD+) was required as a co-factor (electron acceptor) in contrast to milk xanthine oxidase which could use molecular oxygen as an electron acceptor.

They adapted the convenient fluorometric assay for xanthine dehydrogenase by Burch *et al.* (1956) to *Drosophila*. They confirmed that partially purified extracts of both *ry* and *ma-l* lacked XDH activity but failed to confirm the earlier observation that *maroon* had low activity. The assay was later modified for use with single flies (Glassman, 1962). These workers also demonstrated that hypoxanthine accumulated in *ry* and *ma-l* mutants, extending the previous observations of s ccumulation of AHP in the same genotypes (Mitchell *et al.*, 195

(3) Do cs of XDH map to *rosy*

Hubby est 1960) performed the first experiments on the effects f *ry* and *ma-l* on XDH. They used the mutants ry^2 and *ma-l* und *ma-l*/+ he rozygotes to have .67 +/+ levels,

$ry^2/+$ to have .53 +/+ level, whereas the double heterozygotes *ma-l*/+ $ry^2/+$ had .36 +/+ level.

The criticism that a leaky allele at a locus may result in departure from the classical gene dosage effect was raised by Grell (1962). He used X-ray induced chromosomal deficiencies and duplications to study the effects of dosage of the ry^+ and *ma-l*$^+$ alleles. He found that females with one, two, or three copies of the *ma-l*$^+$ gene and males with one or two copies had the same level of XDH activity -- differing from the earlier report of Hubby and Forrest (1960). However, the enzyme appeared to be sensitive to different doses of the ry^+ gene, with three doses producing about 1.4X the activity of two doses, and 1 dose about half the normal activity. He suggested two hypotheses to explain these observations: one, that the location of the *ma-l* locus on the X-chromosome resulted in selection for dosage insensitivity so that males and females would have the same activity; and two, that the amount of product produced by ry^+ and *ma-l*$^+$ differed such that the smaller amount of the ry^+ product was rate-limiting.

Independently, Glassman *et al*. (1962) confirmed that *ry/+* heterozygotes had about 70% of the normal wild type Oregon-R activity, while *ma-l* did not have a dosage effect. They attributed the deviation from the 50% expected value for *ry/+* to heterozygosity at other loci. The interpretation of a rate-limiting role for the ry^+ product with an excess of *ma-l*$^+$ product was also made. The above suggests that the structural gene for XDH is *rosy* not *ma-l* as gene dosage dependency is usual for structural genes in eukaryotes (O'Brien and Gethmann, 1973; Hodgetts, 1975).

(4) Electrophoretic variants of XDH map to *rosy*

Four electrophoretic variants of XDH were found by surveying wild type strains of *Drosophila* (Yen and Glassman, 1965). The hybrids between strains with a fast and a slow variant were shown to produce three bands of XDH, one of each parental mobility and a darker intermediate band. Crosses of these variants to mutants at the three loci then known to affect XDH activity, *ma-l*, *ry*, and *lxd* (*low xanthine dehydrogenase*, see discussion of this mutant in part III of Introduction) showed that only the *ry* locus was involved with variation in mobility. The *ry* locus was further implicated by an experiment which mapped the mobility of XDH to a region on the third chromosome between *W* (*Wrinkled* 3-46.0) and *Sb* (*Stubble* 3-58.2) which includes the *ry* locus (52.0) but not *lxd* (33.0).

(5) Purine sensitivity of *ry* and *ma-l* mutants

The use of chemicals in the media to regulate the eye phenotype and/or lethality was suggested by the observation that phenocopies of the *ma-l* or *ry* eye color could be produced by growing wild type flies on media containing 4-hydroxypyrazolo (3,4-d) pyrimidine (HPP), an inhibitor of XDH (Glassman, 1965). The greater sensitivity of *ry* flies to higher temperature (29°C) and malpighian tubule abnormalities observed by Hadorn and Schwink (1956 a,b) were thought to be due to the accumulation of hypoxanthine. Therefore, Glassman tested purine for its toxicity to *ry*, *ma-l*, and wild type flies. He found that both *ry* and *ma-l* failed to complete development at purine concentrations which did not affect wild type flies. The importance of this tool for studying recombination within these loci was emphasized (Glassman, 1965).

This purine lethal screen has been used extensively by other workers for fine structure mapping at both loci (see Finnerty, Baillie, and Chovnick, 1970 and Chovnick *et al.*, 1970, 1971 for some examples.).

(6) Genetic fine structure map of the *rosy* locus

The first attempts to localize the site of a given mutation within the *ry* locus were made by Chovnick *et al.*(1962, 1964) and Chovnick (1966) using a system of closely linked recessive lethal markers. Crosses were made between heterozygous females and males such that only chromosomes having undergone recombination would be recovered. Females bearing a chromosome with a *ry* allele and two left-flanking recessive lethals ($l_1\ l_2\ ry^x\ +\ +$) and a chromosome with a different *ry* allele and two right-flanking recessive lethals ($+\ +\ ry^y\ l_3\ l_4$) were crossed to males carrying another *ry* allele, l_1 and l_3 on one chromosome ($l_1\ +\ ry\ l_3\ +$) and *ry*, l_2, and l_4 on the other ($+\ l_2\ ry\ +\ l_4$). Another cross was made involving females bearing $l_1\ l_2\ ry^y\ +\ +$ and $+\ +\ ry^x\ l_3\ l_4$ chromosomes to the same males. The specific lethals used were *Minute-34 (M34)* and *Deformed (Dfd)* to the left of *ry* and *Stubble (Sb)* and *Ultrabithorax (Ubx)* to the right. Additional non-selected markers were sometimes used, *curled (cu)*, *karmoisen (kar)*, and *lethal-26 (l26)*, to further identify the position of a crossover event. This system permitted the recovery of a substantial proportion of the recombination events in the *ry* region while 95% of the total zygotes in the crosses were unable to reach eclosion. The 5% surviving flies could be scored for rosy phenotype and ry^+ recombinants tested for outside markers. Use of a higher temperature (29°C) at which *ry* flies are sensitive increases the efficiency of the screen. In this way,

the order ry^1 - ry^{26} - ry^2 was first established. Thirteen other X-ray induced mutants were then tested against ry^{26} to give a left-right positioning.

Further separation of sites was attained by *inter se* crosses within the left cluster and within the right cluster giving rise to six distinct sites arranged in a linear order with ry^{23} as the left-most marker and a right cluster of five alleles (ry^{41}, 25, 7, 6, and 3); ry^1 was thought to be an intracistronic rearrangement involving the left end of the cistron. Later studies utilizing the purine screen included more alleles and gave the *ry* structural unit at 5.0 X 10^{-3} map units. More detailed information on positions of various alleles was also given. (Gelbart *et al.*, 1976 has the most recent information.)

(7) Cytological and genetic map of the *ry* region

Several dominant visibles with recessive lethal effects in the region of *ry* had been described in Bridges and Brehme (1944) including *Deformed (Dfd)*, *Lyra (Ly)*, *Minute-34 (M34)*, *Moiré (Mé)*, *Stubble (Sb)*, *Ultrabithorax (Ubx)*, and *Xasta (Xa)*. A recessive visible eye color mutation, *karmoison (kar)*, was also described. Chovnick *et al.* (1962) separated *lethal-26 (l26)* from ry^{26}, induced by X-rays, and mapped it to the right of *ry*. Schalet *et al.* (1964) examined X-ray induced *ry* mutants with lethal effects and found that in all cases the lethal phenotype could be attributed to a deficiency covering more than the *ry* locus. They used two of these deficiencies, ry^k and ry^{27}, to uncover non-*ry* visible and lethal mutations in the region and found recessive semi-lethal *messy (mes)* mutants affecting bristles and wings 0.5 map units to the left of *ry* and nine mutants (8 lethals and 1 visible) in

four complementation groups to the right of *ry*. The visible mutant, at 0.5 map units to the right of *ry*, was designated *piccolo (pic)* and resembles the *bobbed* bristle phenotype.

Lefevre (1971) examined cytologically several of the *ry* deficiencies and found missing bands in the 87 D region of the polytene chromosome map. One of the mutants, ry^{74}, appeared to be a very short deletion in 87 D8-12, probably involving only one of these bands, thus delimiting the *ry* locus to one of five bands between 87 D8-12. Other longer *ry* deficiencies invariably were also missing bands in this region.

(8) Evidence for conversion at the *ry* locus

The development of the purine selective screen for ry^+ recombinants (Glassman, 1965; Finnerty, Baillie, and Chovnick, 1970) and of compound autosomes (Baldwin and Chovnick, 1967) led, respectively, to the ability to analyze larger numbers of meiotic events and to the recovery of recombinant half-tetrads. Reversed metacentric compo third chromosomes were used in females heterozygous for two *ry* alleles on the right arm of the compound third. In a cross between females heterozygous for ry^5 and ry^{41} and males homozygous for ry^2, 21 ry^+ progeny were recovered. (Chovnick *et al.*, 1970, 1971 and Ballantyne and Chovnick, 1971) Eighteen of these chromosomes were detached by X-rays and analyzed for outside markers. Six chromosomes could be interpreted as the results of single intragenic cross-over events; ten resulted from the conversion of ry^{41} and two from the conversion of ry^5. (The term conversion is borrowed from the fungal geneticists and refers to those intragenic recombinational events which are parental for outside markers, suggesting that the mutant allele, in the case of *ry*, is

changed to the wild type allele by a mechanism other than classical exchange. This event results in aberrant marker segregation in tetrads, eg. 3:1 instead of 2:2 from a heterozygote.) All of these recombinants and con tants appeared indistinguishable from wild type in terms of XDH activity. In addition, the *ry* detached arms from the ten half-tetrads appearing by flanking marker identity to be ry^{41} convertants were shown to carry the ry^{5} allele as expected if the event was non-reciprocal. (Ballantyne and Chovnick, 1971)

The later production of a series of *ry* null alleles on different electrophoretic backgrounds (See Mapping of electrophoretic sites, below), ry^{+0} and ry^{+1}, allowed a further check on the origin of the ry^{+} exceptionals from crosses between *ry* heterozygous females and *ry* males. These experiments showed that all convertant products (as defined by outside marker exchange) exhibited the same parental electrophoretic mobility, for example. conversion of a ry^{8} allele which was induced on a ry^{+0} background, gave a mobility identical to ry^{+0} and conversion of ry^{102}, from a ry^{+1} background, gave a ry^{+1} mobility. This demonstration argued against a mutational origin of exceptional ry^{+} chromosomes. (McCarron, Gelbart, and Chovnick, 1974)

(9) Mapping of electrophoretic sites within the *rosy* locus

The identification of XDH molecules with varying electrophoretic mobility in natural populations of *Drosophila* (Yen and Glassman, 1965) was expanded by Chovnick and his co-workers (McCarron, Gelbart, and Chovnick, 1974; Gelbart, McCarron, Pandey, and Chovnick, 1974; and Chovnick *et al.*,1975). Eight ry^{+} isoalleles with five distinguishable electrophoretic mobilities were described. The isoalleles $ry^{+0\rightarrow+5}$

were subjected to mutagenesis by either X-rays or EMS and the *ry* null mutants thus obtained were used for recombination studies. The basic premise was that ry^+ recombinants derived from females heterozygous for null alleles induced on differing electrophoretic backgrounds could be tested for electrophoretic mobility to determine at what site the two wild type isoalleles differed. These electrophoretic sites and flanking markers were thus carried along in the cross as nonselected markers.

The electrophoretic sites identified by the above procedure were found to fall into two clusters at either end of the genetic map; *e507*, *e217*, and *e303* mapped in the region of ry^5 and ry^8 at the left end of the locus; whereas *e508*, *e111*, *e302*, and *e408* mapped on the right near ry^{41}. This distribution indicated that all of the *ry* null mutants were located in the structural gene for XDH, that is, none were mutants of a regulatory nature. The sites *e217*, *e507*, and *e508* were later mapped more precisely to position them among the *ry* nulls; *e217* and *e507* mapped with ry^{502} and *e508* at ry^{501}. It is thought that the electrophoretic sites fall closely beside the null mutant sites, although to which side is as yet unclear.

At present, 12 isoalleles with 7 mobilities and some differences in amount of activity have been characterized. Some mobilities mapped to two different sites, as for ry^{+5} which differed from ry^{+0} at the *e507* and *e508* sites. (Gelbart *et al.*,1976)

(10) Complementation within the *rosy* locus

The early *rosy* mutants failed to show any intragenic complementation (Schalet *et al.*, 1964). However, the fact that XDH was a homodimer (See evidence for this under XDH characterization) suggested that

intragenic complementation should be possible, with two mutant polypeptides forming a mutant dimer able to carry out some of the functions of wild type XDH. A test for complementation based on the restoration of wild type eye color in trans heterozygotes was conducted among thirty EMS induced and forty X-ray induced null mutants. Seven EMS and four X-ray induced mutants were found to complement (Gelbart, McCarron, Collis, and Chovnick, 1974; Gelbart *et al.*, 1976).

All of the complementing mutants were crossed *inter se* and produced 15 (out of a possible 55) complementing combinations. Some of these pairs showed an intermediate eye color rather than the restoration of a complete wild type phenotype. These pairs were analyzed for XDH activity and levels from 0.2% to 16% of wild type were found. One combination, ry^{606}/ry^{609}, (2.5% of wild type activity) produced an XDH with faster migration (1.02) that the wild type ry^{+6} enzyme (1.00). This indicates that at least one of the null alleles had an amino acid substitution resulting in a different charge from the wild type.

(11) Purine sensitive *ry* mutants

Leaky mutants at the *ry* locus with low levels of XDH activity were selected on the basis of heightened sensitivity to purine. A total of 28 mutants were produced with EMS mutagenesis; 13 had the typical *ry* eye color; another 15 had a ry^{+} eye color. Three of the latter were tested more fully: for recessivity to ry^{+}, exposure by deficiencies covering the *ry* locus, allelism to *rosy* nulls, and level of XDH activity. The mutant $ps\ (purine\ sensitive)^{611}$ exhibited 1% of the *+6* activity, ps^{612} 5%, and ps^{214} 24% of *+2* activity; all of the mutants exhibited virtually 100% XDH cross-reacting material as determined by Laurell

rocket gel electrophoresis implying that these are missense mutants. These mutants are killed at doses of purine (approximately 0.2%) which have almost no effect on ry^{+2} or ry^{+6} flies, which exhibit 80-100% eclosion.

The *ps* mutants have been mapped against the *ry* null alleles using standard procedures including information on electrophoretic sites. The mutant ps^{214} was found to map between ry^{606} and *e507* in the left end of the *rosy* locus. The other two alleles, ps^{611} and ^{612}, mapped in the middle of the *rosy* locus distal to ry^{8}. (Gelbart *et al* 1976; Chovnick *et al.*, 1977)

The present information from mapping of *ry* nulls, complementers, electrophoretic sites, and *ps* mutants has been summarized in a map by Gelbart *et al.* (1976) as shown in Figure 1.

(12) Evidence for a controlling element adjacent to the *ry* locus

A wild type strain, ry^{+4}, was found to have much greater levels of XDH activity (approximately 3-4 fold greater) than other wild type strains. (Chovnick *et al.*, 1976) By thermolability and enzyme kinetic studies (Edwards *et al.*, 1977) the ry^{+4} XDH could not be distinguished from that of other strains. However, by immunological criteria, it was established that this strain had increased amounts of protein as well as XDH activity. These data suggested a difference in regulation of XDH protein synthesis rather than production of a more active structurally changed protein. Mapping experiments were carried out using null alleles produced on the ry^{+4} background, with respect to other *ry* nulls, electrophoretic variants, and purine sensitive alleles. These indicated that the site responsible for the high activity

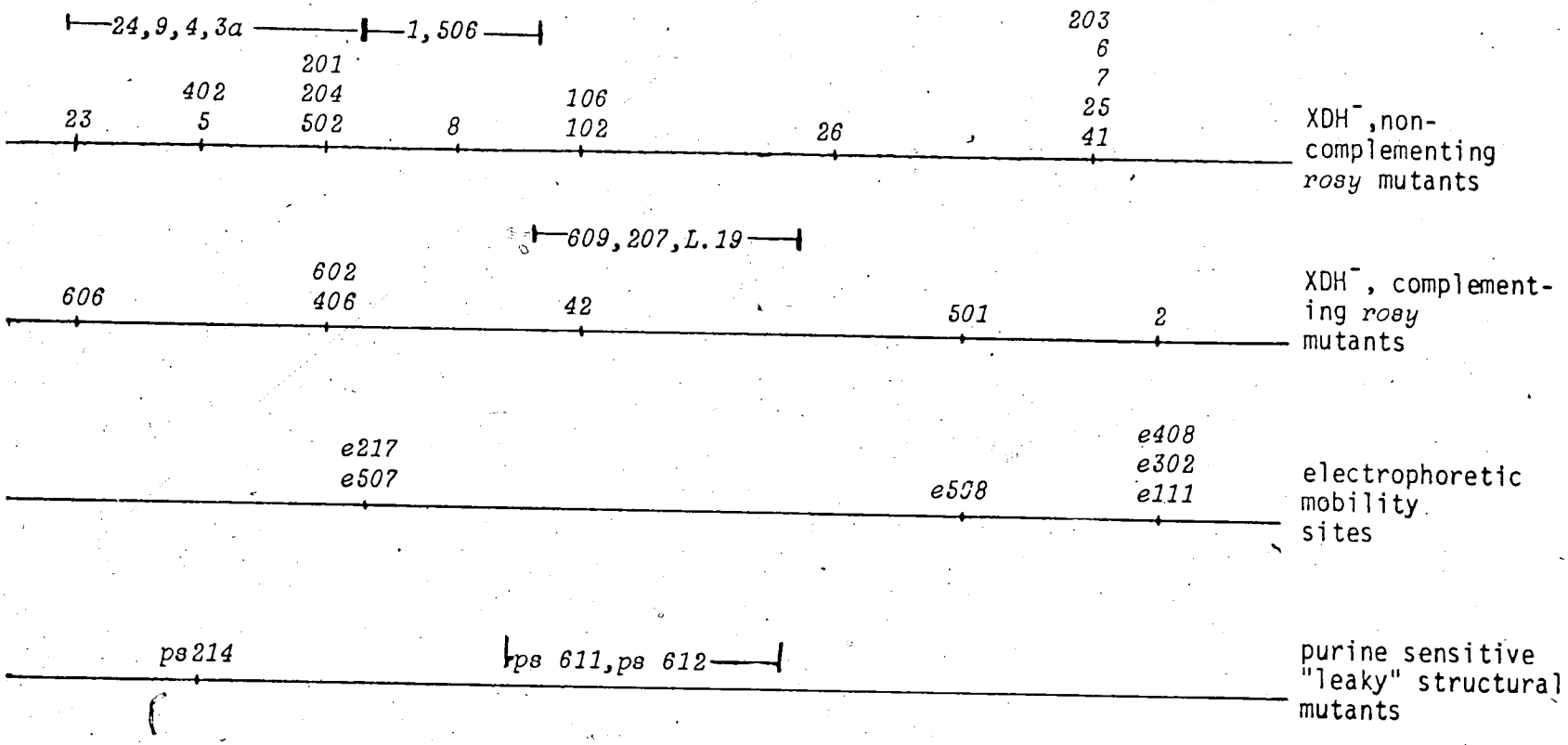


Fig. 1. Genetic fine structure map of the *rosy* locus (from Chovnick *et al.*,1976)

is located to the left of all known markers within the *ry* locus.

The site was then mapped against *l(3) S12*, an X-ray induced mutant which maps between *mes* and *ry* and is believed to be a mutant in the first genetic unit to the left of *ry*. Heterozygous females, either $ry^{406}/+$ or $ry^{402}/+$ both null alleles mapping in the left end of *ry*, *l(3) S12*, and other outside markers were crossed to males of a genotype such that only recombinants in the *ry* region would survive. These flies, with a crossover between *l(3) S12* and ry^{406} or ry^{402}, were classified for high or low activity for XDH; 10 had high activity; 6 had low activity. This indicated that the site of difference, designated *i409H* or *L*, was between *l(3) S12* and the left end of the XDH structural gene element.

This intensity variant is thought not to be due to duplication of the XDH structural gene as no cytological abnormalites are found and the genotype is quite stable. Thus, it is thought to be a mutation in the control element for XDH. (Chovnick *et al.*, 1976, 1977)

III. Other loci affecting XDH and related enzymes

(1) *maroon-like (ma-l)*

The first mutation at the *maroon-like* locus (*ma-l*,1-64.8) was X-ray induced in 1935 by Oliver (Bridges and Brehme, 1944) and found to lack XDH activity by Forrest *et al.* (1956). The early work of Glassman and Mitchell (1959) showed that *ry* flies lacked cross-reacting material to anti-XDH sera whereas *ma-l* flies possessed CRM. The work of Grell (1962) on dosage effects and Yen and Glassman (1965) on electrophoretic variants of XDH indicated that *ry*, not *ma-l*, was the

major if not only structural gene for XDH. Two other enzymes, pyridoxal oxidase (PO) and aldehyde oxidase (AO), were subsequently found to be lacking in *ma-l* flies (Forrest *et al.*, 1961 and Courtright, 1967) but not in *ry* mutants. The two genes were further distinguished by differences in maternal effect, that is, *ma-l* homozygotes or hemizygotes from *ma-l/ma-l*$^+$ mothers had wild type eyes. This is not true of *ry* progeny from *ry/ry*$^+$ females. (Hubby and Forrest, 1960)

An array of mutants at the *ma-l* locus was produced by X-ray and chemical mutagenesis (Schalet and Finnerty, 1968a). The *ma-l* locus was found to show intragenic complementation (Schalet and Finnerty, 1968b; Finnerty and Chovnick, 1970) with restoration of 5-66% of XDH activity and similar restoration of aldehyde oxidase activity (Finnerty, 1976). The alleles were mapped using the purine selection scheme for *ma-l*$^+$ recombinants. (Finnerty, Duck, and Chovnick, 1970) Both single crossover events (41% of *ma-l*$^+$ chromosomes) and conversions (59% of *ma-l*$^+$ chromosomes) were seen. The length of the locus was determined to be 8.3×10^{-4} map units, approximately one-tenth the size of the *ry* locus.

The alleles at the *ma-l* locus fell into five complementation groups, groups III, IV, and V acting as independent cistrons with complementation between groups; the group I mutants could be considered as deletions or polar mutants covering groups III, IV, and V; group II mutants were similar to group I, but covered only groups IV and V. The genetic and complementation maps were colinear. The question of the number of cistrons involved was resolved by the use of a triple mutant: *ma-l*^{F4} (III) *ma-l*^{F3} (V) / *ma-l*^{1} (IV). On a three cistron model this

heterozygote would be expected to show complementation, whereas on a one cistron model complementation may or may not be exhibited. Four double mutants (*ma-l*^{F4} *ma-l*^{F3}) which did not show complementation with *ma-l*^{1} were produced, indicating one cistron at this locus (Finnerty and Chovnick, 1970).

This work was later criticized by Duck and Chovnick (1975) on the basis that unequal exchanges might have occurred in the production of the double mutants such that complementation could not take place. They therefore tested the triple mutant *F4 + F3/+ 1 +* combination for ability to undergo conversion of the *ma-l*^{1} site. This ability was shown, indicating that the *F4 + F3* chromosome was not deficient for any information between the two mutant sites. The argument for a single cistron at the *ma-l* locus as put forward by Finnerty and Chovnick (1970) was thus accepted.

Two models to explain the effect of *ma-l* on XDH and AO have been put forward: one, that *ma-l* codes for a polypeptide subunit common to both XDH and AO; two, that *ma-l*$^{+}$ controls a co-factor common to both enzymes (Glassman, 1965). Possible co-factors are FAD, molybdenum, and iron as found in vertebrate xanthine oxidases. The first model was partially refuted by the findings of Yen and Glassman (1965) and Gelbart, McCarron, Pandey, and Chovnick (1974) that no known electrophoretic variants of XDH map to the *ma-l* locus. The additional information that *ma-l* mutants contain 100% XDH CRM further disfavors this hypothesis.

Andres (1976) has performed an elegant series of experiments to test the function of the *ma-l* gene. He purified XDH and AO from wild

type flies, produced separate antibody preparations to the enzymes, and bound the antibodies to sepharose columns. Andres used these immuno-adsorption columns to purify XDH and AO CRM from *ma-l* flies. The extracts were found to contain XDH CRM but did not contain AO CRM. The XDH CRM appeared to be identical to wild type active XDH by immuno-diffusion, gel electrophoresis, presence of FAD, molybdenum, and iron. Thus, it appears that the role of *ma-l* is not simply the production of a subunit, nor that *ma-l* mutants are unable to incorporate one of the known co-factors of XDH. Other hypotheses not ruled out by these studies include synthesis or incorporation of an unknown co-factor or activation of a co-factor.

(2) *low xanthine dehydrogenase (lxd)*

Keller and Glassman (1964) screened 98 wild type strains for xanthine dehydrogenase with the purpose of finding a class of mutants involved in regulation of enzyme activity. One strain, an inbred stock from Pennsylvania State University, was observed to have low activity -- about 25% of the average (Oregon-R was used as a standard). The genetic analysis of this strain showed that the gene responsible for the difference was on the third chromosome at 3-33±. The gene was designated *low xanthine dehydrogenase (lxd)*.

This gene was later found to reduce both AO and PO (Courtright, 1967; Glassman, 1965). The homozygotes, *lxd/lxd* appeared however to produce near normal levels of XDH CRM (Karam, 1965) but reduced AO CRM (Courtright, 1967). Heterozygous *lxd/+* flies have normal levels of XDH activity but reduced levels (60%) of AO activity (Courtright, 1967).

The *lxd* gene was furthermore found to interact with the expression of the *ma-l* and *cinnamon* (*cin*) phenotypes. Whereas *ma-l* progeny from *ma-l/+* mothers and *cin* progeny from *cin/+* mothers normally show wild type eyes, such flies which are also *lxd/lxd* show dull red eyes (Courtright, 1975). This effect could sometimes be removed by the addition of certain concentrations of molybdenum to the media although most of the flies which emerged had the mutant eye phenotype.

(3) *cinnamon (cin)*

The first mutation at the *cinnamon* (*cin* 1-0.0) locus was induced by EMS in a search for meiotic mutants (Baker, 1973). The mutant was found to be similar in phenotype to *ry* and *ma-l* mutants with a reduction in drosopterins resulting in dull red eye color, and accumulation of AHP, hypoxanthine, guanosine, inosine, and xanthine. The cin^+ product (either maternally or zygotically) appeared to be necessary for completion of development, that is, *cin* progeny of *cin* mothers were inviable, whereas cin^+ progeny of *cin* mothers or *cin* progeny of cin^+ mothers were viable and had wild type eyes.

The *cin* phenotype and maternal effects were compared to those of *ma-l* by Browder and Williamson (1976). They showed that in maternally affected *ma-l* individuals XDH activity was detectable in first and second instar larvae, reduced in third instar larvae and pupae, and undetectable in adults. Maternally affected *cin* individuals, however, had more XDH activity in larval stages and in early pupae but none detectable in aged adults. These authors also showed that *cin* had an effect on AO and PO activities, similar to that of *ma-l*. This effect was such that low but detectable amounts of AO were found from

late third instar larvae to aged (7 day) adults, but no PO activity was observed at any of these stages. A second allele at this locus, cin^2, was also produced by these workers, and observed not to have the conditional female sterility of the *cin* mutant.

Padilla and Nash (1977) have used allopurinol (HPP, described earlier) to screen for more *cin* mutations: with the addition of allopurinol to the media, maternally-affected *cin* offspring are mutant in eye color. Two new EMS induced mutants, cin^3 and c [illegible]ere thus obtained. The sensitivities to allopurinol of the t[illegible] mutants were tested as defined by the concentration of allopuri[illegible]uired to produce 50% mutant eye color; the order (from most sensitive to least sensitive) was found to be cin^3 -- *cin* -- cin^4. This sensitivity appeared to be correlated with the female sterility phenotype, that is, cin^3/cin^3 females mated to cin^3/Y males never produce progeny, *cin/cin* females mated to *cin*/Y males produce about 8% progeny, and cin^4/cin^4 females are completely fertile when mated to cin^4/Y males. Using deficiency and duplication mapping, the *cin* locus was also ordered with respect to other genes in the region, mapping to the left of *y* at the tip of the X-chromosome.

(4) Structural genes for AO and PO

In the above discussion, several references have been made to the enzymes aldehyde oxidase and pyridoxal oxidase. Both of these activities were discovered incidentally during work on XDH and both appear to be influenced along with XDH by the *ma-l*, *lxd*, and *cin* loci. The structural gene for AO was mapped to the third chromosome at about 64 by analogy to the *D. simulans aldox* locus at 3-74.5 (Courtright, 1967). Dickinson

(1970) discovered electrophoretic variants of aldehyde oxidase and used these to map the *aldox* locus to 3-56.6. Null mutants at the locus were also produced; in addition, Collins *et al.* (1971) reported on *low aldehyde oxidase* (*lao*) a mutant which reduced AO activity and mapped to the same region as *aldox*. It has been suggested that this is a leaky allele of the *aldox* locus. (Dickinson and Sullivan, 1975)

The structural gene for pyridoxal oxidase is thought to be *low pyridoxal oxidase* (*lpo* 3-57±) (Collins and Glassman, 1969). Mutants at this locus have less than 5% of wild type PO activity, but neither electrophoretic variants nor dosage studies using deficiencies have been reported.

Thus, it appears that the structural loci for three related enzymes map together within five map units on the third chromosome and that their activities are influenced by three unlinked loci scattered throughout the rest of the genome. The further elucidation of the roles of each locus can only prove this system to be even more interesting.

IV. XDH Purification and Characterization

(1) XDH purification

Glassman and Mitchell (1959) described a procedure for the purification of XDH involving treatment with charcoal (Norite-A), 30-50% ammonium sulfate precipitation, a calcium phosphate gel, and a further ammonium sulfate precipitation which gave an XDH purification of 10 to 50-fold. This extract was used successfully to elicit antibodies in rabbits.

A purification scheme which produced a 500-fold pure extract was

developed by Parzen and Fox (1964). An homogenate of adult flies was treated with Norite-A; the supernatant was adjusted to pH 5.0 and then re-adjusted to pH 8.0; the supernatant was put through a 25-50% ammonium sulfate precipitation; the re-dissolved pellet was passed over a DEAE-cellulose column and the pooled fractions were concentrated with Sephadex G-25. The yield was about 50%. No tests of the homogeneity of the preparation were reported.

Karam (1965) used three methods to purify XDH; the method producing the highest purification is outlined below. A crude homogenate of adult flies was subjected to a pH 5.0 precipitation; the supernatant was adjusted back to pH 8.0 and put through an ammonium sulfate (35-45%) precipitation. This extract was then put through a Sephadex G-25 column to get rid of eye pigments and the protein band was then chromatographed on a DEAE-cellulose column. The resulting pooled fractions of activity represented a 20% yield with a 123-fold purification.

A technique which resulted in a homogeneous preparation of XDH was reported by Seybold (1974). He also started with an homogenate of adult flies which was subjected to treatment with Norite-A. The charcoal treated extract was put through a 40-60% ammonium sulfate treatment; the re-dissolved pellet was subjected to a 5 minute heat treatment at 68°C and the supernatant dialyzed overnight. This dialyzed preparation was then put through DEAE-cellulose chromatography, pooled, and concentrated. The DEAE concentrate was further chromatographed on an hydroxyapatite column and the concentrate from this was applied to a Sephadex G-200 column. The resulting preparation was approximately

400-fold pure with a yield of 10%. This preparation was judged homogeneous by analytical gel electrofocusing and polyacrylamide disc gel electrophoresis.

Both Andres (1976) and Edwards *et al.* (1977) have used immunoaffinity chromatography to purify XDH. For this procedure, antibodies to conventionally purified XDH are linked to Sepharose CL-4B. This is accomplished by activating the Sepharose with cyanogen bromide, adding the antibodies, and shaking the mixture with glycine in sodium bicarbonate buffer; the gel is then washed with NaCl in sodium bicarbonate, sodium acetate, and again with sodium bicarbonate; a further wash with Tris buffer is required. XDH is prepared by conventional steps up to DEAE chromatography before being applied to the column and is eluted with either KI (Andres, 1976) or with NH_4SCN (Edwards *et al.*, 1977). This procedure also yields a homogeneous XDH preparation.

It should be noted that the estimates of purity and yield produced by the Parzen and Fox (1964) method appear to be suspect in light of the findings of both Karam (1965) and Seybold (1974). The latter workers found a purification of roughly 120-fold after DEAE chromatography of an ammonium sulfate treated preparation.

(2) Molecular weight determinations

The molecular weight of XDH has been determined by gel filtration to be approximately 300,000. Two subunits of 130,000-140,000 have been observed upon sodium dodecyl sulfate gel electrophoresis (Seybold, 1974). The same value of 300,000 molecular weight for the native XDH enzyme as determined by gel filtration on Sepharose-4B was also obtained by Andres (1976) with a subunit molecular weight of 150,000.

(3) XDH is a homodimer

The determinations of the molecular weight of XDH and its subunits suggest, but do not prove, that XDH is a dimer consisting of two identical subunits, that is, a homodimer. This model has been tested by Gelbart, McCarron, Pandey, and Chovnick (1974) using two electrophoretic variants of XDH. The isoalleles ry^{+0} and ry^{+5} differ at two sites, *e507* and *e508*; the former maps to the left cluster of electrophoretic sites; the latter maps to the right. The distance between them spans most of the known *ry* structural element. If XDH is a heterodimer composed of two subunits α and β and *e507* maps in α, and *e508* in β, then both cis and trans heterozygotes of ry^{+0}/ry^{+5} should give three bands of XDH in an electropherogram. If, however, XDH is a homodimer, a cis heterozygote *(e507S e508S/e507F e508F)* should be able to produce three bands (S/S, S/F, F/F) in a gel whereas the trans heterozygote (*e507S e508F/e507F e508S*) should only produce an intermediate band. The latter "cis-trans" effect was observed indicating that XDH is a homodimer.

(4) Kinetic studies of XDH

The Michaelis-Menten constants (K_m's) have been determined for each of the substrates of XDH and for NAD+. The determinations made by various workers have been consistent. The K_m for AHP is 6.7×10^{-6}M; the K_m for hypoxanthine is 2.1×10^{-5}M (Glassman and Mitchell, 1959), 2.36×10^{-5}M (Parzen and Fox, 1964), 2.0×10^{-5}M (Edwards *et al.*, 1977). The K_m of XDH for NAD+ is 4.0×10^{-5}M as determined by Edwards *et al.* (1977). The K_m for xanthine is 2.5×10^{-5}M (Glassman and Mitchell, 1959), 2.36×10^{-5}M (Parzen and Fox, 1964), or 2.4×10^{-5}M (Edwards *et al.*, 1977).

(5) XDH requires co-factors

A crude fly extract which had been subjected to charcoal treatment to remove endogenous pteridines and purines was found to require the addition of an electron acceptor such as NAD+, methylene blue, or 2.6-dichlorophenol for restoration of XDH activity (Glassman and Mitchell, 1959). Andres (1976) has shown that both XDH and XDH CRM (prepared from *ma-l*) contain the co-factors flavinadenine dinucleotide (FAD), molybdenum, and iron. No other co-factors are known at present.

(6) Heat stability

XDH has been found to be heat-labile at 50°C in the presence of its substrates AHP or hypoxanthine (Glassman, 1966). However, other workers (Seybold, 1974; Edwards *et al.*, 1977) have found that treatment of XDH with heat at temperatures of 68 - 70°C for five minutes produces little loss of activity and a significant increase in purification by getting rid of more heat-labile proteins.

(7) Multiple molecular forms of XDH

Shinoda and Glassman (1968) have shown that partially purified extracts of *Drosophila* contain two forms of XDH which are separable by DEAE chromatography or polyacrylamide gel electrophoresis. The first peak of XDH from DEAE chromatography, XDH-I, can be converted *in vitro* to XDH-II by incubation with *ry* or *ma-l* extracts. XDH-I appears to be the form of XDH present *in vivo* as it is the only form present in crude extracts. However, flies raised on a high protein diet have increased levels of XDH activity without increased CRM and this appears to be of the XDH-II type. It should be noted that none of the experiments

showing two peaks of XDH on DEAE chromatography used a heat step prior to the chromatography; Seybold (1974) obtained only one form of XDH from DEAE following a heat treatment. The biological significance of the two forms of XDH is unknown.

MATERIALS AND METHODS

I. *Drosophila melanogaster* stocks

Drosophila stocks were routinely cultured at room temperature, 22 ± 1°C, in half-pint milk bottles containing standard media. The media consisted of 10% yeast, 10% sucrose, 1.5% agar, 1% propionic acid, and .01% chloramphenicol. Some of the crosses were cultured in vials containing the same standard media.

Amherst was used as the wild-type strain for all experiments. Several *rosy* mutants were obtained from A. Chovnick's laboratory including ry^2, a spontaneous mutant, and three alleles, ry^8, ry^{26}, and ry^{41}, induced by X-rays in the *cu kar* chromosome (Lindsley and Grell, 1968). Three other mutants, ry^{601}, ry^{603}, and ry^{604}, received from the same laboratory were induced by EMS in the ry^{+6} electrophoretic background (Chovnick, pers. communication). A stock containing third chromosomes with multiple markers was also obtained from A. Chovnick: *Tp(3) MKRS M(3) S(34) kar* ry^2 *Sb/kar*^{2} *Df(3)* ry^{75} (a deficiency which covers the *pic* locus; Chovnick *et al.*, 1976) and is hereafter referred to as the *MKRS* stock (McCarron *et al.*, 1974). The two eye color mutations *kar* and *ry* give an orange eye color which is easily distinguished from *ry* alone in young flies. These chromosomes comprise a balanced lethal system in that *Sb* and the *Df(3)* ry^{75} are lethal in the homozygous state. Two other stocks: *ru lxd by* (*roughoid, low xanthine dehydrogenase, blistery*) and *ma-l* were used as controls for some experiments.

II. Mutagenesis

Mutants at the *ry* locus were obtained using ethyl methane sulfonate (EMS) as the mutagenic agent. The procedure used was that of Lewis and Bacher (1968). Young Amherst adult males were collected and starved in empty bottles for 8-12 hours. They were then transferred to bottles containing tissues saturated with 9-10 ml. of a 0.025 M solution of EMS (Terochem Laboratories) in sterile 1% sucrose. The flies were allowed to feed for 18-20 hours at 22°C after which they were transferred to new bottles containing standard medium. The bottles and glassware were decontaminated by rinsing with a solution of 0.5% thioglycolate in 1 N NaOH.

The males were mated to virgin females of the *MKRS* genotype and the F_1 flies were examined for eye color. All *ry* phenotype flies were saved and mated back to *MKRS* flies of the appropriate sex in order to produce a stock of the new mutant balanced over the *Tp (3) MKRS* chromosome. Those mutants which did not contain recessive lethals on the balanced region of their 3rd chromosome were allowed to become homozygous for the new *rosy* allele by selecting out non-*Sb* flies from the mutant/*MKRS* stock.

III. Complementation tests

Intragenic complementation within the *rosy* locus was tested for by making non-reciprocal crosses between all the *rosy* mutants. For all combinations excluding $ry^{a9,a10,a11}$, two bottles (each containing 20 virgin females and 20 males) were set up. Progeny from these matings were scored for mutant versus non-mutant eye color. For crosses

involving newly induced mutants balanced over the *MKRS* chromosome, only non-*Sb* progeny were considered. The appropriate flies were collected and tested for XDH activity using the fluorometric assay. For this assay, 200 mg of <4 day old live flies were extracted in 2 ml of extraction buffer (described under fluorometric assay) plus 50 mg. Norite, settled 10 minutes, and centrifuged 2 X 10 minutes at 14,600 x g. From this extract, two assays of 0.75 ml supernatant with no additional buffer were obtained.

Crosses involving $ry^{a9,a10,a11}$ were done in vials with 10 females and 10 males, and the progeny scored for eye color, but no assays for XDH were made. However, those crosses which produced flies of wild-type or intermediate eye color were repeated, and extracts prepared for gel and fluorometric assays.

IV. Xanthine dehydrogenase assays

(1) Fluorometric assay. The assay procedure is based upon the differential fluorescence of isoxanthopterin (IXP) and 2-amino 4-hydroxy pterine (AHP). As XDH catalyzes the conversion of AHP to IXP, its activity can be measured by the increase in fluorescence of a reaction mixture containing fly extract, substrate, cofactor, and buffer (Glassman, 1962).

A solution of AHP (Sigma) was made up by dissolving 3 X 10^{-3} M AHP in 1N NaOH and diluting this mixture to 3 X 10^{-4} M in the cold and dark as AHP is heat and light sensitive. Aliquots of 0.5 to 1 ml were then frozen for future use. The cofactor, β-nicotinamide adenine dinucleotide (NAD^+, Sigma) was dissolved in Tris buffer (10 mg/5 ml) and such solutions were made up as required.

The flies were homogenized in extraction buffer: 1M Tris-Cl (pH 8.0) containing 2.5 X 10^{-4} M EDTA and 1 mM dithiothreitol (DTT) at a weight/volume ratio of 100 mg flies/ml buffer. After grinding, Sigma untreated activated charcoal (Norite) was added to the extract at a ratio of 1 mg charcoal/4 mg adults or 1 mg charcoal/8 mg larvae. The mixture was stirred and allowed to settle for 10 minutes, then centrifuged at 14,600 x g for a minimum of 10 minutes. The supernatants were filtered through nylon bolting cloth (50 micron mesh) or glass wool and centrifuged for a further 10 minute period. At times, a third centrifugation was required to get rid of all the charcoal.

Assays were carried out in an Aminco-Bowman spectrofluorometer with an activation wavelength of 338 nm and a fluorescent wavelength of 405 nm. AHP has a minimum fluorescence level at these settings whereas IXP has a peak fluorescence when activated at 338 nm. The sensitivity level of the photomultiplier was originally set at the sensitivity at which a solution of 1 X 10^{-6} M quinine sulfate in .1N H_2SO_4 gave a reading of 30% transmission.

The assays were based on 1 ml of solution. For crude extracts, 0.5 ml of the fly extract and 0.48 ml of Tris-Cl buffer were allowed to prewarm (for 5 minutes) in the cuvette placed in a temp-block module heater (Scientific Products) set at 30°C. After warming, 10 µl of NAD and 10-30µl of AHP were added to the reaction mixture. After shaking, the cuvette was placed in the fluorometer and the change in % transmission was recorded on a Beckman recorder for 1 to 5 minutes. The change in relative fluorescent intensity, ΔRFI, was calculated by multiplying the %T X meter multiplier setting. The ΔRFI observed was

converted to μm of AHP oxidized per 1% fluorescence by use of a mixed dilution curve constructed by varying the amounts of AHP and IXP in buffer solution and reading the fluorescence obtained.

(2) Gel assay. Polyacrylamide and agarose gels were routinely stained for XDH using hypoxanthine as a substrate as given by Yen and Glassman (1965). The stain consisted of 0.2 M Trizma base, 10^{-3} M hypoxanthine, 4.5 X 10^{-4} M NAD, 2.6 X 10^{-4} M phenazine methosulfate, and 3.9 X 10^{-4} M nitrobluetetrazolium (all from Sigma). The chemicals were preweighed and kept in the dark; they were dissolved in distilled water immediately prior to use. A modification of this stain was used for some of the polyacrylamide gels and all of the agarose gels using .1 M Tris-Cl, pH 8.0 buffer in place of the dry Tris and water. This gave a staining mixture of pH 8 as compared with pH 10 for the former mixture and produced a better stain.

The substrate AHP was sometimes substituted for hypoxanthine in these stains. AHP (32.64 mg) was dissolved in 1 ml of 1 N NaOH and added to 100 ml of .1 M Tris-Cl, pH 8.0 to give a final concentration of 2 X 10^{-3} M AHP in the stain. The other components, NAD, PMS, and NBT, were then added to the mixture.

The gels were allowed to stain in the dark for 30 minutes or longer and then rinsed with water.

V. Vertical slab polyacrylamide gel electrophoresis

An 8% polyacrylamide running gel with a 6% stacking gel was routinely used. A stock solution of acrylamide: bis (30:0.8) was prepared by dissolving 30 g of acrylamide (Terochem Laboratories) and

0.8 g of N,N'-methylene bis acrylamide (Eastman Organic Chemicals) in 100 ml of distilled water, filtering the solution and storing it in the dark at 4°C. The electrode buffer, Tris-borate-EDTA pH 9.2, was prepared using 21.08 g Trizma base, 1.08 g boric acid, 1.86 g disodium EDTA in 2 liters of distilled water (Yen and Glassman, 1965).

Polyacrylamide gels were prepared between two glass plates (14 by 16 cm) with one plate containing a 2 cm X 13 cm notch at the top. Lucite strips were used as spacers between the plates and these were held in place with large paper clamps. The strips were sealed to the plates by dripping 1.5% molten agar along the sides and bottom of the gel former. For an 8% gel, 30 ml of solution was prepared by mixing 8 ml of stock acrylamide solution, 22 ml Electrode Buffer, 15 µl TEMED (N,N,N', N' Tetramethylethylenediamine, Eastman Kodak), and 30 mg ammonium persulfate (EC Apparatus Corporation). This solution was poured between the prepared glass plates. A lucite spacer was placed between the glass plates at the top of the gel to prepare a flat bed for the subsequent stacking gel. Polymerization was carried out at room temperature for at least 30 minutes before this spacer was removed. Then a 6% polyacrylamide mixture was prepared as above with 6 ml of stock solution, 24 ml electrode buffer, plus TEMED and AP. After pipetting this solution onto the top of the running gel, a lucite comb forming 13 pockets was placed between the plates.

After polymerization for a minimum of 30 minutes, the clamps, comb, and bottom lucite strip were removed and the gel clamped to a vertical slab gel electrophoresis chamber (Aqueboque Machine and Repair Shop, N.Y.). The upper notch of the glass plate was sealed to the

upper buffer chamber with 1.5% molten agar. Electrode buffer was poured into the upper chamber to a level above the gel pockets and checked for leakage to the bottom chamber. Electrode buffer was then added to the lower chamber so that the bottom of the gel was immersed in buffer. The gel was then prerun for 30 minutes to 1 hour at 10 mA constant current to get rid of excess ammonium persulfate.

Crude fly extracts were prepared by homogenizing 100 mg/1 ml in electrode buffer + 1 M sucrose. The extracts were centrifuged for 10 minutes at 14,600 X g. Samples from XDH purification steps were brought to 10% glycerol for use in gels. Samples were loaded into the pockets with a Hamilton syringe and 3-5 µl of .002% bromphenol blue was added to one pocket as a tracking dye. The gels were run for a minimum of 3 hours at a constant current of 10 mA. They were then removed from the apparatus and placed in a tray containing 100 ml of stain (see Gel assay). After a minimum of 30 minutes, the gels were destained with water.

VI. XDH purification

XDH was partially purified from Amherst third instar larvae using a modification of the procedure of Seybold (1974). Large quantities of Amherst larvae were obtained from plastic trays, containing standard media plus live yeast, which had been placed in a population cage containing adults. Females were allowed to lay eggs on the food for up to 1 day; trays were removed and left at room temperature until mature third instar larvae had crawled up the sides of the trays. These were collected in an ice bucket by running a stream of tap water over the

sides of the trays. The larvae were rinsed several times with tap water until no more pupae floated in the bucket. They were collected in a sieve and left to dry on paper towels; when dry, they were either used immediately or frozen at -70°C.

All extraction steps were carried out in the cold, at approximately 4°C. A crude extract of the larvae was prepared by grinding up to 200 g wet weight of larvae with a mortar and pestle in extraction buffer as described under XDH assays. The ratio of larvae to buffer was 100 g/1000 ml in earlier experiments and increased two-fold in later experiments (see Results for details). In later experiments, larvae were homogenized more efficiently by use of a Waring blender. Originally, the crude extract was centrifuged for 15-20 minutes at 14,600 X g and the supernatant was subjected to Norite tr in order to remove endogenous purines and pteridines. Later, first centrifugation was omitted and the entire extract treated with Norite. In both cases the ratio of Norite/larvae was 1 g/8 g. The charcoal was added to the mixture and stirred with a glass rod. The mixture was allowed to settle for 10 minutes, was filtered through a nylon mesh into centrifugation bottles, and spun at 14,600 g for 15 minutes. The supernatants were poured through nylon mesh and recentrifuged. When necessary, this filtration and centrifugation was repeated a third time.

After removing an aliquot of crude extract for samples, the remainder was subjected to an ammonium sulfate precipitation. A 43-58% cut was routinely used; this was a narrower range than that described by Seybold. Solid ammonium sulfate (J.T. Baker Co.) was added slowly

to a stirring preparation at 23.9 g/100 ml extract. The mixture was stirred for at least 30 minutes, then centrifuged for 15 minutes at 14,600 X g. The pellets were discarded and the supernatants pooled and brought to 58% saturation with the addition of 9.6 g/100 ml solution. After stirring and centrifugation as before, the supernatants were discarded and the pellets redissolved in extraction buffer.

The ammonium sulfate was then removed from the preparation by dialysis against 2 changes of 2700 ml of .1 M Tris-Cl, pH 8.0 over a 4 hour period. The dialyzed extract was then put through a heat treatment. Not more than 100 ml of extract were placed in a preheated 250 ml beaker and set in a water bath at 60°C for 3 minutes with swirling at 30 second intervals. The preparation was cooled in ice and immediately poured into cold centrifuge tubes for centrifugation at 14,600 X g for 15 minutes. The supernatants were collected and brought to 16% polyethylene glycol (PEG, ave m.w. 6000-7500, MC/B) by the slow addition of 40% wt./vol. PEG in Tris-Cl. The mixture was allowed to stir in the cold for at least 20 minutes and centrifuged for 15 minutes. The 16% PEG supernatant was collected and either used immediately for further purification or frozen at -70°C. The enzyme was found to be quite stable in PEG either frozen, or when left overnight in an ice bucket in the cold room.

The PEG supernatant was further purified by column chromatography. A column of diameter 2.5 cm and 25 cm height was poured to a height of 20 cm with DEAE-cellulose (Sigma, coarse mesh) prepared by washing with HCl, NaOH, and NaCl prior to use. The column was washed with

.1M Tris-Cl, pH 8.0 plus 1 mM DTT and equilibrated with .05 M NaCl in the same buffer. The PEG supernatant was then pumped onto the column at a speed of approximately 1 ml/minute and washed on with an equal volume of starting buffer. The column was then eluted with a 500 ml gradient of .05 M - .5 M NaCl in the same buffer. Fractions of approximately 5 ml volumes were collected and every fifth fraction assayed for XDH activity and absorbance at A_{280} in a Beckman DB-G spectrophotometer. Additional fractions around the XDH peak were assayed and the peak fractions pooled, brought to 10% glycerol, and concentrated. For concentration, an Amicon ultrafiltration cell model 202 was used for large volumes and a model 10-PA with a PM10 membrane was used for volumes less than 15 ml.

The DEAE concentrate was further purified by application to an hydroxyapatite column. Bio-Gel HTP (Biorad) was prepared in 1 mM sodium phosphate buffer, pH 6.7 and 1 mM DTT and poured into a column of diameter 1.5 cm to a height of 40 cm. It was equilibrated with a large amount of the same buffer (approximately 10 column volumes). The DEAE concentrate was pumped onto the column and washed on with a volume twice that of the sample; both steps were done at a flow rate of about 4 ml/min. The column was then eluted at a lower speed with a 500 ml linear gradient of 1 mM- 250 mM sodium phosphate buffer, pH 6.7 + 1 mM DTT. Fractions were collected, assayed, and concentrated in 10% glycerol as described for the DEAE step.

VII. Protein determination

Protein was determined by a modification of the procedure of Lowry *et al.* (1951). Reagents used were 12.5% sodium carbonate, 0.1% anhydrous cupric sulfate, and 2 N Folin-Ciocalteau reagent diluted 1:2 immediately prior to use (all from Fisher Scientific Co.). The fly extracts were dialyzed overnight against H_2O to get rid of dithiothreitol which was found to be Lowry-positive. A volume of 0.2 ml of appropriate dilutions of the samples was added to test tubes containing 2.0 ml of Na_2CO_3 and 0.5 ml of $CuSO_4$ and vortexed. After letting the mixture stand for 10 minutes at room temperature, 0.5 ml of the Folin reagent was added, while vortexing, to the tubes. The color was allowed to develop for a further 20 minutes before the absorbance at 660 nm was read on a Beckman DB-G spectrophotometer. Bovine serum albumin (BSA, Sigma) diluted with water was used as a standard and the blank contained H_2O plus the reagents.

VIII. Disc gel electrophoresis

Disc gel electrophoresis with a continuous buffer system was performed on the partially purified XDH preparations. Glass cylinders of 10 cm length and 6 mm diameter were sealed with parafilm at one end and placed in holders (rubber stoppers placed upside down). An 8% solution of acrylamide:bis as used for vertical slab gel electrophoresis was prepared and pipetted to a height of 7.5 cm in the cylinders. A layer of distilled water was carefully added to the top of the gel and polymerization allowed to take place for 30 minutes. The water layer was then removed and a 6% stacking gel pipetted on top to a full height of 8.2 cm for the complete gel. Water was carefully layered on top of

this gel in order to allow complete polymerization of the stacking gel. After 30 minutes the water and parafilm were removed.

The cylinders were placed in a disc gel apparatus (Buchler Instruments) and electrode buffer added to the top and bottom chambers. The gels were prerun at 1.5 mA constant current/tube for 30 minutes to get rid of excess ammonium persulfate. The buffer was then changed and 100-200 µl of sample was applied to each tube with a Hamilton syringe. The gels were run for 4½ hours at 1.5 mA per tube, removed, and stained for protein or XDH activity.

IX. Gel stain for protein

Acrylamide and agarose gels were stained for protein using .05% Coomassie brilliant blue (Sigma) in 25% isopropyl alcohol and 10% acetic acid. Gels were covered with this staining solution and left at room temperature for 1 hour (agarose gels) or overnight (acrylamide gels). They were then destained with several changes of 7% acetic acid.

X. Preparation of rabbit anti-XDH serum

Two female San Juan Island wild type rabbits were immunized against XDH preparations. Before immunization, control sera were obtained from both rabbits by bleeding them from the middle ear artery using a 10 ml syringe equipped with a #23 gauge needle. The blood was allowed to clot at 4°C for 24 hours and the sera drawn off. Additional sera were obtained by centrifugation at 14,600xg for 15 minutes; the sera were then frozen.

Rabbit 5GJ6 was immunized with 2 ml of DEAE-step XDH preparation mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories) injected half intramuscularly and half subcutaneously. The immunization was repeated at weekly intervals for 3 weeks using 1 ml of DEAE preparation and 1 ml of Freund's incomplete adjuvant (Difco). One, two, and three weeks after the last immunization the rabbit was bled for immune serum.

Rabbit 5HE3 was originally immunized with 1.8 ml of HAP preparation mixed with 2 ml of Freund's complete adjuvant injected intramuscularly and subcutaneously. Three booster shots of 1 ml HAP preparations in incomplete adjuvant were given at weekly intervals, and serum was obtained 8 and 16 days after the final boost.

Both rabbits were later boosted with HAP-step XDH preparations and bled by cardiac puncture to obtain about 40 ml of serum per rabbit. The sera were prepared for use in Laurell rocket gel electrophoresis by an ammonium sulfate precipitation. Five ml of sera were brought to 45% saturation by the addition of 1.29 g of ammonium sulfate, vortexed, and centrifuged. The pellet was re-dissolved in 5 ml of extraction buffer and frozen for future use.

XI. Laurell rocket gel electrophoresis

1% weight/volume Sigma Agarose Type I, Low EEO, was dissolved in 25 ml of Camag High Resolution electrophoresis buffer (Tris-barbiturate, pH 8.8, 1 pkg/1800 ml) by heating in a boiling water bath. After melting, the liquid agarose was allowed to cool to 45°C at which temperature 25 µl of ammonium sulfate treated rabbit anti-XDH sera was added to the agarose and stirred. The liquid was immediately pipetted

with a prewarmed pipette into a gel former prepared as described previously for vertical slab polyacrylamide gel electrophoresis.

The gel was allowed to cool in a vertical position for a minimum of one hour after which time one of the glass plates and the lucite strips were removed. With the gel laid on a flat surface, 12 holes were punched in the gel in a row approximately 2 cm from the bottom of the gel and 1.1 cm apart with a #2 cork borer (5 mm diameter).

The gel and supporting glass plate were then placed on a Camag Universal Electrophoresis Chamber and Tris-barbiturate buffer added to the chambers. The gel was connected to the chambers by using buffer-soaked 1 mm thick filter papers as wicks between the gel and the buffer. Samples of wild-type (Amherst) and mutant flies or buffer were loaded into the holes using a 10 µl Hamilton syringe. These extracts were prepared in Tris-borate-EDTA-sucrose buffer, pH 9.2 (Electrode Buffer and 1 M sucrose) as for polyacrylamide gel electrophoresis.

The gel was electrophoresed at a constant voltage of 120 volts (10-15 mA) for a minimum of 9 hours. The gel was then removed and stained in the dark for XDH for at least two hours. The XDH stain is described under Gel assay. The heights of the XDH rockets were measured from the top edge of the hole to the top of the rocket and a standard curve of wild type extract versus height of rocket was plotted for each gel. The heights of the rockets containing both mutant and wild type extracts were compared to the height obtained by wild type alone. From this information determinations of the amount of cross-reacting material to anti-XDH sera, if any, were obtained for *lxd ma-l* and for each of the *ry* mutants.

RESULTS AND DISCUSSION

I. Mutagenesis

Several putative *ry* mutants were recovered in the F_1 generation after EMS mutagenesis of males following the mating scheme shown in Figure 2. These mutants were identified on the basis of dull red eye color and were established as stocks over the *MKRS* chromosome. Some mutants segregated out other eye colors such as *white* and were not further analyzed. Those which segregated as *ry* alleles were assayed for XDH activity in the fluorometer. The results of these assays are given in Table 1. All of the mutants except for ry^B, ry^C, and ry^F appeared to have no activity. The values for ry^B and ry^C are around the 50% level expected for heterozygotes at the *ry* locus as the ry^2 allele does not exhibit activity. These flies appear to be rosy in eye color at eclosion but become more wild type with age. It has been suggested that these may be purine-sensitive alleles at the *ry* locus (A. Hilliker, personal communication), as the phenotype is similar to that of some of the *ps ry* mutants found in Chovnick's laboratory (Gelbart *et al.*,1976). The third mutant, $ry^F/MKRS$ has slightly less activity than expected for a heterozygote but was not further analyzed. A fourth mutant, $ry^L/MKRS$, was not included in later studies due to its relative inviability. A control strain, *lxd*, gave comparable activity to that found by Keller and Glassman (1964) of 40% of Oregon-R wild type activity when compared to either Amherst or Canton-S wild type strain.

These mutants which appeared as nulls in the test tube assay were

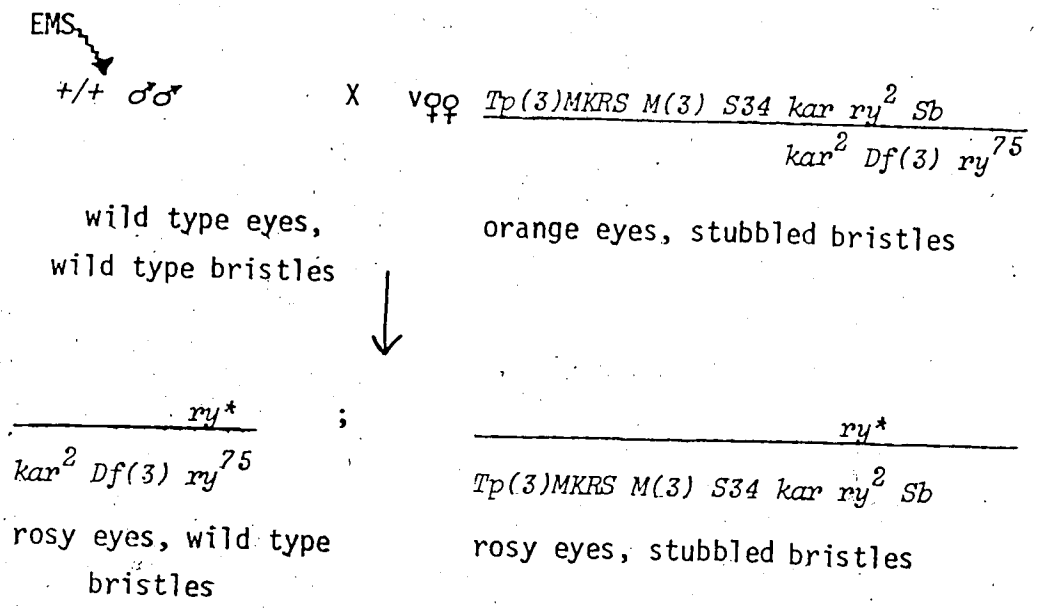


Fig. 2. Mutagenesis Scheme for the Induction of *ry* Mutants

The *ry** represents a newly-induced mutant allele in the F_1 progeny which can be distinguished from non-virgin progeny by eye color; the *MKRS* stock has two eye color mutations which results in an orange eye phenotype. Both *Sb* and non-*Sb* flies were crossed back to flies of the *MKRS* genotype to establish a stock over the *MKRS* balancer chromosome.

Table 1. XDH Activity of Newly Induced Mutants in the Fluorometric Assay

Genotype	% wild type activity
ry^{a1}	0
ry^{a2}/MKRS	0
ry^{a3}	0
ry^{a4}/MKRS	0
ry^{a5}/MKRS	0
ry^{a6}/MKRS	0
ry^{a7}/MKRS	0
ry^{a8}/MKRS	0
ry^{a9}/MKRS	0
ry^{a10}/MKRS	0
ry^{a11}/MKRS	0
"ry"^{B}/MKRS	55.3
"ry"^{C}/MKRS	52.6
"ry"^{F}/MKRS	37.8 *
ry^{L}/MKRS	0
ru lxd by (control)	27.0
ru lxd by (control)	26.1 *

All mutants were assayed as adults with Amb[illegible]s the control strain except as noted (*) where Canton-S was used as the control. The flies were ground in extraction buffer (100 mg/ml), treated with Norite, centrifuged, and the supernatants were assayed in the fluorometer as in Materials and Methods. For each genotype the average of three replicates is represented.

then tested for XDH activity in gel assays. The gel assay was found to be more sensitive than the fluorometric assay in one instance, that is, $ry^{a4}/MKRS$ showed activity in the gels but not in the fluorometer. The results of the gels are shown in Table 2. The mutant ry^{a4} produced a faint band of activity which was electrophoretically distinguishable from the Amherst band (Figure 3). This mutant, ry^{a4}, also gave a band in combination with $Df(3)ry^{75}$ and must therefore be considered a "leaky" mutant at the *ry* locus which, however, retains the typical rosy phenotype. A mutation which simultaneously affects both activity and electrophoretic mobility has been observed previously in the *ry* locus: the complementing pair, ry^{606}/ry^{609}, from Chovnick's laboratory was observed to have a mobility different from that of the ry^{+6} isoallele (Gelbart *et al.*, 1976). However, this is the first instance of a mutant phenotype associated with a detectable level of XDH activity. Previously, it has been considered that only a "modicum" (less than 1%) of XDH activity is required to produce a wild type eye color (Gelbart *et al.*, 1976).

II. Complementation tests

The *ry* null mutants plus the collection from Chovnick's laboratory were tested for interallelic complementation as depicted in the complementation matrix, Table 3. This table is based on a phenotypic expression of complementation -- the production of an eye color which is more wild type than the parental eye color. Five instances of such complementation were detected involving only those mutants induced in this laboratory. Two of the heterozygous pairs, ry^{a3}/ry^{a4} and ry^{a3}/ry^{a5},

Table 2. XDH Activity of *ry* Mutants in Polyacrylamide Gel Assays

Genotype	% wild type
$ry^{a1, a3}$	0
$ry^{a4}/MKRS$	2.4
$ry^{a4}/Df(3)ry^{75}$	0.4
$*ry^{a2, a5, a6, a7, a8, a9, a10, a11}$	0
MKRS	0
$ry^{2,8,26,41,601,603,604}$	0

* assayed as heterozygotes with *MKRS*

Adult flies were homogenized in electrode buffer + sucrose (100 mg/ml) and centrifuged 10 min at 14,600 x g. The supernatants were loaded into the pockets of an 8% polyacrylamide gel (6% stacking gel) using 30μl volumes for mutant extracts and 5μl for wild type. The gels were electrophoresed for 3-4 hours, then stained for XDH using either hypoxanthine or AHP as the substrate as in Materials and Methods. The bands were quantitated by densitometry.

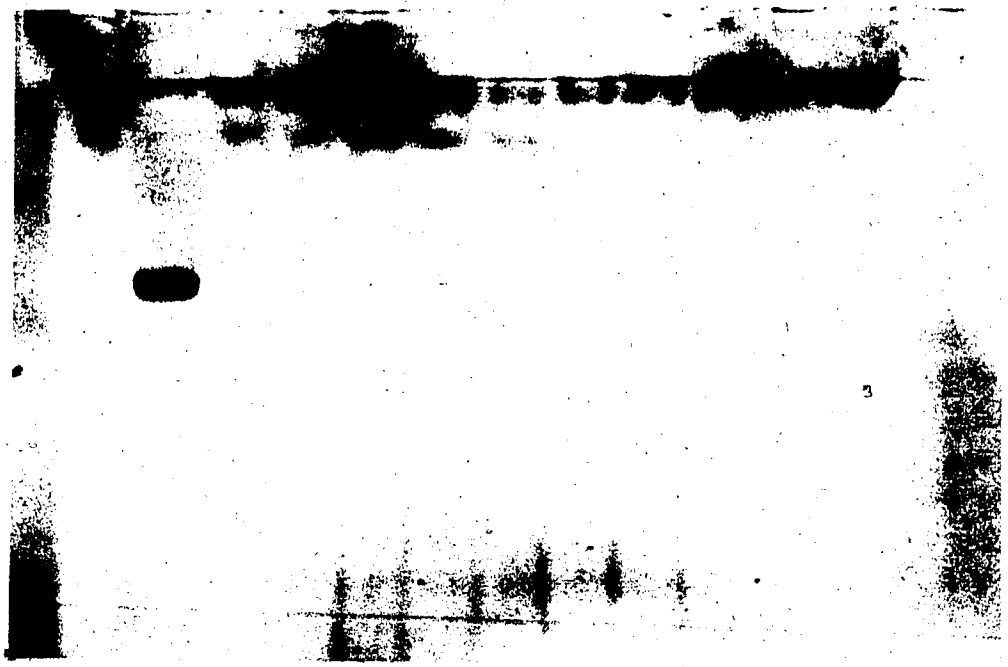


Fig. 3. Polyacrylamide Gel Stained for XDH Activity Using Hypoxanthine as Substrate

From left to right, the slots contain *MKRS*, Amherst, $ry^{a4}/Df(3)ry^{75}$, $ry^{a4}/MKRS$, ry^{a3}/ry^{a4}, ry^{a3}, ry^{a3}/ry^{a5}, $ry^{a5}/Df(3)ry^{75}$, $ry^{a5}/MKRS$, ry^{a5}/ry^{a8}, $ry^{a8}/Df(3)ry^{75}$, $ry^{a8}/MKRS$, and tracking dye. For all mutant genotypes, 30µl of crude supernatant were applied; for wild type, 5µl of extract were used. The gel was run and stained as described in Materials and Methods.

Table 3. Interallelic Complementation at the *rosy* Locus

8																	
26	-																
41	-	-															
601	-	-	-														
603	-	-	-	-													
604	-	-	-	-	-												
a1	-	-	-	-	-	-											
a2	-	-	-	-	-	-	-										
a3	-	-	-	-	-	-	-	-									
a4	-	-	-	-	-	-	-	-	+/-								
a5	-	-	-	-	-	-	-	-	+/-	-							
a6	-	-	-	-	-	-	-	-	-	-	-						
a7	-	-	-	-	-	-	-	-	-	-	-	-					
a8	-	-	-	-	-	-	-	-	-	-	+	-	-				
a9	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
a10	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+		
a11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
My mutant	8	26	41	601	603	604	a1	a2	a3	a4	a5	a6	a7	a8	a9	a10	a11

\- rosy eye color

+/- intermediate eye color

\+ wild type eye color

The presence or absence of complementation is based on the phenotype of the heterozygote, that is, mutant versus non-mutant eye color.

exhibited an intermediate eye color, that is, some of the flies were classified as "rosy" and others as wild type. Three pairs, *a4/a10*, *a5/a8*, and *a9/a10* gave eye colors indistinguishable from wild type.

All interallelic combinations of the matrix excluding those involving *a9*, *a10*, and *a11* were individually assayed in the fluorometer for XDH activity regardless of eye color. No further instances of activity were uncovered by this test, in fact, some of the complementing *ry* pairs failed to show XDH activity by this assay (Table 4). This may be due to increased lability of mutant XDH compared to wild type XDH under the conditions of extraction. Attempts were made to show XDH activity under potentially more favorable conditions by increasing the concentration of substrate, addition of glycerol to the extraction buffer, and assaying at room temperature instead of 30°C. One mutant combination, ry^{a5}/ry^{a8}, which failed to show activity at the normal substrate concentration, showed low activity (1.6%) at a higher concentration of substrate. The *in vivo* test appears to be the most sensitive indicator of XDH activity in most cases.

All combinations which gave intermediate or wild type eyes were then produced from reciprocal crosses, again scored for eye color, and assayed in both fluorometric and gel assays. Only combinations including the ry^{a4} allele showed activity in the gels. Duplicates of each gel were run and one stained using AHP as substrate, the other using Hx as substrate, as it was thought originally that ry^{a4} might be a substrate specific mutant. For example, if *a4* could oxidize Hx but not AHP, this would account for the mutant eye phenotype. However, with the development of a good staining procedure using AHP, it was found that $ry^{a4}/MKRS$,

Table 4. XDH Activity of Complementing Mutant Pairs

Genotype	Eye Color	XDH Activity (Gel Assay)	XDH Activity (Fluorometric Assay)
ry^{a3}/ry^{a4}	+/-	0.2%	-
ry^{a3}/ry^{a5}	+/-	-	6.5%
ry^{a5}/ry^{a8}	+	-	1.6% *
ry^{a4}/ry^{a10}	+	+ (not quantitated)	-
ry^{a9}/ry^{a10}	+	-	-

* requires 50λ AHP (3×10^{-4}M) instead of 30μl (usual assay)

The eye color is described as +/- (intermediate) if some flies appeared more mutant than wild type, and + (wild type) if all flies appeared non-mutant. Gel and fluorometric assays are based on extracts of 100 mg/ml as described in the text under Materials and Methods

$ry^{a4}/Df(3)ry^{75}$, ry^{a3}/ry^{a4}, and ry^{a4}/ry^{a10}, that is, all combinations of ry^{a4} tested, produced a band using either substrate. An attempt was made to quantitate by densitometry the levels of XDH activity represented by the bands in Figure 3. The combination ry^{a3}/ry^{a4} represented [illegible] Amherst; $ry^{a4}/Df(3)ry^{75}$ 0.4% of Amherst; $ry^{a4}/MKRS$ 2.1% of [illegible] The differences between these genotypes may be exagger[illegible]andom staining differences in the gel; ideally, several replicates of each genotype should have been estimated. However, it would appear that the level of XDH activity observed *in vitro* does not necessarily follow the eye color observed *in vivo* as the combination with the least activity (*a3/a4* in Figure 3) is the only one with a non-mutant eye color.

The pattern of complementation observed appears to be random, as expected for intragenic complementation involving a homodimer. For a heterodimer, it is expected that all non-polar mutations in the gene coding for one subunit would complement all such mutations in the other subunit. However, for a homodimer, both polypeptides will always be mutant and the level of activity restored in a heterozygote is unpredictable. The level of restored activity in these results is low, in agreement with the results of Gelbart *et al.* (1976) who observed levels of XDH activity from 0.2%-16% in complementing pairs of *ry* alleles. They also found the complementation map to be non-linear, and in fact, produced a circular map of complementation.

The complementation tests confirmed the results of Gelbart *et al.* (1976) and provided a useful tool in screening for possible nonsense

mutations in that it is assumed that all alleles exhibiting complementation are likely to be of the missense variety.

III. XDH purification

Several initial experiments were conducted in order to determine a suitable procedure for purification of XDH in this laboratory. Some modifications of the Seybold (1974) procedure were based on these experiments.

Ammonium sulfate cuts were done on a crude extract of frozen larvae. In a 30-40-50-60% series of saturations, the re-dissolved pellets were found to contain 0.38, 0.81, 11.25, and 16.8 ΔRFI/min/ml activity respectively with the 60% supernatant having little activity (0.15 ΔRFI/min/ml). This indicated that Seybold's choice of a 40-60% cut was adequate. However, in view of a second series of 35-45-55% saturations which gave 0.36, 2.25, and 20.0 ΔRFI/min/ml activities in the re-dissolved pellets and 0.51 ΔRFI/min/ml in the 55% supernatant, it was evident that most of the activity precipitated between 45-55% saturation. A 43-58% ammonium sulfate cut was therefore chosen for the purification scheme.

The next step, heat treatment, posed more difficulties. At no time was a purification of 5-6 fold as obtained by Seybold attained. The XDH appeared to be much more heat-sensitive than observed by Seybold. An attempt to stabilized the enzyme by addition of its co-factor, NAD^+, and/or its substrate, AHP, before heating yielded no better result. In fact, AHP made the XDH much more heat-labile and NAD^+ produced no difference. These results are similar to those found

by Glassman (1966).

A heat treatment of 4 minutes at 68°C produced better results (1.25 fold purification) than the same temperature for 5 or 6 minutes (1.08 fold). Later the temperature was reduced to 60°C which gave somewhat better results. The heat treatment remained one of the most variable steps in the purification procedure as may be seen in Table 9. The step was retained in order that one peak of XDH would be recovered from DEAE chromatography (see Introduction under multiple molecular forms of XDH).

A further purification step was added to the protocol, taking advantage of the differential solubility of proteins in polyethylene glycol (PEG). Table 5 shows the results of successive additions of 40% PEG to a heat-treated extract and centrifugation of the extract. The pellets obtained with 0-4%, 4-8%, and 8-12% wt/vol PEG were negligible, with all of the XDH activity remaining in the supernatants. An appreciable pellet was obtained with a 16% treatment with some loss of activity to the pellet; at 18% and 20% even more XDH activity precipitated. At 16%, 83% of the activity (106.4 ΔRFI/128.9 ΔRFI) was retained in the supernatant and 17% in the pellet, thus a 16% PEG precipitation was incorporated into the purification scheme.

No accurate estimate of the purification gained by this treatment has been made due to the difficulty in obtaining an accurate protein estimate in the presence of PEG which is strongly Lowry-positive. The presumption of purification achieved with PEG is based on obtaining a fairly large pellet and a good yield of XDH in the supernatant. An additional advantage of the use of PEG comes from the increased

Table 5. PEG Treatment of a Heat-treated Extract of XDH

Sample	Δ RFI/min/ml *
0% PEG (heat-treated extract)	15.3
4% PEG supernatant	18.0
8% PEG supernatant	16.2
12% PEG supernatant	17.9
16% PEG supernatant	12.7
18% PEG supernatant	11.7
20% PEG supernatant	7.6

* corrected for dilution factor

Polyethylene glycol in Tris-buffer (40% wt/vol) was added slowly to an XDH extract, stirred, and centrifuged; the supernatants were assayed for XDH activity in the fluorometer.

stability of the enzyme in its presence.

An attempt was made to use affinity chromatography for the purification of XDH taking advantage of its requirement for NAD+. Agarose-hexane-nicotinamide adenine dinucleotide (Agnad-Type 1, P-L Biochemicals) was used as described in the purification of pork liver fuconate oxidoreductase (Nwokoro and Schacter, 1975). The XDH extract was prepared through the first steps of conventional purification and applied to the Agnad column. Figure 4 shows that XDH is not bound to the column but elutes with the bulk protein. Thus, XDH does not appear to have high affinity for its co-factor, NAD+, and this step was not incorporated into the purification scheme. D. Baillie (personal communication) also found that the Agnad column was not useful in purifying XDH.

The other steps as outline above, plus DEAE-cellulose chromatography and hydroxyapatite chromatography were used to purify XDH. The results of the final XDH extraction used for production of antisera are detailed in Table 6. The Norite treated extract is used as the base-line for measuring purification because this extract exhibits more XDH activity than a crude extract due to the removal of endogenous purines and pteridines which compete with AHP for XDH. The ammonium sulfate treatment here shows a purification of 2.5 fold which is a typical result(Seybold, 1974). The dialysis does not affect purification but is necessary to get rid of the ammonium sulfate before DEAE chromatography. The choice of dialysis before or after heat and PEG treatment is optional, but it was routinely done before these treatments. The heat step in this particular example gave a fair yield (81%, Table 7)

Fig. 4. Elution Profile from NAD+-Agarose Column

● represents XDH activity; ▲ represents A_{280}

XDH was eluted from the column with the bulk protein indicating that it does not have a high affinity for its co-factor NAD+.

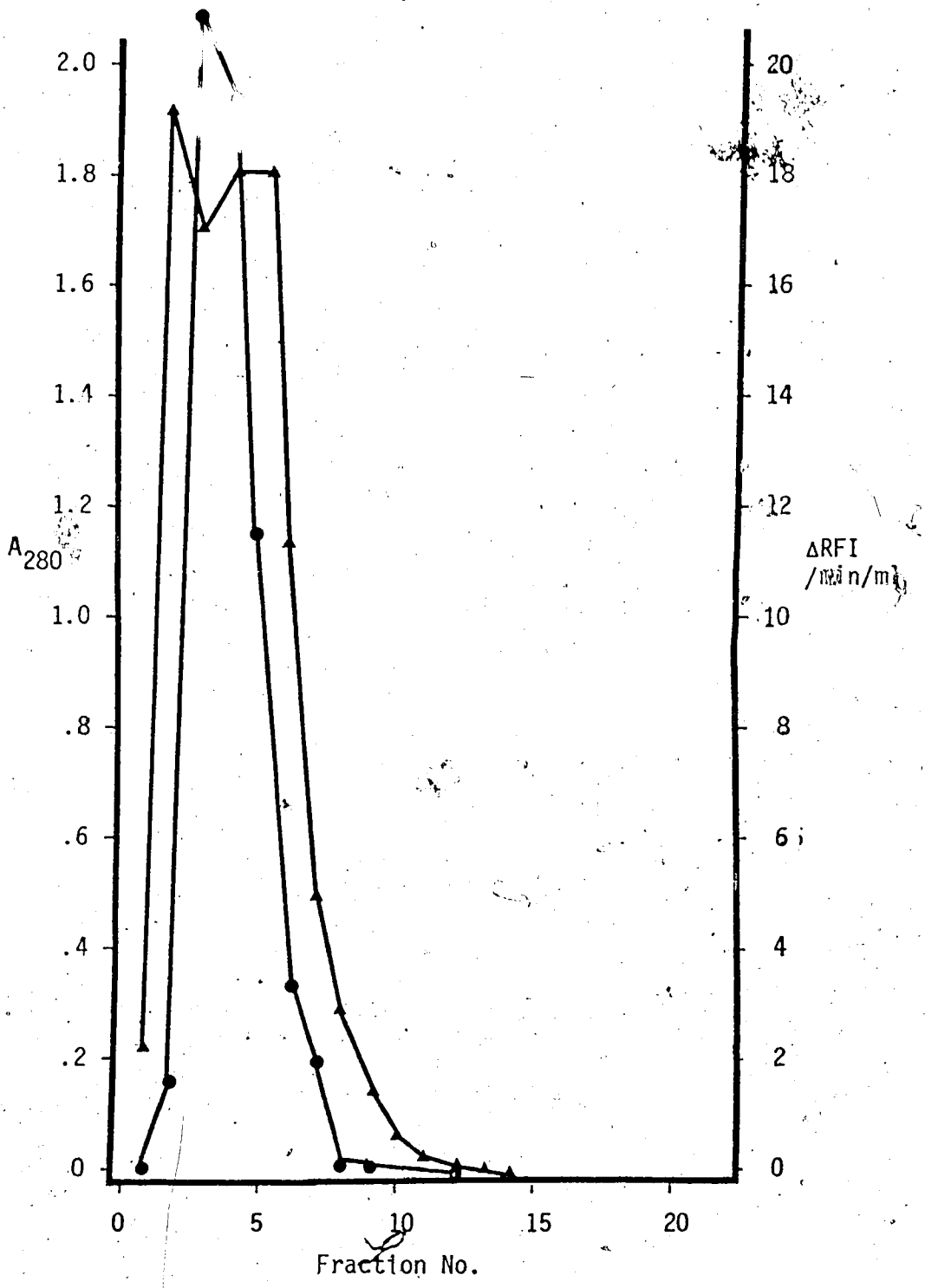

2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
A_{280}
20
18
16
14
12
10
8
6
4
2
0
ΔRFI
/min/ml
0
5
10
15
20
Fraction No.

Table 6. XDH Purification Scheme; Sample #5

Step	mg protein/ ml	activity units/ml *	Specific act. units/mg prot.	Fold-Purif. (Step)	Fold-Purif. (Cumulative)
Norite-treated	3.6	1.22	0.34	1	1
Amm. Sulf. treated	13.8	11.8	0.86	2.5	2.5
Dialyzed	9.4	8.0	0.85	1.0	2.5
Heat treated	6.1	7.14	0.76	0.89	2.2
PEG supernatant	ND	3.58	-	-	-
DEAE pool	0.46	9.16	19.9	26.2 **	58.7
HAP pool	0.045	2.64	58.7	2.9	173.

* defined as μM AHP oxidized/min/ml

** includes both PEG treatment and DEAE chromatography

Each step was assayed for activity and protein in three replicates; the mean is shown.

but did not result in a purification. The PEG step gave comparable results to those described above.

The DEAE-cellulose chromatography gave a narrow peak of XDH activity against a background of relatively high absorbance (Figure 5). Some of the early A_{280} (fractions 1-34) is due to the PEG in the extract. The XDH activity was eluted from the column with NaCl at 190-230 mM NaCl; the peak fractions were pooled and concentrated as described in Materials and Methods.

The DEAE concentrate was applied to an hydroxyapatite column and found to elute in a relatively narrow peak at about 160 mM sodium phosphate. The absorbance, A_{280}, in these fractions was very low as shown in the elution profile, Figure 6. The pooled fractions were assayed, concentrated, and frozen in 10% glycerol for use in preparation of antisera.

The yield at each step, Table 7, was calculated taking into account the total activity put into each step and that regained from the step. The cumulative yield was calculated as the product of the yield at that step and the previous step. Thus the yield does not consider the volumes used for samples.

The activity as expressed in fluorescence units, ΔRFI/min/ml, was translated into units of activity by use of a mixed dilution curve. The curve was plotted from the data in Table 8 and is shown in Figure 7. The change in fluorescence units was divided by the slope of the curve to give the μM of AHP oxidized/min/ml. This activity was then expressed as specific activity by dividing by the number of mg protein/ml as obtained from the Lowry protein test.

Fig. 5. Elution Profile from DEAE-cellulose Column

● represents A_{280}; o represents XDH activity (ΔRFI/min/ml)
The NaCl gradient was started at fraction #34, and XDH was eluted from the column at approximately 200 mM of NaCl.

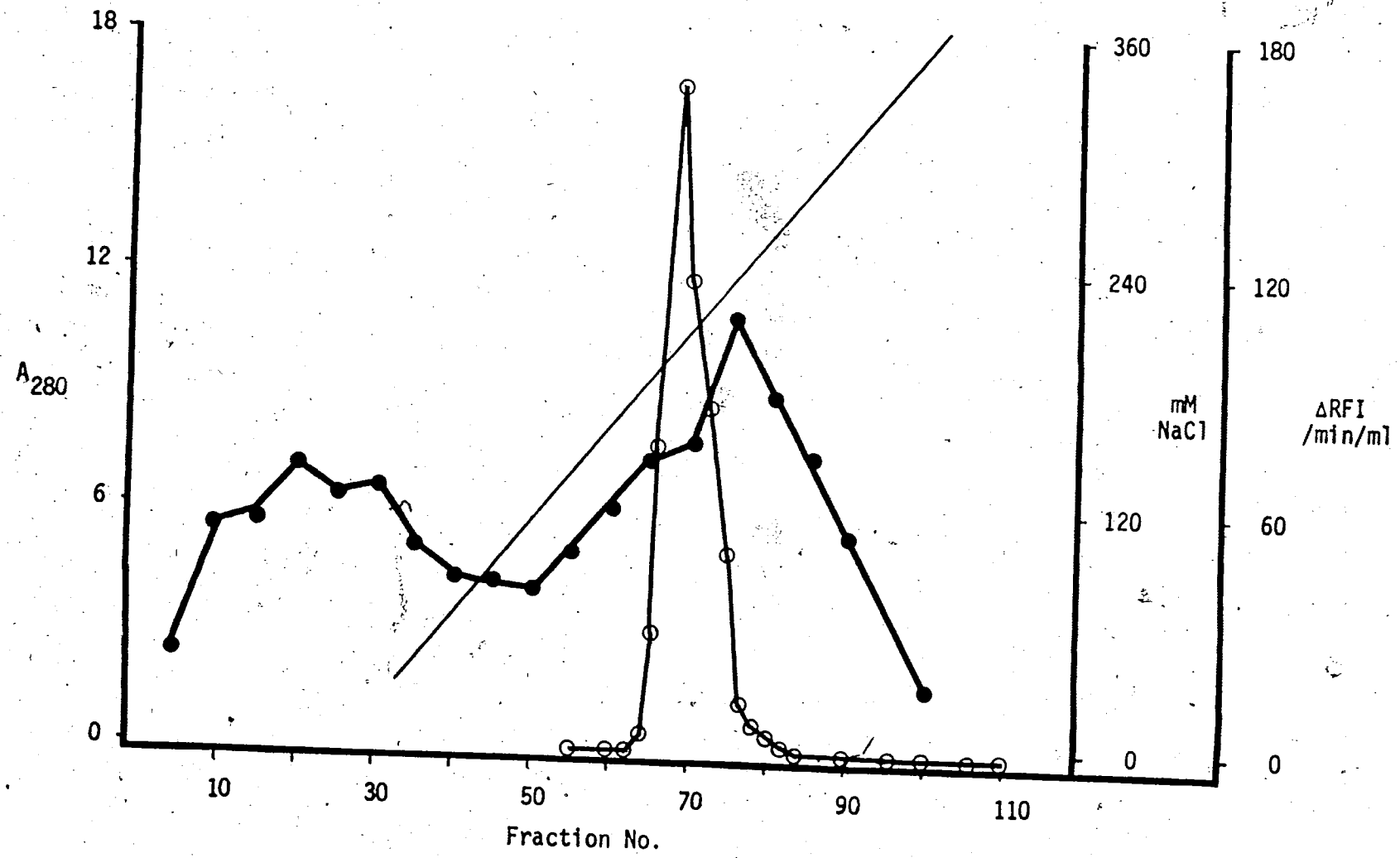

18
12
6
0
A_{280}
360
240
120
0
mM
NaCl
180
120
60
0
ΔRFI
/min/ml
10
30
50
70
90
110
Fraction No.

Fig. 6. Elution Profile from Hydroxyapatite Column

▲ represents A_{280}; ● represents XDH activity (ΔRFI/min/ml) The sodium phosphate gradient was started at fraction #12 and the XDH was eluted from the column at approximately 160 mM of sodium phosphate.

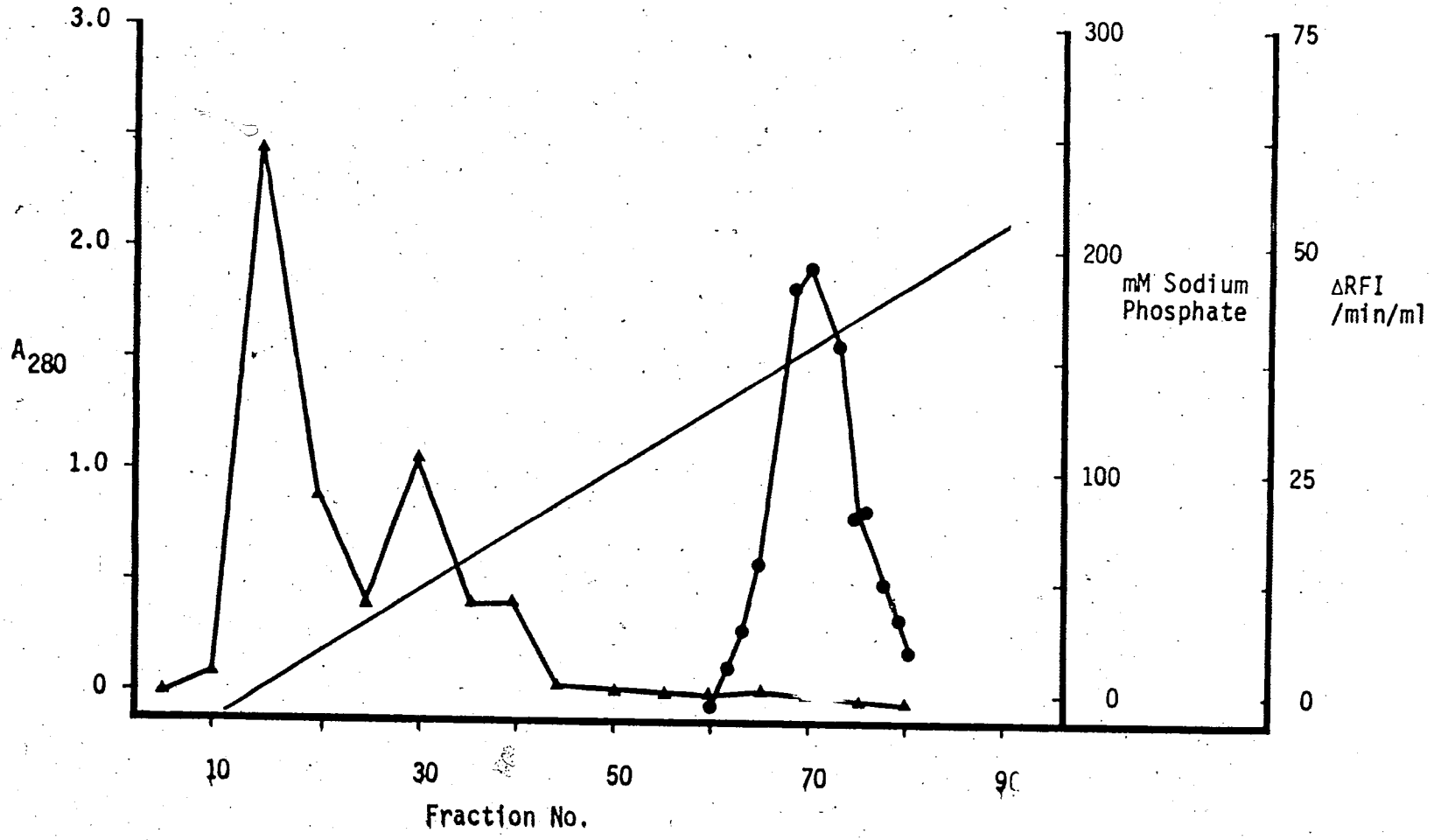

3.0
2.0
1.0
0
A_{280}
300
200
100
0
mM Sodium Phosphate
75
50
25
0
ΔRFI /min/ml
10
30
50
70
90
Fraction No.

Table 7. XDH Purification: Calculation of Yield for Sample #5

Step	* Act. into /ml	ml into	Total act. into	*Act. out /ml	ml out	Total act. out	Yield (Step)	Yield (Cumulative)
Norite-treated	-	-	-	16.9	850	14,365	100%	100%
Amm. Sulf. treated	16.9	845	14,280.	163.7	89	14,569	102%	102%
Dialyzed	163.7	86	14,078.	111.0	114	12,654	90%	92%
Heat treated	111.0	112	12,432.	99.0	102	10,098	81%	74.5%
16% PEG Super.	99.0	100	9,900.	49.7	158	7,853	79%	59%
DEAE pool	49.7	158	7,853	127.	44.	5,588	71%	42%
DEAE pool, conc	127.	42	5,334	336	16.2	5,443	102%	43%
HAP pool	336.	16.2	5,443	36.6	44	1,610	30%	13%

* Activity expressed as ΔRFI/min/ml

Table 8. Mixed Dilution Curve of AHP and IXP

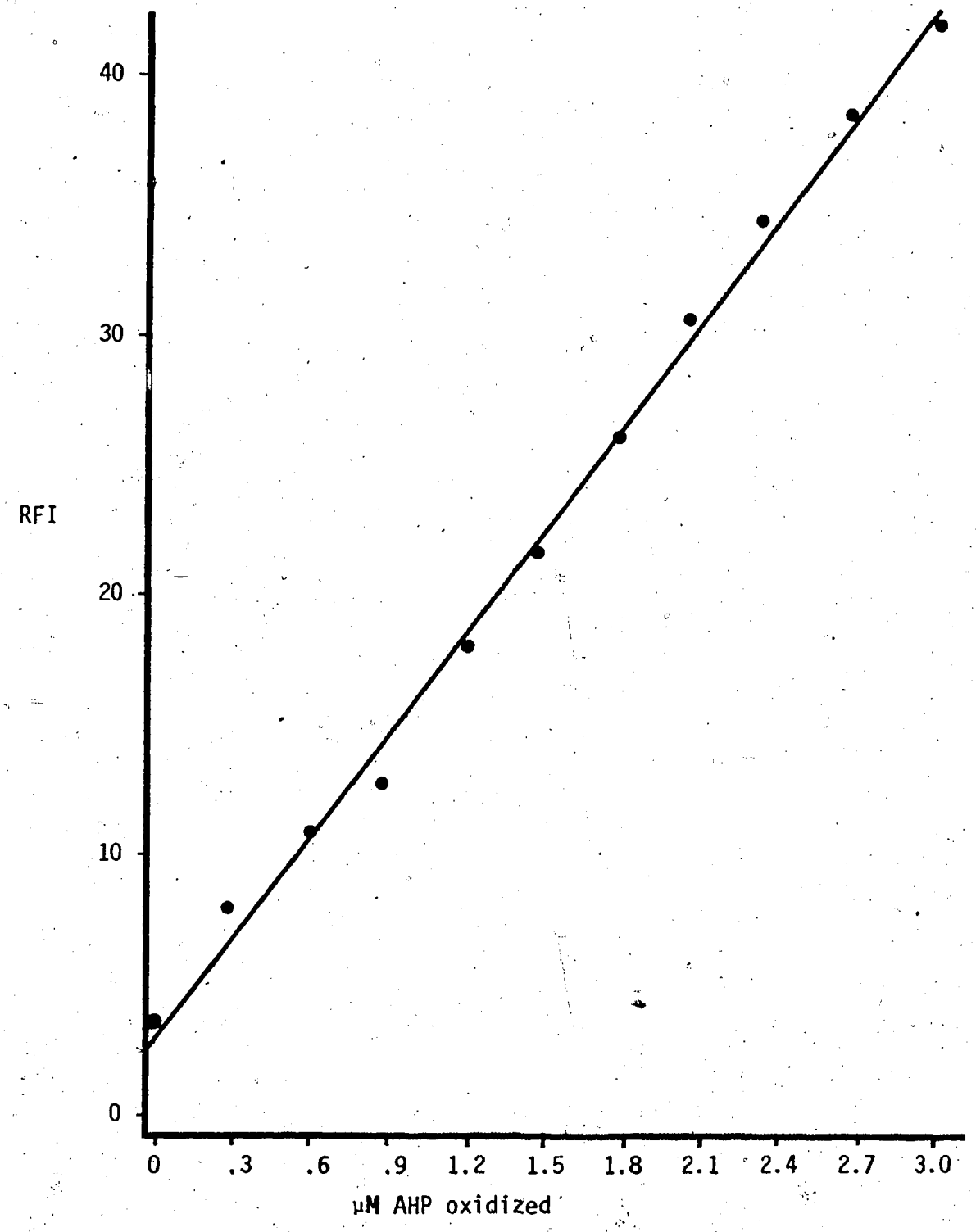
*µl AHP	*µl IXP	uM AHP "oxidized"	**Ave. RFI
100	0	0	3.6
90	10	0.3	8.0
80	20	0.6	11.1
70	30	0.9	13.0
60	40	1.2	18.9
50	50	1.5	23.4
40	60	1.8	27.7
30	70	2.1	33.8
20	80	2.4	37.5
10	90	2.7	40.5
0	100	3.0	43.4

* represents µl of stock solutions of 3 X 10^{-4}M AHP and IXP added to 9.9 ml 0.1M Tris-Cl, pH8.0; 1 ml samples were used for determinations of RFI in the fluorometer.

** represents the average of 3 determinations

Fig. 7. Mixed Dilution Curve of AHP and IXP

● represents relative fluorescent intensity (RFI) = % Transmission X Meter Multiplier. The RFI observed in 1 ml samples of various mixtures of AHP and IXP (see Table 8 for details) were recorded. The sensitivity of the fluorometer was set at 45.5; wavelengths of 338 nm (activation) and 405 nm (fluorescent) were used. The slope of this curve, 13.87, was used to convert ΔRFI to μM of AHP oxidized.


40
30
RFI
20
10
0
0
.3
.6
.9
1.2
1.5
1.8
2.1
2.4
2.7
3.0
µM AHP oxidized

The five partially purified XDH preparations used for eliciting antibodies have been summarized in Table 9. The first preparation was taken only through DEAE-cellulose chromatography; the remainder were purified through HAP chromatography. None of the preparations were fully purified. This can be seen in Figure 8 which shows disc gels containing 200 µl of HAP concentrate samples from each of the final three XDH preparations. All contain four to five distinct bands of protein.

The purification results obtained agree fairly well with those of Seybold (1974) with the exception of the heat treatment. One possible explanation for the differences encountered in this step is Seybold's use of adults as material and the use herein of larval material. Although adults have more XDH activity (Sayles *et al.*, 1973 and this laboratory) the collection of large quantities (up to 200g/extract) of adults poses considerable problems compared with the collection of large amounts of larvae. Singh *et al.* (1976) have suggested that XDH in older flies (*D. pseudoobscura*) is less heat sensitive than XDH from young flies and that electrophoretically indistinguishable XDH from different wild type stocks varies greatly in heat sensitivity. If these observations extend to *D. melanogaster*, the difficulties encountered in the heat treatment may be due to the choice of wild type strain and/or the use of larval XDH.

IV. Preparation and testing of XDH antisera

The two immunized rabbits were bled on several occasions for immune sera as described in Materials and Methods. All of this sera was tested for titre of antibody to XDH, however only the results of the final bleeding are shown here as only this sera was used for the

Table 9. Summary of XDH Purification Steps of Samples Used to Elicit Antibodies

Preparation #	1		2		3		4		5	
g larvae	36.9		75		140		200		185	
ml extraction buffe	370		750		1400		1000		925	
Step	Spec. Act	Purif.	Spec. Act.	Purif.	Spec. Act	Purif.	Spec. Act.	Purif.	Spec. Act	Purif.
Crude extract	.027	-	.21	-	0.57	-	.11	-	ND	-
Norite-treated	.067	1	.42	1	1.01	1	.28	1	0.34	1
Amm. Sulf. treated	-	-	1.96	4.7	-	-	.57	2.0	0.86	2.5
Dialyzed	.203	3.0	1.03	2.5	2.46	2.4	.61	2.2	0.85	2.5
Heat-treated	.205	3.1	1.95	4.6	5.86	5.8	1.53	5.5	0.76	2.2
PEG supernatant	-	-	-		-	-	-	-	-	-
DEAE pool	4.01	64.3	-		8.89	8.8	8.2	29.3	19.9	58.7
DEAE pool, conc.	3.71	55.4	-		-	-	-	-	-	-
HAP pool	ND	ND		-	76.67	76.67	72.9	260	58.7	173.1
HAP pool, conc.	ND	ND	15.37	36.6	-	-	84.0	300	-	-
Yield calculated	12.2% (DEAE pool, conc.)		11.2% (HAP pool, not conc.)		6.7% (HAP pool, conc.)		17.6% (HAP pool, conc.)		13% (HAP pool, not conc.)	

Spec. Act. = specific activity, μM AHP oxidized/mg prot.

Purif. = cumulative fold of purification at that step

ND = step not done - = not [redacted]ined

Fig. 8. Disc Gels of Partially Purified XDH Preparations

From left to right, 200 µl of HAP concentrated preparations were loaded as follows: prep #3 (.007 mg. prot.); prep #4 (.030 mg. prot.); and prep #5 (.008 mg. prot.). The gels were run for 4.5 hours at 1.5 mA per tube and stained for protein overnight using Coomassie blue. The sharp line at the bottom of each gel represents the tracking dye.

CRM studies. Table 10 shows the results for antisera from the two rabbits with incubation against both larval and adult Amherst extracts. The crude sera was precipitated by 45% ammonium sulfate and the pellets re-dissolved in a volume of extraction buffer equivalent to the original volume of crude sera. The 5HE3 serum was able to completely inhibit an equal volume of larval extract at dilutions up to 1:100 and adult extracts at dilutions up to 1:40. The 5GJ6 titre was much lower. The supernatant from the 45% precipitation appeared to have some inhibitory effect, however, the appropriate control, control sera 45% supernatant, was omitted, thus this inhibition may be due to the presence of the ammonium sulfate rather than antibodies.

The titre curves for 5HE3 serum are plotted in Figure 9. From the table and figure it is obvious that 5HE3 serum should be chosen for further experiments and that adult fly preparations should be used as the level of XDH CRM is higher than in larval preparations. It also appears that there is a narrow range from which inhibition goes from complete to very low. This may be due to XDH antigen-antibody complexes retaining XDH activity (confirmed by gel electrophoresis) and the difficulty in excluding by centrifugation these complexes from the test tube assay. The variability encountered suggested that the test tube incubation and assay might not be very satisfactory.

V. CRM results

It was decided to test for cross-reacting material to anti-XDH sera in the *ry* mutants by the method of Laurell rocket gel electrophoresis (Laurell, 1966; Weeke, 1973; Gavin, 1975) as it is a quantitative procedure for measuring CRM. This method employs an

ble 10. Titre Curves of Rabbit Anti-sera, Final Bleeding

XDH Preparation	Serum Preparation	Serum Dilution	% Activity Remaining 5HE3	% Activity Remaining 5GJ6	% Inhibition 5HE3	% Inhibition 5GJ6
Amherst larvae	45% Amm. Sulf. pellet in extraction buffer	1:50	0	0	100	100
		1:75	0	40.6	100	59.4
		1:100	0	60.9	100	39.1
		1:150	40.2	75.4	59.8	25.6
		1:200	59.8	79.7	40.2	20.3
Amherst adults	45% Amm. Sulf. pellet in extraction buffer	1:10	0	0	100	100
		1:20	0	11.7	100	88.3
		1:40	0	84.8	100	15.2
		1:50	94.4	82.8	5.6	17.2
		1:75	70.6	72.4	29.4	27.6
		1:100	81.1	73.1	18.9	26.9
		1:150	84.6	65.5	15.4	34.5
Amherst larvae	45% Amm. Sulf. supernatant	1:10	60.9	89.6	39.1	10.4
		1:50	75.9	87.0	24.1	13.0
		1:100	75.9	81.2	24.1	18.8

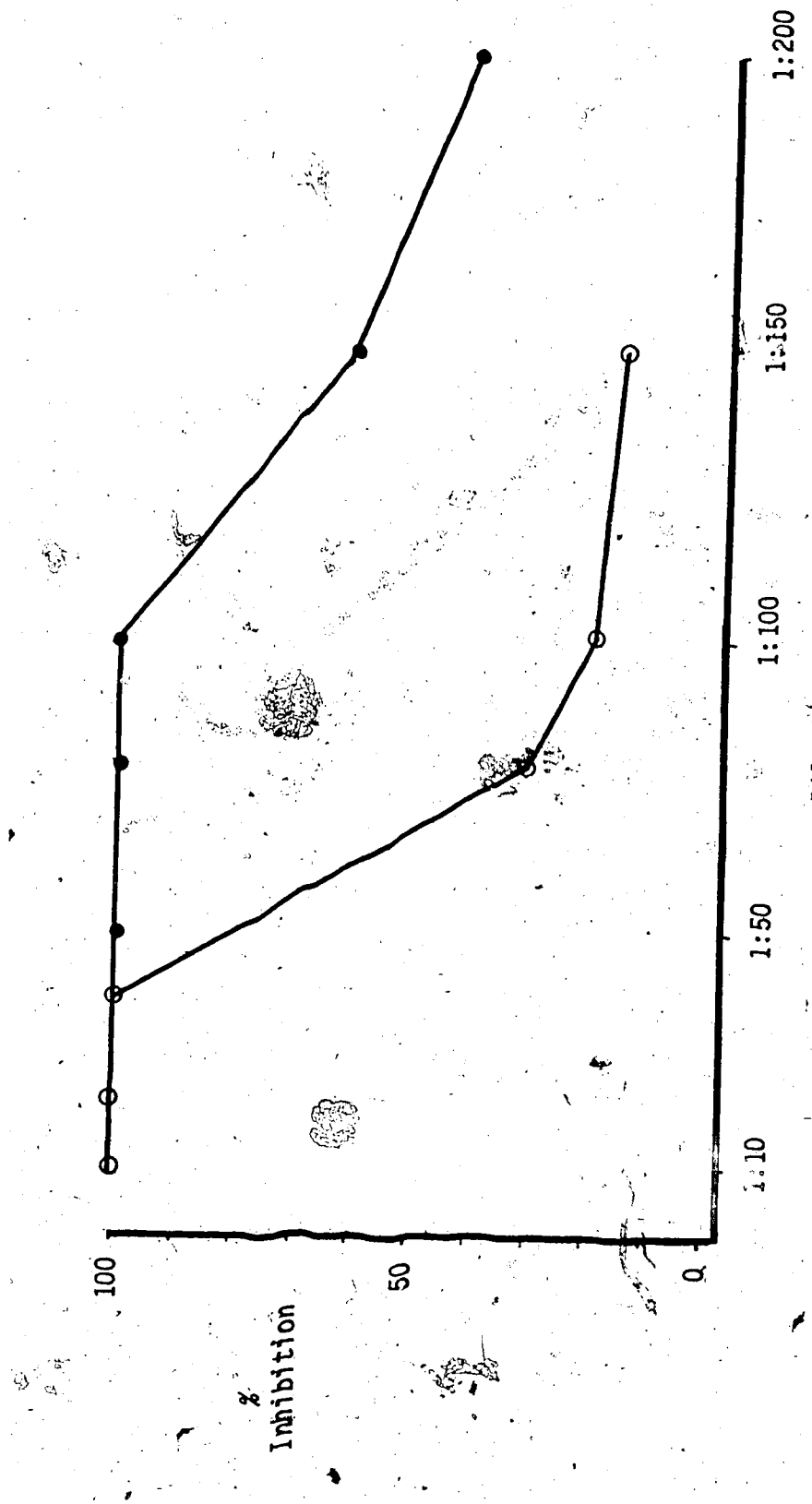
XDH extracts of larvae and adults were prepared following standard procedures for fluorometric assays and 0.3 ml of extract added to 0.3 ml of serum dilutions. The mixtures were incubated at 4°C for 18 hours, centrifuged 15 minutes at 14,600xg, and the supernatants assayed for XDH activity. The % remaining activity is based on activity remaining after incubation with control sera which had also been treated with ammonium sulfate.

Fig. 9. Titre Curves from Final Bleeding of 5HE3 Rabbit

● represents Amherst larval extract (100 mg/ml) incubated with re-dissolved and diluted 45% ammonium sulfate pellet of 5HE3 immune serum.

o represents Amherst adult extract (100 mg/ml) incubated with re-dissolved and diluted 45% ammonium sulfate pellet of 5HE3 immune serum.

All extracts were incubated with serum for 18 hours at 4°C, centrifuged, and the supernatants assayed for XDH activity.


% Inhibition
100
50
0
1:10
1:50
1:100
1:150
1:200
Dilution of Serum

agarose gel containing antiserum through which antigen is electrophoresed. Whereas free antigen may move readily through the gel, the antibody antigen complexes migrate slowly, if at all, and form a precipitate in the shape of a rocket; these are visualized by staining. Ideally, a monospecific antiserum is used so that the gel can be stained for total protein. The CRM of any given sample is estimated by the distance travelled by the tip of the rocket from the pocket, that is, the height of the rocket. The accuracy of this measurement relies upon uniformity in the width of the rocket and the thickness of the gel. With uniformity, volume can be expressed as height only and the area under the rocket does not need to be measured.

The amount of extract which could be applied to the gel is restricted by the size of the holes punched in the gel, thus an antiserum concentration was chosen (25 µl/25 ml) which allowed the rockets to reach a height of approximately 2 to 3 cm. It has been shown that the best range for quantitative work is 1-5 cm in which there is an error of 0.4-2% (Weeke,1973). Figure 10 shows a gel to which 0.5-17.5 µl of Amherst adult extracts were applied and run for 11 hours. The heights of the rockets observed are tabulated in Table 11. The next figure, Figure 11, shows a plot of the standard curve derived from this data. The best fitting line is plotted with a slope of 4.66 and intercept of 3.10 and r^2 (regression co-efficient) of 1.00. The following figure, Figure 12, shows the importance of length of running time to obtaining a satisfactory standard curve. Running times of 7.5 and 9 hours proved insufficient to allow the higher volumes to reach their peak height; 10.5 hours provided linearity

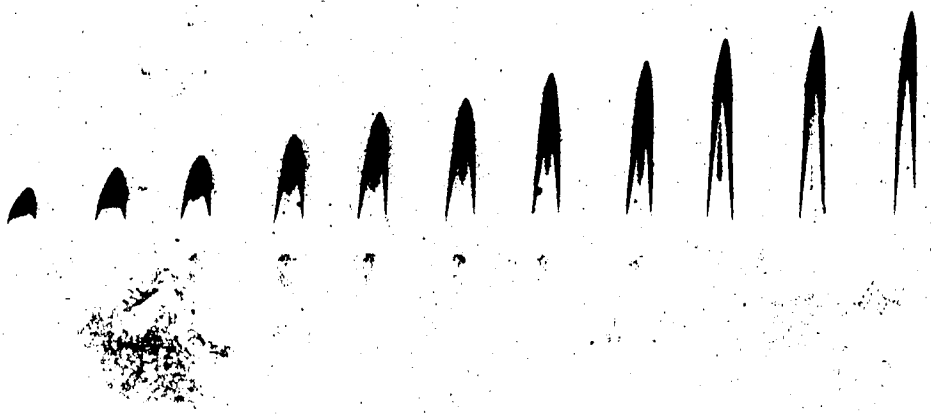


Fig. 10. Standard Curve: Laurell Rocket Gel Electrophoresis

From left to right, the wells contained 1.0, 2.5, 4.0, 5.5, 7.0, 8.5, 10.0, 11.5, 13.0, 14.5, 16.0, and 17.5µl of Amherst crude extract. Electrode buffer plus sucrose was added to each well to give a final volume of 17.5µl for each sample. The gel was electrophoresed at a constant voltage of 120 v for 11 hours, removed, and stained for XDH activity.

throughout the range of volumes to 16 µl. All gels were therefore run 10-11 hours, and the standard curves from each gel were found to fit a linear regression curve with regression co-efficients of 0.99 - 1.00. Some curves were not linear in the range of 16 µl even though they were run for the standard time; for these gels, the higher volumes of Amherst were not included in the calculations as the height of the rocket from 8 µl of Amherst was the only one of interest and the curves were always linear in that range.

It should be noted that the intercepts (Figures 11, 12) consistently fail to go through zero. This is probably because the antigen-antibody complexes are able to migrate, although much more slowly than free antigen. The conditions of electrophoresis (buffer system, pH) are such that free antibody migrates very little in the gel (Weeke, 1973). However, it would be difficult to fulfil conditions of non-migration of both free antibody and the complexes simultaneously.

Due to the presence of contaminants in the original XDH preparations, which resulted in the production of antibodies to several proteins, several rockets were observed over each well when a protein stain was used. Therefore, it was decided to stain specifically for XDH activity since other antigen-antibody complexes were known to retain activity, and indeed XDH also (McCarron *et al.*, 1976 and this laboratory); this necessitated the presence of wild type extract in all pockets. Both mutant and active enzyme preparations were thus placed in the same well; it was thought that if the mutant extract contained any XDH CRM, the wild-type peak should be

Table 11. Laurell° Rocket Gel Electrophoresis: Standard Curve

Slot #	µl buffer	µl Amherst	height of XDH rocket (mm)
1	16.5	1.0	4.5
2	15.0	2.5	8.0
3	13.5	4.0	10.0
4	12.0	5.5	11.75
5	10.5	7.0	14.5
6	9.0	8.5	17.25
7	7.5	10.0	19.0
8	6.0	11.5	22.0
9	4.5	13.0	24.5
10	3.0	14.5	27.75
11	1.5	16.0	29.75
12	0	17.5	32.0

The rockets in the gel depicted in Fig. 10 were measured to the nearest .25 mm from the top of the pocket to the tip of the rocket.

Fig. 11. Laurell Rocket Gel Electrophoresis: Standard Curve

■ represents the distance travelled (in mm) by various volumes of crude Amherst extract. The gel was run for 11 hours and stained for XDH activity. The best-fitting line describing the set of points is drawn in according to the formula $y = 3.10 + 1.66 \times (r^2 = 1.00)$.

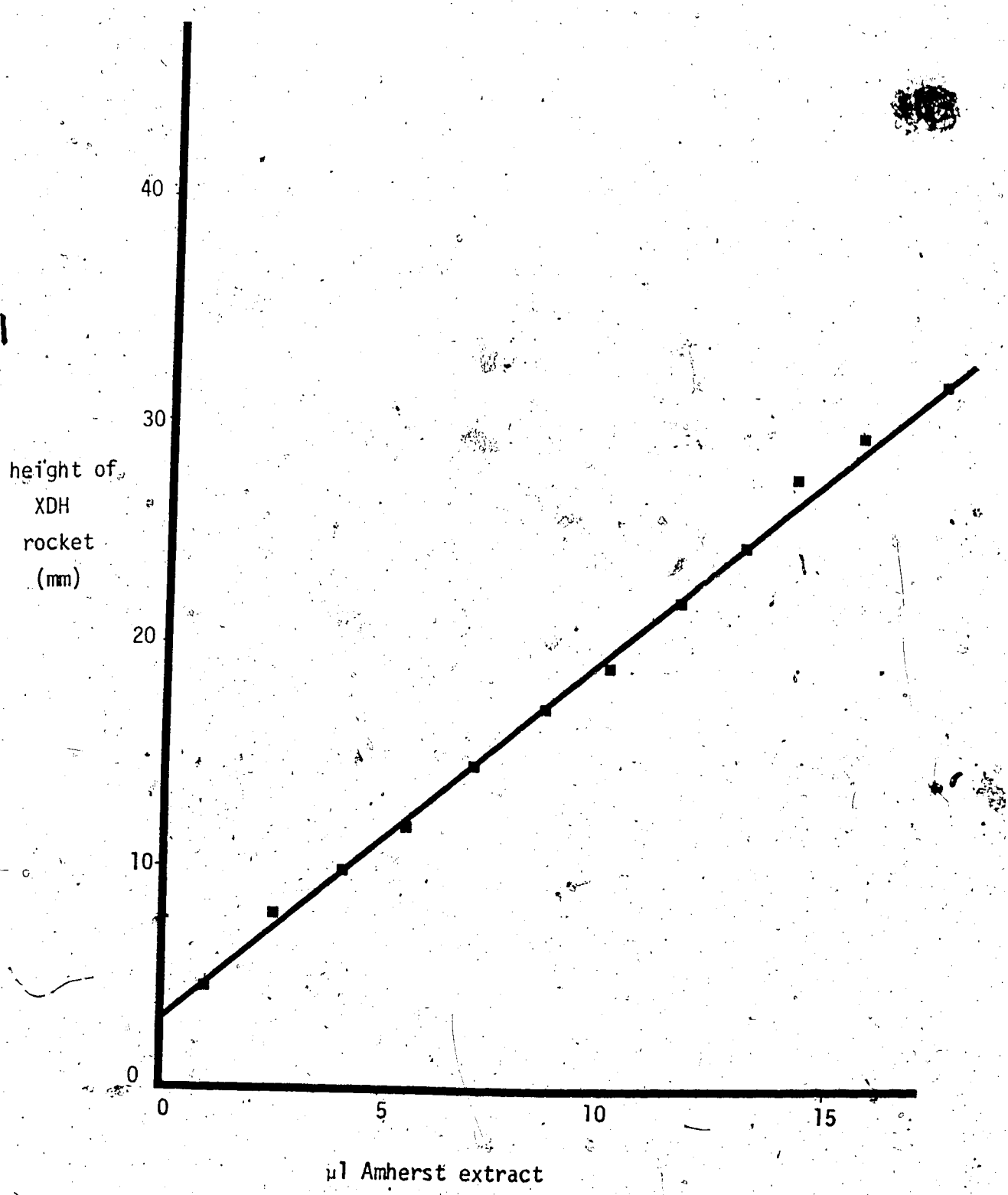

40
30
20
10
0
height of XDH rocket (mm)
0
5
10
15
µl Amherst extract

Fig. 12. Laurell Rocket Gel Electrophoresis: Standard Curves from Gels Run Different Lengths of Time

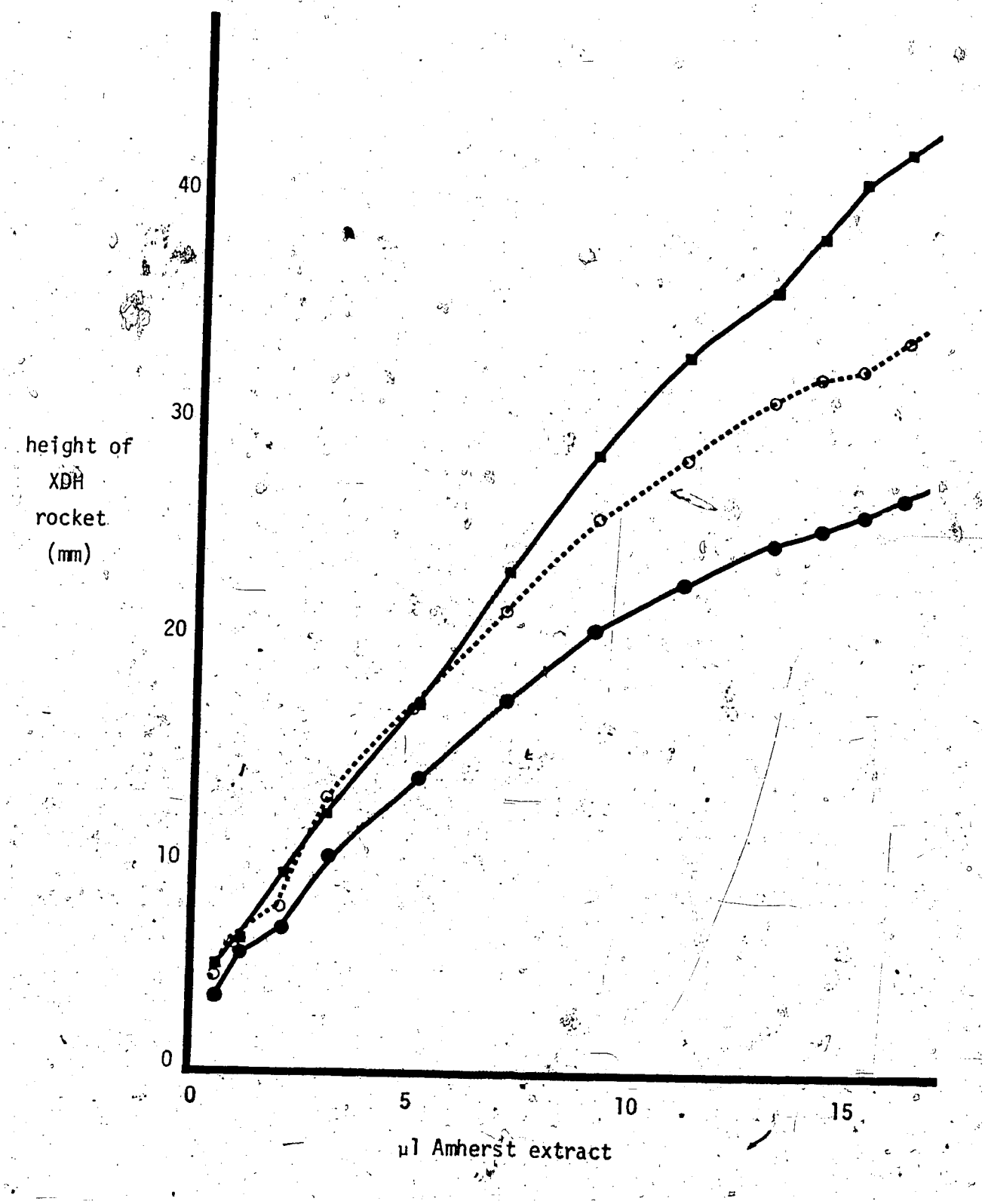
The distances run by various volumes of crude Amherst extracts in gels electrophoresed for varying times:

■ represents heights in gel run 10.5 hours.

○ represents heights in gel run 9 hours.

● represents heights in gel run 7.5 hours.

All gels were run at a constant voltage of 120 v. and stained for XDH activity immediately after running.


height of
XDH
rocket
(mm)
40
30
20
10
0
0
5
10
15
µl Amherst extract

higher than with wild type alone. It was therefore expedient to ascertain the validity of this method. For this purpose the mutant *lxd* was useful. This mutant has lower levels of activity (Keller and Glassman, 1964 a,b) than CRM (Karam, 1965). Figure 13 is a photograph of a gel in which a standard curve of Amherst (wells # 1,3,5,7,11) is interspersed with wells containing 6 µl of Amherst, 6 µl of *lxd*(#2,6,10) and wells with 16 µl of *lxd* alone (#4,8,12). The heights of the rockets were measured (Table 12) and the "equivalent µl Amherst" determined, that is the heights are equivalent to the heights obtained with "x" µl of Amherst. This determination was made by calculating the best fitting formula for the standard curve and deriving the corresponding µl of Amherst to each height observed over wells containing mutant extracts. Slots #2,6,10 gave an average of 9.35 equivalent µl Amherst whereas only 6 µl of Amherst was actually placed in the well. Thus the 6 µl of *lxd* must have contributed 3.35 equivalent µl Amherst or 55.8% as much as the wild type. In slots #4,8,12, an average of 8.52 µl Amherst with 16 µl of *lxd* was attained, or (8.52/16) 53.3% of the wild type level. Thus both methods gave similar results and the combination of mutant and wild type appears valid.

Using the above methods of gel preparation, running, and calculations, all *ry* mutants in this laboratory were tested for CRM. Standard preparations of antiserum and wild type extract (frozen in aliquots) were used in all experiments. No calculations of protein levels in crude extracts were made however. Each mutant was run in 3 replicates in at least one gel (two mutants/gel) with a standard curve interspersed.

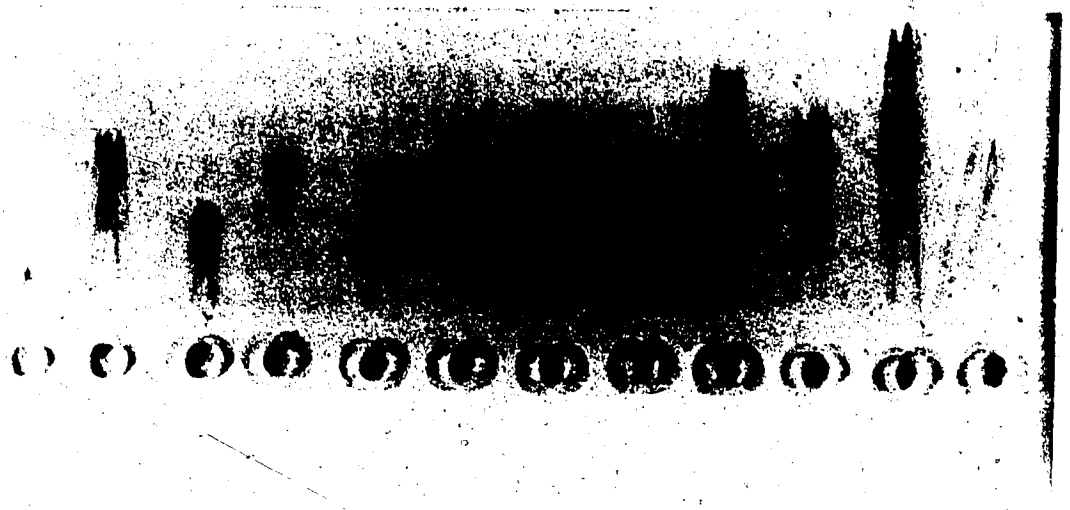


Fig. 13. Laurell Rocket Gel Containing *lxd* Extracts

From left to right, the wells contained: (1) 1 µl Amherst; (2) 6 µl Amherst, 6 µl *lxd*: (3) 4 µl Amherst; (4) 16 µl *lxd*; (5) 7 µl Amherst; (6) 6 µl Amherst, 6 µl *lxd*; (7) 10 µl Amherst; (8) 16 µl *lxd*; (9) 13 µl Amherst; (10) 6 µl Amherst, 6 µl *lxd*; (11) 16 µl Amherst; and (12) 16 µl *lxd*.

The gel was electrophoresed and stained for XDH activity. The rockets rockets above the wells containing only *lxd* extracts are much lighter staining due to the lower activity of the *lxd* mutant.

Table 12. CRM Level of *lxd (low xanthine dehydrogenase)*

Slot #	µl Amherst	µl *lxd*	ht. of rocket (mm)	equivalent µl Amherst
1	1	-	9.75	-
2	6	6	26.25	8.52
3	4	-	17.0	-
4	-	16	25.25	8.05
5	7	-	23.0	-
6	6	6	27.5	9.11
7	10	-	30.75	-
8	-	16	26.25	8.52
9	13	-	35.25	-
10	6	6	30.25	10.42
11	16	-	41.25	-
12	-	16	27.25	8.99

From the gel in Fig. 12, the heights of the rockets have been measured to the nearest .25 mm and the rockets containing *lxd* extracts have been compared to the standard curve to determine what volume of Amherst that height represented (equivalent µl Amherst). From the slots containing both Amherst and *lxd* (#2,6,10), the average is 9.35 µl Amherst. Only 6µl Amherst were applied, thus 6µl *lxd* added 3.35 µl to the height of the rocket for (3.35/6) 55.8% CRM. Similarly from slots containing only *lxd* extracts (#4,8,12), the average is 8.52µl Amherst from 16µl *lxd* applied, for (8.52/16) 53.3% CRM.

The interspersion of the standard curve was to minimize local differences, if any, in the gel. Figure 14 shows a gel containing the mutants ry^{a6} and ry^{a8} from which CRM information was derived. All of the gels were similar to this one. Table 13 shows the mean proportion of the height of the Amherst rocket reached by each mutant. Two controls are included, *ma-l* and *lxd*; *ma-l* shows a height twice that of Amherst alone as it is ry^{+} and produces an inactive XDH protein.

Note that many of the mutant and wild type combinations do not reach the expected height of wild type alone. No explanation for this consistent observation has been found. A possibility, that XDH substrates present in the *ry* flies (non-charcoal treated) are competing with the antibody for binding sites on XDH, would explain the phenomenon. This seems unlikely however since XDH bound to antibodies retains activity, suggesting that the substrate and antibody binding sites are different. This questions remains unresolved. Those mutants which gave heights less than or equal to Amherst alone were considered to be CRM⁻. Table 14 gives the values of CRM determined for each mutant as derived from Table 13. The positive values for those mutants which were tested as heterozygotes with the *MKRS* chromosome were multiplied by a factor of 2 under the assumption that ry^{2} did not produce any CRM (*MKRS*: $ry^{2}/Df(3)ry^{75}$ and ry^{2} homozygotes failed to produce CRM) however, "complementing" CRM cannot be ruled out. The controls, *ma-l*, *lxd*, and $ry^{+}/Df(3)ry^{75}$ (one dose of the ry^{+} gene), gave results consistent with other observations; *ma-l* has been shown to have 100% CRM (Andres, 1976), *lxd* was shown to have 85% of Oregon-R CRM (Karam, 1965). Any quantitative differences between published and observed results may be due to differences in method of determin-

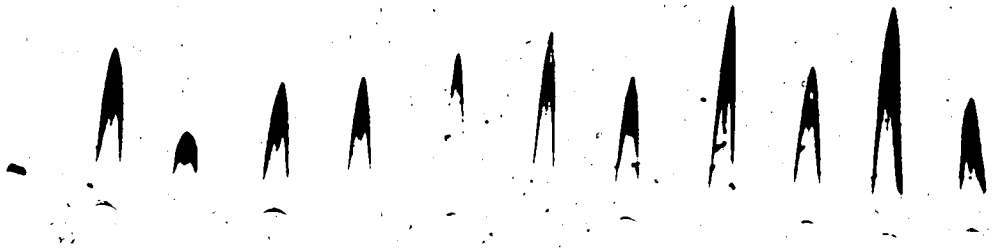


Fig. 14. Laurell Rocket Gel Containing ry^{a6}/*MKRS* and ry^{a8}/*MKRS* Extracts

From left to right the slots contained: (1) 1 µl Amherst (5.0 mm); (2) 8 µl Amherst, 8 µl ry^{a6}/*MKRS* (20.0 mm); (3) 4 µl Amherst (10.0 mm); (4) 8 µl Amherst, 8 µl ry^{a8}/*MKRS* (17.0 mm); (5) 7 µl Amherst (17.5 mm); (6) 8 µl Amherst, 8 µl ry^{a6}/*MKRS* (20.75 mm); (7) 10 µl Amherst (23.25 mm); (8) 8 µl Amherst, 8 µl ry^{a8}/*MKRS* (18.75 mm); (9) 13 µl Amherst (28.0 mm); (10) 8 µl Amherst, 8 µl ry^{a6}/*MKRS* (20.5 mm); (11) 16 µl Amherst (29.25 mm); and (12) 8 µl Amherst, 8 µl ry^{a8}/*MKRS* (17.0 mm).

The gel was run and stained for XDH following standard procedures.

Table 13. Proportion of the Height of the Amherst Rocket Reached By Equal Volumes of Mutant and Amherst Extracts

mutant	mean proportion	standard deviation
ry^{a1}	0.736	.023
ry^{a2} *	0.813	.115
ry^{a3}	1.062	.090
ry^{a4} *	1.091	.105
ry^{a5} *	1.097	.103
ry^{a6} *	1.169	.027
ry^{a7} *	0.759	.020
ry^{a8} *	0.928	.063
ry^{a9} *	1.248	.099
ry^{a10} *	1.355	.072
ry^{a11} *	0.726	.090
ry^{2}	0.810	.100
ry^{8}	0.760	.045
ry^{26}	0.828	.035
ry^{41}	1.376	.086
ry^{601}	0.841	.057
ry^{603}	0.778	.042
ry^{604}	0.850	.013
MKRS $(ry^{2}/Df(3)ry^{75})$	0.872	.050
lxd	1.533	.029
ma-l	2.171	.240

*Tested as heterozygote with ry^{2}

The height of each rocket was compared to the height of the rocket containing Amherst alone (from the standard curve of each gel). Mutants which increased the height of the Amherst rocket (proportion >1) are considered to by CRM^{+}. The standard deviation takes into account the variation between the three replicates of each mutant run per gel.

Table 14. % Cross-reacting Material to Anti-XDH Serum

Mutant(s)	%XDH CRM
$ry^{a2,a7,a8,a11}$ *	0
$ry^{a1,2,8,26,601,603,604}$	0
MKRS ($ry^{2}/Df(3)ry^{75}$)	0
ry^{a3}	6.2
ry^{a4} *	18.2
ry^{a5} *	19.4
ry^{a6} *	33.8
ry^{a9} *	49.6
ry^{a10} *	71.0
ry^{41}	37.6
$ry^{+}/Df(3)ry^{75}$	49.3
lxd	55.8
ma-l	117.1
lxd ry^{2}/*lxd* ry^{+}	32.2

* assayed as heterozygote with ry^{2}

ation of CRM as well as differences in the wild type standard strains used. By gene-dosage theory (Grell, 1962), $ry^{+}/Df(3)ry^{75}$ should show 50% CRM and 49.3% was observed.

The CRM studies have proved to be a useful tool in detecting inactive XDH polypeptides in that two non-complementing mutants as well as most of the complementing mutants appeared to have CRM. Thus, ry^{41} and ry^{a6} as well as the complementing mutants may be of the missense variety rather than nonsense mutants.

CONCLUSIONS

The results of the XDH assays, complementation tests, and CRM studies have been summarized in Table 15. These tests have screened for the presence of inactive XDH protein in mutant strains and have detected evidence of such in nine of eighteen strains examined. Of the fourteen EMS induced mutants, seven appear to be of the missense variety. Inactive protein was also observed in one of the three X-ray induced mutants and in the only spontaneous mutant examined.

Those mutants which are non-complementing and non-CRM are likely candidates for nonsense mutants. Mutants induced by EMS are preferable to X-ray induced mutants as approximately one-half of the latter may be base-pair deletions or insertions (Malling and de Serres, 1973) rather than base-pair substitutions; EMS is believed to cause primarily mutations of the substitution type in *D. melanogaster* (Lim and Snyder, 1974).

These strains can now be subjected to further EMS mutagenesis with selection for ry^+ flies among the progeny. A large number of mutagenized chromosmes can be tested using the purine screen which allows only ry^+ flies to survive. Purine-resistance may, however, result from mutations at other loci, perhaps coding for permeases, which allow the fly to develop on media containing purine; these flies would still have *ry* eyes. Some true revertants may also be picked up in this test; these would have high levels of XDH activity.

The purine screen is a very important tool in such experiments

Table 15. Summary of *rosy* Mutants

ry mutant	origin	activity	complementation	CRM
2	spontaneous	-	+*	-
8	X-ray	-	-	-
26	X-ray	-	-	-
41	X-ray	-	-	+
*601***	EMS	-	-	-
*603***	EMS	-	-	-
*604***	EMS	-	-	-
*a1***	EMS	-	-	-
*a2***	EMS	-	-	-
a3	EMS	-	+	+
a4	EMS	+(gels)	+	+
a5	EMS	-	+	+
a6	EMS	-	-	+
*a7***	EMS	-	-	-
a8	EMS	-	+	-
a9	EMS	-	+	+
a10	EMS	-	+	+
*a11***	EMS	-	-	-

* complements ry^{406} (Gelbart *et al.*, 1976)

** non-complementing, non-CRM, EMS induced null allele

as some minimum number of mutagenized chromosomes of each putative nonsense allele must be tested before it is concluded that the allele cannot be suppressed. It is crucial to choose a level of purine which allows flies with low levels of XDH to survive. The complementing pair ry^{a3}/ry^{a5} (6.5% activity) fails to complete development at the level of purine used in Chovnick's laboratory for recombination studies. It may be necessary to use a level of purine which allows some *ry* flies to survive (escapers) in order to obtain flies with low levels of XDH.

Interesting flies coming out of the purine screen would be expected to have wild type eyes and fairly low levels of XDH activity. Any viable tRNA suppressor would have to be "leaky", that is, fairly inefficient in suppression of a nonsense mutant. Greater efficiency would interfere with normal termination signals and result in lethality. A correlation between efficiency of suppression and lethality has been observed in yeast (Capecchi *et al.*, 1975; Gesteland *et al.*, 1976). Since only low levels of XDH activity are necessary for wild type eye color, the flies would exhibit a non-mutant phenotype.

The mutation should also be dominant rather than recessive since only one mutant gene is necessary to produce a mutant tRNA; indeed, two mutant copies of any tRNA might be lethal. The mutation must also map outside the *ry* locus; reversions or intra-locus suppression by a second lesion (for example, a base-pair insertion which restores the reading frame of an earlier deletion) would not be of interest. These would probably have high levels of XDH activity and could be

eliminated by that criterion.

A further genetic test could be employed: the simultaneous suppression of putative nonsense alleles at more than one locus. Good choices of alleles to examine are the *Acph-1* null mutants already determined to be CRM^- and complementation negative (Bell and MacIntyre, 1973) and the *Adh* alleles screened in a similar manner (Schwartz and Sofer, 1976). The latter could also be mutagenized in attempts to obtain a tRNA suppressor mutation since a lethal screen exists for *Adh* similar to that for *ry*: flies of the Adh^- genotype are sensitive to ethanol in the media.

Those mutants which met the above genetic criteria should be further analyzed for the presence of mutant tRNA. This might be accomplished by mapping of the lesion to a known tRNA locus (see Grigliatti *et al.*, 1977). Those tRNA's whose anticodons could be mutated by a single base to read a nonsense codon would be the first candidates for such examination. However, in order for a suppressor mutation to be viable and thus observable, there may be a need for several isoaccepting forms of tRNA for a given amino acid (that is, redundancy) in that species. In yeast, eight loci can mutate to produce efficient suppression; all of these loci code for $tRNA^{tyr}$ since tyrosine is the amino acid inserted into the polypeptide chain. Several other loci can produce inefficient suppression; these suppressors insert serine and are recessive lethal mutations (Hawthorne and Mortimer, 1963; Gilmore and Mortimer, 1966; Hawthorne and Leupold, 1974; Brandiss *et al.*, 1975).

Those tRNA's which are likely candidates for suppressor molecules could be examined directly for the presence of a base change. In at least some cases, a single base change may result in a different elution profile from the wild type on reversed-phase chromatography.

The detection of a mutant tRNA which is able to suppress alleles at loci coding for proteins in non-related pathways would be good evidence for the existence of tRNA suppression in *D. melanogaster*. The final proof might be the ability of this tRNA to translate known nonsense codons of yeast or bacteria in an *in vitro* protein synthesis system such as has been done with yeast tRNA to finally prove that tRNA is the vehicle of informational suppression in that organism (Capecchi *et al.*, 1975; Gesteland *et al.*, 1976).

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