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A COMPARATIVE STUDY OF ALDEHYDE OXIDASE FROM
TUMOROUS-HEAD AND OREGON-R-C STRAINS OF
DROSOPHILA MELANOGASTER

BY

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B.S., Florida Technological University, 1975

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science: Biological Sciences
in the Graduate Studies Program of the College of Natural Sciences
of Florida Technological University

Orlando, Florida
1977

ABSTRACT

Aldehyde oxidase has been partially purified from Oregon-R-C and tuh(ASU) strains of *Drosophila melanogaster* using an affinity technique. The two enzymes were subjected to a partial kinetic analysis and found to be very similar to one another. This indicated the problem of elevated aldehyde oxidase activity in tuh(ASU) at key developmental stages (Kuhn and Cunningham, 1976) is due to an abnormal regulation. A comparative isozyme study through the developmental stages showed no major differences between the enzymes indirectly supporting the idea of an abnormal regulation. A comparison of tuh(ASU) with four wild-type strains indicates it may be a fourth allozyme of aldehyde oxidase.

ACKNOWLEDGEMENTS

I would first of all like to thank Drs. Cunningham and Kuhn for their invaluable assistance and guidance in the completion of this thesis. I would also like to thank my parents for their undying love and faith in me and my abilities even when I was at my low educational ebb. Finally, and most importantly, I want to thank my wife, Jan, for her love and patience when the hours were long and the rewards small. It is to you I dedicate my work.

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INTRODUCTION

The original strain of tumorous-head Drosophila melanogaster was collected at Acahuizotla, Mexico, in 1941 by field workers from the University of Texas. Amorphous head growths were first noted in the spring of 1945, and in 1946 workers at the University of Utah obtained the strain and named it tumorous-head (symbolized tuh). From there, the strain was sent to several localities around the United States where subsequent genetic divergence occurred (Woolf, 1965; Kuhn, 1971b).

The distribution and morphology of the abnormal growths have been described by Newby (1949) with both internal and external malformations observed. Some of the external malformations appeared as modified legs. Postlethwait et al. (1972) reevaluated this homoeotic effect (i.e. the transformation of one organ or tissue-type to another organ or tissue type not located in that area) of tumorous-head and observed alterations from antenna to leg, eye or head to abdomen, and rostralthaut to genital structures. Kuhn and Dorgan (1975) have examined inbred tumorous-head strains showing higher penetrance and expressivity and observed the same alterations noted by Postlethwait et al. (1972). They have also observed transformations of eye to genital tissue, eye to leg, and rostralthaut transformations to structures that appear to be abdominal tissue. Gateff and Schneiderman (1974) have suggested that since the tumorous-head mutant is homoeotic, the term tumor is not accurate.

Gardner and Woolf (1949) have shown that the amorphous head

growths are caused by a third-chromosome semidominant gene symbolized as tu-3 which has been mapped by Gardner (1959) to 3-58.5±. Gardner et al. (1952) have shown that the penetrance of tu-3 can be increased by a sex-linked recessive gene (symbolized tu-1^a) that is responsible for a maternal effect, and by modifier genes found in laboratory stocks as well as in natural populations. Pyati (1976) recently mapped the tu-1^a gene to 65.8 on the X-chromosome. All flies that show the tumorous-head trait are homozygous for these genes.

A third chromosome dimorphism, symbolized as 3A and 3B, has been found in all but one of the tumorous-head strains (Woolf and Phelps, 1960). Chromosome 3A contains tu-3, while chromosome 3B possesses the Payne inversion with the recessive gene for scarlet eyes (symbolized In(3L)P,st). The tu-3 gene is located in the right arm, while In(3L)P,st is positioned in the left arm. Since the 3B/3B combination is homozygous lethal, only two types of adult flies exist: homokaryotypes (3A/3A) and heterokaryotypes (3A/3B). Due to the adaptive advantage of the heterokaryotypes, over 80% of the adult flies possess the 3A/3B combination, even though 3B is homozygous lethal (Woolf and Church, 1963). Most flies used in this study are 3A/3B (Kuhn, unpublished).

A reduced productivity of female homokaryotypes versus female heterokaryotypes (Woolf and Church, 1963; Woolf et al., 1964) results from a polygenic system involving the second chromosome and the left arm of chromosome 3A (Woolf and Knowles, 1964). A viability maternal effect controlled by the left arm of chromosome 3A (Woolf, 1967) may

have something to do with the reduced homokaryotype productivity. The greater productivity of female heterokaryotypes is due to superior fertility and fecundity (Knowles, 1967). A second chromosome maternal effect is the primary cause of reduced fertility in homokaryotypes, while a region near roughoid (0.0) in the left arm of chromosome 3A reduced homokaryotype fecundity (Knowles, 1967). All that was needed to override the detrimental effects of genes reducing fecundity and genes reducing longevity was heterozygosity for the left arm chromosome 3, whether it be In(3L)P,st, or In(3L)P,gm, or left arm derived from Urbana or Abilene laboratory strains (Kuhn, 1970). However, to override the deleterious effects on fertility, a coadaptation of In(3L)P,st with chromosome 2 appears to be necessary. Coadaptation is the process in which a particular portion of a chromosome unique to a strain (i.e. to an inversion) is incorporated without deleterious effects into a related strain not carrying that portion of the chromosome. The importance of In(3L)P,st was shown when the inversion was incorporated in an Urbana laboratory strain. The homokaryotypes and heterokaryotypes showed no difference in fitness when the inversion was first incorporated. After two years, in which coadaptation of In(3L)P,st gradually occurred, a significant increase in overall fitness of the Urbana In(3L)P,st strain was observed (Kuhn, 1971a).

Maternal effects influencing abnormal growths in the head region, viability, and fertility have thus far been discussed for tumorous-head. A fourth maternal effect has been described by Woolf (1968). Abnormal testis development in 20 to 40 percent of the males occurs in the

presence of $tu-1^b$ (a naturally occurring allele to $tu-1^a$), and is maternally affected. The maternal effect exists when $tu-1^b$ is in the presence of second chromosome modifiers with $tu-3$ (Woolf, 1968).

Kuhn (1971b, 1973) has shown a fifth maternal effect associated with sex-ratio in favor of males. This sex-ratio abnormality is mainly controlled by genes in chromosome 3B with modifying genes located in the second chromosome (Kuhn, 1971b).

Studies by Kuhn (1974) and Woolf and Lott (1965) have shown that extensive genetic interactions exist between eclosion, sex ratio, karyotype and penetrance of the tumorous-head trait, and that none of the above traits are independent in their expression. This is explained on the basis of females being more likely to develop the tumorous-head trait than males. Also, the heterozygosity for chromosome 3B increases the probability that female and male zygotes will survive to the adult stage despite the abnormal development resulting in the tumorous-head trait.

The Genetics of Xanthine Dehydrogenase, Pyridoxal Oxidase and Aldehyde Oxidase

Three closely related enzymes in Drosophila melanogaster have been found to be genetically controlled in a similar manner. The three enzymes are xanthine dehydrogenase, pyridoxal oxidase and aldehyde oxidase. Of particular interest to this study is the enzyme aldehyde oxidase, but all three will be discussed to show their genetic relationship to each other.

In Drosophila melanogaster, xanthine dehydrogenase is produced

and regulated by at least four different loci. They are maroon-like (ma-1), rosy (ry), low xanthine dehydrogenase (lxd), and cinnamon (cin). Flies with the ma-1 eye mutation, characterized by a brownish eye color, show no activity for xanthine dehydrogenase (Forrest et al., 1956). The ma-1 gene has been mapped to 1-64.8± (Lindsley and Grell, 1968). The recessive gene ry is also characterized by a brownish eye color, and is located at 3-52.3± (Lindsley and Grell, 1968). Tests have shown that the ry mutant is devoid of xanthine dehydrogenase activity (Glassman and Mitchell, 1959). Glassman (1965) purified xanthine dehydrogenase from wild-type flies in order to produce antibodies against it so immunological tests could be run with various mutant strains. Glassman suggested that the structural gene for xanthine dehydrogenase is located at the ry locus since rosy flies contained very little of the cross reacting material (CRM). Ma-1 flies, however, appeared to contain an equivalent amount of CRM with low xanthine dehydrogenase. Since no active xanthine dehydrogenase was detected, the function of the ma-1 locus is still uncertain. Sayles, et al. (1973) recently found that in rosy flies the functional ma-1 complementation factor is present in the eggs, and during early embryogenesis.

A third locus, low xanthine dehydrogenase (lxd), affects xanthine dehydrogenase (Keller and Glassman, 1964). This mutant gene (3-33±; Lindsley and Grell, 1968) decreases xanthine dehydrogenase activity to 25% when flies are homozygous for it. The lxd flies exhibit normal, or wild-type, eye color suggesting sufficient quanti-

ties of xanthine dehydrogenase are present for pteridine metabolism. As with the *ma-1* mutant, equal amounts of the CRM for xanthine dehydrogenase can be found in *lxd* flies. No inhibitor for wild-type xanthine dehydrogenase was found by Keller and Glassman (1964) in their *lxd* flies. Electrophoretic mobilities of enzymes from wild-type and *lxd* flies were essentially the same (Keller *et al.*, 1963). The suspected purpose of *lxd* is of a regulatory nature at possibly the post translation level (Courtright, 1975). Keller and Glassman (1964) found that from about 150 wild-type strains from all over the world tested for the presence of *lxd*, only six of 17 inbred strains from a single sample were shown to possess it.

A fourth locus, termed cinnamon (*cin*), has been found to reduce xanthine dehydrogenase activity (Baker, 1973; Browder and Williamson, 1976). The *cin* mutant is characterized by a brown eye color, and has been tentatively mapped near 1-0.0 (Baker, 1973). Browder and Williamson (1976) as well as Baker (1973) have found that the *cin* locus behaves similarly to the maroon-like locus (maternal transmission). However, xanthine dehydrogenase activity is detectable in higher amounts in *cin* flies during the third instar through early pupal development than in *ma-1* flies. Adults, however, are devoid of xanthine dehydrogenase activity in both mutant strains.

Another enzyme, pyridoxal oxidase, exhibits many similarities to xanthine dehydrogenase. A low pyridoxal oxidase (*lpo*) mutant has been reported by Collins and Glassman (1969). The enzyme level of pyridoxal oxidase in *lpo* mutants is only 2% of wild-type, while normal quantities

of xanthine dehydrogenase are produced. Ma-1 flies show no pyridoxal oxidase (Forrest et al., 1961), while flies with lxd show only approximately 5% normal pyridoxal oxidase activity (Collins and Glassman, 1969). The lpo locus has recently been mapped to 3-57.1± by Dickinson and Weisbrod (1976), and it has been suggested that the locus may be a structural gene for pyridoxal oxidase. The cin locus also exhibits an effect on pyridoxal oxidase since cin mutants are essentially devoid of activity from third instar through adult (Browder and Williamson, 1976).

Two hypothesis have been proposed to explain the similarities between xanthine dehydrogenase and pyridoxal oxidase. One states that xanthine dehydrogenase is possibly produced by polypeptides ry⁺, ma-1⁺, and regulated by lxd⁺ (Glassman, 1965) while pyridoxal oxidase consists of subunits from ma-1⁺, lxd⁺, and lpo⁺ (Collins and Glassman, 1969). An alternate hypothesis is the cofactor theory (Glassman, 1965). Xanthine dehydrogenase and pyridoxal oxidase consist of separate structural genes while the products of ma-1⁺ and lxd⁺ are involved in the formation of some cofactor required for the activity of both enzymes. The cofactor theory appears to be preferred for both enzymes (Glassman et al., 1968; Collins and Glassman, 1969; and Andres, 1976).

Another enzyme that is under similar genetic control is aldehyde oxidase (Courtright, 1967). As with xanthine dehydrogenase, aldehyde oxidase utilized benzaldehyde as a substrate, although it appears to have a broader substrate spectrum. In Drosophila simulans, the structural gene for aldehyde oxidase has been mapped to 3-74.5±. Dickinson

and Weisbrod (1976) have recently mapped the aldehyde oxidase locus in Drosophila melanogaster to 3-57.2±0.3, which is to the right of lpo. It had earlier been mapped to the left of lpo at 3-56.6±0.7 (Dickinson, 1969; 1970). Collins, Duke and Glassman (1971) have postulated a regulatory gene termed low aldehyde oxidase (lao) at 3-56.0±. Glassman (1962) suggested that aldehyde oxidase might be the ma-1⁺ complementing factor since it is in reduced quantities in lxd flies. Courtright (1967), however, showed that norite treatment removed the ma-1⁺ complementing factor, but did not remove aldehyde oxidase establishing an important difference between the enzymes. Courtright indicates that the product of the ma-1 gene is a component of both aldehyde oxidase and the ma-1⁺ complementing factor. As with xanthine dehydrogenase and pyridoxal oxidase, the functional aldehyde oxidase enzyme results from an association between aldehyde oxidase and ma-1⁺, and is controlled by the lxd locus. When Drosophila melanogaster females were crossed with Drosophila simulans males, the paternal aldehyde oxidase was not detected until the third instar stage of development (Courtright, 1967). Ma-1 females were then crossed with Oregon-R males with the results showing that aldehyde oxidase activity is detected in very low amounts until third instar stage of development. These two experiments showed that aldehyde oxidase is a maternal effect enzyme giving it an added similarity with xanthine dehydrogenase (Courtright, 1967).

The cin locus affects aldehyde oxidase activity in a similar manner as the ma-1 locus (Browder and Williamson, 1976). These results

suggest that the *cin* locus controls aldehyde oxidase in a different manner than xanthine dehydrogenase and pyridoxal oxidase, since in comparison with *ma-1*, xanthine dehydrogenase had higher amounts of activity, while pyridoxal oxidase has essentially none.

The maternal effect activity reported by Courtright (1967) has been further studied by Dickinson (1970). Using aldehyde oxidase negative (*aldoxⁿ*) mutants, he found that the enzyme plays no major role in the *ma-1* maternal effect. Male *ma-1* flies produced from *aldoxⁿ* mothers still had the *ma-1* maternal effect even though no maternally transmitted aldehyde oxidase was present in the egg. Dickinson (1971) reexamined the aldehyde oxidase maternal effect previously described by Courtright (1967), and found that enzyme activity could be detected as early as the first instar stage of development. It has been previously thought that synthesis did not begin until the third instar stage of development, indicating the maternal effect is confined to the first twenty-four hours of development. Dickinson (1971) determined the tissue specificity of aldehyde oxidase in the third instar larvae and in adult males, and proposed a regulation gene next to the structural gene (Dickinson, 1975). Histochemical examination of the larvae demonstrated that most activity occurred in the hypodermis with activity in imaginal discs, gut and Malpighian tubules. Of particular interest is the aldehyde oxidase activity associated with eye-antennal imaginal discs. Janning (1973) has demonstrated heavy aldehyde oxidase activity associated with the antenna portion of the disc with no activity in the eye portion of the disc in third instar Canton-S larvae.

Adults showed the most activity in the gut, Malpighian tubules, and genital structures. It is interesting to note that about half of the total activity in females is associated with the ovaries (Dickinson, 1970).

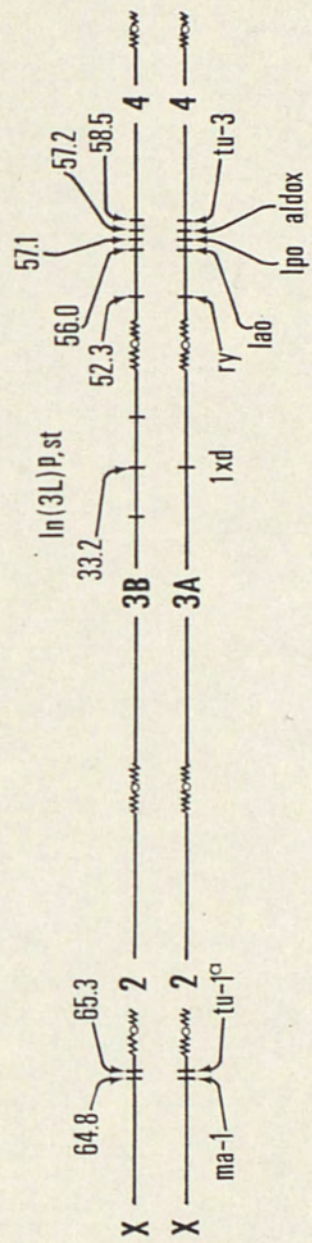
A Review of Aldehyde Oxidase

It has been noted that the genes responsible for the tumorous-head trait are mapped to similar locations to genes responsible for aldehyde oxidase, pyridoxal oxidase, and one gene for xanthine dehydrogenase (Figure 1).

Aldehyde oxidase activity has been followed throughout the life cycle in tumorous-head and wild-type Oregon-R-C as well as in other stocks (Kuhn and Cunningham, 1976). Tumorous-head larvae and adults possess higher specific activity for aldehyde oxidase than those observed for any of five laboratory strains. Since levels of enzyme activity in Oregon-R-C flies reflected the calculated average for the strains, it was selected as the standard and compared with tumorous-head at various stages of development. During embryogenesis, tumorous-head eggs possessed 100% higher aldehyde oxidase specific activity than the wild type Oregon-R-C strain. This difference is important since the thickenings of the epidermis that will invaginate to form the eye-antennal imaginal disc complex are determined within the first three to seven hours of embryogenesis (Fristrom, 1970). A second period of elevated enzyme activity occurs from late third instar and continues until eclosion. Cells in the eye-antennal imaginal discs remained morphologically undifferentiated except for the ommatidial cell clusters and have grown only

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

ry = rosy, lao = low aldehyde oxidase, lxd = low xanthine dehydrogenase, lpo = low pyridoxal oxidase, tu-1^a and tu-3 = tumorous-head



by cell division throughout the larval stages. At metamorphosis, the epithelium undergoes morphogenetic movement known as eversion, after which time differentiation and cuticle synthesis follow (Postlethwait and Schneiderman, 1973). Therefore, the highest levels of aldehyde oxidase occur at the two most crucial development times for eye-antennal development, which are determination and differentiation.

Aldehyde oxidase is a maternally affected enzyme (Courtright, 1967) which is apparently not synthesized during embryogenesis (Dickinson, 1971). Since the tumorous-head phenotype is maternally affected (Gardner and Woolf, 1949) it is reasonable to speculate that tumorous-head genes act at determination (Postlethwait and Schneiderman, 1973) by switching the normal developmental program to different developmental programs. If aldehyde oxidase is in any way involved with the developmental switching mechanism responsible for the homoeotic effect of tumorous-head genes with aldehyde oxidase synthesis (Kuhn and Cunningham, 1976). As demonstrated by Janning (1973) the antennal disc is aldehyde oxidase positive while the eye disc is negative. Many tumorous-head eye imaginal discs, however, possess aldehyde oxidase. This aldehyde oxidase activity may be correlated to specific homoeotic transformations since the genital disc and nests of abdominal histoblasts from third instar Oregon-R-C larvae also show aldehyde oxidase activity. Tumorous-head eye imaginal discs that show aldehyde oxidase activity are individually unique, and show positive staining in the eye portion of the disc approximately 64% of the time in all of the third instar larvae examined (Kuhn and Cunningham, 1976). If biochem-

ical abnormalities could be determined in the antennal portion, then phenotypic variability in penetrance and expressivity of the trait, which appears in about 85% of the tumorous-head adults (Kuhn and Dorgan, 1975) would most likely correlate with enzymatic variability.

A possible explanation for the aldehyde oxidase abnormality in tumorous-head could be provided by the Britten-Davidson model for gene regulation (Britten and Davidson, 1969). Tu-3 may be a mutation in a sensor gene, resulting in the wrong integrator genes being transcribed. After translation, the postulated activator proteins interact with receptor sequences that are not normally activated in the eye disc or antennal disc (Davidson and Britten, 1973). Attached structural genes are then transcribed and translated. Aldehyde oxidase may represent one of a variety of these structural genes that were misread. It may also represent a cytological marker for detecting where the mutant gene has been activated. No cause-effect relationship has yet been determined for aldehyde oxidase in relation to the tumorous-head affect, but the possibility has not been completely ruled out.

Kuhn and Cunningham (1977a,b) have also observed that aldehyde oxidase is distributed in third instar larval wing discs of wild-type and mutant strains of Drosophila melanogaster in a specific manner. Garcia-Bellido et al. (1973) have observed what they have termed developmental compartments in the wing of Drosophila. They used mitotic recombination and cell-marker mutants which form clones of cells that are easily identified. When the mutants were irradiated at varying times of development to induce recombination, they found that the clones

fit into specific boundaries that depended on the stage of development the fly was irradiated. This indicated that specific compartments (i.e. anterior versus posterior) were being formed at specific times in development. Based on the work done with the engrailed and bithorax series of mutants, the aldehyde oxidase distribution in the wing discs appears to correlate to specific developmental compartments. The anterior side of the wing pouch stains aldehyde oxidase positive, while the posterior side is aldehyde oxidase negative. An arching bridge of aldehyde oxidase activity separates the dorsal compartment of the disc from the ventral compartment (Kuhn and Cunningham, 1977a).

Kuhn and Cunningham (1977c) have removed and stained all the different types of imaginal discs and other imaginal cell groups present in third instar larvae to determine presence or absence of aldehyde oxidase in them. They have found that the various imaginal discs can be differentiated according to their aldehyde oxidase staining pattern. Since these patterns are unique and reproducible, it shows that at least on the enzyme level, aldehyde oxidase is an excellent biochemical marker of stages of determination.

Aldehyde oxidase is a molybdoflavoprotein with a molecular weight between 250,000 and 280,000 (Courtright, 1967; Andres, 1976). It appears to be at least a dimer (Dickinson, 1970; Andres, 1976) consisting of 0.47 moles flavin adenine dinucleotide (FAD), 0.49 moles Iron, and 0.35 moles of molybdenum per mole of enzyme (Andres, 1976). Aldehyde oxidase is able to catalyze a number of aldehydes including acetaldehyde, benzaldehyde and salicylaldehyde, and does not require

nicotinamide adenine dinucleotide (NAD^+) as a cofactor (Courtright, 1967; Dickinson, 1970; Collins et al., 1971). Dickinson (1969) and Dickinson and Sullivan (1975) reported a K_m for acetaldehyde as 10^{-2} , while for benzaldehyde it was 10^{-5} . They also found that the V_{max} for acetaldehyde was greater than that for benzaldehyde. The physiological substrate for aldehyde oxidase remains unknown, although Madhavan et al. (1973) have shown that farnesol serves as a substrate, and have suggested a role in juvenile hormone metabolism. The viability of mutants lacking aldehyde oxidase indicates that such a role, if real, is not vital (Dickinson and Sullivan, 1975). Sprey (1977) has found that in all the species he has studied, another enzyme, 5'-nucleotidase, was present when aldehyde oxidase was not. This could indicate that the two enzymes have similar functions in morphogenesis since the activity patterns observed were somewhat similar. Based on these results, Sprey (1977) concluded that the positioning of the cells at determination (i.e. anterior side versus posterior side) will determine whether the enzymes will or will not be synthesized.

Aldehyde oxidase has been purified by Courtright (1967) and Dickinson (1970) with a 20% and 15% recovery respectively. Andres (1976) has also purified the enzyme using an immunoabsorption technique, but the final yield was only 12%. Aldehyde oxidase appears to exist in two interconvertible forms that are under coordinate genetic control (Courtright, 1967). The expressivity of one form over the other is determined by the age of the culture. Dickinson (1970) has also found by using electrophoretic methods that there are three

major alleles (allozymes) for aldehyde oxidase.

The purpose of this study is to continue the biochemical investigation of aldehyde oxidase in the tumorous-head (ASU) and Oregon-R-C strains of *Drosophila melanogaster* begun by Drs. Kuhn and Cunningham (1976). The investigation will be approached in the following manner; A) to devise a quick and efficient method of purification necessary to facilitate comparative studies between strains; B) to partially characterize the enzyme from tumorous-head and Oregon-R-C for comparative purposes, and C) to perform an isozyme study through the developmental cycle of tumorous-head and Oregon-R-C to determine if the abnormally high levels of aldehyde oxidase in tumorous-head, at key stages, are due to structural gene differences, or an abnormal regulation of the known isozymes.

MATERIALS AND METHODS

Genetic Stocks and General Procedure

The following strains (as described in Lindsley and Grell, 1968) of Drosophila melanogaster were used in this study: 1) a tumorous-head strain from Arizona State University, symbolized tuh(ASU), in which flies showing the tumorous-head trait were used to propagate the strain; 2) an Oregon-R-C laboratory strain obtained from California Institute of Technology stock center (CT); 3) a Canton-S laboratory strain from CT; 4) a Swedish-C laboratory strain from CT; and 5) an Oregon-R laboratory strain from CT.

Experimental cultures were maintained at 25°C in one-half pint milk bottles on freshly prepared Drosophila medium containing cornmeal-agar-dried yeast-sucrose-molasses with propionic acid added as a mold inhibitor (Kuhn and Cunningham, 1976). For the ontogeny study, the method of Mitchell and Mitchell (1964) was used for harvesting. For the egg stage, females were allowed to lay eggs for twelve hours giving an average time of six hours. All the later stages of development were begun from eggs collected after six hours. First and second instar larval stages were begun by placing the eggs harvested after six hours on new media trays similar to the ones used for the initial egg collection. Late third instar, early, middle and late pupae stages were begun by placing the eggs harvested after six hours into one half pint milk bottles containing the previously described standard Drosophila medium. The stages were allowed to progress to the following average

times (Kuhn and Cunningham, 1976):

egg stage	6 hours
first instar larvae	31 hours
second instar larvae	58 hours
late third instar larvae	110 hours
early pupae	130 hours
middle pupae	168 hours
late pupae	205 hours.

Spectrophotometric Assay for Aldehyde Oxidase

All assays were performed using a Unicam SP 1800 Ultraviolet spectrophotometer equipped with a Unicam AR 25 linear recorder.

Aldehyde oxidase activity was assayed using essentially the same procedure as described by Courtright (1967). The assay mixture contained in a total volume of 1.1 ml, 0.66 ml of 0.5 M KH_2PO_4 - K_2HPO_4 , pH 7.5, buffer containing 0.1% bovine serum albumin (Sigma) and 1 mM ethylene-diamine tetraacetic acid (EDTA), 0.2 ml dichlorophenol-indophenol (Sigma), 0.04 ml of 1 mg/ml phenazine methosulfate (Aldrich), 0.1 ml of enzyme solution diluted so as not to give an absorbance change exceeding 0.250 absorbance units per minute, and 0.1 ml of a 1 M acetaldehyde (Aldrich) solution prepared fresh daily. The reduction of dichlorophenolindophenol was monitored at 600 nm, with one unit defined as that amount of enzyme causing a decrease of 1.00 A_{600} per minute at 25°C (Courtright, 1967). Protein was measured using the procedure of Lowry et al. (1951). Specific activity is expressed as units per milligram protein.

Sample Preparation (4°C)

Cell-free extracts for the purification procedure were prepared by placing three grams of frozen flies into a 40 ml glass homogenizer

with 10 ml of 0.1 M Tris-HCl buffer, pH 8.1 with 1 mM EDTA. The pre-cooled material was thoroughly homogenized, and allowed to set in 0.3 grams of neutralized, activated charcoal (Sigma) for one hour. The above procedure was also used for fresh materials (eggs, instar larvae, pupae, etc), excluding the activated charcoal step. The sample was then centrifuged at 42,000 x g for 30 minutes at 4°C. This sample preparation procedure was used as the first step in all experimental procedures used.

Purification Procedure for Aldehyde Oxidase (at 4°C)

All previously reported procedures for the purification of aldehyde oxidase required large gram quantities of flies due to the poor yield of pure enzyme obtained (Courtright, 1967; Dickinson, 1970; and Andres, 1976). For this study, a more efficient procedure was desired.

1) Ammonium Sulfate Fractionization (modified from Andres, 1976)

A saturated solution of ammonium sulfate (J. T. Baker) in 0.1 M Tris-HCl buffer, pH 8.1, with 1 mM EDTA was added dropwise at 4°C to the previously prepared sample until a 50% saturation was achieved. The solution was allowed to mix slowly for 30 minutes to allow complete equilibration. After setting overnight, the 50% fraction was centrifuged at 42,000 x g for 30 minutes. The aldehyde oxidase was then precipitated out in the 50-95% fraction by adding an appropriate amount of solid ammonium sulfate. The active sample was desalted using an Amicon ultrafiltration apparatus with a PM 30 filter at 20 psi's. The

flow rate was 20 mls per hour.

2) QAE Sephadex Anion Exchange Chromatography

The concentrated, desalted, sample was then applied to a QAE Sephadex A-25 (Sigma) column (0.9 x 24.5 cm) equilibrated in 0.02 M Tris-HCl buffer, pH 7.8, with 0.02 M NaCl (Mallinckrodt). The enzyme was eluted using a linear gradient (total volume of 300 mls) of 0.02 to 0.3 M NaCl. A head pressure of 14 cm was applied with 4 ml eluent collected per hour. The active fractions were reconcentrated by Amicon ultrafiltration using a PM 30 filter at 20 psi's with a flow rate of 25 ml per hour. The column was then flushed with 150 ml of 1.0 M NaCl (in 0.02 M Tris-HCl, pH 7.8) before reequilibrating it in the initial buffer.

3) Affinity Chromatography (modified from Chu, 1973)

a.) Ligand Synthesis

N-(4-bromoethyl)-benzyl-6-methylnicotinamide was prepared by adding two grams of 6-methyl-nicotinamide (Ash Stevens) to 7.5 grams p-dibromoxylene (Aldrich) dissolved in 250 ml tetrahydrofuran (MCB) and stirring the mixture under reflux overnight. The white precipitate formed was extracted with methanol as described by Chu (1973) to purify the ligand, with no apparent result. It decomposed at 250°C. Dr. John Idoux (Organic Chemist) felt that with those reaction conditions and reagents involved, the white precipitate was the desired product. As a result, it was used in this state for

attachment to the space-arm.

b.) Spacer-arm synthesis

1,2-di(diaminodecanyl) ethylene dibromide was prepared by adding 5 grams of ethylene dibromide (Aldrich) dropwise (one drop per 10 minutes) to a solution of 10 grams of 1,10-diaminodecane (Aldrich) in 50 ml of methanol. During the addition of the ethylene dibromide, and for the next four hours, the mixture was stirred and heated under reflux. The product was retrieved from tetrahydrofuran (MCB) by flash-evaporation, using a Renco flash-evaporator. The desired compound melted in the range of 120-125°C.

c.) Affinity matrix synthesis

Five grams of cyanogen bromide activated Sepharose 4-B (Sigma) was reswollen in a 1 mM HCl solution, placed on a Buchner funnel, and washed with the same solution (200 ml per gram activated gel) for 15 minutes. The rehydrated gel is then equilibrated to a pH of 9.0 with a 0.1 M sodium bicarbonate buffer, pH 9.0. Two and one half grams of 1,2-di(diaminodecanyl) ethylene dibromide was dissolved in 200 ml of cold 0.1 M sodium bicarbonate buffer, pH 9.0, and added to the activated Sepharose. The slurry was then stirred gently overnight at 4°C. The residue was collected and washed with 10 volumes (in ml) of anhydrous methyl per bed volume (in ml) of Sepharose-spacer arm complex. Five grams of the ligand salt was then dissolved in 200 ml methanol,

filtered and added to the dried beads. The resulting slurry was stirred gently overnight at room temperature. Just prior to use, the affinity matrix was rehydrated overnight in the starting buffer (0.05 M glycine-KOH buffer, pH 8.3, with 1 mM EDTA).

d.) Pouring the column

The affinity matrix was then diluted 1:10 with Sepharose 6-B (Sigma), and the column poured. Sepharose 6-B was used since it had a higher allowable head pressure and resulted in less diffusion due to the smaller porosity of the gel.

e.) Elution parameters

The reconcentrated sample from the anion exchange step was applied to the affinity column (1.5 x 26 cm) in a 0.05 M glycine-KOH buffer, pH 8.3, with 1 mM EDTA and 0.05 M NaCl. The column was washed with the initial buffer until protein was no longer eluted off at a head pressure of 60 cm with 20 ml of eluent collected per hour. Aldehyde oxidase was then removed from the column using the same buffer containing 0.13 M NaCl, and the active fractions are reconcentrated using an Amicon ultrafiltration device with a PM 30 filter.

4) Gel Filtration Column Chromatography

The reconcentrated sample from the affinity column was applied to a column packed with Ultrogel ACA 34 (LKB) (1.5 x 28 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.1, containing 1 mM EDTA and 0.2 g ultrapure sucrose (Schwarz/Mann) per ml buffer.

The sucrose was added to stabilize the enzyme. A head pressure of 6 cm was applied with 8 ml of eluent collected per hour. The active fractions were then reconcentrated by Amicon ultrafiltration with a PM 30 filter as described previously.

All column chromatographic steps were monitored at 280 nm using an Altex model 153 Analytical U.V. monitor with a 200 μ l Biochemical cell. A Linear Model 260 recorder was used to record the results from each step.

Enzyme Characterization

The following characterizations of aldehyde oxidase from tuh(ASU) and Oregon-R-C were performed on the partially purified sample obtained from the QAE step, using the previously described assay procedure. All data were plotted according to the Lineweaver-Burke method (Segel, 1975), and applied to the least-square-fit analysis (Snedecor, 1959).

1) K_m Determination for Acetaldehyde

The K_m for acetaldehyde was determined by varying the amounts of acetaldehyde from appropriate stock solutions. The assay buffer was adjusted when needed to obtain a final volume of 1.1 ml.

2) Inhibitor Studies

Three inhibitors of aldehyde oxidase were used, with the effects determined at a single concentration level. Acetaldehyde was used as the substrate at the concentrations used for the K_m determination. The three inhibitors used were Quinicine-HCl (0.1 mM), 2,3-dihydroxypyridien (1.1 mM), and 3-acetylpyridine

(10 mM). All inhibitors were obtained from Aldrich.

Polyacrylamide Gel Electrophoresis

All procedures involving polyacrylamide gel electrophoresis (unless otherwise noted) were carried out on a 5.5% gel prepared from the following solutions:

- (a) two parts solution consisting of 10.45 gm Acrylamide (Aldrich) to 0.55 gm N,N'-methylenebisacrylamide (Isolab) per 100 mls
- (b) one part solution 2 consisting of 2.20 g boric acid (Sigma), 4.31 g Tris (hydroxymethyl) aminomethane (Sigma), and 0.37 m EDTA (Sigma) per 100 ml of glass distilled H₂O
- (c) one part solution 3 consisting of 0.4 grams ammonium persulfate (Isolab) per 100 ml H₂O and
- (d) 0.01% N,N,N',N'-tetramethylethylenediamine (J. T. Baker) by volume.

Photoactivated gels were prepared by substituting 0.1% solution of riboflavin (Eastman), at 1 volume/40 volumes gel solution, for solution 3 and N,N,N',N'-tetramethylethylenediamine. The gel polymerization is initiated by placing a 25 watt fluorescent lamp 2-3 cm away from the poured gel solution (Richards and Coll, 1965).

An Ortec Model 4200 Slab electrophoresis system was used, with both gel and chamber buffer composed of tris-borate. The chamber buffer is a 1:4 dilution of solution 2 (described above). The final pH was 8.4. One and one half grams of ultrapure sucrose was added to the 30 ml gel solution to eliminate mixing when water is layered over

the surface. Ultrapure sucrose, 0.15 g, was dissolved in 1 ml of crude extract to facilitate layering on the gel. Bromophenol blue was used as a marker to observe the migration of the sample.

Gels were stained for aldehyde oxidase activity by a method similar to that of Courtright (1967). The solution consisted of 5.70 ml acetaldehyde (99% + pure), 0.020 g of phenazine methosulfate, 0.035 g of nitroblue tetrazolium (Aldrich), 40 ml of 0.5 M Tris-HCl buffer, pH 7.5, and enough glass distilled H₂O to give a final volume of 100 ml. Gels were run at 65 mamps for three hours at 4°C, and staining in the dark for 1-6 hours. Gels were then placed in a 7.5% acetic acid solution until photographed.

The method of Reisner et al. (1975) was used for general protein staining. The final stain solution consisted of 3.5% perchlorid acid containing 0.04% Coomassie Brilliant Blue G-250 (Isolab). Between 10 and 200 µg of protein was needed for adequate detection, with 120 µg giving optimal results. The sensitivity of the stain, however, was as low as 1.5 µg in a single band. Gels for general protein determination were run for one and one-half hours at 65 mamps, and placed in the stain solution overnight. If destaining is required, a 7.5% acetic acid solution was used, with changes performed periodically until the gel is clear. All gels were left in 7.5% acetic acid until they were photographed.

Isozyme Study through the Developmental Stages of tuh(ASU) and Oregon-R-C (at 4°C)

Gels were prepared, electrophoresed and stained for aldehyde

oxidase activity as previously described. The times used, and the method of collection, for the various stages of development have been described under general procedures. Oregon-R-C and tuh(ASU) were electrophoresed at the same time under the same conditions. The enzyme activities applied were approximately the same as determined by the spectrophotometric assay procedure described previously.

RESULTS

Purification Procedure for Aldehyde Oxidase

The results of the purification procedure are summarized in Table I. A comparison of the initial specific activities shows that Oregon-R-C has a slightly lower value than tuh(ASU) (11.2 versus 13.1 units/mg protein). The following is a description of the results from the purification of aldehyde oxidase from Oregon-R-C.

The initial crude extract yielded 1680.0 units of activity (from three grams of flies homogenized in 10 ml of buffer) and 150.0 mg of protein. The resulting specific activity (units/mg protein) was 11.2. A 91% yield was obtained in the ammonium sulfate fractionating step with 1528.8 units of aldehyde oxidase activity recovered. Total protein present is 42.6 mg resulting in a specific activity of 35.9. The fold purification obtained is 3.2.

In the QAE Sephadex anion exchange step, a 96% yield of active enzyme was achieved with the total number of active units at 1467.6. The resulting protein concentration is 28.4 mg with the specific activity of aldehyde oxidase at 51.7 units/mg. The overall fold purification is now at 4.6. Figure 2 depicts the elution profile of aldehyde oxidase from the QAE column. The active aldehyde oxidase was eluted off very close to the void volume.

The affinity chromatography step resulted in approximately a 12 fold increase in purity of the active enzyme. A 65% yield was obtained with 953.9 units of activity present. The protein concentration is now

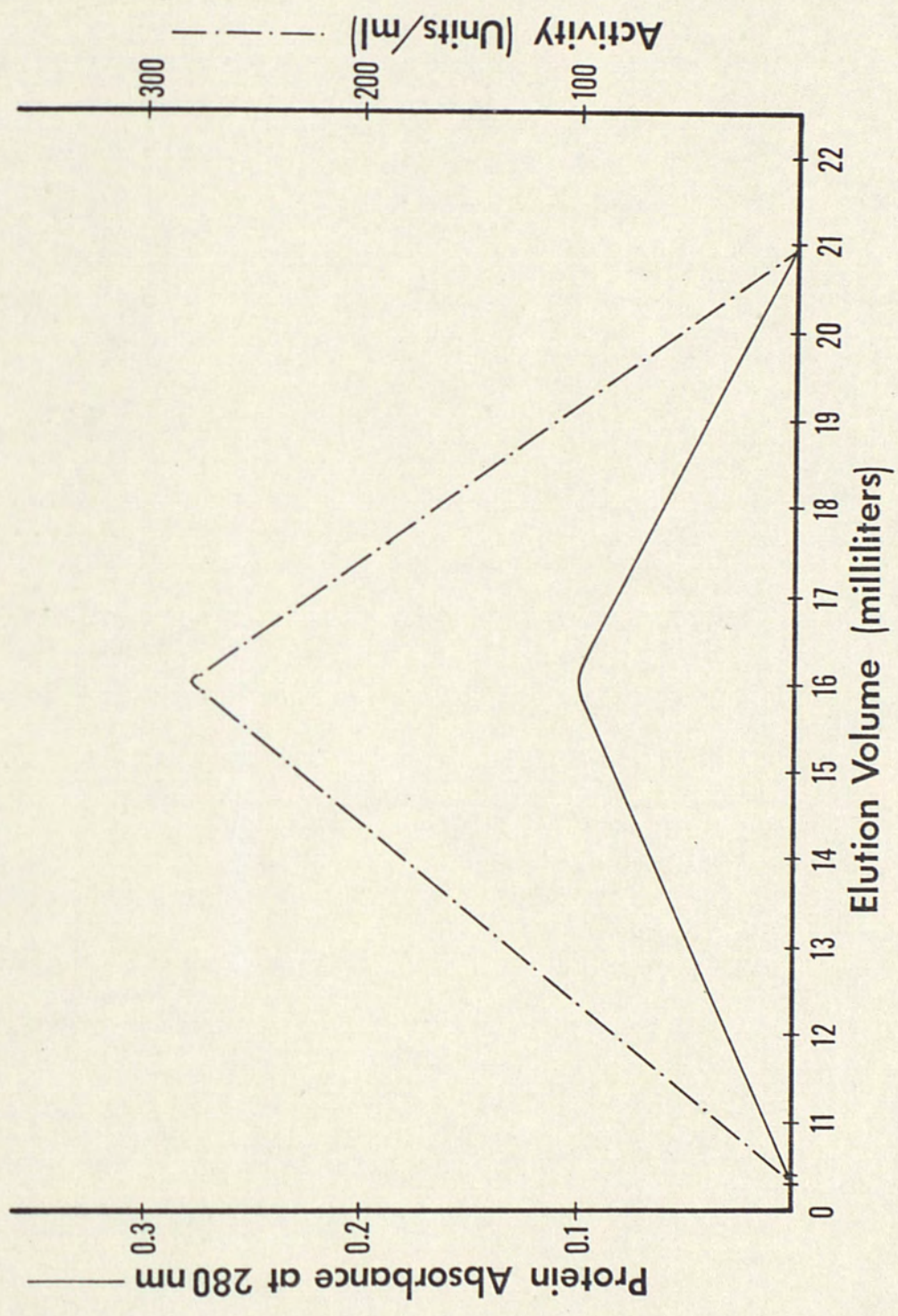
TABLE I

Purification of aldehyde oxidase from the Oregon-R-C strain of Drosophila melanogaster.

Fraction	Total Protein Mg	Total Activity Units	Specific Activity Units/mg	Purification Overall	Yield %
Crude Extract	150.0	1680.0	11.2	1	100
Ammonium Sulfate	42.6	1528.8	35.9	3.2	91
QAE-Sephadex	28.4	1467.6	51.7	4.6	87
Affinity Chromatography	1.5	953.9	620.8	55.4	56.5
Gel Filtration	—	810.8	Very High	—	48.0

Figure 2. Elution profile for QAE-Sephadex A-25 anion exchange chromatography. Activity was eluted off with 0.02 M Tris-HCl buffer, pH 7.8, containing 0.02 M NaCl.

———— = protein absorbance (280 nm), —·—·— = aldehyde oxidase



at 1.5 mg giving a specific activity of 620.8 units/mg. The elution profile for the affinity step is shown in Figure 3. The enzyme did not elute off the column until approximately 320 mls after the eluting gradient (0.05 M glycine-KOH buffer, pH 8.3, with 0.13 M NaCl) was begun.

Preliminary work with the gel filtration step indicates it will be a very promising step in the purification procedure. The percent yield obtained was 85, with a total of 8.0.8 units of aldehyde oxidase activity present. An electrophoretic analysis of the results from the affinity and gel chromatography steps (Figure 5) show that there has been a significant increase in purity. The protein concentration could not be determined by the Lowry or 260/280 technique due to the limited amount present. The elution profile (Figure 4) shows that the active peak comes off the column very close to the void volume.

As can be seen from the electrophoretic evaluation of the results (Figure 5), two major bands of contaminating protein are present besides the active aldehyde oxidase band of protein. The two contaminating bands of protein were reduced significantly when the sample was passed through the gel filtration column.

Enzyme Characterization

The results of the characterizations performed on aldehyde oxidase from Oregon-R-C and tuh(ASU) are summarized in Tables II and III. The K_m and V_{max} values obtained for the substrate acetaldehyde indicate that aldehyde oxidase from tuh(ASU) and Oregon-R-C are very similar with respect to the acceptance of this particular substrate.

Figure 3. Elution profile for affinity chromatography. Activity was eluted with 0.05 M glycine-KOH buffer containing 1 mM EDTA and 0.13 M NaCl.

———— = protein absorbance (280 nm),

— · — · — · = aldehyde oxidase

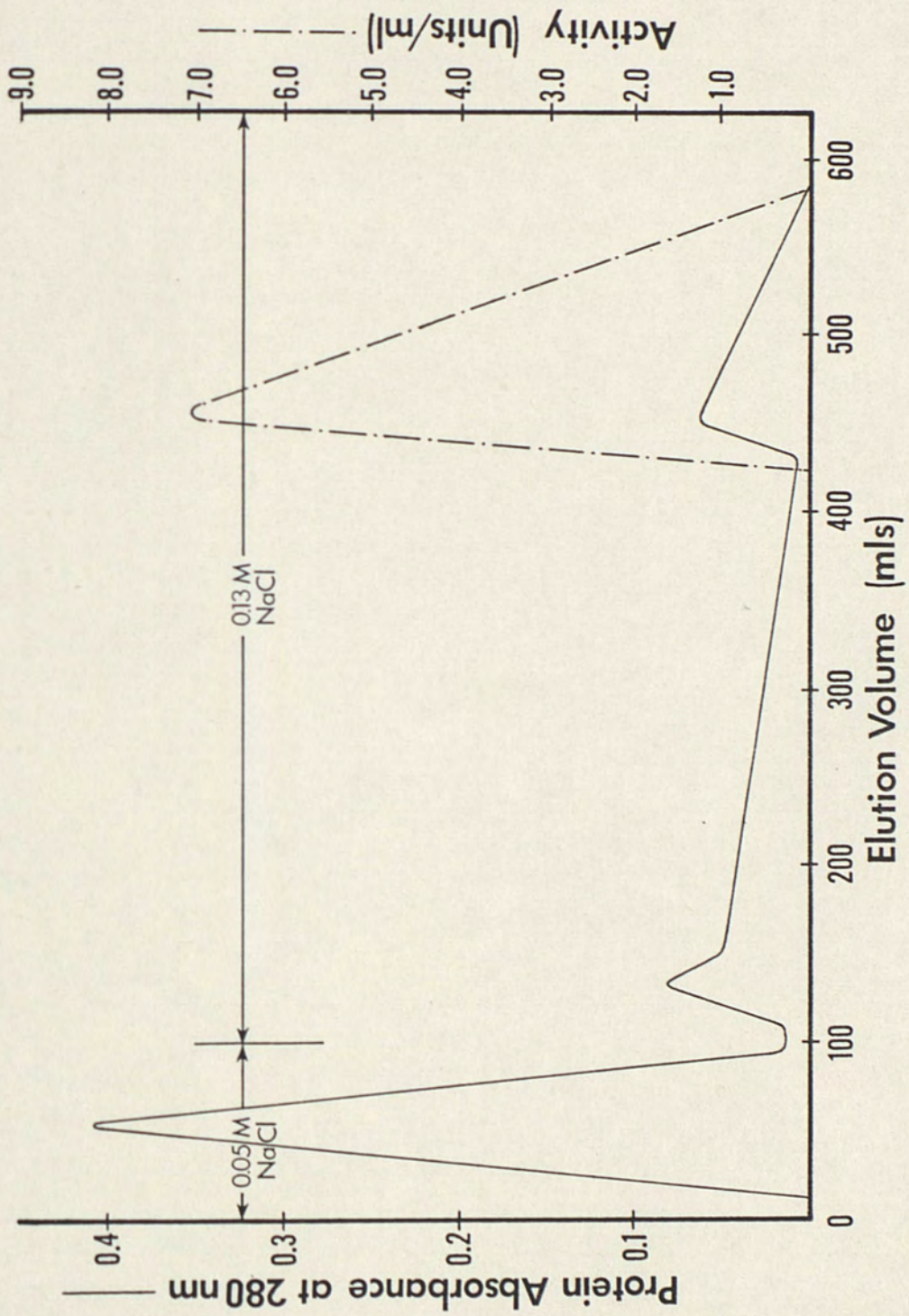


Figure 4. Elution profile for ultrogel ACA 34 gel filtration column. Activity was eluted off with 0.1 M Tris-HCl buffer, pH 8.1, containing 1 mM EDTA and 20% sucrose.

———— = protein absorbance (280 nm),

— · — · — · — · — · — · = aldehyde oxidase

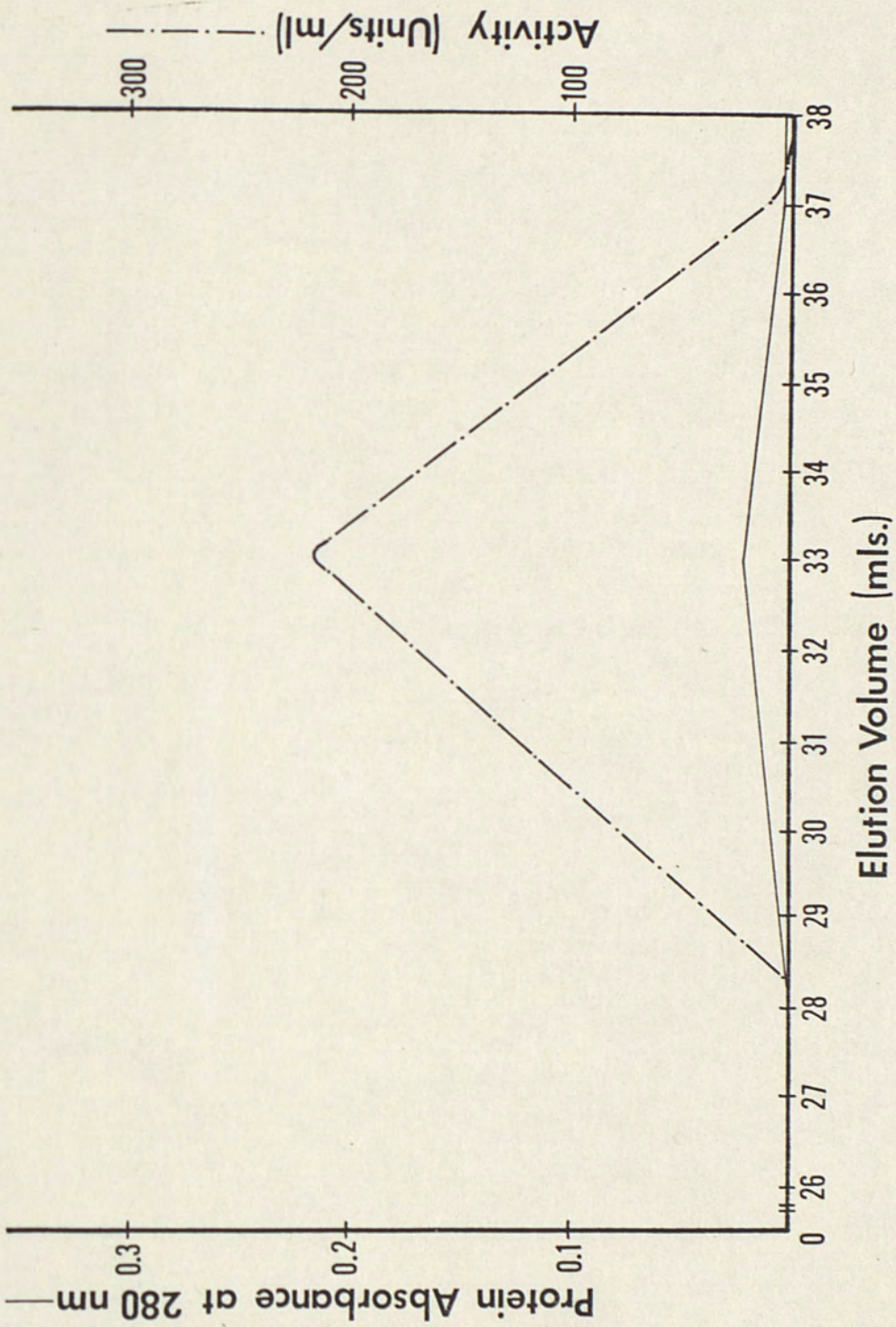


Figure 5. General protein and activity stains for aldehyde oxidase from the purification procedure.

1 and 2 = contaminating protein,

3 = aldehyde oxidase stained for general protein,

4 = aldehyde oxidase stained for activity,

A = sample from affinity step, B = sample from gel filtration step.

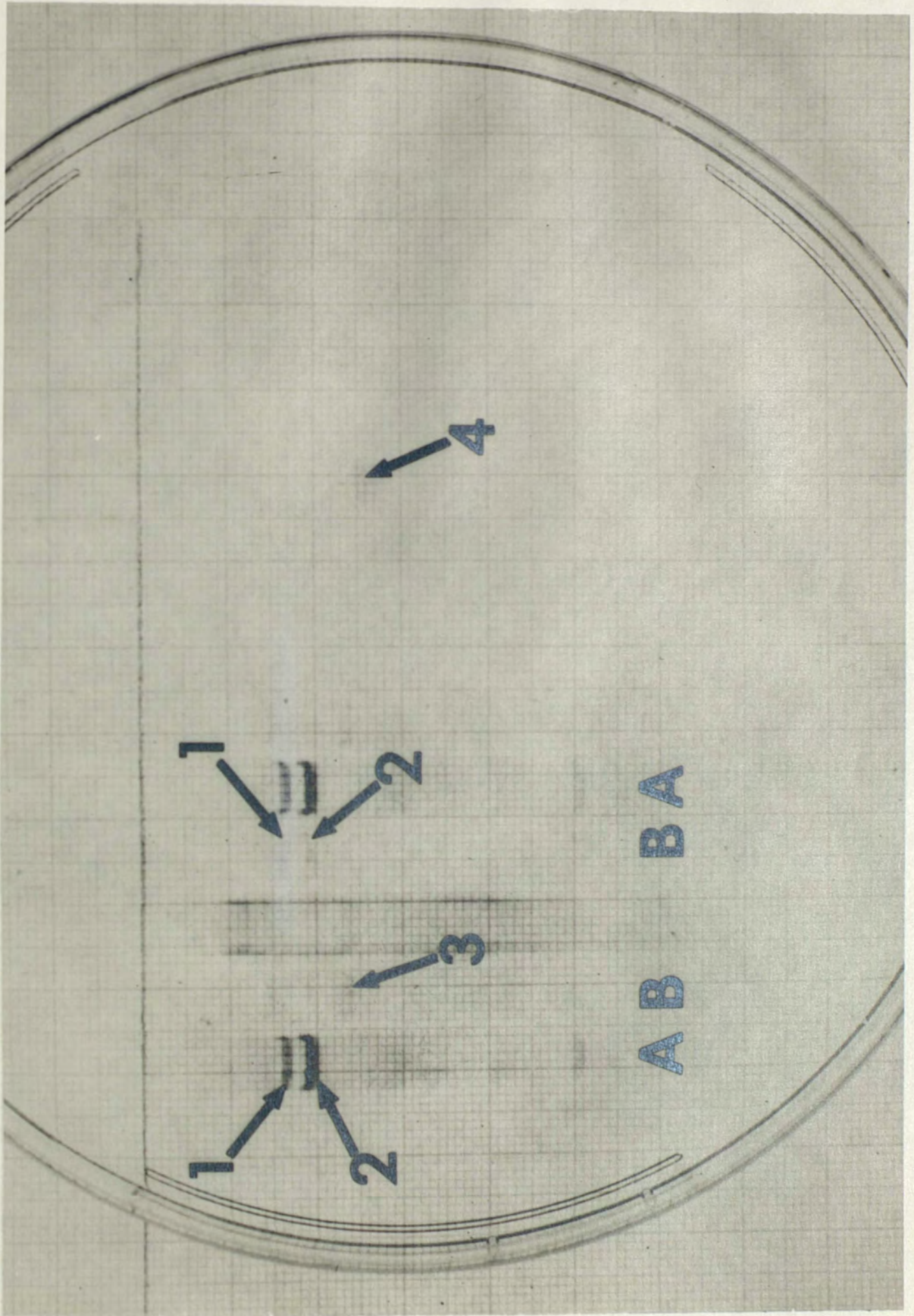


TABLE II

Summary of kinetic characteristics for aldehyde oxidase from Oregon-R-C

Inhibitor or Substrate	Km (M)	Vmax (Abs/min)	Inhibition Type
Acetaldehyde	1.1×10^{-2}	0.293	—
Quinicrine-HCl	—	—	Mixed
2,3-Dihydroxypyridine	—	—	Mixed
3-Acetylpyridine	—	—	Mixed

TABLE III

Summary of kinetic characteristics for aldehyde oxidase from tuh(ASU)

Substrate or Inhibitor	Km (M)	Vmax (Abs/min)	Inhibition Type
Acetaldehyde	1.3×10^{-2}	0.273	—
Quinicrine-HCl	—	—	Mixed
2,3-Dihydroxypyridine	—	—	Mixed
3-Acetylpyridine	—	—	Mixed

The inhibitor studies using 0.2 mM quinicine-HCl (Figures 6 and 7), 1.1 mM 2,3-dihydropyridine (Figures 8 and 9), and 10 mM 3-acetylpyridine (Figures 10 and 11) further substantiated this similarity between the enzymes.

Isozyme Study

The results of the isozyme study are shown in Figure 12. As can be seen from the data obtained, no major isozymes were apparent in Oregon-R-C or tumorous-head. It should be noted, however, that when the gels were loaded with extracts containing high levels of aldehyde oxidase activity, a minor active band below the major band became visible in both strains (Figure 12-g, odd numbered slots). It was apparent that the two enzymes migrated differently with tuh(ASU) aldehyde oxidase migrating at a slower rate than Oregon-R-C aldehyde oxidase. When tumorous-head aldehyde oxidase was tested against four wild-type strains (Figure 12-h), it was found that it migrated slightly slower than all of the four tested strains. Swedish-C migrated 32 mm, Oregon-R-C and Oregon-R migrated 31 mm, Canton-S migrated 30 mm and tuh(ASU) migrated 29 mm (although not readily apparent from Figure 12-h, Oregon-R-C was found to migrate with Oregon-R in previous electrophoretic determinations). Based on this comparison, four electrophoretic variants of aldehyde oxidase (allozymes) were found. The relative migration distances are summarized in Table IV.

Figure 6. The kinetics of aldehyde oxidase from Oregon-R-C in the presence of 0.2 mM quinicine-HCl. A sample reaction mixture contained 0.66 ml of 0.5 M KH_2PO_4 - K_2HPO_4 buffer, pH 7.5, containing 0.1% BSA and 1 mM EDTA, 0.05 ml enzyme, 0.05 ml of 4.1 mM quinicine-HCl solution, and varying amounts of acetaldehyde.

● = without inhibitor, x = with inhibitor

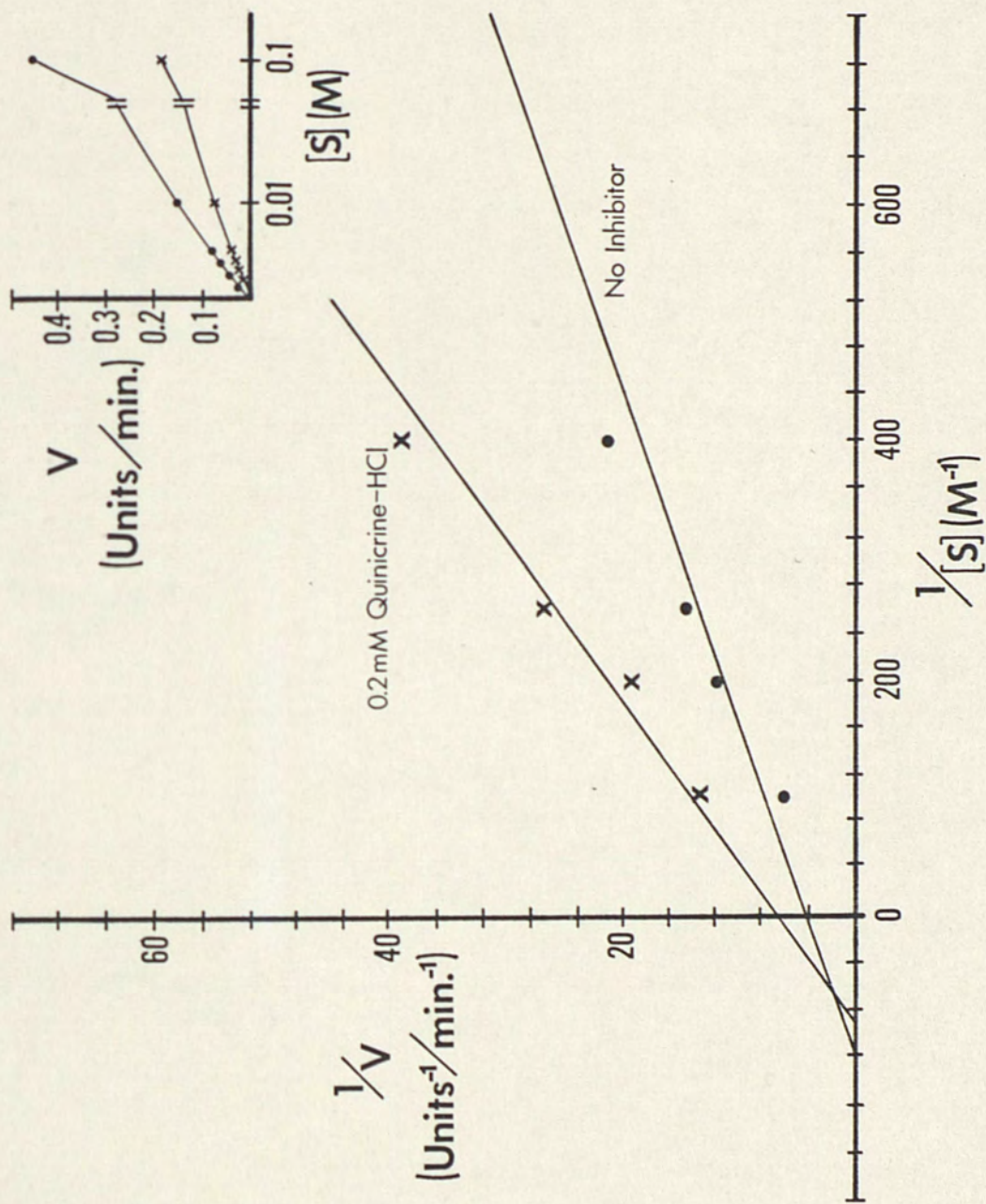


Figure 7. The kinetics of aldehyde oxidase from tuh(ASU) in the presence of 0.2 mM quinicine-HCl. Sample reaction mixture is the same as Figure 6.

o = without inhibitor, ▲ = with inhibitor.

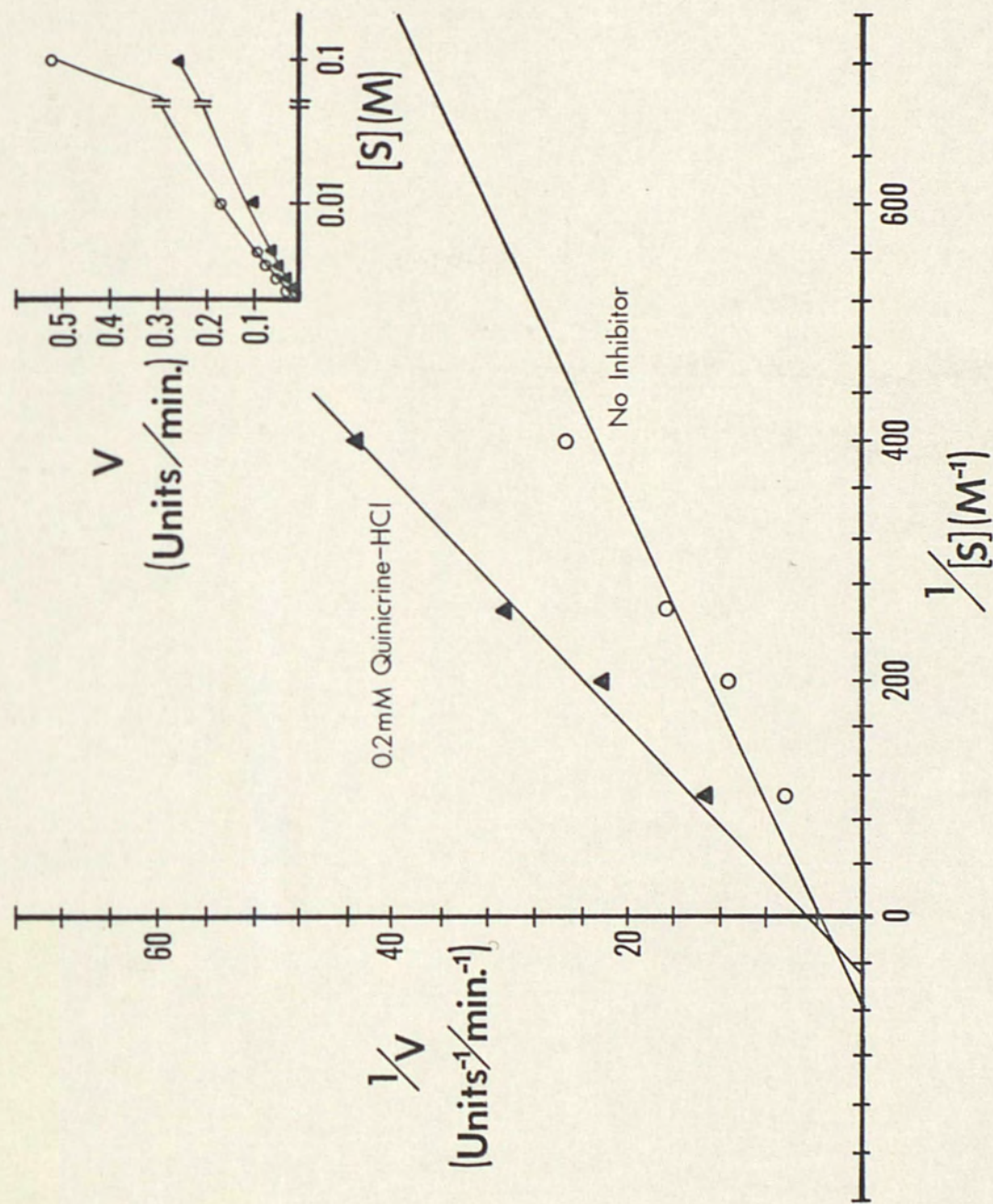


Figure 8. The kinetics of aldehyde oxidase from Oregon-R-C in the presence of 1.1 mM 2,3-dihydroxypyridine. Sample reaction mixture is the same as in Figure 6 except 0.05 ml of a 22.0 mM stock solution of 2,3-dihydroxypyridine was used as the inhibitor.

● = without inhibitor, x = with inhibitor

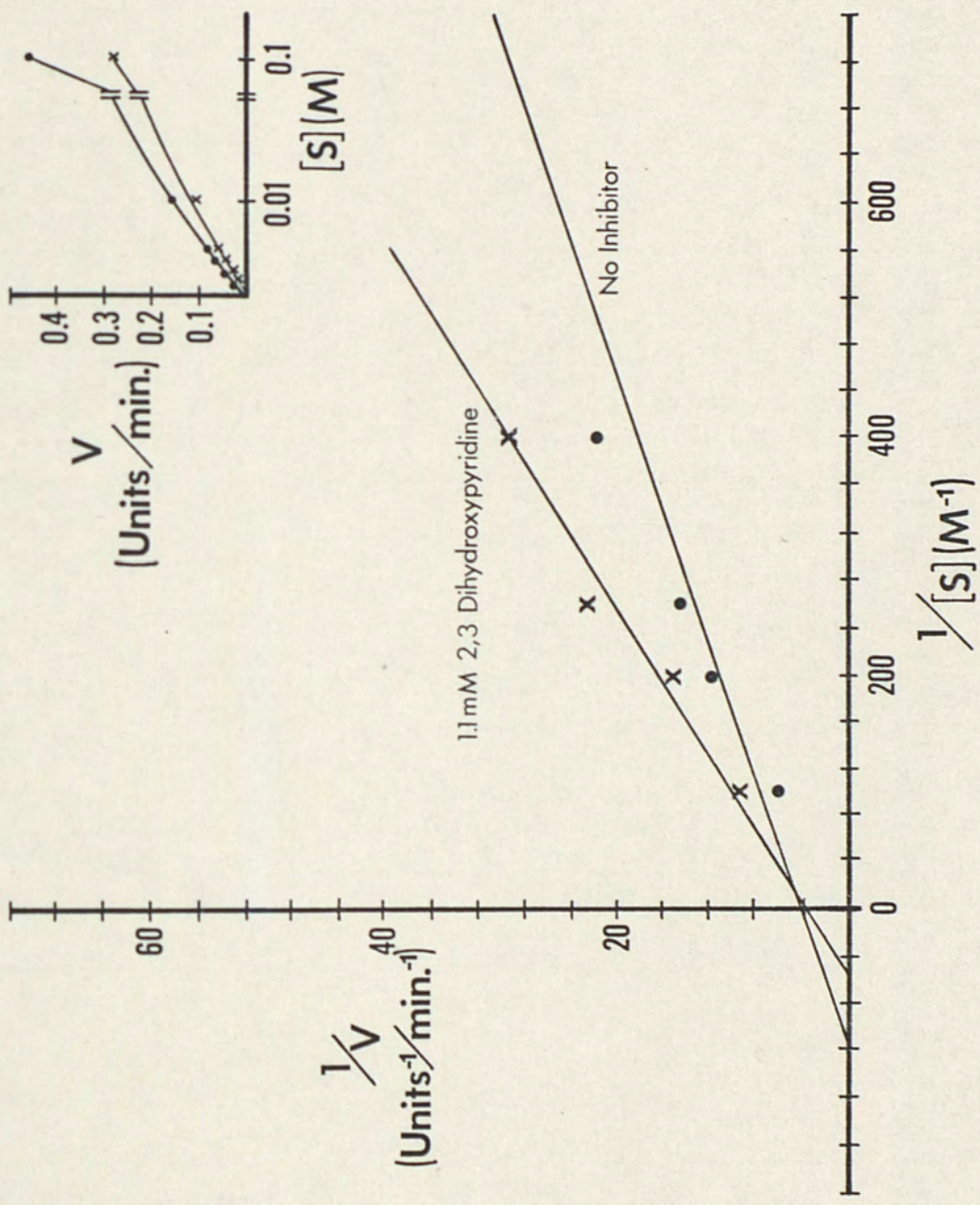


Figure 9. The kinetics of aldehyde oxidase from tuh(ASU) in the presence of 1.1 mM 2,3-dihydroxypyridine. Sample reaction mixture is as described in Figure 8.

o = without inhibitor, ▲ = with inhibitor

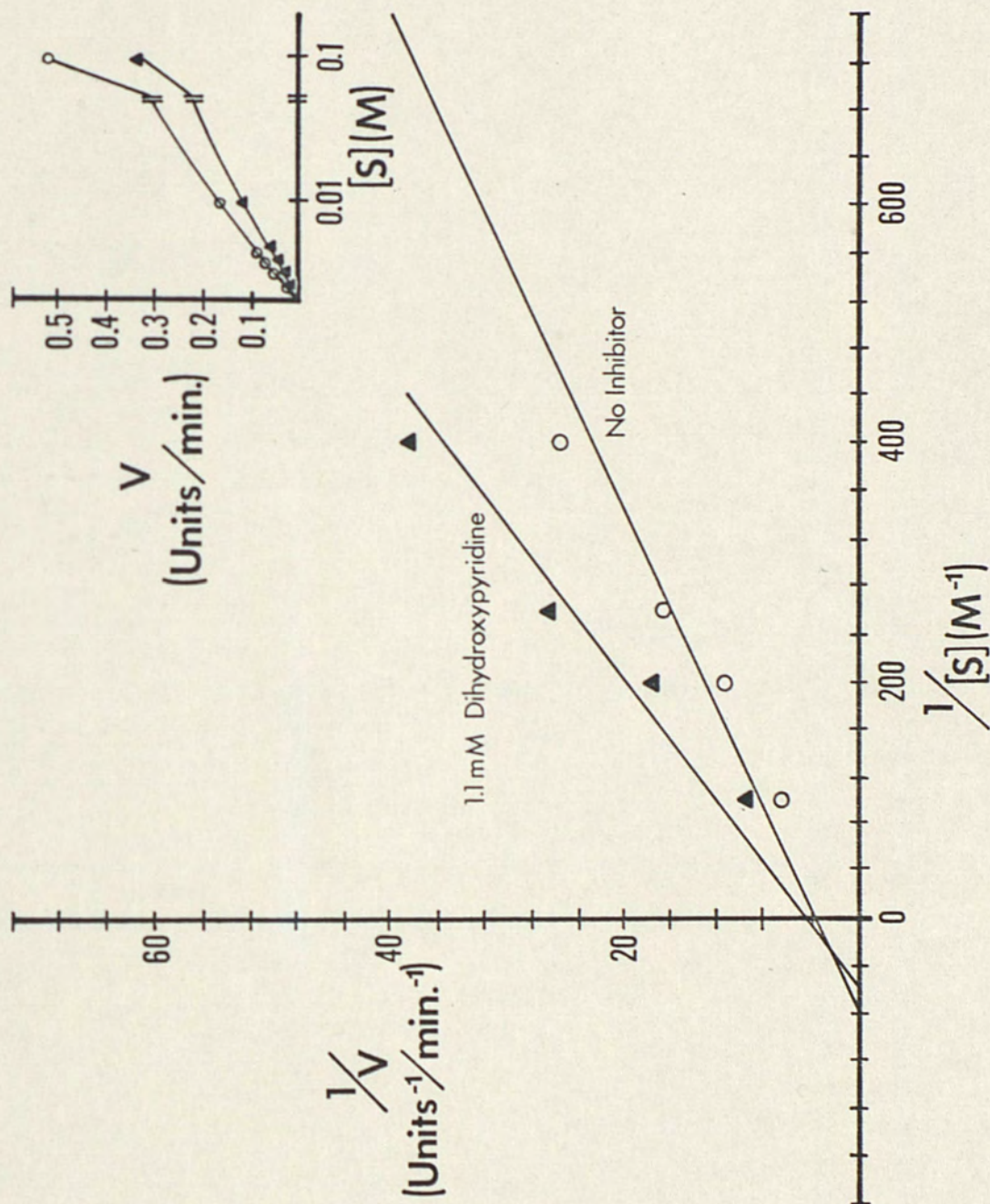


Figure 10. The kinetics of aldehyde oxidase from Oregon-R-C in the presence of 10 mM 3-acetylpyridine. Sample reaction mixture is the same as described in Figure 6 except 0.1 ml of a 0.1 M solution of 3-acetylpyridine was used as the inhibitor.

● = without inhibitor, x = with inhibitor.

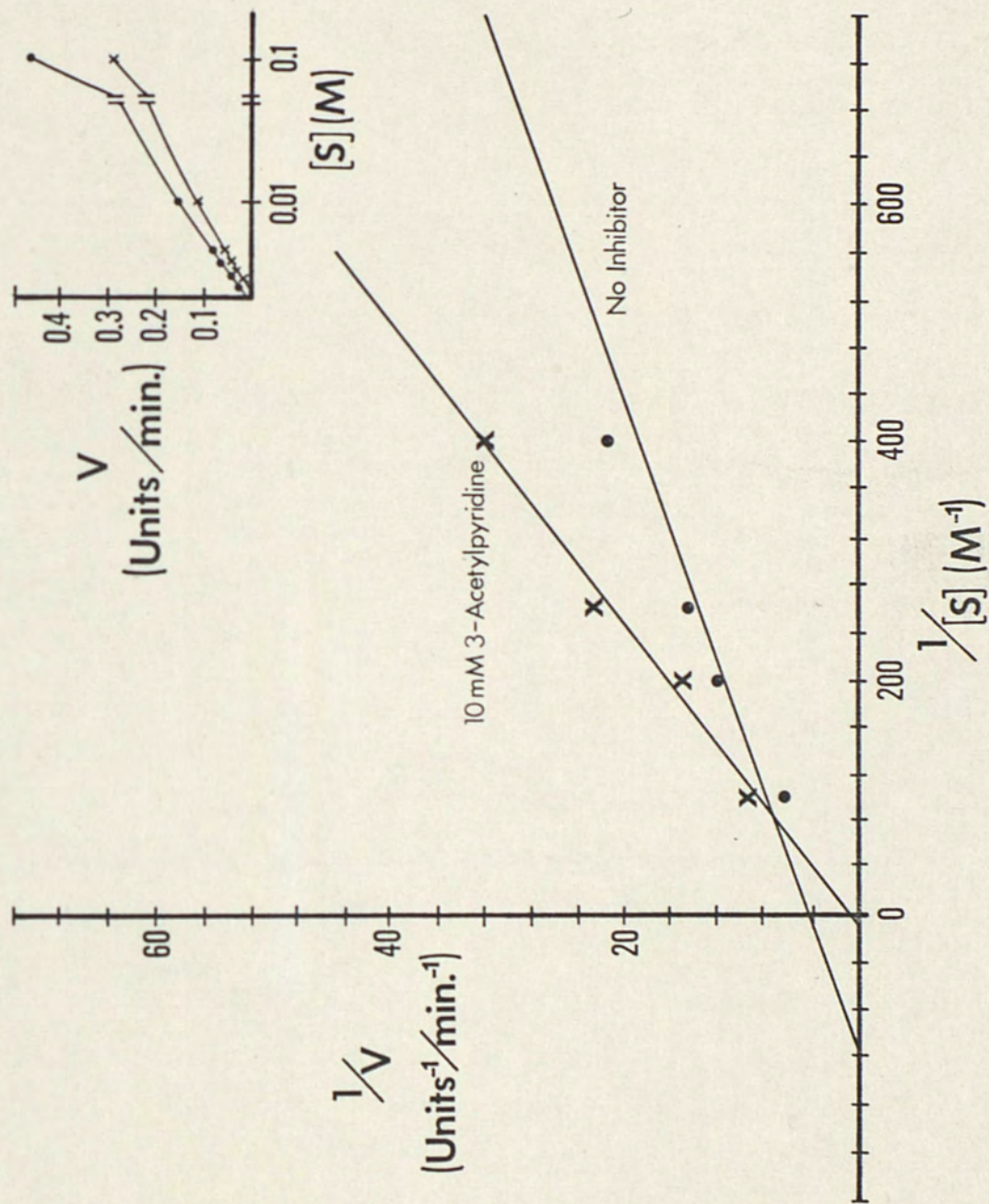


Figure 11. The kinetics of aldehyde oxidase from tuh(ASU) in the presence of 10 mM 3-acetylpyridine sample reaction mixture is the same as in Figure 10.

o = without inhibitor, ▲ = with inhibitor

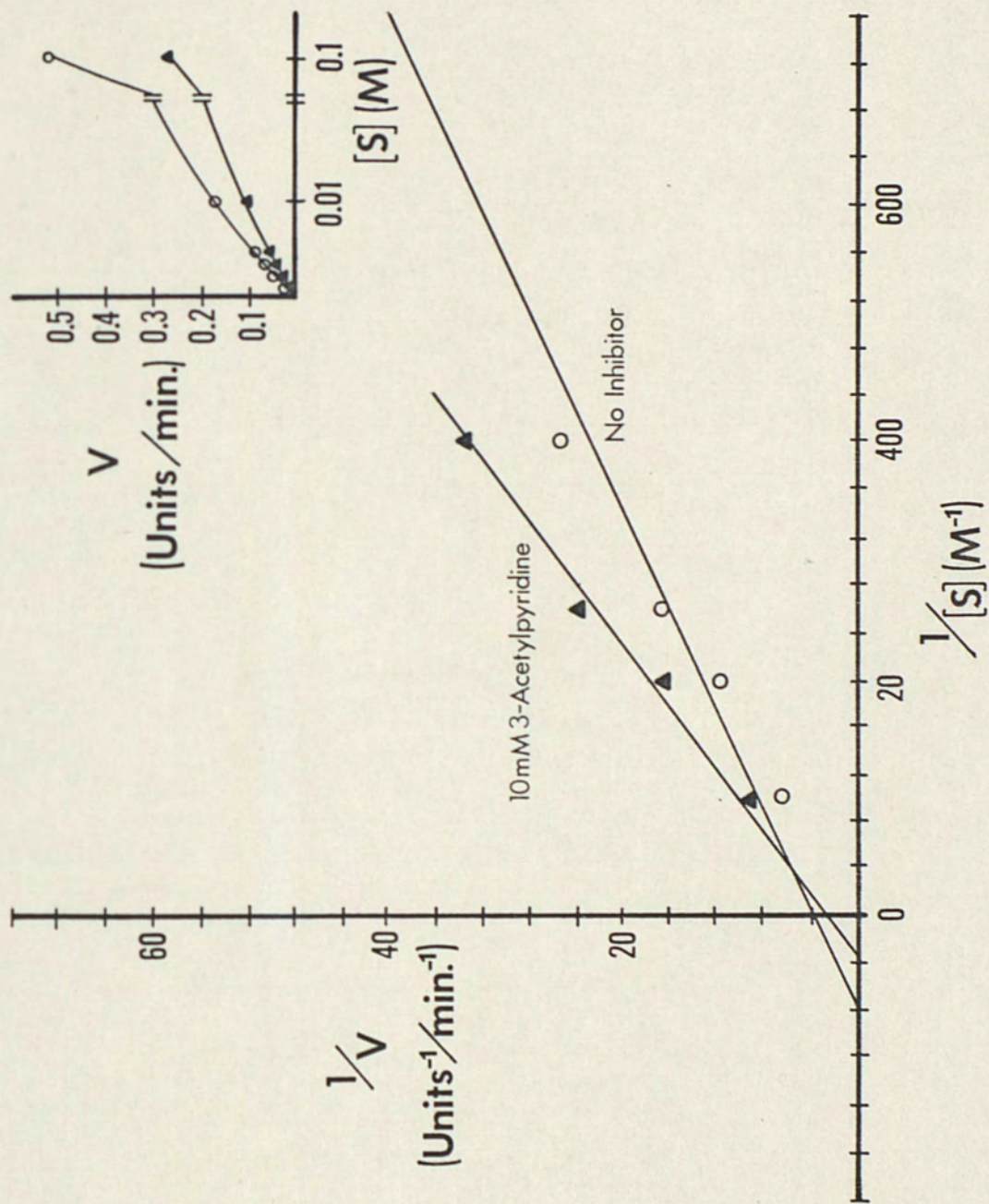


Figure 12. Polyacrylamide gel electrophoresis on various strains of Drosophila melanogaster. (a) through (g) are a comparison of aldehyde oxidase from Oregon-R-C and tuh(ASU) through the developmental cycle, (odd numbered slots = tuh(ASU), even numbered slots = Oregon-R-C).

Fig. 12(h) is a comparison of aldehyde oxidase from tuh(ASU) with 4 wild-type laboratory strains. 1, 4, 9, 12 = tuh(ASU); 3, 6 = Oregon-R-C; 8, 11 = Canton-S; 2, 5 = Swedish-C and 7, 10 = Oregon-R.

Each small grid represents 1 mm.

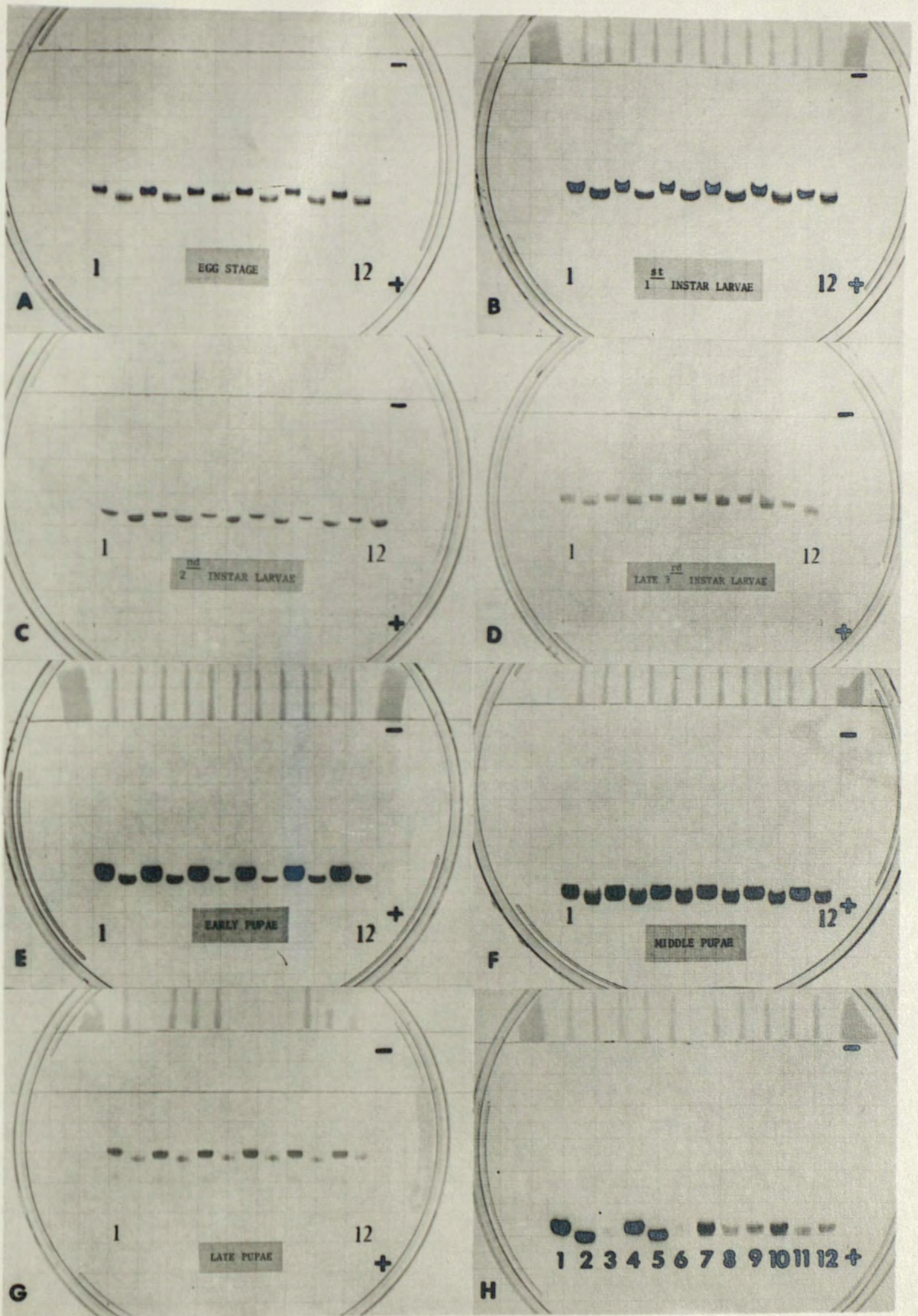


TABLE IV

(From Figure 12-h) Relative migration rate of aldehyde oxidase in five strains of Drosophila melanogaster

Strain	Migration Dist. (mm)	Slot #
tuh (ASU)	29	1, 4, 9, 12
Oregon-R-C	31	3, 6
Canton-S	30	8, 11
Swedish-C	32	2, 5
Oregon-R	31	7, 10

DISCUSSION

The results obtained in this comparative study of aldehyde oxidase will be approached in the same order as the data was given in the results section. The purification procedure will be first, followed by the kinetic analysis, and finally the isozyme study. An experiment will then be proposed to determine whether a quinone-like component is involved in the electron transport system of aldehyde oxidase.

The sequence for the purification procedure was selected due to the following observations. Aldehyde oxidase was found to be more stable after being passed through the QAE Sephadex A-25 packed column. Because of this, the QAE anion exchange step was chosen as the first procedure after the ammonium sulfate fractionating step. The QAE Sephadex material, which is a stronger anion exchanger than DEAE Sephadex, was used instead of the DEAE Sephadex anion exchanger used by previous investigators (Courtright, 1967; Dickinson, 1970; and Andres, 1976) due to a much greater percent yield of active enzyme (97% recovery versus 60% recovery). The fold purification was not quite as good with the QAE anion exchanger as with the DEAE anion exchanger (1.4 versus 2.0), but the significant increase in the percent yield of active enzyme was felt to more than compensate for this difference. The fold purification of 12 obtained from the affinity chromatographic step alone more than compensated for the slight reduction in efficiency due to the use of the QAE Sephadex column. To our knowledge the important affinity chromatography step has not been

previously employed in purification of Drosophila enzymes.

The purification procedure used for this study yielded some interesting information concerning aldehyde oxidase. When the parameters for the QAE anion exchange chromatography step were being refined, it was found that increasing the pH of the elution buffer beyond 7.9 resulted in strong binding of the enzyme to the column. Since the buffer is at a basic pH, the enzyme has a net charge in the negative range. At a pH of 7.8, this negative charge was very weak, or absent, since the enzyme eluted off the column at a rather low ionic strength (0.02 M Tris-HCl buffer, pH 7.8, with 0.02 M NaCl). When the pH of the eluting buffer was increased to 8.1, the enzyme was not eluted off the column until a concentration of 0.15 M NaCl was applied. At a pH of 8.6, aldehyde oxidase was not removed from the column until a concentration greater than 0.3 M NaCl was used. These results indicate that aldehyde oxidase undergoes a dramatic shift in charge from a pH of 7.8 to 8.6. Based on the pK_R values for the various amino acids (Lehninger, 1975), three amino acids may be involved in this large shift to the negative side. One of the amino acids, cysteine, has an R group with a negative charge above a pH of 8.3. The only problem here, however, was that the R group, -SH, tends to form a dimer when in close proximity with another -SH group. However, in a protein most potential disulfide bonds would already have been formed. Tyrosine is another viable alternative, but only if a pK_R shift had occurred to lower the pK (dissociation constant) of the hydroxyl (-OH) from 10.0 to around 8.5. A shift in pK can occur if the chemical

environment is such in the folded polypeptide that certain electron withdrawing or electron donating groups are in close proximity to the group undergoing the pK shift. Lysine would also be another choice. The lysine epsilon amino group, which has a pK of around 10, could also be shifted to a lower pK so a charge change of + to 0 on that R group would occur resulting in an overall increase in the negative charge of the protein. The possibility also exists that all three are present and exert their effects together on the overall charge of the enzyme.

The results obtained from the affinity column indicate that the Drosophila aldehyde oxidase was similar in some ways to the mammalian aldehyde oxidase. Chu (1973), however, was able to get a 100 fold purification of aldehyde oxidase, whereas only a 12 fold purification was obtained in this study. In order to elute the enzyme, Chu needed a combined salt and pH gradient, with the enzyme eluting off at a pH of 10.5 and 0.56 M NaCl. For this study, however, only an increase in NaCl from 0.05 M to 0.13 M at a constant pH of 8.3 was required to elute the enzyme. This indicates that the affinity of aldehyde oxidase from Drosophila for the ligand was much less than that of the mammalian enzyme. A ligand is essentially a competitive inhibitor. The enzyme's affinity for the ligand should be such that it can bind, and be removed, under non-denaturing conditions. This observed difference was probably due to a difference in the active site in the Drosophila aldehyde oxidase which will not allow it to bind to the bulky ligand very easily. Purine, which is an excellent substrate for the mammalian aldehyde

oxidase, was a very poor substrate for Drosophila aldehyde oxidase requiring high concentrations to show any changes in the reduction of dichlorophenol-indophenol. This further substantiated the difference in active sites between the two aldehyde oxidases. For Drosophila aldehyde oxidase, a less bulky ligand might be desirable. A benzaldehyde or acetaldehyde analog would probably fit this criteria very well. Acetaldehyde, however, did not have a high $K_m(10^{-2})$ as a substrate, so a benzaldehyde analog (benzaldehyde has a K_m of 10^{-5}) might be the best. To determine which would be the best, a series of inhibitor studies would have to be performed so that K_i could be calculated to give relative inhibitor affinities for the enzyme. If a benzaldehyde analog was used, the possibility exists that both aldehyde oxidase and xanthine dehydrogenase could be purified at the same time from the same column. If the affinity step is pursued, a spacer-arm will have to be used similar to the one used in this study to eliminate any steric hindrance that might occur from the Sepharose bead. In the mammalian system, Chu (1973) found that the relative positioning of the charged (amino) groups, as well as the length, of the spacer-arm influenced the binding of aldehyde oxidase to the ligand. This phenomenon, as well as mutual affinity for the ligand, allowed him to purify both aldehyde oxidase and xanthine dehydrogenase at the same time. Experimental results of the present study were not able to determine if this spacing of the charged groups or the arm length influenced the binding of Drosophila aldehyde oxidase. A more effective ligand would have to be found before any real tests of the spacer-arm effect can

be conducted.

The final purification step involved the use of gel filtration with ultrogel ACA 34 as the column packing material. It has an upper molecular weight fractionating limit of around 350,000. This means that any globular protein that has a molecular weight of 350,000 or greater will not enter into the beads. As a result, it will come out with the initial elution volume (at the end of the void volume). The smaller the molecular weight of the globular protein, the longer it will be retained inside the bead (Morris and Morris, 1975). For this study, two columns of different lengths were tried. When the aldehyde oxidase sample was applied to the shorter of the two columns (1.5 x 26 cm), it came off close to the void volume. When applied to the longer column (1.5 x 60 cm), it did not come off until approximately 10 ml after the void volume. These results indicate that with the shorter column, aldehyde oxidase was not retained sufficiently to see a distinction between the void volume or something close to the void volume. Even when the column was extended to an optimal fractionating length, the aldehyde oxidase was retained only slightly by the beads. These results indicate that aldehyde oxidase has a molecular weight somewhere near, but less than, the 350,000 molecular weight fractionating limit of the ultrogel beads, assuming a globular protein. These results are consistent with reported molecular weights for aldehyde oxidase of between 250,000 and 280,000 (Courtright, 1967; Andres, 1976).

Since the results obtained in the purification of aldehyde

oxidase from both Oregon-R-C and tumorous-head were essentially the same, then it can be assumed that at least in the overall form and charge the enzymes are similar to each other.

The results from the purification procedure indicate that two main contaminating bands of protein are still present besides the aldehyde oxidase band after the gel filtration step (Figure 5). Based on the stain uptake by the three bands of protein present, it appears that the two contaminating bands are there in lesser amounts than the aldehyde oxidase band (Figure 5). Assuming that this is the case, a two-fold increase in purity would be needed for aldehyde oxidase homogeneity. The possibility also exists that the contaminating bands of protein are inactive forms of aldehyde oxidase. Relating this to the equivalent stage of purification of previous investigators (two-fold to achieve purity), then the results of only Courtright (1967) were comparable to the percent yield of active enzyme obtained in this study (48%) at the same relative stage of purification. He had a 47% yield, while Dickinson (1970) had only a 24% yield, and Andres (1976) a yield of 37% (using standard procedure). In the subsequent step of their purification procedure, all three investigators took a drastic loss in activity with a final yield of 21% for Courtright (1967), 16% for Dickinson (1970), and 18% for Andres (1976).

Of importance to the present study is the fact that the two contaminants were reduced significantly when the sample obtained from the affinity column was fractionated by the gel filtration column. Both the short and long gel filtration columns gave similar results

in respect to the elimination of the contaminating protein bands. The shorter column (1.5 x 26 cm) was chosen since the yield of active enzyme was 90%, while for the longer column it was only 50%. This difference in percent yield of aldehyde oxidase was due to the increased retention time of the longer column. In order to eliminate totally the contaminating bands of protein, another passage through the gel filtration column might be tried. If this is not effective, one or more of the procedures to be discussed in the next few paragraphs could be attempted.

The first viable alternative would be the pH step as described by Andres (1976). It involves taking the pellet obtained from the 50-95% ammonium sulfate fractionating step, and dissolving it in 0.1 M Tris-maleate buffer, pH 6.0. The pH is then lowered to 5.0 by adding 1 M maleic acid, with the resulting sample allowed to set for fifteen minutes. The pH is then adjusted to 7.5 by adding 1 M Tris (free base) and the sample is centrifuged at 42,000 x g for thirty minutes. All procedures should be done at 4°C when possible. The one problem with this step is aldehyde oxidase is relatively unstable at pH's below 7.0. This was found by earlier investigators in our laboratory who tried the pH step as described by Dickinson (1970). The investigator will have to be careful in controlling all the conditions described, and to do the procedure as quickly as possible.

The heat step and hydroxyapatite adsorption chromatography steps, as described by Andres (1976) were tried with little success in removing the contaminating protein. Another possible alternative is a

preparative isoelectric focusing step involving a Sephadex G-75 granulated gel. Preliminary results indicate activity in a very defined region of protein in the granulated gel. If this band of protein is indeed aldehyde oxidase, then its isoelectric point is at pH of approximately 7.0. This would roughly coincide with the results obtained from the QAE Sephadex anion exchange step since it showed that a pH of 7.8, the enzyme is very weakly negatively charged. Application of the procedure necessitates maintaining everything at 4.0°C to prevent denaturation of the protein (aldehyde oxidase) when the high voltage is applied to separate the protein. It will also be important to determine the stability of the enzyme in the presence of the Ampholines used for the isoelectric focusing.

One other alternative is the immunoabsorption step as described by Andres (1976). The yield of active enzyme could be improved by using both a pH and salt gradient instead of just a high salt concentration used by Andres (1976). Preliminary studies indicate that aldehyde oxidase remains stable at elevated pH's up to 9.0 with salt concentrations as great as 0.2 M (Respass, unpublished). By using a combined salt and pH gradient as described by Chu (1973), it might be possible to induce a conformational change in the binding antibody thus allowing the release of the aldehyde oxidase without inactivating a large portion of the active enzyme. The main stumbling block for this procedure is the requirement of pure aldehyde oxidase to produce the antibodies needed to synthesize the column.

The aldehyde oxidase sample obtained from the purification

procedure was then subjected to the following kinetic analysis. A comparison of the results obtained from the kinetic study using the QAE and affinity samples showed no major differences. As a result, the QAE sample was used for this study due to higher activity and greater stability.

The results obtained from the comparative kinetic study have shown that aldehyde oxidase from Oregon-R-C and tumorous-head are very similar. The K_m 's obtained using acetaldehyde as a substrate were 1.1×10^{-2} M for Oregon-R-C and 1.3×10^{-2} M for tumorous-head. Therefore, K_m for acetaldehyde is almost the same for the two enzymes as would be the affinity for acetaldehyde. This is a very important finding since it indicates the elevated levels of aldehyde oxidase found by Kuhn and Cunningham (1976) in tumorous-head were not due to a major difference in the active sites, but due to a larger number of enzyme molecules present. This suggests that the problem is in the regulation of the enzyme at the genetic level. It should be noted, however, that although the K_m 's for the two aldehyde oxidases were similar, this does not rule out the possibility that compensating changes in the forward and reverse reaction rates may have occurred since

$$K_m = \frac{K_{-1} + K_{+2}}{K_1}$$

In other words, one form of the enzyme may be able to turn over larger amounts of product (K_2), but cannot bind the substrate (K_1) as quickly as the other.

A review of literature has found a reported K_m of 10^{-2} for acetaldehyde (Dickinson, 1969; Dickinson and Sullivan, 1975). This is completely compatible with the results obtained in this study (Tables II and III). Rajagopalan (1962) has reported for mammalian aldehyde oxidase a K_m of 1×10^{-3} for acetaldehyde. The V_{max} values obtained (0.293 units/minute for tumorous-head) also indicates that the two enzymes are fairly similar to one another.

The inhibitor study using quinicrine-HCl, 2,3-dihydroxypyridine, and 3-acetylpyridine gave similar inhibitions, of which all three appear to be of the mixed non-competitive type (Segel, 1975). This indicates that the inhibitor is binding to more than one site on the enzyme non-competitively and not at the active site itself. Classical non-competitive inhibition, on the other hand, involves the binding of the inhibitor to a single site other than the active site. Both inhibition types are not reversed simply by increasing the substrate concentration. When quinicrine-HCl was used, the two enzymes gave nearly identical inhibition types (Figures 6 and 7) with the inhibitor and no inhibitor lines intersecting to the left of the Y axis, above the X axis. The inhibitor 2,3-dihydroxypyridine also yielded similar inhibition types (Figures 8 and 9) although the Oregon-R-C intersecting point was just to the right of the Y axis, while tumorous-head intersected slightly to the left. For 3-acetylpyridine (Figures 10 and 11), the points of intersection were to the right of the Y axis for both enzymes. The Oregon-R-C intersecting point was a little farther to the right than was the intersecting point for tumorous-head. If the

inhibitions depicted are of the mixed non-competitive type, more than one level of concentration of inhibitor will be needed to calculate the K_i values (affinity of inhibitor for the enzyme) (Segel, 1975). Of paramount importance was the fact that the mode or type of inhibition obtained for the three inhibitors were similar for the aldehyde oxidases purified from Oregon-R-C and tumorous-head. These results indirectly support the idea that the problem of elevated activity in tumorous-head is due to a regulatory problem, and not some major difference in the structure of the enzyme.

The comparative isozyme study through the developmental stages of Oregon-R-C and tumorous-head yielded no major aldehyde oxidase isozymes unique to either of the strains. These results also indirectly substantiated that the elevated levels of aldehyde oxidase in tumorous-head were due to a problem in genetic regulation. It is important to note at this time that when the gels were loaded with high levels of enzyme activity, a minor active band was observed in both strains below the major active band (Figure 12-h). Since it appears to be under coordinate genetic control with the major active band (Courtright, 1967), then it is probably related to, or a portion of the major aldehyde oxidase band of activity. It could be a monomer if the major band is a dimer, or a dimer if the major band is a tetramer assuming the greater mobility of the minor band is due to the sieving effects of the polyacrylamide gel. The reverse could of course be possible if a charge difference is the major reason for the migration difference. The minor band also may be the result of a

post-translational modification of the main enzyme resulting in a change in the charge or molecular weight. The possibility also exists that the minor and major bands are different combinations of proteins coded for by more than one structural gene. To date, only one structural gene for aldehyde oxidase has been found, and it codes for a peptide with an estimated molecular weight of only 150,000 (Dickinson, 1970). Although no major isozymes were found, the aldehyde oxidase from Oregon-R-C migrated at a faster rate towards the anode than did tumorous-head aldehyde oxidase (Figure 12) indicating different allozymes in the two strains. The kinetic data indicates that the amino acid substitutions that have occurred do not affect the active site conformations. Since a difference was found in the migration rates of the two enzymes, a comparison with four wild-type laboratory strains was performed. It was found that Swedish-C migrated the fastest, Oregon-R-C and Oregon-R migrated the next fastest, Canton-S was the third fastest, and tumorous-head was the slowest toward the anode (Figure 12). Dickinson (1970) has reported three allelic variants of aldehyde oxidase. Based on the above results, the tumorous-head enzyme may be yet another allelic variation (allozyme) of aldehyde oxidase.

One area of controversy considering aldehyde oxidase, xanthine dehydrogenase, and pyridoxal oxidase are the modes of control of these enzymes by the maroon-like locus. Andres (1976) and Courtright (1967) have found that the $ma-1^+$ complementing factor is less than 10,000 in molecular weight. Andres (1976) has further shown that this

factor is not flavin adenine dinucleotide (FAD), molybdenum or iron since both ma-1 cross-reacting materials and the active forms of the enzyme contained these cofactors. Andres (1976) also ruled out the fact that the ma-1 locus controls the simple incorporation of a polypeptide. One area that has yet to be considered is that the ma-1⁺ complementing factor might code for an enzyme involved in the incorporation, or synthesis, of the quinone-like compound. Studies by Rajagopalan et al. (1962) have found that a quinone-like compound (coenzyme Q) was an integral part in the mammalian aldehyde oxidase electron transport system. The following is a proposed study of Drosophila aldehyde oxidase to determine whether a quinone-like compound might be involved in its electron transport system. The enzyme will be purified using the previously described method, and analyzed using the following experiments (Rajagopalan et al., 1962).

1) First, a determination of the absorption spectrum for aldehyde oxidase in the oxidized and reduced state will be performed (from 240 nm to 560 nm). Under anaerobic conditions the reaction will be initiated by placing 0.001 M acetaldehyde into the cuvette with the enzyme. A difference spectrum (arithmetic difference in absorbance between an aerobic and anaerobic solution of enzyme) will then be set up from these results.

2) Second, an inhibitor study using amytal (5×10^{-4} M) and antimycin A (1 μ g/ml) which are known to inhibit the reduction and reoxidation, respectively, of coenzyme Q in mitochondrial

transport.

3) Third, an inhibitor study using menadione (2×10^{-7} M) which gives results similar to the inhibition of the succinoxidase system by various naphthoquinones which is reversed by coenzyme Q.

4) Finally, an inhibition of the enzyme by Triton X-100 (2×10^{-4} M) which has been used for the aqueous dispersion of coenzyme Q.

If the difference spectrum shows a strong peak at 275 nm, then a quinone might be suspected to be present. Additional evidence that a quinone is involved in the aldehyde oxidase system would be provided if the kinetic studies listed above (parts 2, 3 and 4) are similar to ones found in other quinone-enzyme systems. A difference spectrum of ma-1 cross-reacting material versus active aldehyde oxidase might also give a clue as to the presence or absence of quinone in the ma-1 CRM.

SUMMARY

Aldehyde oxidase has been purified to near homogeneity from both Oregon-R-C and tumorous-head strains of Drosophila melanogaster. A new procedure involving affinity chromatography was used which gave a twelve-fold increase in enzyme purity in a single step. A comparative kinetic study was done on the two partially purified aldehyde oxidases to determine if there were any major structural differences between them. The results indicate that they were very similar to each other showing that the elevated levels of aldehyde oxidase found at key developmental stages in tumorous-head (Kuhn and Cunningham, 1976) were due to abnormal genetic regulation. No major isozymes were found unique to either strain when an isozyme study was performed through the developmental stages. This also indirectly indicated the problem found in tumorous-head was a regulatory effect on one major isozyme. The two enzymes migrated at different rates and, when compared with other wild-type strains, tumorous-head aldehyde oxidase migrated slowest toward the positive pole. These results indicate that tumorous-head aldehyde oxidase is a fourth allozyme, since only three electrophoretic variants had been previously reported (Dickinson, 1970).

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