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Xanthine Dehydrogenase Is Transported to the Drosophila Eye

Andrew G. Reaume, Stephen H. Clark and Arthur Chovnick

Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06268

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ABSTRACT

The *rosy* (*ry*) locus in *Drosophila melanogaster* codes for the enzyme xanthine dehydrogenase. Mutants that have no enzyme activity are characterized by a brownish eye color phenotype reflecting a deficiency in the red eye pigment. This report demonstrates that enzyme which is synthesized in some tissue other than the eye is transported and sequestered at the eye. Previous studies find that no leader sequence is associated with this molecule but a peroxisomal targeting sequence has been noted, and the enzyme has been localized to peroxisomes. This represents a rare example of an enzyme involved in intermediary metabolism being transported from one tissue to another and may also be the first example of a peroxisomal protein being secreted from a cell.

THE *rosy* gene in *Drosophila melanogaster* (*ry*) is located at 3–52.0 on the recombination map and 87D11–12 on the polytene chromosome map. It codes for the enzyme xanthine dehydrogenase (XDH) and has been the subject of extensive genetic, molecular, and biochemical characterization (for review see DUTTON and CHOVNICK 1988). The gene has been sequenced (LEE *et al.* 1987; KEITH *et al.* 1987), and two cis-regulatory sites have been identified (CLARK *et al.* 1984; CURTIS *et al.* 1989). XDH is a molybdoenzyme (FINNERTY and WARNER 1981) and a homodimer with subunit molecular weight of 150 kD (EDWARDS, CANDIDO and CHOVNICK 1977; GELBART *et al.* 1974). The enzyme catalyzes reactions that include the purine degradation steps: hypoxanthine to xanthine, and xanthine to uric acid. Null enzyme mutants complete development, and adults are characterized by a brownish eye color in comparison to the normal wild type dark red eye color. The mutant eye color phenotype is the result of a deficiency of the red pterin pigment relative to the level of brown ommochrome pigment which is unchanged from normal. The relationship between the eye color phenotype and XDH activity is unclear. While XDH catalyzes at least one pterin reaction (2-amino-4-hydroxypterin to isoxanthopterin) it has never been shown to catalyze a pterin reaction in the red pigment pathway. SCHWINCK (1965) suggested that redox reactions associated with XDH were coupled to drosopterin synthesis. However, this notion remains unsubstantiated (for discussion see PHILLIPS and FORREST 1980; NASH and HENDERSON 1982).

Another unsettled issue relates to notions about the tissue distribution of *rosy* locus expression. Classical tissue transplantation studies, involving larval tissues, that examine resultant adult eye color, lead to the conclusion that the *rosy* locus is expressed only in Malpighian tubules and fat body (reviewed by HADORN 1956). XDH enzyme activity during development also is limited primarily to Malpighian tubules and fat body (URSPRUNG and HADORN 1961; MUNZ 1964). Upon finding XDH activity in preparations of dissected adult, wild type eyes, BARRETT and DAVIDSON (1975) suggested that XDH was transported to the adult eye from its sites of synthesis in the malpighian tubules and fat body.

The following facts lead us to a reexamination of the question of tissue distribution of *rosy* locus expression: (1) Several genes are known whose products are post-translational modifiers of XDH, and whose mutations lead to modification, or indeed, inactivation of XDH activity (FINNERTY 1976; O'BRIEN and MACINTYRE 1978). Thus, XDH activity in a tissue requires concordant expression of all of these genes as well as the *rosy* locus. (2) As noted above, the relationship of *rosy* expression to eye color is unclear and finally, (3) there is no indication in the translation sequence (LEE *et al.* 1987; Keith *et al.* 1987) for a putative leader peptide, characteristic of secreted proteins.

The present report describes experiments that were designed to rigorously examine the tissue distribution of *rosy* locus expression. We confirm that XDH is present at the site of the adult eye. Additionally, we demonstrate that it is not synthesized there but rather is transported and sequestered there. A mechanism of transport is discussed.

MATERIALS AND METHODS

Stocks used: The wild-type strain used in these experiments is a derivative of the Oregon-R strain which has been

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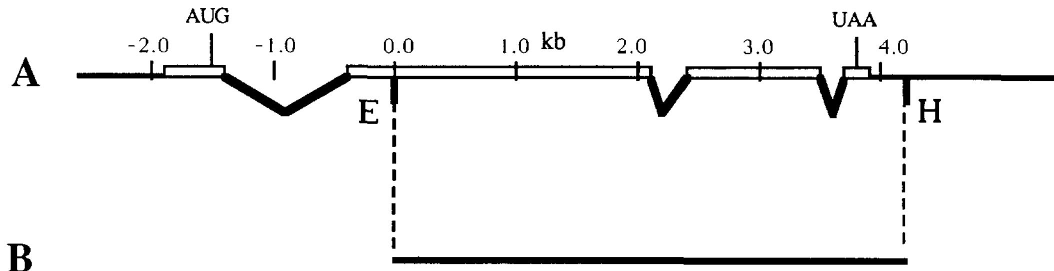

FIGURE 1.— Probes used for *in situ* hybridization to tissue sections. A map of the *ry* gene is shown in (A). Open bar indicates exonic sequence, V-shaped solid bar shows intron region and horizontal solid bar indicated flanking genomic DNA. A 4.1-kb *Eco*RI-*Hin*dIII fragment (B) was used as a probe by inserting it adjacent to an SP6 promoter in both orientations in the plasmids pSP64 and pSP65. cRNA was transcribed in the presence of [^{35}S]UTP as described by HARTLEY, XU and ARTAVANIS-TSAKONAS (1987). E, *Eco*RI. H, *Hin*dIII.

made isogenic for its third chromosome, designated ry^{+5}. This strain produces normal levels of XDH relative to several other isogenic wild-type chromosomes.

The overproducer strain used in the *in situ* hybridization experiment has eight extra copies of the *ry* gene by virtue of *P* element insertions (RUBIN and SPRADLING 1983). Additionally the genotype is homozygous for the ry^{+4} chromosome which is an overproducer variant (CHOVNICK *et al.* 1978; CLARK *et al.* 1984). Whole body XDH activity of this strain has been checked and shown to be six times that of a standard wild type genotype with two copies of the gene.

The mutant strain used in these experiments was ry^{506} which is a deficiency of about one third of the coding region generated on the ry^{+5} chromosome (COTÉ *et al.* 1986). The mutant ry^{545} is a point mutation of the 3′ acceptor site of the 5′ intron on the ry^{+5} chromosome (LEE *et al.* 1987).

All other mutants are described in LINDSLEY and GRELL (1968).

Flies were cultured on a standard cornmeal medium at 22°-25°.

***In situ* Hybridization:** Pupae were collected in ½-pint plastic bottles and aged for the appropriate length of time. Adults were collected 24 hr after clearing a collection bottle. All pupae and adults were fixed in Carnoy's fixative (6 isopropanol: 3 chloroform: 1 formic acid) for 1 hr, then dehydrated in a graded series of alcohol, cleared in xylenes and embedded in Paraplast. Sections were cut 10 μm thick and dried on poly-L-lysine coated slides. The prehybridization and hybridization methods are essentially as described in HARTLEY, XU and ARTAVANIS-TSAKONAS (1987). See Figure 1.

Histochemistry: Frozen sections (10 μm) were cut on a Slee cryostat, collected on poly-L-lysine coated slides and air dried for 20 to 30 min. A staining mix consisting of the following was then applied to the sections: 1 mg/ml nitroblue tetrazolium, 0.3 mg/ml phenazine methosulfate, 0.5 mg/ml hypoxanthine and 6% gelatin. The slides were allowed to sit at room temperature for several hours in a humidified chamber. Specificity for XDH activity is conferred by including hypoxanthine as a substrate in the above mix. The gel then was cleared by dipping the slides in H_2O at 50°-60°. Cover slips were then directly mounted in 70% glycerol or the sections were dehydrated, cleared and mounted in Permount. Controls for specificity include: (1) omission of the hypoxanthine substrate from the mix and (2) staining *ry* mutant tissue.

Antibody staining: Frozen sections (10 μm) were cut, collected and air-dried as described above. The sections were then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (130 mM NaCl, 10 mM phosphate (pH 7.4)) for 1 hr on ice. Slides were then washed three times in PBS and blocked in 0.1% bovine serum albumin (BSA), 1.5% normal goat serum and 0.03% Triton X-100 in PBS. Primary antibody was mouse anti-XDH diluted to 1:1000 together with 0.1% BSA and 1.5% normal goat serum in PBS. Incubations for primary antibody were overnight at 4°. Slides were then washed three times for 10 min each time at room temperature with 0.1% BSA and either 0.03% Triton X-100 (first and third washes) or 0.1% Triton X-100 (second wash) in PBS. Secondary antibody was biotinylated goat anti-mouse immunoglobulin G. Secondary antibody was applied in PBS with 0.1% BSA and 1.5% normal goat serum for 1 hr at room temperature. Then, three washes (as described following the primary antibody application) were followed by avidin biotin complex (ABC) labeling (Vector Laboratories) as described by the manufacturer except that 0.1% BSA was included in the mix and incubation time was for 30 min at room temperature. This was followed by three washes for 10 min each at room temperature in PBS with 0.1% BSA and 0.03% Triton X-100, and then two washes for ten minutes each in PBS alone. The stain was developed in 0.5 mg/ml diaminobenzidine and 0.03% hydrogen peroxide in PBS. Tissues were then dehydrated through a graded series of ethanol, cleared and mounted in Permount.

RESULTS

***rosy* expression is not evident in the eye:** In order to assess whether or not the *rosy* locus is expressed in the eye, the spatial distribution of *ry* mRNA was examined by *in situ* hybridization to paraffin sections of pupae and adults. Several steps were taken to make the technique as sensitive as possible: (1) cRNA probes were used since they are demonstrably more sensitive than DNA probes (COX *et al.* 1984). (2) A large probe was used in order to maximize target size and hence sensitivity. (3) The RNA probe was size-reduced by alkaline hydrolysis to provide better penetration into the tissue sections. (4) Transcript was assayed in an oveproducer genotype that makes six times the amount of XDH relative to a typical wild type strain (see MATERIALS AND METHODS).

A 4.1-kb *Eco*RI-*Hin*dIII genomic fragment of the *ry* gene was linked to an SP6 promoter in the Riboprobe vectors pSP64 and pSP65. cRNA probes were transcribed in the presence of [^{35}S]UTP. These sense and antisense RNAs were used to probe paraffin sections of pupae and adults from the overproducer strain.

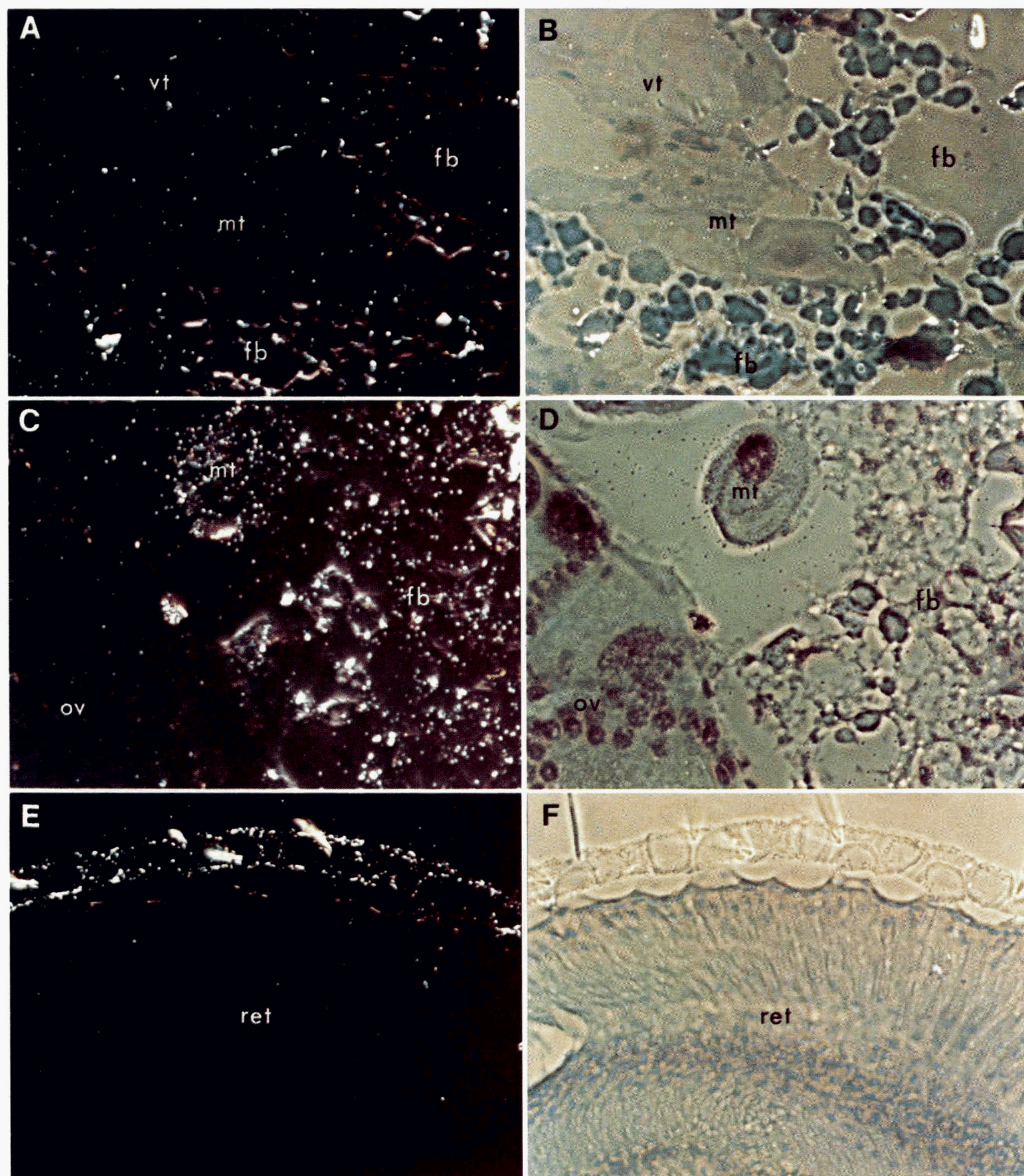

FIGURE 2.—*In situ* hybridization to *ry* message in tissue sections. (A, C, E) Darkfield microscopy. (B, D, F) Phase contrast microscopy. (A, B) A sense-strand probe was used as a control to show signal specificity. (C, D) *In situ* hybridization of antisense strand probe to wild type reveals signal over Malpighian tubules and fat body but not ovary. No signal was ever detected in the eye of either wild type or overproducer (see text) pupae or adults. (E,F) This figure shows the result of *in situ* hybridization of antisense probe in an overproducer (RC2) strain adult. Note that the cuticle making up the lens of each ommatidium exhibits nonspecific binding since it binds to the sense-probe (not shown) as well as to the antisense probe. (fb = fat body; mt = Malpighian tubules, ov = ovary, vt = ventriculus, ret = retina).

The stages examined include 24-hr pupae, 48-hr pupae, 72-hr pupae and 0–24-hr adults. Figure 2C demonstrates that the antisense-strand probe is able to detect *ry* message in the fat body and Malpighian tubules of adults. The control for signal specificity was the sense-strand probe which showed no localization of signal in these tissues (Figure 2A).

ry message was never detected in the developing eyes of pupae or adults (Figure 2E). Note that cuticular structures such as the lens of the eye bind both the sense and antisense-strand probes, thus representing nonspecific signal.

XDH is present in the eye: We next questioned the presence of XDH in the eye both in terms of XDH enzyme activity and response to XDH-specific antibody.

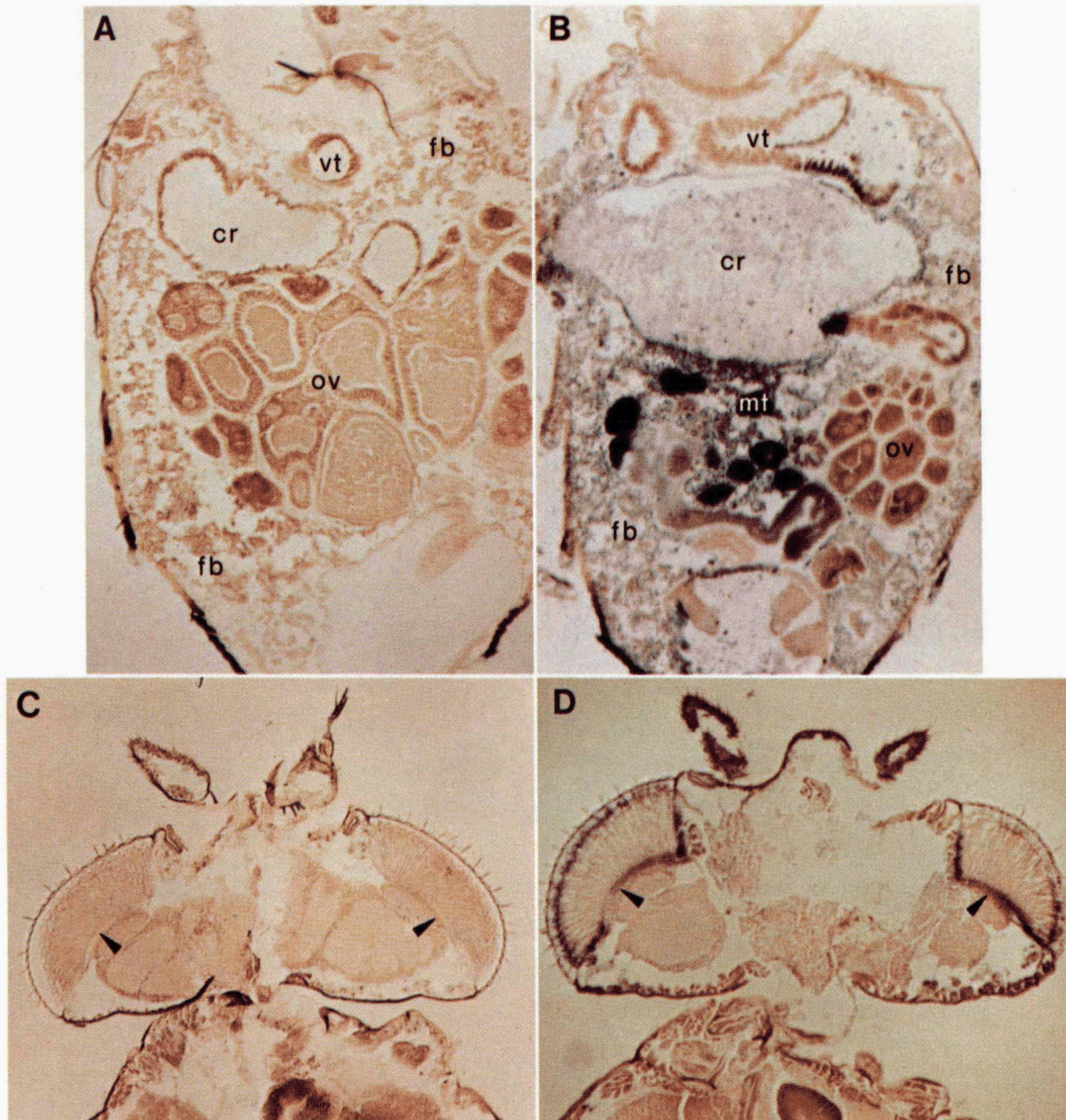

FIGURE 3.—Histochemical staining for XDH activity. A nitroblue tetrazolium method was used to locate XDH activity in frozen sections. Note that activity is indicated by blue stain. (A, B) abdominal cross sections. (C, D) head cross sections. (A, C) No staining occurs in a ry^{506} mutant strain. (B) In the abdomen, the activity is localized to fat body, Malpighian tubules, and ventriculus. (D) In the head, the activity is localized to the fat body behind the optic lobe of the brain (not shown) and the basement layer of the retina (arrow). (fb = fat body; mt = Malpighian tubules; ov = ovary; cr = crop; vt = ventriculus.

Enzyme activity was examined by a nitroblue tetrazolium histochemical technique applied to frozen sections of adults. Figure 3B confirms that in the adult abdomen, XDH activity is found primarily in the fat body and malpighian tubules. Within the adult head, XDH activity is found in the fat body located behind the optic lobes of the brain and at the basement region of the retina (Figure 3D). Occasionally, we find activity in the apical region of the eye (perhaps cone cells or primary pigment cells) and the retina (perhaps the receptor cells or secondary pigment cells). Specificity of this technique is demonstrated by testing it on *ry* mutants (Figure 3, A and C) and by leaving substrate out of the staining reaction (not shown; see materials and methods). Hundreds of flies have now been examined by this technique and it has proven to be a convenient and reproducible method to demonstrate XDH activity in the eye as well as in the other tissues mentioned.

This result is corroborated by staining frozen sections of adults with antibody against XDH. This procedure also shows an accumulation of XDH antigen at the interface between the retina and lamina (Figure 4C). As with the activity staining we also see occasional staining in the apical region of the eye and the retina. Staining of *ry* mutants (Figure 4B) or with preimmune serum (not shown) reveals no signal in this region.

Despite our inability to detect *ry* locus transcripts in the eye, we note that the amount of XDH detected in this region of the eye is comparable to amounts seen in the fat body and Malpighian tubules.

XDH is transported to the eye: To settle the issue

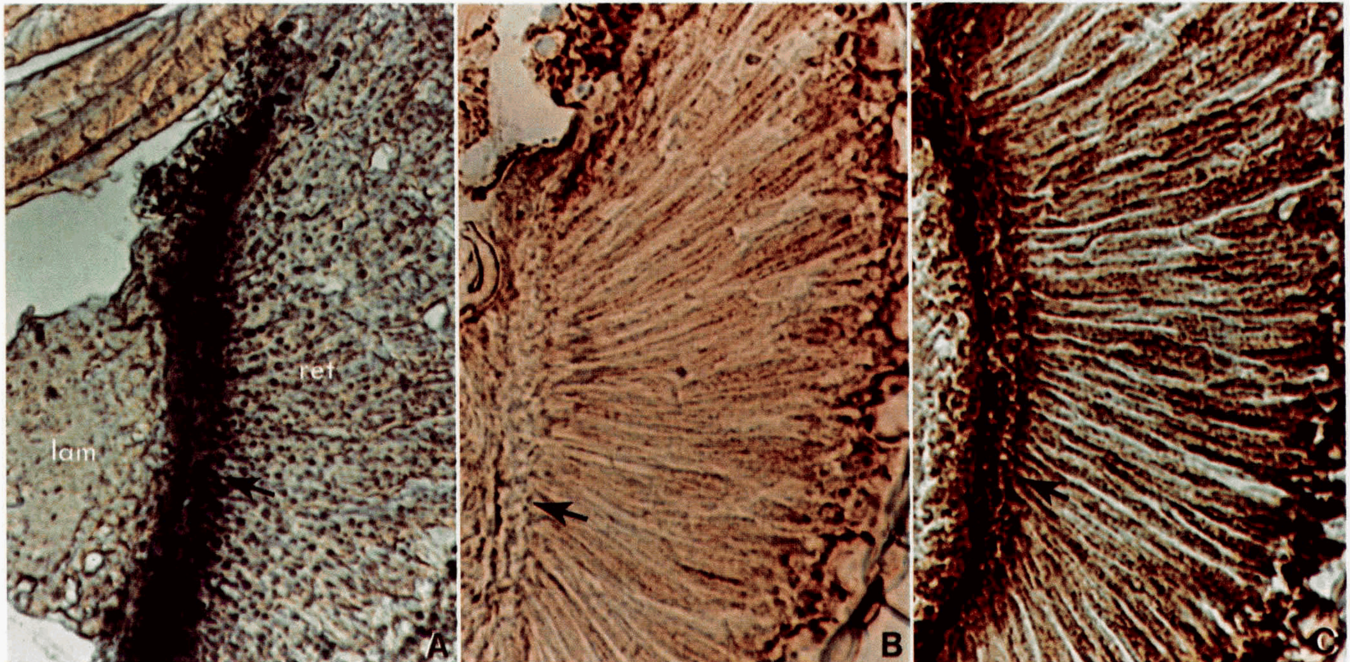

FIGURE 4.— Antibody staining to XDH in tissue sections of heads. (A) Histochemical staining for XDH activity is shown for orientation purposes. In this figure an adult (0–24 hr old) head shows staining localized to the basement layer of the retina. (B) The control for specificity of this technique was the staining pattern seen in ry^{545} mutants. Arrow indicates lamina/retina border where histochemical staining (A) and antibody staining (C) reveal XDH. lam = lamina; ret = retina.

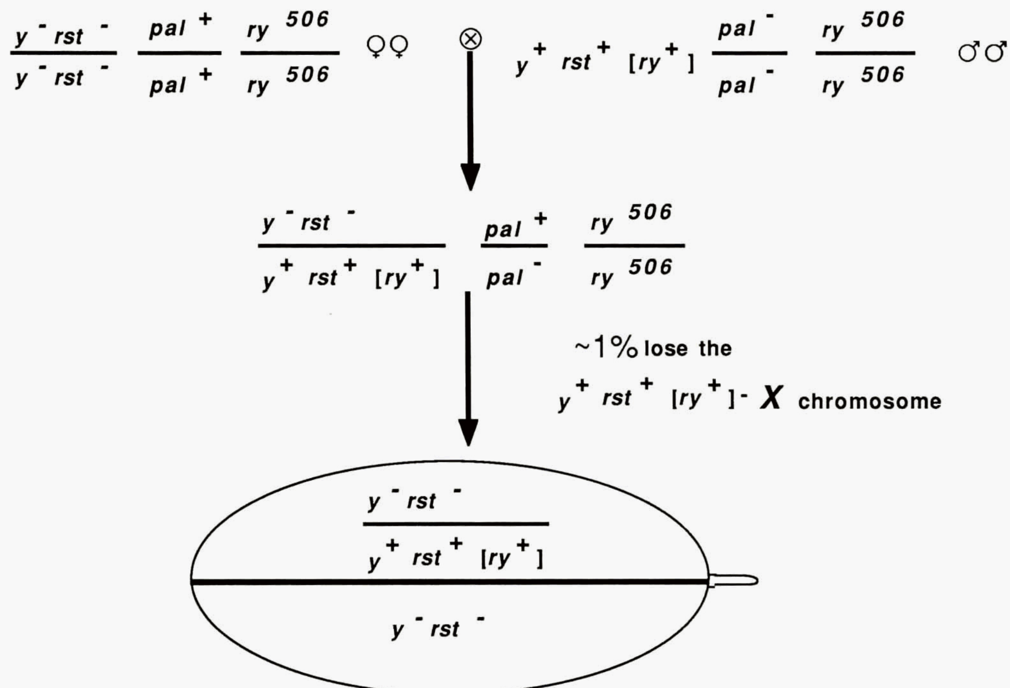

FIGURE 5.—Genetic mosaic construction. Flies mosaic for the *ry* gene were generated by the following scheme:

y rst; ry^{506} ♀♀ ⊗ y^+ rst^+ [ry^+]; *pal*; ry^{506} ♂♂

The *rst* mutation in the females serves as an autonomous eye marker to positively identify the genotype of eye tissue, and *y* marks the cuticular structures. *pal* is a mutation which causes elevated levels of paternal chromosome loss at one of the early mitoses in the zygote resulting in a genetic mosaic (BAKER 1975). The y^+rst^+ [ry^+] chromosome is an *X*-chromosome which bears a copy of the ry^+ gene inserted by *P* element transformation. Therefore, zygotes from the above cross that receive a *y rst* chromosome from their mothers and an *X* from their fathers will occasionally loose the paternal *X* at the first, second or third mitosis resulting in mosaic adults.

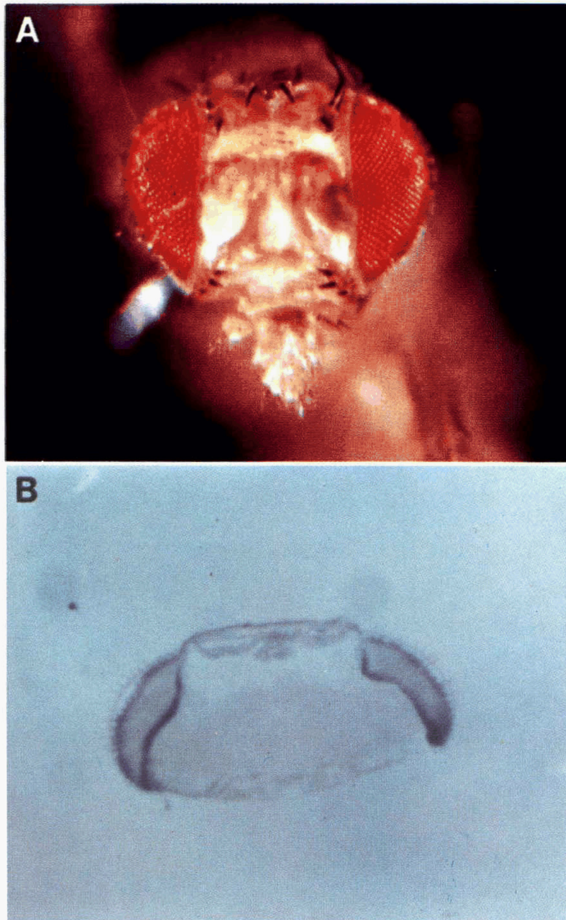

FIGURE 6.—ry^+/ry^- mosaics. (A) Whole mount view of a typical mosaic head. Note the disordered arrangement of facets in the left (rst^- ry^-) eye relative to the right eye (rst^+ ry^+). The color of both eyes is wild type. When these mosaic heads are examined by histochemistry and antibody staining techniques it is found that both eyes have XDH. (B) A histochemical preparation from the same individual shown in (A). The left eye is rst^-ry^- while the right eye is rst^+ ry^+.

of whether or not XDH is transported to the eye, genetic mosaics were constructed following the breeding protocol of Figure 5. In one experiment, 43 mosaics were scored among 8480 progeny. Figure 6A illustrates a typical bilateral head mosaic in which one eye carries the paternally transmitted *X* chromosome (y^+ rst^+ [ry^+]), and exhibits the regular order and size of eye facets characteristic of the rst^+ allele (LINDSLEY and GRELL 1968), while the other eye has lost the paternal *X*, and exhibits the *rst* phenotype (irregular order and size of facets) reflecting the presence of the maternal *X* chromosome bearing the ry^- rst^- mutant markers. Since the third chromosomes carry the ry^{506} mutation, which has a large deletion of the 3′ end of the XDH coding sequence (COTÉ *et al.* 1986), the only ry^+ allele is present on the paternally transmitted *X*. A histochemically stained preparation of tissue (Figure 6B) from the same mosaic individual shown in Figure 6A demonstrates that XDH is present in the genetically ry^- eye as well as in the genetically ry^+ eye. Clearly, this result is explicable only on the notion that the XDH present in the ry^- eye must have been transported there from some genetically competent ry^+ tissue where it was synthesized.

DISCUSSION

The present report describes the results of experiments demonstrating that XDH is synthesized particularly in fat body and Malpighian tubules, and transported to the eye where it is sequestered at the basement layer of the retina. It should be noted that our present methods do not determine whether XDH is located within cells in the eye or in an extracellular space.

The notion that an enzyme may be synthesized in one tissue and used in another is not entirely novel. XDH has already been shown to be present in pupal and adult hemolymph (MUNZ 1964). Moreover, its mammalian counterpart, xanthine oxidase, is also present extracellularly in milk (GILBERT and BERGEL 1964). Finally, there is evidence suggesting that other fly enzymes are transported from one tissue to another (GEIGER and MITCHELL 1966; PRICE 1974). The mechanism by which this enzyme is exported to the hemolymph and to the eye is of interest since no leader sequence is evident. However, the same is true of all of the peroxisomal enzymes that have been sequenced thus far. In these cases, transport of the protein occurs by a post-translational process that requires ATP, and may or may not require a proton motive force (FUJIKI and LAZAROW 1986; BELLION and GOODMAN 1987). BEARD and HOLTZMAN (1987) have localized Drosophila XDH in the Malpighian tubules to peroxisome-like vesicles. Drosophila XDH has also been shown to have a probable 3′ peroxisome targeting sequence (PTS) GOULD, KELLER and SUBRAMANI1988). It seems likely that XDH in the Malpighian tubule peroxisomes is transported there by the post-translational process described for other peroxisomal proteins. Perhaps export of XDH from cells also occurs by a similar post-translational transport mechanism. This could occur either directly at the cell membrane or XDH could be pumped into transport vesicles which then fuse with the cell membrane. Perhaps peroxisomes are even fused with the cell membrane and deposit XDH and other peroxisomal enzymes outside the cell.

The begging question is why the fly would utilize such a system for transporting and sequestering the enzyme at the eye. Approximately 30% of total adult XDH activity is associated with the eyes (BARRETT and DAVIDSON 1975). Surely purine catabolism cannot be the primary basis for such an accumulation of the enzyme. We have reason to believe that the enzyme serves as a carrier molecule bringing an eye pigment

precursor in the form of an enzyme substrate to the eye at the time of pigment formation. Work is in progress to examine this issue further.

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