The *rosy* Locus in *Drosophila melanogaster*: Xanthine Dehydrogenase and Eye Pigments

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ABSTRACT

The *rosy* gene in *Drosophila melanogaster* codes for the enzyme xanthine dehydrogenase (XDH). Mutants that have no enzyme activity are characterized by a brownish eye color phenotype reflecting a deficiency in the red eye pigment. Xanthine dehydrogenase is not synthesized in the eye, but rather is transported there. The present report describes the ultrastructural localization of XDH in the Drosophila eye. Three lines of evidence are presented demonstrating that XDH is sequestered within specific vacuoles, the type II pigment granules. Histochemical and antibody staining of frozen sections, as well as thin layer chromatography studies of several adult genotypes serve to examine some of the factors and genic interactions that may be involved in transport of XDH, and in eye pigment formation. While a specific function for XDH in the synthesis of the red, pteridine eye pigments remains unknown, these studies present evidence that: (1) the incorporation of XDH into the pigment granules requires specific interaction between a normal XDH molecule and one or more transport proteins; (2) the structural integrity of the pigment granule itself is dependent upon the presence of a normal balance of eye pigments, a notion advanced earlier.

DESPITE the many investigations carried out over many years, a real understanding of the interactions leading to the production of the wild type eye color phenotype in *Drosophila melanogaster* remains elusive. The *rosy* gene of *D. melanogaster* (*ry* :3-52.0) is one of more than 83 different genes for which mutations are associated with an altered eye color phenotype (PHILLIPS and FORREST 1980). *Rosy* codes for the enzyme xanthine dehydrogenase (XDH: EC 1.2.1.37.), and has been the subject of extensive genetic, molecular, and biochemical characterization (for review see DUTTON and CHOVNICK 1988). Homozygous null mutant adults are characterized by a brownish eye color resulting from a partial deficiency of the red, pteridine pigments compared to relatively normal levels of brown, xanthommatin pigment (NOLTE 1955; FERRÉ *et al.* 1986). While XDH catalyzes at least one pteridine reaction (2-amino-4-hydroxypteridine to isoxanthopterin) it has never been shown to catalyze a pteridine reaction in the red pigment pathway which takes place in the eye [for discussion see PHILLIPS and FORREST (1980) and NASH and HENDERSON (1982)]. The XDH pteridine substrates and products have been found in significant quantities only in the Malpighian tubules and testes (HADORN and MITCHELL 1951). Moreover, injection of XDH-deficient flies with isoxanthopterin, xanthine, or uric acid, failed to rescue the eye color phenotype (HADORN and GRAF 1958; HUBBY and FORREST 1960).

In a prior report, we demonstrated that the *ry* gene is transcribed and the enzyme synthesized in fat body, Malpighian tubules, and gut cells of both larvae and adults. In contrast, the *ry* gene is not expressed at the transcript level in cells of the adult eye or its developmental precursors, despite the fact that the normal adult eye contains significant quantities of XDH (REAUME, CLARK and CHOVNICK 1989). Moreover, this report demonstrated, using genetic mosaics, that an adult eye, homozygous for a deletion of the *ry* gene, possesses XDH that was synthesized in the fat body and other competent tissues of the mosaic individual, and then is transported to the eye.

The adult eye, as illustrated in Figure 1, is composed of approximately 800 facets or ommatidia. Each ommatidium is, in turn, made up of 14 cells: 8 photoreceptors, 4 cone cells and 2 primary pigment cells. In addition, each ommatidium shares with its neighbors: 6 secondary pigment cells, 6 tertiary pigment cells and a bristle element (4 cells). The secondary pigment cells contain type II pigment granules that are composed of bright red drosopterin pigment and brown xanthommatin pigment. Type I granules, which contain only xanthommatin, are found in the primary pigment cells, the cone cell feet at the base of the retina, and in receptor cells (SHOUP 1966; CAGAN and READY 1989).

XDH is a large molecule (300 kD) that must be

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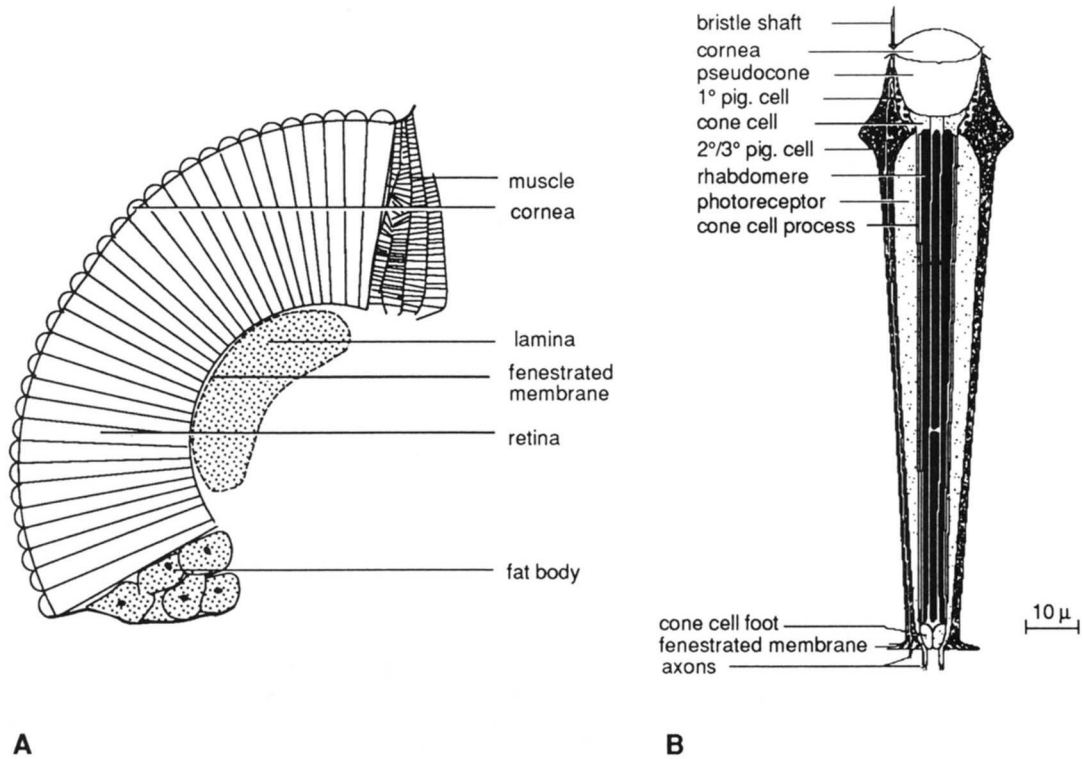

FIGURE 1.—The adult eye. (A) A cross-section of the entire retina. Anterior is toward the top. The retina is composed of approximately 800 ommatidia. Muscle, associated with the antennae, borders the anterior side, while fat body borders the posterior side. The lamina of the brain lies underneath. (B) Schematic longitudinal view of a single ommatidium drawn to scale (derived from CAGAN and READY 1989). Red pigments (pteridines) are found only within the type II pigment granules of the secondary pigment cells. Brown pigments (ommochromes) are found in the type I granules of primary pigment cells, the photoreceptors, and the clone cell feet as well as in the type II pigment granules of the secondary pigment cells. Abbreviations: 1° pig. cell, primary pigment cell; 2° pig. cell, secondary pigment cell. Anterior is to the right.

transported from its cellular site of synthesis to the eye. The first part of this investigation pursues the specific cellular localization of XDH in adult eye tissue. The second part of this investigation examines some of the factors and genic interactions that may be involved in transport of XDH, and in eye pigment formation.

MATERIALS AND METHODS

Strains and mutations: The wild-type strain used in these experiments carries an isogenic third chromosome designated ry^{+5} which was extracted from a *zeste* mutant laboratory stock. This strain produces normal levels of XDH and exhibits characteristic XDH thermostability and electrophoretic mobility (MCCARRON, GELBART and CHOVNICK 1974; MCCARRON *et al.* 1979). the ry^{506} allele is a deficiency of the 3′ half of the coding region (COTÉ *et al.* 1986). It arose as a mutation of the ry^{+5} allele, and the third chromosomes of these strains are otherwise identical. The RC2 strain has eight extra copies of ry^{+} by virtue of P-mediated transposon insertions (RUBIN and SPRADLING 1982). Additionally, the strain is homozygous for the ry^{+4} allele which is a natural overproducer (CHOVNICK *et al.* 1976; CLARK *et al.* 1984); thus, whole body XDH activity is at least six times that of a standard wild-type genotype with two copies of ry^{+}. The mutations bw^{AR}, ca^{AR}, ltd^{AR} and $ma\text{-}l^{AR}$ were generated by the senior author in an ethylmethanesulfonate (EMS) mutagenesis of an Oregon-R, wild type strain. All other mutations are described in LINDSLEY and GRELL (1968).

XDH enzyme histochemistry: 10 μm frozen sections were cut on a Slee cryostat, collected on poly-L-lysine coated slides, air dried and stained by a nitroblue tetrazolium method described in REAUME, CLARK and CHOVNICK (1989).

XDH antibody staining for light microscopy: Frozen sections (10 μm) of isolated heads that had not been previously fixed were cut, collected, and air-dried. The sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hr on ice. Slides were then washed three times in PBS and blocked in 1% bovine serum albumin (BSA), 1.0% normal horse serum and 0.03% Triton X-100 in PBS. The primary antibody reagent used in these experiments is a mouse IgG monoclonal (Mab ry 16E10) specific for purified *Drosophila melanogaster* XDH (A. G. REAUME, D. A. KNECHT and A. CHOVNICK, manuscript in preparation). Primary antibody was administered as 1:1,000 dilution of Mab ry 16E10 ascites fluid in PBS (130 mM NaCl, 10 mM phosphate, pH 7.4) containing 1% BSA and 1% normal horse serum, and incubated overnight at 4°C. Slides were then washed three times for 10 min each at room temperature with 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 [biotinylated horse anti-mouse IgG (Vector Laboratories)] 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 for the primary antibody application) were followed by avidin biotin complex labeling (Vector Laboratories) as described by the manufacturer except that 0.1% BSA was included in the mix. 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. Finally

the slides were stained in 0.5 mg/ml diaminobenzidine (DAB) 0.08% $NiCl_2$ and 0.03% hydrogen peroxide in PBS. They were mounted in Aquamount (Polysciences).

XDH assays of subcellular fractions: This method is derived from HEARL and JACOBSON (1984). Heads from 0.5 g of flies were detached from bodies by freezing the flies in a test tube containing liquid nitrogen and vigorously shaking the frozen animals. Heads were hand separated from the bodies and homogenized in 5 ml of 50 mM Pipes (pH 7.0), 0.09 M NaCl in a Dounce glass homogenizer. Coarse particulates were removed by centrifuging at 200 × *g* for 5 min. The supernatant was collected and centrifuged at 2000 × *g* for 20 min. The pellet was a crude pigment granule preparation. It was resuspended in 1 ml of the above buffer and then mixed with 10 ml of 90% Percoll (Pharmacia) in 0.23 M sucrose. A Percoll gradient was formed by centrifuging this mixture at 25,000 × *g* for 2 h. The gradient was then fractionated by aspiration of samples from the top.

Each sample received a mild detergent treatment by adding Triton X-100 to a concentration of 1%. The samples were then pipetted up and down repeatedly for about 30 sec each and then centrifuged for 5 min. The supernatants were measured for XDH activity by the following fluorometric assay.

An aliquot of the sample to be tested was added to the following reaction mix: 300 μM aminohydroxypteridine (AHP), 0.1 M Tris (pH 8.5), 1.4 mM NAD (added fresh before using). Conversion of AHP to isoxanthopterin was followed over time with an excitation wavelength of 334 nm and monitoring the emission at a wavelength of 412 nm. One unit of activity is arbitrarily defined here as the amount of XDH required to produce a change of one fluorometric unit in 1 min.

Descriptive ultrastructure: To maintain cell structural integrity for study of the eye, the preparative procedures followed those of CAGAN and READY (1989). Heads were bisected in PBS and then fixed overnight in 2% glutaraldehyde diluted in PBS. They were then washed in several changes of PBS over the course of 3 h and postfixed in 2% $KMnO_4$ for 4 hr. Next, the tissues were dehydrated in an ethanol series, cleared in propylene oxide for 30 min, in propylene oxide/Epon-Araldite (1:1) overnight and then embedded in Epon-Araldite for several hours before hardening in blocks.

Ultrathin sections (700–900 Å) were cut on a glass knife. Sections were collected on copper grids and post-stained in uranyl acetate and lead citrate. Specimens were observed with a Phillips EM300 operating at 80 kV.

XDH antibody staining for EM: Ultrastructural localization of XDH involved antibody staining in a manner similar to that used for antibody staining for the light microscope. 20-μm sections of unfixed heads were cut and floated onto 4% fresh formaldehyde prepared in PBS. After antibody staining and development of the stain with DAB, the colored product was made electron dense using a OsO_4-thiocarbohydrazide sandwich technique. The tissues were first thoroughly washed in PBS and then treated with alternate incubations in 2% OsO_4 and 0.1% thiocarbohydrazide (both prepared in PBS) as follows: 15 min in OsO_4, 15 min in thiocarbohydrazide, 30 min in OsO_4, 15 min in thiocarbohydrazide, 15 min in OsO_4. It is critical that the tissues be thoroughly washed between each of these changes. Next, the 20-μm sections were dehydrated in an ethanol series, cleared in propylene oxide for 30 min, in propylene oxide/Epon-Araldite (1:1) overnight, and embedded in Epon-Araldite for several hours before hardening in blocks. Ultrathin sections were cut on a glass knife and sections collected on copper grids. No poststaining was performed on the sections.

Thin layer chromatography (TLC) of eye pigments: TLC followed the procedure described by FERRÉ *et al.* (1986) with minor modifications. Flies were collected over a 24-hr period and aged for 8 days. Heads from 10 males, 10 females and 3 male bodies were homogenized in 50 ml of methanol/glacial acetic acid/water (4:1:5 by volume) under dim lighting conditions. After centrifugation for 5 min, 12 ml of the supernatant were loaded onto an Avicel microcrystalline cellulose TLC plate (20 cm × 20 cm) under a sodium vapor safelight. Samples were run in two dimensions as follows. The first dimension was developed in isopropanol/2% aqueous ammonium acetate (1:1, by volume) for 3 hr. The plates were thoroughly dried over the course of 2–3 hr. The second dimension was run in 3% ammonium chloride for 50 min. The development and drying was carried out in the dark. The separated pterin compounds were examined under UV light.

RESULTS

XDH distribution in adult eye tissue: Several lines of evidence indicate that XDH is localized specifically to type II pigment granules. The first is the correspondence of XDH distribution in the eye, as judged by light microscopy of antibody or histochemically stained tissues, with the distribution of pigment granules. Figure 2 illustrates the concentration of XDH at the border between the retina of the eye and the lamina of the brain, and is clearly found on both sides of the basement membrane (see arrows, Figure 2A) that separate these two tissues. A low magnification electron micrograph illustrates the distribution of pigment granules in this region (Figure 3). Note that the granules are heavily clustered on the retinal side of the basement membrane, but are definitely present on the lamina side as well. Although the two types of pigment granules are not clearly distinguished in Figure 3, it has been established that those lying just above the basement membrane consist of type I and type II while only type II granules lie below the basement membrane (NOLTE 1950; SHOUP 1966; STARK and SAPP 1987; A. REAUME, unpublished observations).

Ultrastructure of the retina/lamina border: As described in the previous section, XDH and pigment granules are localized to both sides of the basement membrane of the eye. While it is understood that pigment on the retinal side of the basement membrane is contained within the core cell feet and the secondary pigment cells (CAGAN and READY 1989), the cellular association of the "postretinal" pigment granules (as they were called by NOLTE 1950) remains uncertain. CAGAN and READY (1989) argue that they may be associated with subretinal cells; although they discuss data from genetic mosaics that is consistent with the view that postretinal granules belong to the secondary pigment cells of the retina. Since this matter is of relevance to XDH tissue distribution, ultrastructure

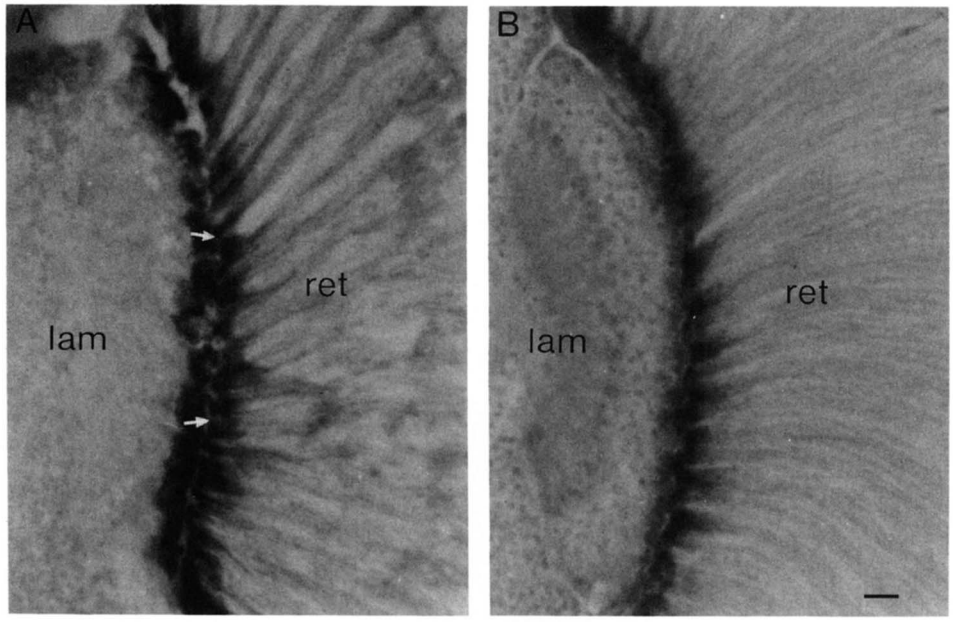

FIGURE 2.—XDH distribution at the retina/lamina border. (A) Young adults were stained for XDH activity as described above (MATERIALS AND METHODS). Note that XDH activity is distributed on both sides of the basement membrane (arrows). (B) The same tissues were also stained with an anti-XDH monoclonal antibody (MAb ry16E10). lam = lamina; ret = retina; scale bar = 10 μm.

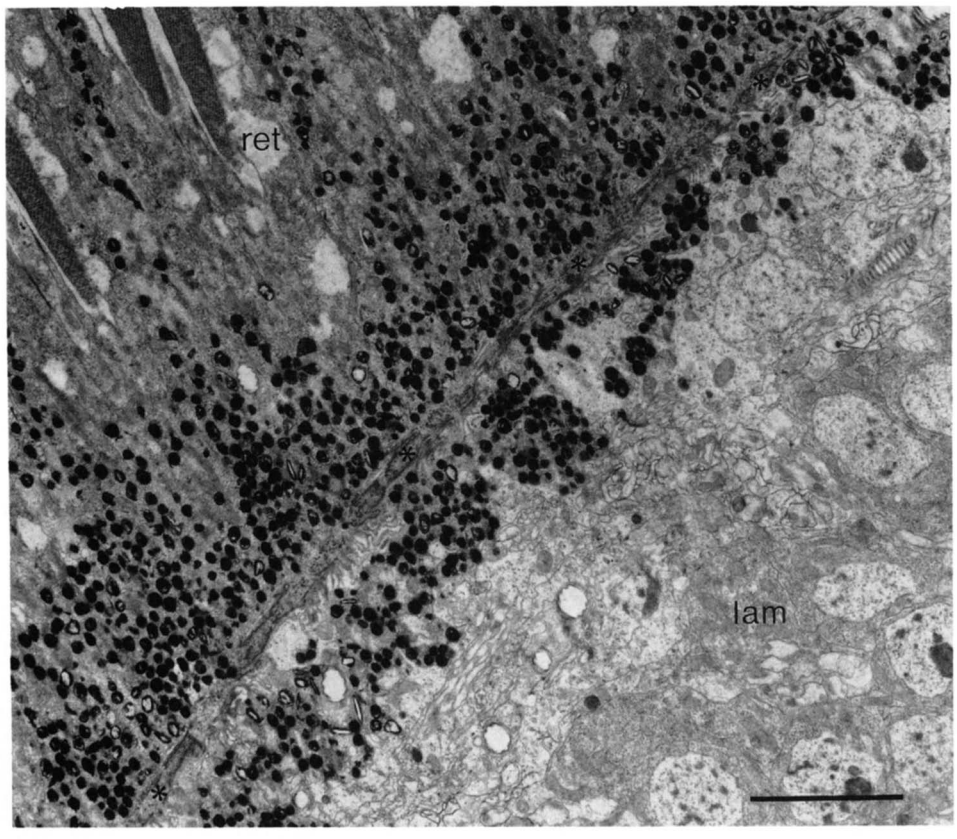

FIGURE 3.—Low magnification electron micrograph of retina/lamina border. The basement membrane (indicated by *) separates the retina from the lamina of the brain. Note that the granules are distributed on both sides of this border. In this figure, type I granules are virtually indistinguishable from type II granules. Some of the granules above the membrane are type I granules associated with the cone cell feet. All of those below the border are type II granules. (NOLTE 1950; SHOUP 1966; STARK and SAPP 1987; A. REAUME, unpublished observations). lam = lamina; ret = retina; scale bar = 10 μm.

studies of the retina/lamina border were undertaken.

ry^{+5} heads were fixed, sectioned longitudinally with respect to the ommatidia, and stained to visualize pigment granules under the electron microscope. The following conclusions are made concerning the "postretinal" pigment granules in these preparations (Figure 4): (1) Pigment granules are found adjacent to mitochondria, indicating that these granules are intracellular. (2) The cytoplasm associated with these granules is continuous with cytoplasm of the secondary pigment cells by virtue of pores in the basement membrane demonstrating that the secondary pigment cells extend through the basement membrane. (3) Granules can be seen within the pore suggesting that they may be free to travel through the basement membrane. Despite the evidence of CAGAN and READY (1989) for the existence of postretinal pigment cells, we are drawn to the conclusion that the postretinal granules are continuous with pigment cells in

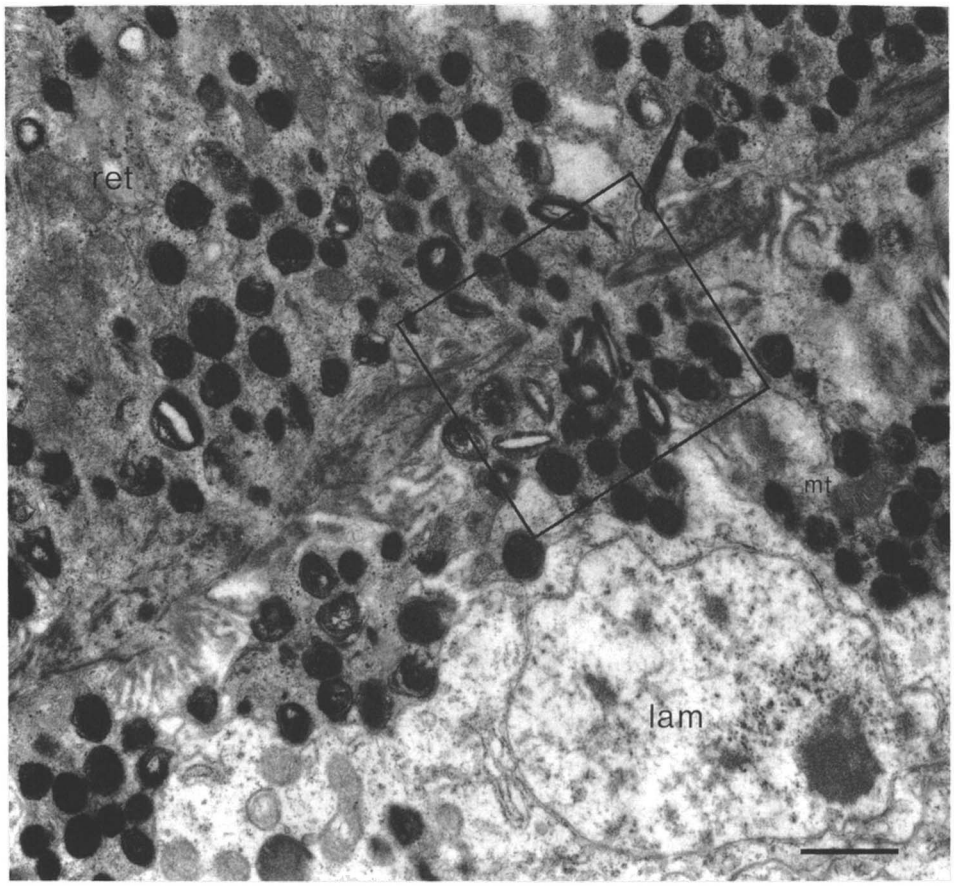

FIGURE 4.—Ultrastructure of retina/lamina border. The same area illustrated in Figure 3 is shown here at higher magnification. The association of mitochondria with the pigment granules reveals that these granules are intracellular. The cytoplasm around the granules is seen to be continuous with the secondary pigment cells by way of a pore in the basement membrane (outlined by the box). Some granules lie within the pore indicating that they may be free to travel through it. lam = lamina; ret = retina; mt = mitochondrion; scale bar = 10 μm.

the retina by way of processes that extend through the basement membrane.

Purification of pigment granules: The second argument for XDH localization to pigment granules comes from examination of purified pigment granules. Heads from approximately 0.5 g of ry^{+5} flies were homogenized and pigment granules were purified as described above (see MATERIALS AND METHODS). Fractions from the Percoll gradient were assayed for XDH activity. Since (1) XDH is found in the fat body of the head as well as in the eye, and (2) the isolated eye pigment granules leak their contents (indicated by the presence of red pigment in the region of the gradient above the band formed by the pigment granules), one expects to find activity in several fractions. However, a peak of activity was observed in the fraction containing pigment granules (Figure 5).

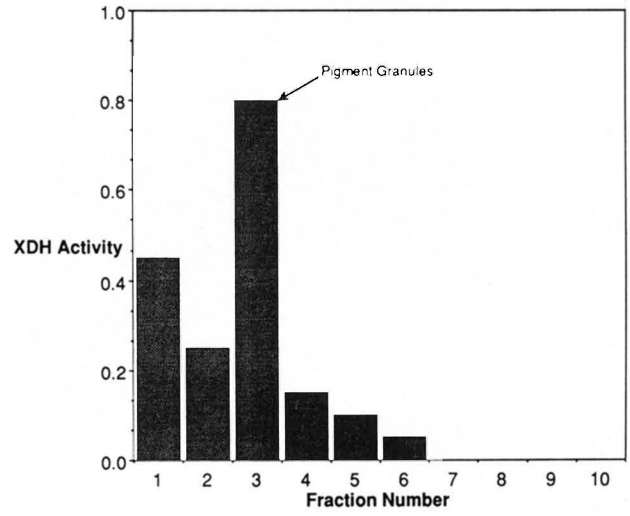

FIGURE 5.—XDH activity in subcellular fractions of the eye. A semipure preparation of concentrated pigment granules was loaded onto a Percoll gradient and centrifuged for 2 hr as described in HEARL and JACOBSON (1984). The fraction containing the pigment granules was easily identified by its color. It corresponded to the fraction with the highest XDH activity.

EM visualization of XDH antigen: The third source of evidence for XDH localization to the type II pigment granules comes from EM visualization of XDH antigen in the adult eye, 0–24 h following eclosion. Examination of this material revealed that the type II granules in ry^{+5} individuals were much more electron dense relative to background as compared to those from ry^{506} individuals (Figure 6, *A* and *B*), indicating that they contain XDH antigen.

Genic interaction in transport to the eye: The observations described above imply the existence of transport processes concerned with export of XDH from the cells where it is synthesized to the secondary pigment cell membrane, passage through the membrane and subsequent incorporation into the type II

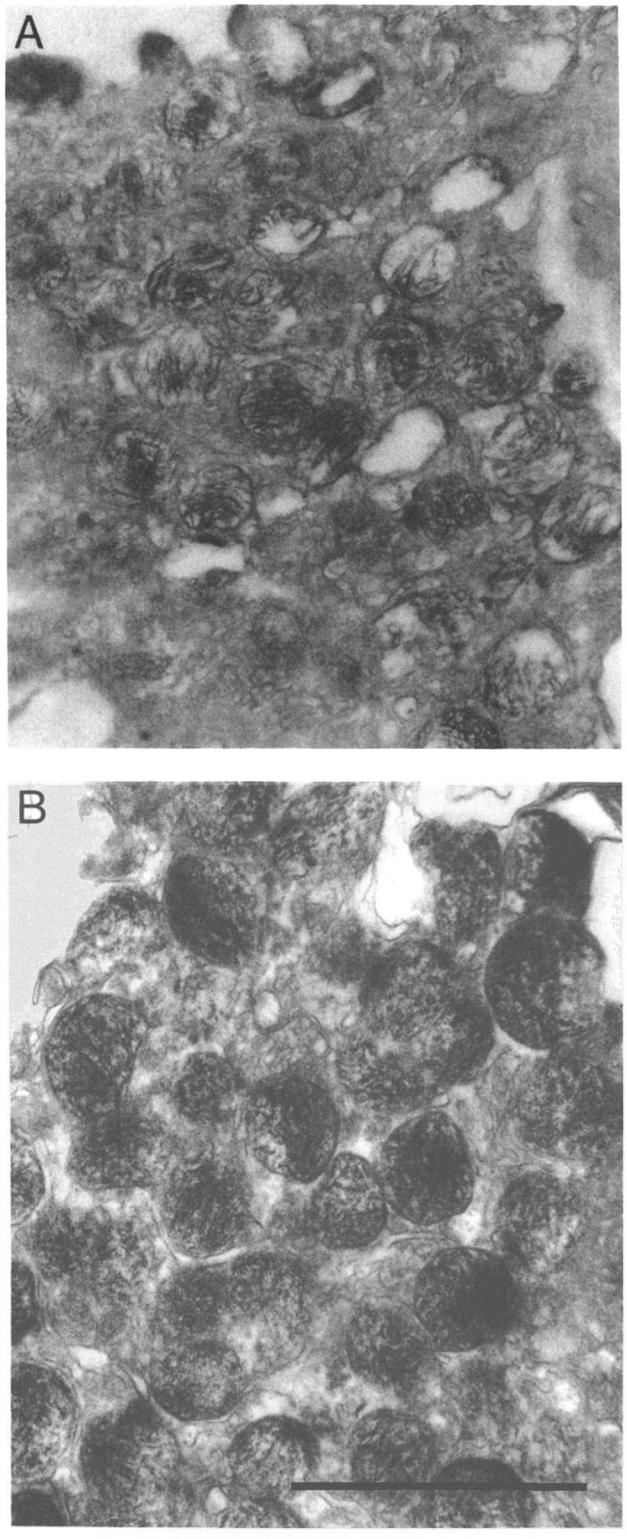

FIGURE 6.—Ultrastructural localization of XDH antigen. (A) in ry^{506} mutant (B) in ry^{+5}. Sections of young adult heads were stained by a procedure similar to that used in staining sections for the light microscope. Following this, the stained material was post stained in OsO_4, dehydrated, embedded in resin, sectioned and viewed under the EM. The granules in ry^{+5} flies stain distinctly darker than those in ry^{506}. Note the membrane boundary and internal structure of type II pigment granules. This is especially apparent in the ry^{506} mutant where stainifg does not obscure these details. Scale bar = 1 μm.

TABLE 1

XDH in eyes of mutants of genes reputed to be associated with transport processes

Mutant[a]	Histochemistry[b]	Antibody stain[b]
bw,bw^{AR}	–	–
ltd,ltd^{AR}	–	–
st,st^{sp}	–	–
$w,w^{a},w^{e},w^{h},w^{st}$	–	–
ca,ca^{2},ca^{AR}	–	–

[a] At least three representatives of each mutant were subjected to each test.

[b] Compared to ry^{+5} control, "–" severe reduction or absence of XDH activity or antigen.

pigment granules. The following several groups of experiments were conducted in an effort to identify factors that may be involved in these processes. Two methods were utilized in these studies. The first involved histochemical examination of XDH activity in frozen section of whole flies. These results were supplemented by antibody staining using our XDH-specific monoclonal antibody.

A number of eye color mutations of several different genes were assayed for their effects on XDH tissue distribution. Many of the mutant strains lacked XDH in their eyes, despite the presence of normal levels of XDH in fat body, Malpighian tubules and gut. Table 1 summarizes the results obtained with one such group of eye color mutants. These involve mutations of genes believed to be associated with transport processes. In these instances, the eye color defects were believed to result from defects in the transport into eye tissue of small molecular weight precursors of the eye pigment synthetic pathways. Thus, *st, w, ltd* and *ca* are defective in ability to transport kynurenine, a brown pigment precursor (SULLIVAN and SULLIVAN 1975), while *w* and *bw* mutants are defective in transport of purine precursors of the red pigment such as xanthine (SULLIVAN *et al.* 1979). Three of these genes [*white* (*w* :1-1.5); *brown* (*bw* :2-104.5); *scarlet* (*st* :3-44.0)] have been sequenced, and attention has been drawn to the relationship of their peptide sequences to a large and important family of *trans*-membrane transport proteins (O'HARE *et al.* 1984; MOUNT 1987; DREESEN, JOHNSON and HENIKOFF 1988; TEARLE *et al.* 1989). The surprising feature of these observations (Table 1) is the absence of XDH in the eyes of these mutants. Not only is XDH missing from the eyes of mutants purported to be defective in transport of red pigment precursors, but the brown pigment transport mutants do not have XDH either. For both groups of mutants, normal levels of XDH are present in the fat body surrounding the eye, as well as in other tissues where it is synthesized. An example is presented in Figure 7 which illustrates the results obtained with the *white* mutant.

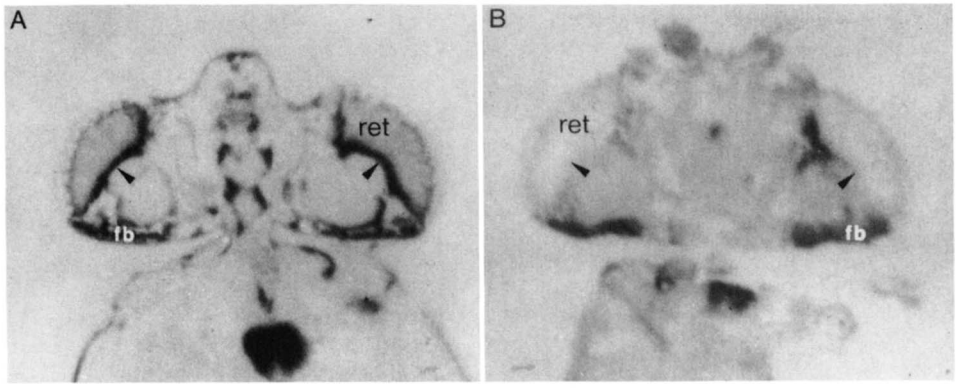

FIGURE 7.—XDH activity in a *w* mutant head. (A) Histochemical stain for XDH activity in ry^{+5} serves as a control. Note that staining is seen in the fat body in the head, as well as the retina/lamina border. (B) Histochemical stain for XDH activity in a *w* mutant. Note that XDH is seen in the fat body surrounding the eye, but not the retina/lamina border. fb = fat body; ret = retina.

From these observations, we suggest that incorporation of XDH into the type II pigment granule requires XDH interaction with the protein products of some or all of the genes represented by the mutants of Table 1.

XDH structure and transport to the eye: Drosophila XDH is one of a class of molybdenum-containing hydroxylases in which molybdenum is bound to the enzyme in the form of a pterin-molybdenum cofactor (MoCF) that binds to a C-terminal segment of the protein (reviewed in WOOTON *et al.* 1991). In purified wildtype extracts of XDH, the MoCF is found in its active form with a S = MoCF complex as well as in an inactive, desulfo, O = MoCF form (HUGHES, BENNETT and BRAY 1991). Reducing substrates, like xanthine, bind to the molybdenum domain of the enzyme, and the ensuing oxidation in the wildtype involves electron transfer to FAD/NAD^+ which is bound to a centrally located segment of the XDH protein (WOOTON *et al.* 1991).

The XDH inhibitor, 4-hydroxypyrazolo[3,4-*d*]pyrimidine (allopurinol or HPP) binds in place of other substrates, and produces a *rosy* mutant phenocopy when fed in appropriate concentrations (KELLER and GLASSMAN 1965; BONI, DELERMA and PARISI 1967). Wild type larvae (ry^{+5}) were raised on standard medium supplemented with HPP at three concentration levels (100, 200, and 300 μg/ml). Enzyme activity diminished in fat body as HPP concentration increased, and was negative at 300 μg/ml. However, XDH peptide levels in fat body remained strong at all HPP concentrations as evidenced by antibody binding and staining. The eye, however, proved to yield very different results. Antibody staining diminishes with increasing HPP levels. At 300 μg/ml, the eye shows no evidence of antibody staining. At this level of HPP, one sees antibody staining in fat body, but not in the eye.

An analogous result was obtained with the *maroon-like* (*ma-1* :1-64.8) mutation, $ma\text{-}l^{AR}$. Mutants carrying this gene, like *rosy* mutants, exhibit an altered eye color phenotype, deficient in the red pigments, and are inactive for XDH. It has long been known that *ma-l; ry*$^+$ individuals possess wildtype levels of XDH protein as antigen (FINNERTY 1976; FINNERTY and WARNER 1981). Several kinds of experiments support the notion that the $ma\text{-}l^+$ product functions to convert the inactive O = MoCF to the active S = MoCF (WAHL *et al.* 1982). Presumably, reducing substrates still bind to the molybdenum domain, but under normal conditions, their oxidation does not take place in the presence of the desulfomolybdenum cofactor, as in *ma-l* mutant individuals. Antibody staining of sections of *ma-l* mutant individuals revealed normal levels of XDH antigen in fat body and other tissues where it is synthesized, but no XDH antigen was found in the eye.

Taken together, the experiments described in this section focus attention upon a key role in the sequestration of XDH to the eye for the specific substrate bound to the molybdenum domain or to the enzyme's catalytic activity.

Effects of pigment biosynthetic enzymes on XDH: Another group of mutants was examined that possess lesions in genes believed to code for specific enzymes in either the biosynthesis of the red eye color pigments [*purple* (*pr* :2-54.5); *Punch* (*Pu* :2-97); *sepia* (*se* :3-26)] or the brown pigment [*cinnabar* (*cn* :2-57.5); *vermilion* (*v* :1-33.0)]. These data (Table 2) conform to expectation for the red pigment genes (*pr, Pu, se*). XDH is found in the eyes of these mutants, confirming that these genes are not involved in the transport process. Differences in the levels of XDH found in the eyes of these strains may relate to differences in their wild type *rosy* alleles compared to our control, ry^{+5} allele. In contrast, mutants of the brown pigment gene (*cn, v*) have no XDH in their eyes, despite the presence of ample quantities of XDH in the surrounding fat body.

XDH distribution in other eye color mutants: Another group of mutants was examined (Table 3) in hope of uncovering evidence for additional factors that might be involved in XDH distribution. The *chocolate* (*cho* :1-5.5) and *prune* (*pn* :1-0.8) mutants were examined (Table 4) because, in both instances, prior reports indicated evidence of normal or excess XDH enzymatic activity, yet reduced levels of red

TABLE 2

XDH in eyes of mutants of genes that code for biosynthetic enzymes in either red or brown pigment pathways

Mutant[a]	Histochemistry[b]	Antibody stain[b]
pr,pr^{bw}	+	+
Pu^{r1},Pu^{r28}	ND	±
se,se^{51j}	+	++
cn,cn^{35k}	−	−
v^{1},v^{36f}	−	−

Red pigment: WILSON and JACOBSON (1977), MACKAY and O'DONNELL (1983) and WEISBERG and O'DONNELL (1986). Brown pigment: BAILLIE and CHOVNICK (1971), SULLIVAN, KITOS and SULLIVAN (1973) and SEARLES and VOELKER (1986).

[a] At least three representatives of each mutant were subjected to each test.

[b] Compared to ry^{+5} control, "+" = normal XDH activity or antigen; "±" = less than normal; "++" = excess over normal; ND = not determined.

TABLE 3

XDH in eyes of miscellaneous eye color mutants

Mutant[a]	Histochemistry[b]	Antibody stain[b]
cho,cho^{2}	−	± (abnormal distribution)
pn	±	±
pn^{2}	±	+ (abnormal distribution)

[a] At least three representatives of each mutant were subjected to each test.

[b] Compared to ry^{+5} control, "−" = severe reduction or absence of XDH activity or antigen; "+" = normal XDH activity or antigen; "±" = less than normal.

pigments (NOLTE 1959; GEARHART and MACINTYRE 1970; MCKAY 1972). For both groups of mutants, the major difference from the normal is seen as a change in the localization of XDH within the eye. There is less antibody staining at the retinal/lamina border, and increased staining in the retina, away from the basement membrane.

Pteridines and eye color mutants: To confirm that our observations on mutants relate to production of the red, drosopterin pigments, and to assess the relationship of *ry* to the other eye color mutations, two-dimensional TLC was carried out on preparations of single and double mutant individuals (see MATERIALS AND METHODS). Table 4 summarizes the results obtained for a representative sample of the single and double mutants.

Major features of these data are: (1) The RC2 strain, carrying eight extra copies of ry^{+} alleles, exhibits much greater XDH activity than does the homozygous ry^{+5} strain when assayed *in vitro* following the pteridine reaction AHP → IXP. However, these strains exhibit no difference in their accumulation of drosopterin eye pigments, nor in their accumulation of the pteridine reactants, AHP and IXP. (2) As expected, all *ry* mutant strains lack detectable isoxanthopterin. (3) The "addition" of a homozygous *ry* mutation to a genotype invariably reduces the amount of all red pigments. (4)

TABLE 4

TLC analysis of eye color mutants

	Drosopterin pigments				Pteridines			
Genotype	AP	DP	IP	NP	AHP	IXP	BP	SP
ry^{+5}	5	5	5	5	2	5	2	1
RC2	5	5	5	5	3	5	2	1
ry^{506}	3	3	3	3	5	0	5	2
bw^{AR}	0	0	0	0	0	0	0	0
$bw^{AR};ry$	0	0	0	0	0	0	0	0
w^{a}	0	0	0	0	0	1	2	0
$w^{a};ry$	0	0	0	0	1	0	2	0
st	5	5	5	5	1	5	2	1
st,ry	1	1	1	1	1	0	2	2
ltd^{AR}	2	2	2	2	1	2	4	1
$ltd^{AR};ry$	1	1	1	1	2	0	4	2
ca	2	2	2	2	1	4	4	2
$ca;ry$	0	0	0	0	1	0	4	2
pr	2	2	2	1	1	5	1	0
$pr;ry$	1	1	1	1	3	0	2	1
Pu^{r1}	0	2	2	2	1	3	2	0
$Pu^{r1};ry$	0	1	1	1	2	0	3	0
se	0	0	0	0	5	5	5	5
$se;ry$	0	0	0	0	5	0	5	5
v^{36f}	5	5	5	5	1	5	2	1
$v^{36f};ry$	2	2	2	2	2	0	3	3
cn	5	5	5	5	1	4	3	1
$cn;ry$	1	1	1	1	2	0	5	2
cho^{2}	2	2	2	2	2	4	4	2
$cho^{2};ry$	1	1	1	1	3	0	4	2
pn^{2}	1	2	2	3	3	5	4	3
$pn^{2};ry$	1	2	2	1	3	0	4	2

Identification of drosopterin pigments and pteridines was by fluorescent color and position on the TLC plate, as described by WILSON and JACOBSON (1977) and FERRÉ *et al.* (1986). Sepiapterin did not appear as described by these authors, probably as a result of degradation during the homogenization step where light conditions were not at an absolute minimum. Nevertheless, its byproduct was positively identified based upon its characteristic quantity in the se mutant, and its fluorescent color (also described by the above authors). We refer to this degradation byproduct as sepiapterin. Quantitation was by visual estimate using an ordinate scale from 1 to 5. All plates were compared to ry^{+5} and ry^{506} control plates which served as reference standards. Strains carrying one eye color mutation were always judged directly against strains that carried both that mutation and ry^{506}. AP = aurodrosopterin; DP = drosopterin; IP = isodrosopterin; NP = neodrosopterin; AHP = 2-amino-4-hydroxypterin; IXP = isoxanthopterin; BP = biopterin; SP = sepiapterin.

In ommochrome (brown pigment) deficient genotypes that possess normal levels of red pigment, (*e.g.*, *cn*, *st*, *v*), the "addition" of a homozygous *ry* mutation to the genotype reduces the level of red pigment to below that of strains that are mutant at only the *ry* locus. (5) The "addition" of a homozygous *ry* mutation to a genotype tends to increase the levels of AHP, biopterin, and sepiapterin.

DISCUSSION

The present report describes studies that demonstrate the localization of XDH in the type II pigment granules of the secondary pigment cells of the eye. Given the fact that XDH is synthesized in other tissues,

the mechanism by which this enzyme is exported to the hemolymph, and subsequently incorporated into the pigment granules presents an intriguing problem in the absence of a leader sequence. There is some evidence that XDH is localized, at least in part, within peroxisomes in the cells where it is synthesized (BEARD and HOLTZMAN 1987). Even this localization may at first seem inconsistent with the absence of a leader sequence. However, the same is true of all of the peroxisomal enzymes that have been sequenced thus far. In these cases, transport of the protein into the peroxisome occurs by a post-translational process that requires ATP, and may require a proton motive force (LAZAROW and FUJIKI 1985; BELLION and GOODMAN 1987). Targeting to the peroxisome is believed to occur by means of a short amino acid sequence, the peroxisomal targeting sequence (PTS), located at the C-terminal end of these proteins (GOULD, KELLER and SUBRAMANI 1988). Drosophila XDH has been shown to have such a sequence at its C terminus (GOULD, KELLER and SUBRAMANI 1988). Thus, it seems likely that XDH is transported to the peroxisomes by the post-translational process described for other peroxisomal proteins. Conceivably, export of XDH from cells may occur by the same or a similar 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 XDH deposited outside the cell.

The other question concerning XDH transport is its uptake at the eye. One possible mode of entry could be for XDH in the hemolymph to pass through membranes at the base of the retina. An attractive model, based upon the present observations, would involve XDH binding to pigment cell membrane proteins, and entering the pigment cell by endocytosis. The endocytic vesicles may then become type II granules. Since the origin of type II pigment granules has never been determined, this mechanism could serve to explain not only how XDH enters the eye, but also how type II pigment granules are formed, and why XDH within the eye is concentrated in these granules.

The unanswered question concerns why XDH is transported to the eye. As much as 30% of total adult XDH activity is associated with the eyes (BARRETT and DAVIDSON 1975), and purine catabolism cannot be the primary basis for such an accumulation of the enzyme. The reactions catalyzed by XDH provide no clue to its involvement in eye pigment synthesis (HADORN and GRAF 1958; HUBBY and FORREST 1960). It is clear that XDH is not essential for eye pigment synthesis since mutants that completely lack the enzyme still possess 60% of normal levels of red pigments (Table 4).

We have demonstrated that a number of eye color mutants lack normal levels of XDH in their eyes. Among these are *w, bw* and *st*. SULLIVAN and SULLIVAN (1975) suggested that the defect in brown pigment associated with *w* and *st* mutants results from a defect in their ability to transport kynurenine, a brown pigment precursor, while *w* and *bw* are defective in their ability to transport xanthine, a red pigment precursor (SULLIVAN *et al.* 1979). There are sequence similarities in *w* and *bw* to the family of *trans*-membrane transport proteins including the bacterial ATP-dependent permeases (O'HARE *et al.* 1984; MOUNT 1987; DREESEN, JOHNSON and HENIKOFF 1988; TEARLE *et al.* 1989). In addition to its catalytic role in purine and pteridine metabolism, we considered that XDH might possibly function in red pigment synthesis by serving to transport xanthine to the pigment cell membrane. Perhaps xanthine is transferred to the *trans*-membrane permease, and pumped into the cell apart from XDH. However, the observation that XDH is found in the pigment cell granules has led to modification of this notion of protein interaction at the pigment cell membrane (see discussion above). We suggest that, under normal conditions, XDH binds specifically to the cell membrane. Defects in the transport proteins (Table 1) or alterations in the XDH molecule (allopurinol treatment, *ma-l* mutant) lead to failure of XDH to bind to the cell membrane. That XDH^- mutants do produce significant quantities of red pigment (Table 4) is taken to mean that significant quantities of xanthine and/or other possible precursors such as guanine or inosine are available from other sources.

Another issue is that mutants with brown pigment defects (*st, cn, v*) lack XDH in their eyes (Tables 1 and 2), yet have wild type levels of red pigments (Table 4). A simple explanation is apparent from the work of SHOUP (1966) and STARK and SAPP (1987). These investigators have shown that type II pigment granules, that are not supplied with a proper balance of pigment compounds, become autophagic type IV granules. Since a small number of type IV granules are seen, even in wild type eyes, a low level of autophagy is the norm, and imbalance of pigment levels is taken to enhance autophagy. Following this scenario, XDH contained in a granule that does not possess a proper pigment balance, is in a proteolytic environment and thus, the levels of XDH in brown pigment mutants are greatly diminished over time. It may be that XDH is capable of completing its function in red pigment synthesis before it is destroyed by proteolysis. Alternatively, a low level of XDH might persist that is sufficient to rescue *ry* function completely. However, when we examine XDH content in these mutants, it is reduced below a detectable level.

The autophagy model is consistent with the results from the TLC analysis of double mutants (Table 4). Double mutants, that lack XDH *and* are mutant as

well for *v, cn, st*, contain significantly less red pigment than flies which only bear a mutation at the *ry* gene. PARISI, CARFAGNA and D'AMORA (1976) also noted this effect. Normally the mutants that eliminate brown pigment synthesis do not have a significant effect on the red pathway. However, when combined with a mutant that partly blocks the red pathway (*i.e., ry*) these "brown pathway" mutants exhibit a more severe reduction in red pigment synthesis. Perhaps, the absence of brown pigments contributes to a proteolytic environment that generally reduces enzyme levels in type II granules. When residual amounts of XDH are removed from the granules, by way of a *ry* mutation, the reduced levels of other enzymes in the red pigment pathway result in less red pigment than the absence of XDH would otherwise bring about alone.

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