A THIRD LOCUS (*lpo*) AFFECTING PYRIDOXAL OXIDASE IN *DROSOPHILA MELANOGASTER*¹

JAMES F. COLLINS² AND EDWARD GLASSMAN³

*Department of Biochemistry and The Genetics Curriculum,
The University of North Carolina at Chapel Hill*

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XANTHINE dehydrogenase (XDH) of *Drosophila melanogaster* is controlled by at least three loci. Electrophoretic evidence (YEN and GLASSMAN 1965) indicates that *ry* (rosy eye color, 3–52.4), a mutant that lacks XDH (GLASSMAN and MITCHELL 1959), is the structural gene for this enzyme. Mutants of the other two loci, *ma-l* (maroon-like eye color, 1–64±) and *lxd* (low xanthine dehydrogenase, 3–33±), have 0 and 25% normal XDH activity, respectively (GLASSMAN and MITCHELL 1959; KELLER and GLASSMAN 1964), and are also deficient in pyridoxal oxidase (FORREST, HANLY and LAGOWSKI 1961; GLASSMAN *et al.*, 1964), and aldehyde oxidase (COURTRIGHT 1967). Table 3, below, shows the distribution of the enzyme activities in the various mutants. Other properties of this enzyme system have been summarized in a review by GLASSMAN (1965).

By assaying many strains, a new mutant *lpo* (low pyridoxal oxidase) has been found which affects only pyridoxal oxidase and may be a structural gene for pyridoxal oxidase. Some of these data have appeared elsewhere in preliminary form (GLASSMAN *et al.* 1968).

MATERIALS AND METHODS

Flies were raised in half-pint milk bottles or in 22 × 93 mm shell vials at 25 ± 1°C on a medium similar to one developed by Dr. E. B. LEWIS (see D.I.S. 34: 117, 1960) that contained approximately 0.8% agar, 5.2% dextrose, 2.6% sucrose, 8.5% cornmeal, 1.5% dried yeast, 0.04% propionic acid, 0.4% phosphoric acid and 0.019% benzyl benzoate in water.

The following mutants and strains were used (see BRIDGES and BREHME 1944): *v* (1–33.0), vermilion eye color; *f* (1–56.7), forked bristles; *Bx*³ (1–59.4), Beadex wing; *ma-l* (1–64±), maroon-like eye color; *ru* (3–0.0), roughoid eye; *lxd* (3–33±), low xanthine dehydrogenase; *by* (3–48.7), blistery wing; *st* (3–44.0), scarlet eye color; *ry* (3–52.4), rosy eye color; PAC, Pacific wild-type strain; OR, an Oregon-R wild-type strain; *Gl* (3–41.4), Glued eye shape; *Sb* (3–58.2), Stubble bristles; *H* (3–69.5), Hairless bristle mutant; *Payne*, inversion of the third chromosome, *Ly* (3–40.5), Lyra wing mutant; *LVM*, inversion of the third chromosome; *Cy*, Curly wing, associated with an inversion of the second chromosome; *h* (3–26.5), hairy, extra hairs on wings and abdomen; *se*^{51j}, an allele of *se*; *se* (3–26.0), sepia eye color; *Pm*, Plum eye color, associated with a second chromosome inversion; *ry*², an allele of *ry*; *cu* (3–50.0), curled

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wing; *cp* (3-45.3), clipped wing; *sr* (3-62.0), stripe, dark stripe along thorax; *e^s* (3-70.7), ebony-sooty body color. The strain *v f Bx^s ma-l* is referred to below as *ma-l*, *ru lxd* by as *lxd* and *st ry* as *ry*.

The *Cy;Pm/H;Sb* stock was obtained from the State University of New York at Stony Brook. The *Gl Sb H/Payne*, *Ly Sb/LVM*, *cu*, *cp*, *se^{51j}* and *se h* stocks were obtained from the Drosophila Stock Center at Bowling Green State University.

Preparation of extracts: 0.2 to 0.6 g of adult flies from 2 week old cultures were homogenized in 2 ml 0.1 M Tris-Cl, pH 8.0, in a 5 ml glass tissue grinder, and the homogenate was centrifuged at $30,000 \times g$ in a Servall refrigerated centrifuge for 25 min. The supernatant was added to 150 mg charcoal (Norit A, neutral, Fisher) in a chilled centrifuge tube and allowed to stand on ice for 1 hr with occasional stirring. The charcoal was removed by three centrifugations at $30,000 \times g$ for 10 min each. The supernatant solution was used as the enzyme source. In experiments where specified, 30 adults were homogenized in 2.1 ml 0.1 M Tris-Cl, pH 7.3, containing 1 mg/ml of bovine plasma albumin (Armour). The homogenate was added directly to 30 mg charcoal in a chilled centrifuge tube and treated as described above.

Enzyme assays: Pyridoxal oxidase was assayed by a modification of the method of HANLY (personal communication). To an $8 \times 8 \times 50$ mm cuvette was added 0.01 to 0.2 ml of extract and enough 0.1 M Tris-Cl, pH 7.3, containing 1 mg/ml albumin, to give a volume of 0.95 ml. The cuvette was incubated in a 30°C dry bath (Thermolyne Corporation) for four min and 0.05 ml of 2.67×10^{-3} M pyridoxal, prepared before the assay by diluting 4×10^{-2} M pyridoxal-HCl (Mann) 15-fold with 0.1 M Tris-Cl, pH 7.3, was then added. The cuvette was inverted to mix the contents, and the fluorescence was read every minute for four to six minutes in a No. 540 Photovolt fluorometer equipped with a 337 m μ primary filter (Photovolt) and a secondary filter composed of the three filters, Numbers 110-813, 110-814, and 110-816 from the G. K. Turner Co. When the extract of 30 flies was assayed, 0.05 ml pyridoxal was added to 0.95 ml extract and the fluorescence was read every other minute for 10 min. The fluorometer was adjusted so that a solution of 1.6×10^{-6} M quinine in 0.1 M sulfuric acid read 100 on the fluorometer scale. A change of one unit on the scale corresponds to the formation of approximately 100 μ moles of pyridoxic acid.

Aldehyde oxidase was assayed fluorometrically by following salicylic acid formation, or by the method of COURTRIGHT (1967) using dichlorophenolindophenol. For the former assay, 0.01 to 0.1 ml extract and 0.95 to 0.86 ml 0.2 M sodium phosphate, pH 6.75, containing 1 mg/ml albumin (final volume = 0.96 ml) were added to a cuvette in a 30°C dry bath. After 4 min, 0.04 ml of 5×10^{-3} M salicylaldehyde (Fisher) was added and the increase in fluorescence was followed every minute for 5 min in the fluorometer. The primary filter was a 340 m μ interference filter and the secondary filter a 449 m μ interference filter. The fluorometer was adjusted such that 5×10^{-7} M quinine in 0.1 M sulfuric acid read 55 on the fluorometer scale. A change of 1 on the scale corresponds to the formation of approximately 435 μ moles of salicylate.

XDH was assayed by a modification of the method of GLASSMAN (1962). Protein was estimated by a modification of the procedure of LOWRY *et al.* (1951).

RESULTS

Preliminary characterization of lpo: By assaying many strains for pyridoxal oxidase a *se* strain was found with a low level of this enzyme. The low level suggested that a biochemical defect related to sepia eye color had been found. However, two strains, *se h* and *se^{51j}*, had levels of pyridoxal oxidase activity comparable to that of wild-type flies. The *se* strain had normal levels of both XDH and aldehyde oxidase. Furthermore, the addition of *se* extract to a mixture containing pyridoxal oxidase from wild-type flies gave no inhibition of pyridoxal oxidase. Thus, extracts of the *se* strain did not contain an inhibitor of pyridoxal oxidase. The *se* strain was then crossed separately to the XDH mutants: *ry*, *ma-l*, and *lxd*.

The pyridoxal oxidase activity of the F_1 progeny was high suggesting that the gene in the *se* strain that affected pyridoxal oxidase activity might be distinct from the genes that affect xanthine dehydrogenase. To determine whether an entirely new locus had been found rather than a special allele of *lxd* or one of the other known genes, the factor causing low activity was localized. This factor was designated *lpo* (low pyridoxal oxidase) during the crosses that were carried out.

Chromosome location: To identify the linkage of *lpo*, the *se* strain was crossed to *Cy/Pm; H/Sb*. F_1 males carrying *Cy* and *Sb* were backcrossed to *se* females. As shown in Table 1, the presence of low activity in the $Cy; Sb^+$ and $Cy^+; Sb^+$ classes of the F_2 generation indicated that *lpo* is on the third chromosome.

Mapping of *lpo*: The preliminary localization of *lpo* was determined by crossing *se* females containing *lpo* to *Gl Sb H/Payne* males. The F_1 *Gl Sb H/se lpo* females were then mated to *se lpo* males and the F_2 generation was examined for flies containing crossovers in the three regions covered in the cross: *se-Gl*, *Gl-Sb*, and *Sb-H*. Since there was no assay of pyridoxal oxidase in single flies there was no way to distinguish *lpo* flies from lpo^+/lpo heterozygotes in the F_2 . However, an assay was developed that could make the distinction when more flies were used. Thus, F_2 males carrying crossovers in each of the three regions were individually mated to 3 *se lpo* females. An extract of 30 of their progeny which carried the chromosome containing the crossover was usually made.

It was possible to distinguish homozygous *lpo* from heterozygous lpo^+/lpo with as few as 10 flies. If at least 10 but not 30 F_3 flies carrying the crossover-containing chromosome were obtained from a cross, the number was made up to 30 with homozygous *se lpo* sibs to keep the amount of protein in the extract constant. Such addition did not interfere with the assay. In control experiments, extracts of 30 *se lpo* flies, which should have activity comparable to 30 F_3 progeny of an F_2 crossover-carrying male homozygous for *lpo*, caused the formation of 0 to 0.4 μ moles of pyridoxic acid in 10 min. Extracts of mixtures of 7 wild-type flies and 23 *se lpo* flies, that simulated the genetic composition of the F_3 progeny of an F_2

TABLE 1
Demonstration of the linkage group of lpo

Class	Pyridoxal oxidase	Enzyme activity Xanthine dehydrogenase	Salicylaldehyde oxidase	Aldehyde oxidase
<i>Cy; Sb</i>	0.93	154.7	21.14	32.3
<i>Cy; Sb⁺</i>	0	168.3	29.19	48.2
<i>Cy⁺; Sb</i>	1.19	153.6	28.10	40.0
<i>Cy⁺; Sb⁺</i>	0.06	172.7	32.80	55.7
PAC	1.68	126.3	16.97	27.7
<i>se lpo</i>	0	202.6	30.93	54.7

se lpo females were crossed to *Cy/Pm; H/Sb* males. The F_1 males heterozygous for *Cy*, *Sb*, and *lpo* were backcrossed to *se lpo* females. The four F_2 classes were separated and extracts of each class were made. In addition, extracts of PAC wild-type and *se* flies were used for comparison. The results are expressed as specific activity, i.e. enzyme units per mg per min. XDH activity is expressed as μ moles product formed, pyridoxal oxidase and salicylaldehyde oxidase as μ moles product formed. Aldehyde oxidase activity is expressed as decrease in optical density at 600 $m\mu$.

lpo⁺/*lpo* male, caused the formation of 0.8 to 2.5 mμmoles of pyridoxic acid. Thus when the F₃ flies were assayed, 0.5 mμmoles of pyridoxic acid formed was used as a cut-off point to distinguish whether the F₂ father was homozygous *lpo* or heterozygous *lpo*⁺/*lpo*.

The length of the chromosome covered in the cross, *se (lpo) × Gl Sb H/Payne*, was from *se* (3–26.0) to *H* (3–69.5); *lpo* was localized between *Gl* and *Sb*. Of the crossover chromosomes carrying *Gl*⁺ and *Sb*, 4 out of 65 carried *lpo*. Of those carrying *Gl* and *Sb*⁺, 20 out of 26 carried *lpo*. Since *Gl* is at 41.4 and *Sb* is at 58.2, *lpo* was placed at 56.4. In this cross there was a reduction in recombination in the *Gl-Sb* region (5.6% observed, 16.8% expected) and in the *se-Gl* region (0.68% observed, 15.4% expected); recombination was normal in the *Sb-H* region (12.6% observed, 11.3% expected).

To determine if the factor causing the reduction in recombination was in the *se* or in the *Gl Sb H/Payne* strain, a similar series of crosses using *Ly Sb/LVM* was made. On the basis of the analysis of 125 crossovers between *Ly* and *Sb*, *lpo* was mapped at 3–56.6. Recombination was also reduced in this cross in both the *Ly-Sb* (7.0% observed, 17.7% expected) and *se-Ly* regions. The factor causing the reduction in recombination thus seems to be in the *se* strain. The reduction in crossing over would cause *lpo* to be mapped to the left of its true position; the error would be slight since *lpo* is at the right end of the interval under consideration. *lpo* is thus placed at 57±.

Since the *ry* gene is also in the interval between *Gl* and *Sb*, a series of crosses was made to demonstrate recombination between *ry*² and *lpo*. *se lpo* females were mated to *st ry*² *sr e*^s males. The F₁ females were mated to *st ry*² *sr e*^s males, and in the F₂ generation, 11 *st ry*²/*st ry*² *sr e*^s males were selected, that contain crossovers between *ry*² and *sr*; some of the *st ry*² chromosomes should contain *lpo*. The males were individually mated to *se lpo* females, and 10 of the sons from each cross were individually mated to *se lpo* females. By the last cross those *st ry*² chromosomes that carried *lpo* would be made homozygous for *lpo*. Thirty of the progeny were analyzed for pyridoxal oxidase. The data showed that 4 of the 11 crossovers between *ry*² and *sr* were also crossovers between *ry*² and *lpo*. On the basis of a map-position of 57±, 5 out of 11 would be expected.

Dosage: ry, the likely structural gene for XDH, exerts a dosage effect on that enzyme (GRELL 1962; GLASSMAN, KARAM, and KELLER 1962). To test the dosage effects of *lpo* on pyridoxal oxidase, *lpo* females were mated to *cp* males and the F₁ males crossed to *cp* females. The F₂ progeny were separated into normal wing (*cp*⁺ *lpo*/*cp lpo*⁺) and clipped wing (*cp lpo*⁺-*cp lpo*⁺) phenotypes, extracts of flies of the two classes were prepared and assayed for pyridoxal oxidase. Similar crosses were made using *cu* instead of *cp*. In four such experiments the heterozygotes (*lpo*/*lpo*⁺) had an average of 56% of the specific activity of the homozygotes (*lpo*⁺/*lpo*⁺). Thus a dosage effect is exhibited. One such experiment is shown in Table 2.

DISCUSSION

In this report we describe a mutant, *lpo*, that causes flies to have a low level of

TABLE 2

The dosage effect of lpo on pyridoxal oxidase activity

Class	Pyridoxal oxidase	Enzyme activity Xanthine dehydrogenase	Salicylaldehyde oxidase
1. a. <i>cp lpo</i> ⁺ / <i>cp</i> ⁺ <i>lpo</i>	0.74	100.8	17.23
b. <i>cp lpo</i> ⁺ / <i>cp lpo</i> ⁺	1.51	108.2	16.49
2. a. <i>cp lpo</i> ⁺ / <i>cp</i> ⁺ <i>lpo</i>	0.98	125.0	20.14
b. <i>cp lpo</i> ⁺ / <i>cp lpo</i> ⁺	1.58	104.1	16.96
OR	1.45	89.5	17.57
<i>cp</i>	2.02	139.0	15.79
<i>se lpo</i>	0	164.6	22.53
<i>ma-l</i>	0	0	0

lpo females were mated to *cp* males, then the F_1 heterozygous *cp/lpo* males were crossed to *cp* females. The two F_2 classes were separated and two extracts were made of each class as well as one extract of standard strains for comparison. The activities are expressed in the same way as in Table 1. The enzyme activity of heterozygous *lpo*⁺/*lpo* was taken as the average of Nos. 1a and 2a and of homozygous *lpo*⁺/*lpo*⁺ as the average of Nos. 1b and 2b. In this experiment the heterozygotes had 56% of the specific activity of pyridoxal oxidase activity of the homozygotes.

pyridoxal oxidase. This is the third locus that is known to affect this enzyme in *D. melanogaster*. *lpo* has been mapped on the third chromosome at $57 \pm$ and is thus between *ry* at 52.4 and the locus homologous to the *aldox* gene of *Drosophila simulans* at $64 \pm$. There was some difficulty in localizing *lpo* since crossover reduction was observed in the left arm of the third chromosome, but the problem is minimized by the proximity of *lpo* to the *Sb* marker on the right arm of the third chromosome, and by the demonstration of crossing over between *ry*² and *lpo*.

lpo may be a structural gene for pyridoxal oxidase. The method of choice to determine this is the mapping of gene(s) responsible for electrophoretic variants of pyridoxal oxidase. No electrophoretic variants of this enzyme are available. *lpo* does exert dosage effects on pyridoxal oxidase. This is one indication that *lpo* may be a structural gene. However, other data indicate that dosage effects are not necessarily confined to structural genes. For example, *lxd* also exerts a dosage effect on pyridoxal oxidase (50% activity in heterozygous *lxd*⁺/*lxd* compared to homozygous *lxd*⁺/*lxd*⁺) as well as on aldehyde oxidase (about 55% activity in the heterozygote). We do not believe that *lxd* is a structural gene for these enzymes.

At least two ideas have been presented which attempt to explain the relationship of the *ry*, *ma-l*, and *lxd* genes to XDH and pyridoxal oxidase. These theories include the polypeptide subunit theory and the co-factor theory (GLASSMAN 1965). The polypeptide subunit theory proposes that *ry*⁺, *ma-l*⁺ and *lxd*⁺ each code for a polypeptide; these combine in certain ways to produce XDH and pyridoxal oxidase. XDH contains all three types of subunits while pyridoxal oxidase consists of subunits coded by the *ma-l*⁺ and *lxd*⁺ genes. The new locus *lpo* would fit into this theory, if we assume that pyridoxal oxidase also contains subunits coded by *lpo*⁺. Thus pyridoxal oxidase and XDH would each have

TABLE 3

The enzymes of the XDH system and their distribution in various mutants

Enzyme	Mutant:	Wild type	<i>ry</i>	<i>lxd</i>	<i>ma-l</i>	<i>lpo</i>
Xanthine dehydrogenase		normal	0	25%	0	normal
Pyridoxal oxidase		normal	normal	<5%	0	2%
Aldehyde oxidase		normal	normal	5-10%	0	normal

unique subunits, i.e. those coded by *lpo*⁺ and *ry*⁺, respectively, as well as two other subunits in common controlled by *ma-l*⁺ and *lxd*⁺. The co-factor theory proposes that XDH and pyridoxal oxidase have separate structural genes while the products of the *ma-l* and *lxd* loci are involved in the formation of a co-factor necessary for the activity of both enzymes. *ry* would be the structural gene for XDH; *lpo* would now be the structural gene for pyridoxal oxidase. Thus *lpo* would exert a dosage effect only on pyridoxal oxidase, since the amount of structural polypeptide present would be a limiting factor in enzyme formation. If the amount of co-factor is also a limiting factor in the production of active enzyme, a dosage effect of *lxd* might be expected. The current knowledge about the XDH system favors the latter hypothesis (see GLASSMAN *et al.* 1968).

Four of the genes affecting XDH, pyridoxal oxidase and aldehyde oxidase, are on the third chromosome. These are *lxd*, *ry* and *lpo* in *D. melanogaster*, and *aldox* in *D. simulans*. We have recently found that another phenotype, low aldehyde oxidase, is also due to a mutant on the third chromosome. Preliminary data indicate that it is not allelic to *aldox*. From electrophoretic evidence, *aldox* is probably a structural gene for aldehyde oxidase in *D. simulans* (COURTRIGHT 1967). The homologous locus in *D. melanogaster* is at 64±. Since *ry* is at 52.4 and *lpo* at 57±, there are three genes, which are probably structural genes, within 15 map units. The enzymes coded by these three genes have an overlapping genetic control; *ma-l* and *lxd* affect all three enzymes. Since the sizes of the three enzymes are approximately the same (GLASSMAN *et al.* 1966; COURTRIGHT 1967) and since the reactions they catalyze are chemically similar, it is possible to speculate that these genes may have arisen through duplication of a common ancestral gene, followed by mutation. Alternatives to this hypothesis have been considered, but at the present time are excessively speculative.

The levels of activity of the enzymes in the mutants of the XDH system are shown in Table 3; "normal" refers to the level of activity in wild-type strains.

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SUMMARY

Two mutants of *D. melanogaster*, *ma-l* and *lxd*, have deficiencies of both xanthine dehydrogenase and pyridoxal oxidase. A third locus, *lpo* (low pyridoxal oxidase), which affects only pyridoxal oxidase, is described in this paper. Homo-

zygous *lpo* flies have approximately 2% the activity of wild type flies. *lpo* has been mapped at $3-57\pm$. It is postulated that *lpo* is a structural gene for pyridoxal oxidase, and because *ma-l* and *lxd* are deficient in pyridoxal oxidase, xanthine dehydrogenase, and aldehyde oxidase, it is postulated that a co-factor necessary for the activities of all three enzymes is lacking in these flies.

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