POLYGENIC CONTROL OF ALDEHYDE OXIDASE IN DROSOPHILA[1]

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Received February 20, 1967

WHEN extracts of wild-type Drosophila are electrophoresed on agar gels and the gels stained with a tetrazolium solution containing benzaldehyde, a formazan band is formed at a location not identical to the xanthine dehydrogenase (XDH) band (COURTRIGHT 1966a). This was a surprising observation since benzaldehyde has been reported to serve as a substrate for XDH (GLASSMAN and MITCHELL 1959a). Since our new band is detected in extracts of *ry* flies, but not *ma-l* flies, the evidence suggested that benzaldehyde was serving as a substrate for pyridoxal oxidase, a possibly related enzyme which also is present in *ry* and absent in *ma-l* flies (FORREST, HANLY and LAGOWSKI 1961). The present paper presents genetic and biochemical evidence that the enzyme in question is neither pyridoxal oxidase nor XDH, but an aldehyde oxidase under similar genetic control. The enzyme also has some properties in common with the so-called *ma-l*$^+$ factor (GLASSMAN 1965, 1966), although its identity to the *ma-l*$^+$ complementing factor is questionable.

MATERIALS AND METHODS

General Procedures: All flies were grown on the cornmeal-agar medium described by GLASSMAN, KARAM and KELLER (1962), at 25°C. After sterilization of the food bottles, 0.5 ml of 0.018% folic acid (Sigma, neutralized with NaOH) in 20% ethanol was applied to each.

All solutions were prepared using glass distilled water. Homogenates were prepared, stored, and processed at temperatures between 0 and 5°C.

For developmental studies eggs were collected using the method of MITCHELL and MITCHELL (1964). Eggs and larvae were homogenized separately in an equal volume of 0.3 M KH_2PO_4—K_2HPO_4, 0.001 M EDTA[3] buffer pH 7.5 in a 2 ml Duall glass homogenizer.

Genetic stocks: The following stocks were used: (a) *Drosophila melanogaster.* Beadex[3] (*Bx*^{3}) 1–59.4, blistery (*by*) 3–48.7, forked (*f*) 1–56.7, low xanthine dehydrogenase (*lxd*) 3–33 (KELLER and GLASSMAN 1964), maroon-like (*ma-l*) 1–64.9 (GLASSMAN 1959), maroon-like^{bronzy} (*ma-l*^{bz}), rosy (*ry*) 3–52.35 (CHOVNICK *et al.* 1964), rough (*ru*) 3–01, vermilion (*v*) 1–33.0, yellow (*y*) 1–0.0, *ru lxd by* (referred to as *lxd*), *ru lxd by ry* (referred to as *lxd ry*), *v f Bx*^{3} *ma-l*; *ru lxd by ry* (referred to as *ma-l*; *lxd ry*), *yf* attached-X, and Oregon-R (OreR) wild type. Unless indicated, map positions are taken from BRIDGES and BREHME (1944).

(b) *Drosophila simulans.* aldehyde oxidase^I (*aldox*^{I}) a fast anodally migrating aldehyde oxidase contained in *jv st pe* and *pe H*^{h}; aldehyde oxidase^{II} (*aldox*^{II}) a slower anodally migrating aldehyde oxidase than aldox^1 present in wild type, from Lima, Peru; javelin (*jv*) 3–0.0 and

[1] This work was supported by Public Health Service Grants GM-504-07 and 1-F1-GM-33, 779-01, and National Science Foundation grant GB-4451.

[2] This work has been presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Abbreviations used in this paper: DCIP—dichloroindophenol, DEAE—diethylaminoethyl, EDTA—ethylenediamine tetraacetic acid, NAD—nicotinamide adenine dinucleotide, NBT—nitro blue tetrazolium, PMS—phenazine methosulfate, Tris—tris(hydroxymethyl)aminomethane.

peach (*pe*) 3–101.0 (STURTEVANT and PLUNKET 1926); Hairy^{hairless} (H^h) 3–61.0 and scarlet (*st*) 3–40.0 (STURTEVANT and NOVITSKI 1941).

Spectrophotometic assays: All assays were performed with a Ziess M4 QIII spectrophotometer equipped with a Gilford multiple sample absorbance recorder. All buffers used for spectrophotometic assays were prewarmed to 30°C. The reaction mixtures for the various enzyme reactions are listed below.

Aldehyde oxidase: The following modification of the flavin-linked aldehyde oxidase assay of MAHLER (1955) was used: 0.03 ml of 1 M redistilled acetaldehyde (Eastman), 0.02 ml of 2 mg/ml PMS (Sigma), 0.20 ml of 0.01% aqueous dichloroindophenol (Mann Research Lab), 0.10 ml of enzyme solution and water, 0.65 ml of 0.5 M KH_2PO_4—K_2HPO_4, 0.001 M EDTA pH 7.5 containing 0.1% bovine serum albumin (Sigma). Under these conditions the enzyme activity is linear with time and enzyme concentration, provided that the assay mixture does not contain more than 0.25 units of enzyme. One unit is defined by MAHLER (1955) at that amount of enzyme causing a decrease of 1.00 OD_{600} per minute at 30°C. In this paper specific activity is units per mg protein.

Catalase: Catalase was assayed using a solution consisting of 0.95 ml of 0.06% H_2O_2 in 0.01 M KH_2PO_4—K_2HPO_4 pH 7.5 and 0.05 ml of enzyme and water. One unit is defined by decomposition of one micromole of hydrogen peroxide per minute. This corresponds to an optical density decrease of 0.043 per minute at 240 mμ.

Preparation of aldehyde oxidase: Single-fly homogenates. For genetic analysis, it was necessary to detect the enzyme in single flies. Routinely, a single fly was homogenized in .003 ml of 0.1 M Tris-HCl, 0.001 M EDTA buffer, pH 8.0 containing 0.1% bovine serum albumin. The homogenizer vessels were round bottom 0.5 ml glass tubes (6 × 50 mm) for which soft glass rods had been shaped and ground with carborundum powder for a good fit. The homogenates were centrifuged at room temperature in a "hematocrit" centrifuge at 12,000 × *g* for 3 minutes. The supernatant solutions were used for electrophoresis.

Preparation of mass homogenates. Two procedures, 1 and 2, were used. (1) Homogenates were prepared by adding 1 ml of 0.1 M Tris-HCl, 0.001 M EDTA, pH 8.0 for each 400 mg of freshly etherized flies. The resulting mixture was homogenized in a Ten Broeck homogenizer and then centrifuged at 48,000 × *g* in a Servall RC-2B refrigerated centrifuge. Alternatively, the homogenization may be done in 0.3 M KH_2PO_4—K_2HPO_4, 0.001 M EDTA, pH 7.5. Such extracts retain aldehyde oxidase activity for several days if stored at 4°C.

(2) Homogenates were prepared by adding 1 ml of 0.1 M Tris-HCl, 0.001 M EDTA, pH 8.0 for each 400 mg of freshly etherized flies. The resulting mixture was homogenized in a Servall Omnimixer operated at full speed for 15 seconds. To the resulting homogenate, 1 M $MnCl_2$ was added dropwise with stirring to a final concentration of 0.05 M to precipitate nucleic acids (SMITH and YANOFSKY 1962). The resulting solution was homogenized for an additional 15 seconds at full speed in the Omnimixer, allowed to sit at 0°C for 2 to 3 hours, and then centrifuged at 48,000 × *g* for 20 minutes in a Servall RC-2B refrigerated centrifuge. The resulting homogenate was either assayed directly or subjected to further purification as described below.

Purification of aldehyde oxidase: The pale yellow supernatant solution from the $MnCl_2$ precipitation step was applied to a 2.5 × 45 cm column of G-25 Sephadex (coarse), equilibrated with 0.1 M Tris-HCl, 0.001 M EDTA, pH 8.0 in order to remove eye pigments. The pale yellow fraction which came off in the void volume of 75 ml had an OD_{280}/OD_{260} ratio of 1.2, and possessed all of the applied aldehyde oxidase activity. For each ml of this fraction, 313 mg of solid, finely ground ammonium sulfate (Baker) recrystallized from 0.001 M EDTA was added with constant stirring. The final pH was adjusted to 7.1 by dropwise addition of 28% ammonium hydroxide (Baker). The resulting solution was stirred at 4°C for one hour, then centrifuged at 48,000 × *g* for 10 minutes and the precipitate discarded. An additional 214 mg $(NH_4)_2\ SO_4$ was added per ml solution, the pH adjusted to 7.1 by dropwise addition of NH_4OH as before. The resulting precipitate, was dissolved in distilled water to a final concentration of 12 mg protein/ml and dialyzed overnight against 200 volumes of 0.01 M KH_2PO_4—K_2HPO_4, 0.001 M EDTA, pH 7.2. The dialyzed sample (110 mg protein/9.2 ml) was applied to a 1.5 × 25 cm column of DEAE-cellulose (Whatman, 1.0 milliequivalents/g) prepared by the methods of

PETERSON and SOBER (1962), and was eluted with a liner gradient of KH_2PO_4—K_2HPO_4, 0.001 M EDTA, pH 7.1 from 0.01 to 0.30 M (total volume equals 360 ml), at a flow rate of 0.32 ml/minute. A volume of approximately 0.1 ml of each 5 ml fraction was applied to the wells of a spot plate containing 0.1 ml of the DCIP assay solution. Fractions containing aldehyde oxidase were detected visually by the disappearance of the blue color of DCIP. The fractions were then assayed for aldehyde oxidase activity. Those fractions with a specific activity greater than 12.00 and a total activity greater than 5.40 units/ml were combined and 561 mg of $(NH_4)_2SO_4$ were added for each ml. The pH was adjusted to 7.2 with NH_4OH as before. The precipitate was dissolved in distilled water and dialyzed overnight against 200 volumes of 0.025 M Tris-H_3PO_4, 0.001 M EDTA, pH 9.0. The dialyzed sample was applied to a 1.5 × 25 cm DEAE-cellulose column equilibrated with 0.025 M Tris-H_3PO_4, 0.001 M EDTA, pH 9.0. The aldehyde oxidase was eluted with a linear gradient from 0.025 M to 1.0 M Tris-H_3PO_4, 0.001 M EDTA, pH 9.0 (total volume equals 400 ml) at a flow rate of 0.32 ml/minute. Five-ml fractions were assayed for aldehyde oxidase activity and those fractions with a specific activity greater than 68.00 and a total activity greater than 4.60 units per ml were concentrated to a volume of 1 ml using suction filtration through an 8-ml collodion bag (Schleicher and Schuell). Approximately 0.2 g sucrose (Baker) was added for each ml, and a total of 925 μg protein contained in 1 ml was applied to a 2.5 × 32 cm column of Sephadex G-150 equilibrated with 0.01 M KH_2PO_4—K_2HPO_4, 0.001 M EDTA, pH 7.2. The enzyme was eluted with the same buffer at a flow rate of 0.45 ml/minute. The 5-ml fractions were analyzed for enzymatic activity by the spectrophotometric assay. The fractions with a specific activity of 340 or greater were pooled and found to be stable at 4°C for at least three weeks. This procedure gives a 200-fold purification from crude extracts, with 20% recovery (Table 1).

Electrophoretic technique: Electrophoresis of extracts was performed as described by URSPRUNG and LEONE (1965), except that "Agarose" (Nutritional Biochemicals), was substituted for agar in the preparation of the gels.

Immunochemical procedures: Antibodies to aldehyde oxidase were elicited by injecting rabbits supra-scapularly with a total of 128 units of aldehyde oxidase, specific activity 411, in Freund's complete adjuvant (Difco) (COHN 1952). Three weeks later the rabbits were injected with the same enzyme preparation emulsified with Freund's incomplete adjuvant. The rabbits were bled 6 to 8 days after the second injection. The gamma globulin was prepared by the method of KEKWICK (1941), except that a 15–18% sodium sulfate cut of whole serum was used, (D. B. ROBERTS, personal communication).

Ouchterlony plates were prepared using a 0.7% agarose solution in 1/20 EBT buffer (URSPRUNG and LEONE 1965). Plates with a well-spacing of 4mm were developed overnight at 37°C, and uncomplexed antigen was washed out by several changes of 0.1 M Tris HCl, 0.001 M EDTA buffer, pH 8.0 over 12 hours. Aldehyde oxidase precipitin lines were detected by staining plates in a solution containing 10 mg NBT (Dajac), 0.4 mg PMS (Sigma), 2 ml acetaldehyde (Eastman) or 0.5 ml benzaldehyde (Fisher) in 25 ml 0.2 M Tris HCl buffer, pH 8.0.

TABLE 1

Purification of aldehyde oxidase

Step	Total ml	Units/ml	Total units	mg protein/ml	Specific activity	Recovery	Purification ×
Crude	34.0	48.0	1632	21.6	2.22	100	1
$MnCl_2$	30.5	120.0	3660	16.0	7.50	225	3.4
G-25	51.0	75.0	3825	8.0	9.38	234	4.2
50–80% $(NH_4)_2SO_4$	9.2	126.0	1160	12.0	10.50	71	4.7
DEAE pH 7.2	51.0	24.0	1225	0.68	35.00	75	15.7
DEAE pH 9.0	66.3	11.74	778	0.07	165.00	47	74.0
G-150	15.3	22.60	345	0.057	396.00	21	178.0

To titrate a given number of units of enzyme, 0.05 ml of enzyme was incubated with varying amounts of antibody to a final volume of 0.10 ml. This solution was incubated at 4°C overnight and the complexed enzyme was removed by centrifugation at 10,000 × *g* for 20 minutes at 4°C. Aliquots of the supernatant solution were then assayed for enzyme activity.

Protein determinations: Protein in crude extracts was determined by the method of LOWRY *et al.* (1951) using bovine serum albumin (Sigma) as a standard. Protein in effluent column fractions was determined by the ultraviolet spectrophotometric method of WARBURG and CHRISTIAN (1941).

Sucrose density centrifugation: Crude extracts of *ry* flies were centrifuged on 5–20% linear sucrose gradients by the method of MARTIN and AMES (1961). Gradients were generated simultaneously in a 20 ml gradient generator (Buchler) and 4.6 ml was collected in each of three 1.27 × 5.08 cm cellulose nitrate tubes, and 0.10 ml of enzyme was carefully layered onto each gradient. To two gradients, 20 units of aldehyde oxidase was applied. Since crude Drosophila extracts contain catalase, 110 units of bovine catalase (Worthington) was applied separately to the third gradient. The tubes were placed in a SW-50L rotor and centrifuged at 39,000 rpm for 12 hours in a model L-2 centrifuge at 4°C. The tubes were punctured with a piercing unit (Buchler) and ten-drop fractions were collected. At least 80% of all of the applied enzyme activity was recovered.

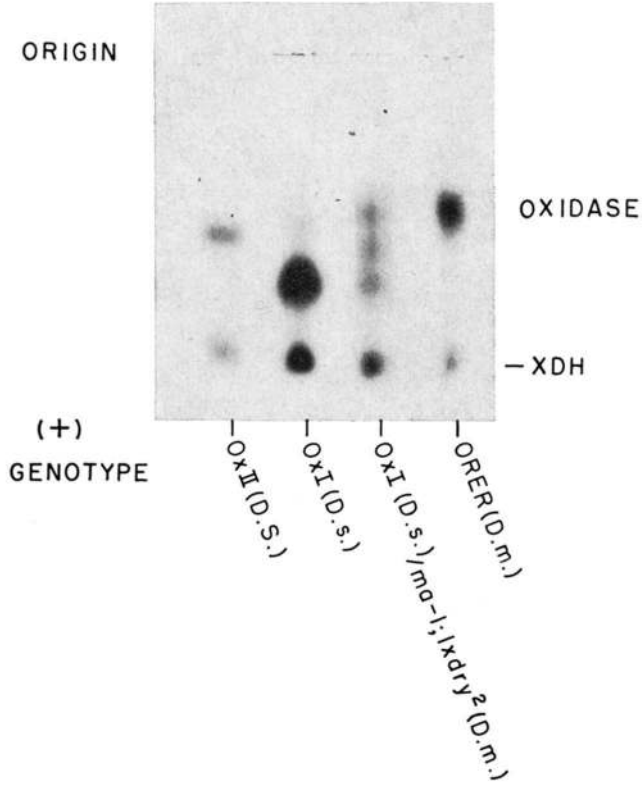

FIGURE 1.—Simultaneous detection of aldehyde oxidase and xanthine dehydrogenase. Extracts of the various genotypes were prepared by method (1) and electrophoresed on agarose gels for 20 minutes at 31 v/cm and 25 ma at 5–15°C. Gels were stained for XDH activity by incubating with 7 mg hypoxanthine (Sigma), 100 mg NAD (Sigma), and the concentrations of PMS, NBT, and Tris buffer given in METHODS. Aldehyde oxidase was detected by staining as in METHODS.

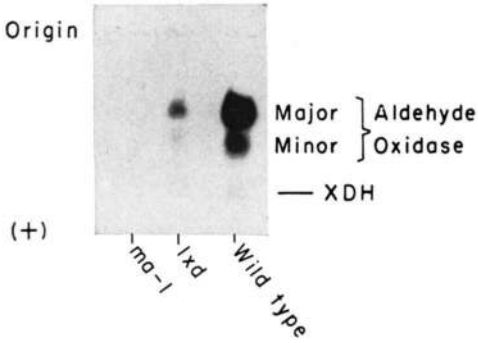

FIGURE 2.—Detection of aldehyde oxidase isozymes of *D. melanogaster* on agarose gels. Mass homogenates of *ma-l*, *lxd*, and wild type were prepared by method (1). The gels were electrophoresed 15 minutes at 31 v/cm and 25 ma at 5–15°C. Aldehyde oxidase activity was detected by immersing the gels in staining solution for one hour.

RESULTS

Identification of aldehyde oxidase by gel electrophoresis: When wild-type extracts of *D. melanogaster* and *D. simulans*, are electrophoresed and simultaneously stained for aldehyde oxidase and xanthine dehydrogenase, two zones of formazan deposition are detected (Figure 1). The faster migrating of the two bands was identified as XDH since it is absent when *ry* extracts are electrophoresed or when hypoxanthine is omitted from the staining solution. The slower migrating component is aldehyde oxidase. It is present in wild-type and *ry* extracts when acetaldehyde or benzaldehyde is supplied as substrate. The independence of aldehyde oxidase and XDH is further indicated by electrophoretic differences in the mobility of aldehyde oxidase in both these two species and their hybrids which are not correlated with equivalent differences in the mobility of the XDH molecules (Figure 1).

When more concentrated extracts are stained, a minor component of aldehyde oxidase is seen on zymograms in addition to the major component (compare Figures 2 and 3). This minor component is under the same genetic control as is

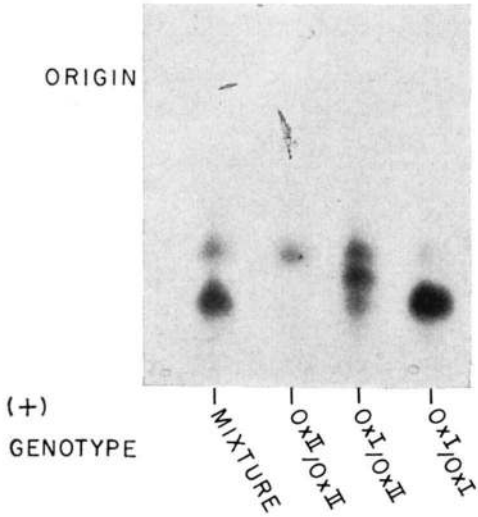

FIGURE 3.—Electrophoretic variants of aldehyde oxidase in *Drosophila simulans*. Extracts of the various genotypes were prepared by method (1) and electrophoresed 20 minutes at 31 v/cm and 25 ma at 5–15°C. Gels were stained for aldehyde oxidase.

TABLE 2

Relative rates of reaction of aldehyde oxidase with various substrates†*

Substrate	Acetaldehyde	Benzaldehyde	Dimethylamino-benzaldehyde	Pyridoxal	Blank
Relative rate	100‡	218	108	<1	<1

* 200-fold pure aldehyde oxidase (see METHODS).
† Reaction rate is the initial rate with excess enzyme in the presence of 0.25 micromoles of each of the substrates in a total volume of 1.0 ml.
‡ 100 under these conditions equals an OD_{600} decrease of 0.08/minute.

the major component (Figure 2). The absence of the major component in *ma-l* and the small amount present in *lxd* flies is paralleled by the absence of pyridoxal oxidase in these genotypes (GLASSMAN *et al.* 1964). However, the major component is not pyridoxal oxidase since extracts purified 200-fold with respect to aldehyde oxidase possess only the major component and react with several aldehydes but not pyridoxal (Table 2). In these extracts there is a component which reduces the tetrazolium and which migrates to the same position as XDH (Figure 2). This component may be XDH since it is absent in *ma-l* extracts.

Genetic control of aldehyde oxidase: The deficiencies in aldehyde oxidase activity in *ma-l* or *lxd* extracts suggested that either the $ma\text{-}l^+$ or lxd^+ locus was the structural gene for aldehyde oxidase. There are three lines of evidence which indicate the polygenic control of this enzyme.

a. *Genetic mapping of aldehyde oxidase.* Although no electrophoretic variants of aldehyde oxidase have been found in more than 25 strains of *D. melanogaster* examined, such variants were found in *D. simulans.* (Figure 3). Reciprocal crosses of *jv st pe* ($aldox^I$) × $aldox^{II}$ established that the gene was not sex linked. The backcross recombinants, + *st pe*/*jv st pe*, were homozygous for $aldox^I$, indicating that the aldehyde oxidase gene was located in the *st pe* region (COURT-

TABLE 3

Crosses of $aldox^{II} \times pe\ H^h$

Generation	Genotype	Number of flies of aldox type		
		I	II	I/II*
P	*pe* ($aldox^I$) H^h	13	0	0
P	+ ($aldox^{II}$) +	0	13	0
F_1	*pe* ($aldox^I$) H^h/+($aldox^{II}$) +	0	0	20
Backcross	*pe* ($aldox^I$) H^h/*pe* ($aldox^I$) H^h	9	0	0
	pe ($aldox^I$) H^h/+ ($aldox^{II}$) +	0	0	8
	pe ($aldox^I$) +/*pe* ($aldox^I$) H^h	29	0	0
	pe ($aldox^{II}$) +/*pe* ($aldox^I$) H^h	0	0	63
	+ ($aldox^I$) H^h/*pe* ($aldox^I$) H^h	69	0	0
	+ ($aldox^{II}$) H^h/*pe* ($aldox^I$) H^h	0	0	38

* I/II type is the $aldox^I/aldox^{II}$ heterozygote (Figure 3).

Number of crossovers between *pe* and *aldox*	132
Number of crossovers between *aldox* and H^h	67
Percent crossing over between *aldox* and H^h	34
Map location	74.5

RIGHT 1966b). To map the *aldox* locus, *pe* H^h (*aldox*^{I}) was crossed to *aldox*^{II} flies, and the F_1 heterozygous females were crossed to *pe* H^h males. All parents were routinely checked for their *aldox* genotype after successful mating. The analysis of the resultant backcross and recombinant progeny (Table 3) indicates that the *aldox* locus is on the *D. simulans* third chromosome at 74.5.

On the basis of these mapping experiments, the homologous *D. melanogaster* *aldox* locus should be located at about 64 on the third chromosome. Two mutants located in this chromosomal region, glassy (63.1) and Henna^{recessive} (64.5) were tested and found negative for deficiencies or altered mobility of aldehyde oxidase molecules. However, the existence of a separate *aldox*$^+$ locus in *D. melanogaster* was confirmed by crossing *ma-l*; *lxd ry* (*D. melanogaster*) females × *aldox*^{I} (*D. simulans*) males. The aldehyde oxidase zymogram obtained from hybrid progeny (Figure 4) is similar to the simple *aldox*^{I}/*aldox*^{II} hybrid zymogram. The position of the slowest aldehyde oxidase band in the interspecies hybrid is the same as that in OreR and shows that there is a functional *aldox*$^+$ locus in *ma-l*; *lxd ry* flies.

Although the aldehyde oxidase structural gene is not allelic to either *ma-l* or *lxd*, both of these loci affect this enzyme (Figure 2). When partially purified extracts of *ma-l* are eluted from a column of G150 Sephadex (Figure 5) no aldehyde oxidase activity is found in any of the fractions, whereas in *lxd* extracts there is a small amount of aldehyde oxidase activity which has the same molecular size as the wild-type aldehyde oxidase (Figure 5).

b. *Aldehyde oxidase in* lxd *flies.* Aldehyde oxidase in *lxd* extracts resemble the wild-type enzyme in size and electrophoretic properties (Figures 2, 5). Its

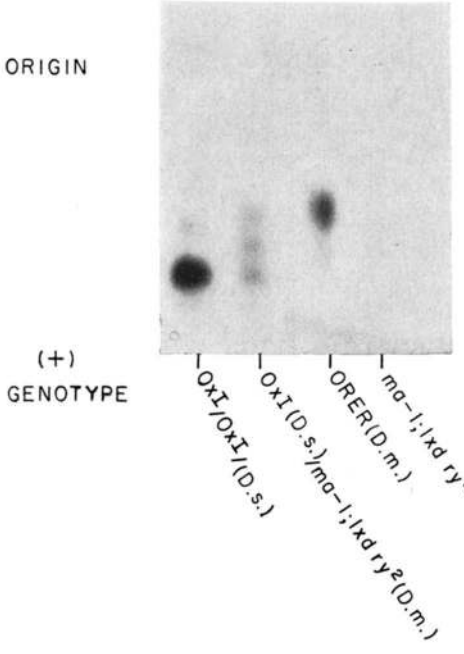

FIGURE 4.—Aldehyde oxidase in species hybrids. Extracts of *D. simulans*, *D. simulans*/*D. melanogaster* (*ma-l*; *lxd ry*), and *D. melanogaster* were prepared by method (1), and electrophoresed 20 minutes at 31 v/cm and 25 ma at 5–15°C. Gels were stained for aldehyde oxidase.

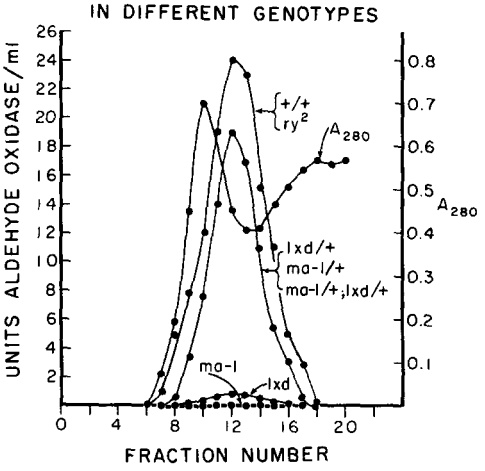

FIGURE 5.—Comparison of aldehyde oxidase activity in various genotypes. Homogenates (5 ml) of various genotypes were prepared by method (2) and were partially purified by eluting from a 2.5 × 32 cm column of Sephadex G-150 and 0.01 M KH_2PO_4–K_2HPO_4, 0.001 M EDTA, pH 7.2 at a flow rate of 0.45 ml/minute. Individual 5-ml fractions were collected and assayed for aldehyde oxidase activity.

lowered amount in *lxd* extracts may be the result of either fewer wild-type molecules or an equal number of catalytically altered molecules. To discriminate between these two alternatives, *lxd* extracts were qualitatively assayed for aldehyde oxidase cross reacting material. (CRM). When approximate equivalence concentrations of antibody are allowed to diffuse against crude extracts of wild type and *lxd*, it is seen that the aldehyde oxidase precipitin line is shifted in favor of antibody excess (Figure 6). According to CROWLE (1961), such a result is to be interpreted as indicating that fewer aldehyde oxidase molecules are produced in *lxd* flies.

In *lxd*/+ heterozygotes, only about 60% of wild-type levels of aldehyde oxidase are produced (Figure 5). To determine if the lower activity was due to a catalytically defective enzyme, equal units of *lxd*/+ and wild-type aldehyde oxidase were titrated against antibody. For both wild-type and *lxd*/+ aldehyde oxidase, an equal amount of antibody is required to precipitate an equal number of enzyme units (Table 4). This result agrees with the observations made on Ouchterlony plates and provides further support for the assumption that the *lxd* locus controls the level of aldehyde oxidase in the fly.

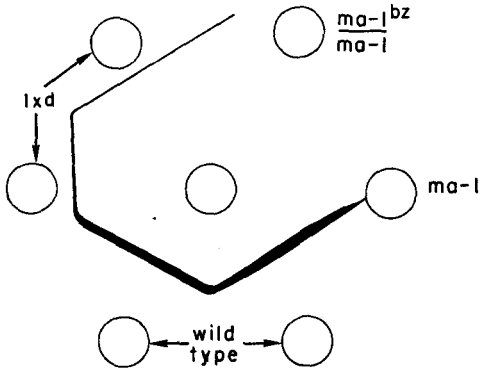

FIGURE 6.—Precipitation of *lxd* aldehyde oxidase on Ouchterlony plates. Crude extracts of various genotypes were prepared by method (1) and 0.03 ml applied to each well as indicated. The center well contained 0.03 ml of antibody diluted to approximate equivalence against wild-type aldehyde oxidase. The gels were developed and stained for aldehyde oxidase activity as in METHODS.

TABLE 4

Precipitation of aldehyde oxidase by antibodies

Microliters antibody	Percent activity remaining in solution		
	ry/ry	*lxd/+*	*ma-l+*
0*	100†	100	100
3	65	63	63
4	20	28	51
5	0	0	29
6	0	0	29
7	0	0	14
8	0	0	9
9	0	0	0

* Gamma globulin prepared from nonimmune rabbits, even when used at 40-fold greater concentrations, did not inhibit or precipitate aldehyde oxidase.
† 100 here equals 10 units of aldehyde oxidase activity per ml.

c. *Lack of aldehyde oxidase in* ma-l *flies.* In *ma-l* flies, no aldehyde oxidase has been detected either on electropherograms or in eluant fractions from G150 Sephadex (Figures 2, 5). When *ma-l* crude extracts are allowed to diffuse against approximate equivalance concentrations of aldehyde oxidase antibody no displacement of the adjacent wild-type aldehyde oxidase precipitin line is observed (Figure 6). Furthermore, when antibody is first incubated with *ma-l* or *ma-l/ma-l^{bz}* extracts and allowed to diffuse against aldehyde oxidase, the staining intensity of the resulting aldehyde oxidase precipitin line is equal to that of unabsorbed antibody (Figure 7). This result shows that there is no or little aldehyde oxidase CRM in *ma-l* extracts. The amount of aldehyde oxidase in *ma-l/+* heterozygotes is about 60% of wild-type levels (Figure 5). However, the *ma-l/+* extract was more effective in precipitating aldehyde oxidase antibodies (Table 4). This might be interpreted as either a lower affinity of a presumed hybrid enzyme for the antibody or a mixture of enzymatically active and inactive molecules in this heterozygote. We have not yet obtained data to decide between these two alternatives.

Although electrophoretic forms of aldehyde oxidase which are allelic to *ma-l* have not been detected, the absence of aldehyde oxidase CRM in *ma-l* stocks suggests that the *ma-l$^+$* locus is either a structural gene for this enzyme or a controlling gene for the *aldox$^+$* locus.

Since the *ma-l$^+$* locus is known to control a factor which complements with the *ry$^+$* locus *in vivo* and *in vitro* to produce XDH (GLASSMAN and MITCHELL 1959b; GLASSMAN 1962), the partial characterization of the *ma-l$^+$* factor by complementation (GLASSMAN 1966; GLASSMAN *et al.* 1966) allows for a comparison of the properties of this factor and aldehyde oxidase, both of which possess in theory a product of the *ma-l$^+$* gene.

i. Maternal transmission: By crossing *D. melanogaster yf* attached-X females to *D. simulans aldox^I* males, one should be able to determine whether aldehyde oxidase is maternally transmitted as is the *ma-l$^+$* factor (GLASSMAN and

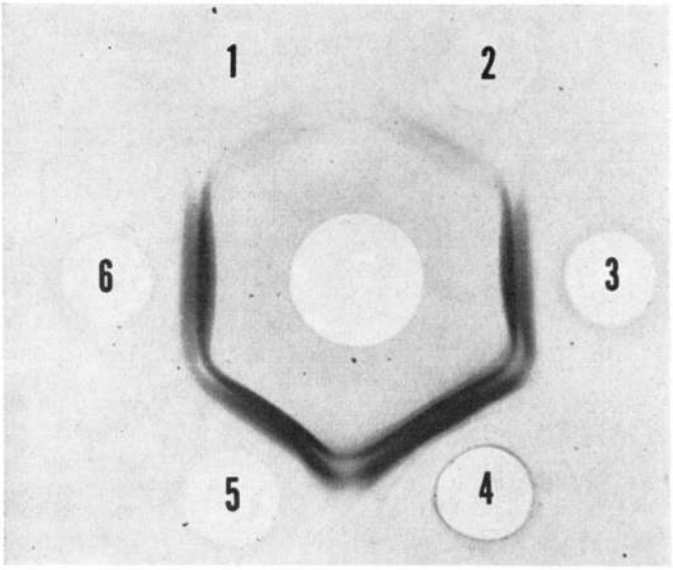

FIGURE 7.—Detection of aldehyde oxidase isozymes. Crude extracts of various genotypes prepared by method (1) were incubated at 37°C for one hour with an equal volume of antibody. Aliquots were then applied to the wells and allowed to diffuse against a wild-type extract prepared by method (1). Gels were developed and stained for aldehyde oxidase activity. Reservoirs 1 and 2 contained wild-type extract; reservoirs 3 and 5 contained *ma-l*/*ma-l*^{hz} extract; reservoir 4 contained 0.1 M Tris HCl, 0.001 M EDTA, pH 8.0; reservoir 6 contained *ma-l*; *lxd ry* extract all preincubated with antibody. The center reservoir contained a crude extract of wild type not preincubated with antibody. The reservoirs were spaced at 10 mm, which resolves the single precipitin line of Figure 6 into two components. These lines may represent the aldehyde oxidase isozymes of Figure 2.

MITCHELL 1959b). Analysis of electropherograms of hybrid progeny for maternal and paternal enzyme mobilities shows that the paternal enzyme is not detected until the late third instar (Figure 8). This absence of the *D. simulans* electrophoretic form in eggs and in first- and second-instar larvae probably indicates that neither the paternal nor the maternal *aldox*$^{+}$ locus is producing functional product.

When *ma-l* females are crossed to OreR males, aldehyde oxidase activity is detected in the eggs, but this activity remains low until third instar (Table 5).

TABLE 5

Aldehyde oxidase activity in ma-l/+ *embryos derived from* ma-l/ma-l *female parents*

	Developmental stage*						
		Larval instar			Pupa		
	Egg	First	Second	Third†	First day	Third day	Adult (*ma-l*/+)
Specific activity	0.82	0.52	0.77	1.36	1.92	3.65	7.31

* Developmental stages include all individuals, from 0–24 hours for any given stage.
† Larvae about 120 hours after hatching.

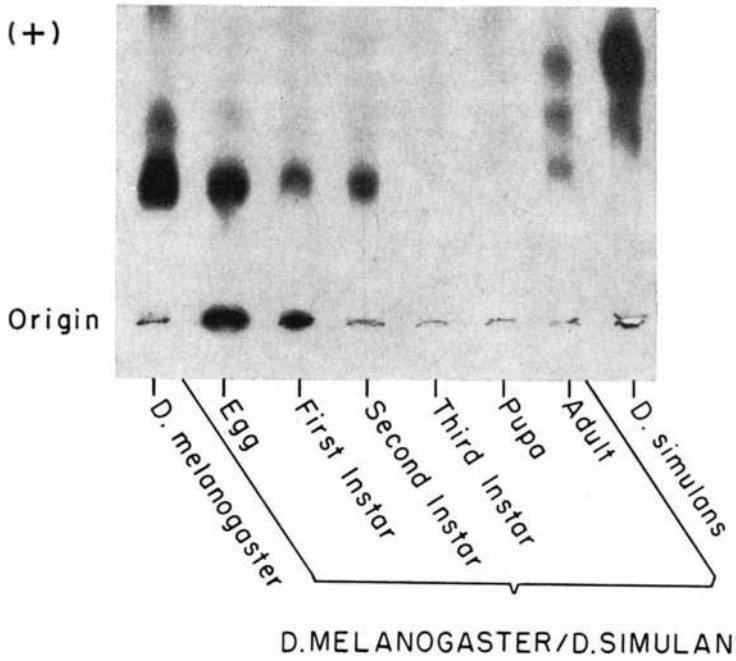

FIGURE 8.—Maternal transmission of aldehyde oxidase. *Drosophila melanogaster*, *D. simulans*, and the various embryonic stages of the hybrid progeny of an interspecies cross (see text) were prepared by method (1), electrophoresed for 20 minutes at 31 v/cm and 25 ma at 5–15°C, and stained for aldehyde oxidase.

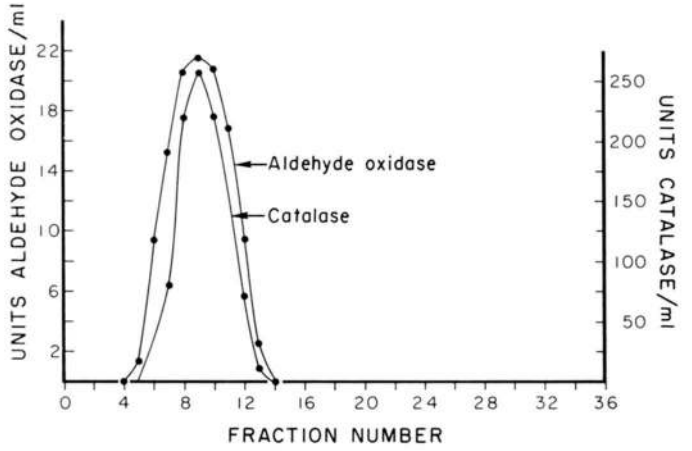

FIGURE 9.—Sedimentation of aldehyde oxidase on a sucrose gradient. Extracts of *ry* flies and bovine catalase were centrifuged and analyzed, and aldehyde oxidase and bovine catalase were assayed as in METHODS. Fraction 36 represents the top of the tube.

The stage of development at which this increase in aldehyde oxidase activity occurs correlates well with the time of detection of the hybrid enzyme in interspecies hybrids.

ii. Sedimentation: GLASSMAN *et al.* (1966) have found that the *ma-l*$^+$ factor sediments in sucrose gradients at about the same rate as bovine catalase. This is also the case for aldehyde oxidase. When *ry* extracts are centrifuged at 39,000 rpm in an SW-50 rotor for 12 hours, aldehyde oxidase is found in the same position as bovine catalase in the gradient (Figure 9).

iii. Trypsin and thermal stability: The *ma-l*$^+$ factor is relatively resistent to trypsin and heat (GLASSMAN 1966). Extracts of *ry* were prepared and treated with trypsin and exposed to 50° and 60°C according to GLASSMAN (1966). Under these conditions, aldehyde oxidase has similar stabilities to the *ma-l*$^+$ factor (Table 6). The enzyme is especially thermostable in dilute solutions. This property was not known to us when we initially purified the enzyme; it might be useful in future purification procedures.

DISCUSSION

Benzaldehyde has been listed as one of the substrates of XDH in Drosophila (GLASSMAN and MITCHELL 1959a). However benzaldehyde is also oxidized by an enzyme with a different electrophoretic mobility (Figure 1). This enzyme does not require NAD$^+$ and is present in *ry* flies, which lack XDH activity; since it reacts with a variety of aldehydes, it has been termed aldehyde oxidase. Its

TABLE 6

A comparison of aldehyde oxidase and ma-l$^+$ *complementing activity after various treatments*

Enzyme source	Aldehyde oxidase units	Treatment	Protein (mg/ml)	Percent aldehyde oxidase remaining	Percent *ma-l*$^+$ complementing activity remaining
ry (pH 5 supernatant solution)‡	1.00	15 min, 50°C	11.80	66	90†
	1.00	15 min, 50°C	1.18	62	. .
	1.00	15 min, 50°C	2.18*	92	. .
	1.00	15 min, 60°C	11.80	4	5†
	1.00	15 min, 60°C	1.18	21	. .
	1.00	15 min, 60°C	2.18*	27	. .
	0.25	60 min, Norite	11.80	168	0.12‡
ry (Methods (2), in 0.01 M potassium phosphate, pH 7.2)	1.12	15 min, trypsin	2.80	100	100†
	1.12	180 min, trypsin	2.80	100	. .
	1.12	15 min, 50°C	2.80	73	. .
	1.12	15 min, 50°C	0.28	73	. .
	1.12	15 min, 50°C	1.28*	86	. .
	1.12	15 min, 60°C	2.80	59	. .
	1.12	15 min, 60°C	0.28	54	. .
	1.12	15 min, 60°C	1.28*	71	. .

* Dilution into 0.1% bovine serum albumin in 0.3 M potassium phosphate, pH 7.5.
† After GLASSMAN (1966).
‡ After GLASSMAN (1962).

genetic control appears to be quite complicated, and at least three loci are involved in its production.

The structural gene responsible for different electrophoretic forms of aldehyde oxidase has been mapped at 74.5 on the third chromosome of *D. simulans* (Table 3). When these electrophoretic variants in *D. simulans* were crossed to *ma-l*; *lxd ry D. melanogaster*, a hybrid aldehyde oxidase zymogram was obtained (Figure 4), indicating that there is a homologous *aldox*$^+$ locus in this genotype.

The *ma-l* locus may also be a structural gene for aldehyde oxidase as evidenced by the absence of aldehyde oxidase CRM in *ma-l/ma-l* flies (Figures 6, 7) and the altered levels of both enzymatic activity and CRM in *ma-l*/+ heterozygotes (Figure 5; Table 4). However, the existence of a second structural gene for aldehyde oxidase should be accepted with caution until electrophoretic variants allelic to *ma-l* are found.

The amount of aldehyde oxidase is lowered by a third gene, *lxd*, which appears to have its effect at the level of enzyme production rather than determination of enzyme structure. Aldehyde oxidase in *lxd* flies has the same electrophoretic mobility at pH 8.7 and the same molecular size as wild-type aldehyde oxidase (Figures 2, 5). In crude extracts of *lxd/lxd* and *lxd*/+flies, the lower amount of aldehyde oxidase CRM suggests that there are fewer wild-type molecules present (Figure 6; Table 4).

Although aldehyde oxidase and pyridoxal oxidase are controlled by the same loci, *ma-l* and *lxd*, the two are known to be separate molecules for several reasons: (1) no pyridoxal oxidase has been detected in *lxd* extracts (GLASSMAN *et al.* 1964) while small amounts of aldehyde oxidase are present in *lxd extracts* (Figures 2, 5); (2) aldehyde oxidase purified 200-fold does not react with pyridoxal, although it reacts readily with several aldehydes; (Table 2); (3) a strain of *D. melanogaster* which has low levels of pyridoxal oxidase (*lpo*) has normal levels of aldehyde oxidase (J. COLLINS, personal communication).

The presence of aldehyde oxidase in *lxd* flies and its absence in *ma-l* flies suggested that it might be the *ma-l*$^+$ complementing factor (GLASSMAN 1962). Although the enzyme has the same pattern of maternal transmission, sedimentation, thermostability, and trypsin resistence as the *ma-l*$^+$ factor (GLASSMAN 1966, GLASSMAN *et al.* 1966; Figures 8, 9; Table 5), since it is not inactivated or removed by Norite (Table 5) nor is it found in *ma-l/ma-l*^{bz} flies which are known to contain 5–10% XDH (Figure 6, 7; GLASSMAN and PINKERTON 1960), its identity with the complementing factor is not established. This evidence does suggest, however, that the product of the *ma-l*$^+$ gene is a component of both aldehyde oxidase and the *ma-l*$^+$ complementing factor.

Thus there are at least three loci involved in the production and regulation of aldehyde oxidase. Our findings are consistent with the assumption that the *aldox*$^+$ and *ma-l*$^+$ loci produce or control subunits of the aldehyde oxidase molecule, while *lxd* regulates the functioning of one or both of these loci.

The author is grateful to DR. H. URSPRUNG for valuable suggestions and advice throughout this investigation, and to DR. S. R. SUSKIND for critical evaluation of the immunochemical procedures and results.

SUMMARY

The genetic control and physiological role of aldehyde oxidase have been examined in *D. melanogaster* and *D. simulans*. The structural gene for aldehyde oxidase is in chromosome 3. The enzyme is low when the gene *lxd* (low xanthine dehydrogenase) is homozygous, and absent when individuals are homozygous for the gene maroon-like (*ma-l*). These nonallelic loci do not alter the aldehyde oxidase molecules, but regulate the amount produced.—Aldehyde oxidase resembles the *ma-l*$^+$ complementing factor in its pattern of maternal transmission, sedimentation, trypsin resistence, and thermostability, but differs from the complementing factor since it is not removed by Norite treatment.

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