IN VITRO COMPLEMENTATION BETWEEN NON-ALLELIC MUTANTS DEFICIENT IN XANTHINE DEHYDROGENASE. II. THE ABSENCE OF THE MA-L$^+$ FACTOR IN LXD MUTANT FLIES.[1]

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In Drosophila melanogaster, the ma-1 (64+, X chromosome) and the ry (52+, 3rd chromosome) eye color mutants are deficient in xanthine dehydrogenase (see Glassman, 1962b, for a discussion of these loci). However, incubating extracts of ma-1 and ry flies together elicits xanthine dehydrogenase activity although none was initially present (Glassman, 1962b). This in vitro complementation suggests that a ry$^+$ complementing "factor" in ma-1 extracts and a ma-1$^+$ complementing "factor" in ry extracts react to produce functional enzyme molecules. The nature of these "factors" is not clear. Recently we have discovered lxd (low xanthine dehydrogenase) at a third locus (33+, 3rd chromosome). These flies have 20 to 25% the xanthine dehydrogenase activity of wild-type (Keller and Glassman, 1964). This paper is a preliminary report describing the effect of this locus on the ma-1$^+$ complementing factor.

METHODS: Drosophila mutants and culture media have been described (Glassman, et al, 1962). Marker genes were used to check on contamination

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of our stocks. Thus, the ma-1 stock is v f Bx^3 ma-1, the ry stock is st ry, the lxd stock is ru lxd by, the ma-1; ry stock is ma-1; st ry^2, the ma-1; lxd stock is v f Bx^3 ma-1; ru lxd by, the lxd ry stock is ru lxd by ry^2, and the ma-1; lxd ry stock is v f Bx^3 ma-1; ru lxd by ry^2 (see Bridges and Brehme, 1944, for a description of these mutant genes). The Pacific (PAC) and the Oregon-R (OR) wild-type (normal) stocks were also used.

The pH5-supernatant was prepared as described previously (Glassman, 1962). Further purification was achieved as follows: solid ammonium sulphate was added to the pH5-supernatant to 30% of saturation. After 60 minutes, the solution was centrifuged and the supernatant solution was brought to 60% saturation with solid ammonium sulphate. After 60 minutes, the precipitate was collected by centrifugation and dissolved in 0.1 M tris(hydroxymethyl)aminomethane buffer (Tris), pH 8, to a final volume 1/20 the volume of the pH5-supernatant used. This is AMM. The AMM was added to a column of Sephadex-75 and eluted with 0.1 M Tris, pH 8. The eluates richest in xanthine dehydrogenase activity were combined and labeled SEPH.

The complementation reaction has been described (Glassman, 1962b). Xanthine dehydrogenase was assayed by the fluorometric method of Glassman (1962a). The "oxidase" activity was assayed qualitatively by detecting the conversion of pyridoxal to pyridoxic acid using the paper chromatographic method of Forrest et al (1961).

RESULTS AND DISCUSSION: The low xanthine dehydrogenase activity in lxd is not due to an inhibitor of this enzyme. When extracts of lxd and wild-type flies are mixed and incubated for varying lengths of time, the activities found are those expected on the basis of simple additivity. In addition, purification procedures were not successful in removing possible inhibitors. Table 1 shows that the relative specific activity of xanthine dehydrogenase in the lxd extracts in each step of the procedure is fairly

constant at about 20% of the PAC extracts, and about 25% of the OR extracts.

Table 1. Relative specific activity of xanthine dehydrogenase in extracts at various stages of purification.

Strain	Fraction	Volume (ml.)	Total Protein (mg.)	Total Enzyme Units	% recovery	Specific Activity (Enz. Units per mg. protein	% Specific Activity of PAC
PAC	CRUDE	21	494	58,800	-	109.2	-
	PH5-SUPER	20	231	57,000	97	248	-
	AMM	0.9	89.0	41,600	71	466	-
	SEPH	2.2	64.2	30,800	52.5	480	-
OR	CRUDE	46	1022	92,000	-	90	82
	PH5-SUPER	45	439	93,000	100	214	86
	AMM	1.6	168	64,400	70	382	82
	SEPH	3.5	92.5	45,600	50	492	103
lxd	CRUDE	13	240	5,200	-	21.6	20
	PH5-SUPER	12.2	114	6,600	100	57.6	23
	AMM	0.7	46.2	2,780	73	82	18
	SEPH	1.6	31.7	3,200	61.5	101	21

Table 2. The absence of the ma-1^+ complementation factor in lxd flies as revealed by the in vitro complementation assay.

	ml. ADDED				
mutant extract →	ma-1	ry	ma-1; lxd	lxd ry	Enzyme units formed per ml.
factor → expected in extract	(ry^+)	($ma\text{-}1^+$)	(ry^+)	($ma\text{-}1^+$)	
TUBE NO.					
1			0.5	0.5	0
2	0.5			0.5	0
3		0.5	0.5		31
4	0.5	0.5			35
5	0.5				0
6		0.5			0
7			0.5		0
8				0.5	0

The complementation assay is carried out using pH5-supernatant extracts. Where necessary the volume is brought to 1.0 ml. with 0.1 M Tris, pH 8. The tubes are incubated at 30° for 60 minutes, after which the solutions are treated with Nonte-A and assayed for xanthine dehydrogenase. The entire procedure is described by Glassman, 1962b.

In order to test the possibility that the low enzyme activity of lxd was due to an effect on one or both of the products of ma-1 and ry loci, the complementation experiment shown in Table 2 was performed. It can be seen that extracts of lxd ry and ma-1; lxd do not produce xanthine dehydrogenase activity when incubated together (tube 1). This is due to a defect of the ma-1^+ complementation factor in the lxd ry extract, since

this extract also fails to complement with active ry^+ factor in the ma-1 extract (tube 2). The ma-1; lxd extract does complement with an active $ma\text{-}1^+$ factor in the ry extract (tube 3) indicating that it contains the ry^+ complementation factor as expected.

These data suggest that extracts of lxd flies are deficient in the $ma\text{-}1^+$ complementation factor. Other data also indicate that this is true. Forrest et al (1961) reported that extracts of wild-type or ry flies could oxidize pyridoxal to pyridoxic acid. In contrast, extracts of ma-1 or ma-1; ry do not. Forrest et al postulate that the $ma\text{-}1^+$ locus codes for this oxidase which may be a subunit of xanthine dehydrogenase. We have been able to confirm these results and show that extracts of lxd flies also lack this oxidase activity (Table 3). In addition, Glassman and Keller (unpublished) have shown that the maternal effect associated with the $ma\text{-}1^+$ gene (Glassman and Mitchell, 1959; Glassman and McLean, 1962) is abolished if the mother or progeny are homozygous for lxd. All these findings indicate a deficiency of the product from the $ma\text{-}1^+$ locus in lxd flies.

Table 3. The absence of the oxidase activity in lxd flies.

Strain	oxidase activity
wild-type (PAC)	+
ry	+
ma-1	0
ma-1; ry	0
lxd	0
ma-1; lxd	0
lxd ry	0
ma-1; lxd ry	0

The data in Table 2 also indicate that the ry^+ complementation factor seems to be normal in lxd flies. In agreement, Karam and Glassman (unpublished) have shown that lxd has the same number of CRM* units per mg. protein as wild-type flies. Since CRM is presumed to be a product of the

* CRM is defined here as any material that crossreacts with rabbit antibody prepared against Drosophila extracts containing xanthine dehydrogenase activity. The technics are described in Glassman and Mitchell (1959).

ry^+ locus (Forrest et al, 1961; Glassman, 1962b), this evidence confirms that this locus and its product are functioning normally in lxd flies.

Since lxd flies lack detectable amounts of the product associated with the $ma\text{-}1^+$ locus, the normal production of the $ma\text{-}1^+$ complementation factor, the oxidase, and the maternal substance must be due to the interactions of the lxd^+ and the $ma\text{-}1^+$ loci. The paradox of the presence of substantial, though less than normal, amounts of xanthine dehydrogenase in lxd in spite of the absence of the product of the $ma\text{-}1^+$ locus can be resolved by various hypotheses. One can postulate that the ry^+, the $ma\text{-}1^+$, and the lxd^+ loci code for three different polypeptide chains, designated R, M, and L, respectively. These polypeptides produce different enzyme activities by polymerizing with each other in different ways. Thus, a polymer of the M and L polypeptides will have the oxidase activity, while any polymer containing R polypeptides will have CRM activity. Only a polymer containing R, M, and L polypeptides will have xanthine dehydrogenase activity. On this basis, the in vitro complementation reaction between ma-1 and ry extracts would be visualized as an interaction between two large polymers containing ML and R subunits, respectively. This is similar to the interaction in vitro between high molecular weight proteins isolated from the am^1 and the am^3 mutants of Neurospora that produces glutamic dehydrogenase activity with little change in molecular weight (Fincham and Coddington, 1963). The deficiencies observed in lxd flies would be due to a defective L polypeptide which can still react in vivo to yield some xanthine dehydrogenase activity, but which is too defective to yield active ML polymers (oxidase). It is of interest that the oxidase activity has a molecular weight almost as great as that of xanthine dehydrogenase (about 250,000) (Moon and Glassman, unpublished).

Alternatively, one can view the lxd gene as a regulator of the activity of either the $ma\text{-}1^+$ gene or its product. In this case, the oxidase would be a polymer composed of only M polypeptides, CRM would again be any

polymer containing R polypeptides, while xanthine dehydrogenase activity would be a polymer of M and R polypeptides. Another possibility is that only the ry^+ locus codes for a subunit of xanthine dehydrogenase, while the products of the $ma\text{-}1^+$ and lxd^+ loci are regulators or activators, not only of the R polypeptide which forms xanthine dehydrogenase, but also of other polypeptides which form the oxidase and other yet unknown enzymes.

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