MUTANTS OF DROSOPHILA MELANOGASTER DEFICIENT IN XANTHINE DEHYDROGENASE

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DESPITE the fact that *Drosophila melanogaster* is widely used in genetic investigations, the difficulties in detecting mutations amenable to enzymatic analysis has resulted in few gene-enzyme studies in this organism. Recently however, HADORN and SCHLINK (1956) reported that the mutant ry^2 lacks isoxanthopterin, while FORREST, GLASSMAN, and MITCHELL (1956) found that this mutant as well as *ma-l* are deficient in the enzyme which converts 2-amino-4-hydroxypteridine to isoxanthopterin in Drosophila. (We could not confirm the previous report that *ma* also lacks the enzyme.) According to LOWRY *et al.* (1949) this type of reaction is catalyzed by xanthine oxidase from milk and, indeed, the enzyme from Drosophila has proven to be xanthine dehydrogenase. Figure 1 summarizes some of the reactions catalyzed by this enzyme. The present paper describes the purification and some properties of the Drosophila enzyme and its deficiency in purified extracts of *ry* and *ma-l*. In addition, the production of rabbit antibodies to the enzyme is described, as well as the presence of a cross-reacting substance in the mutants. (The following abbreviations of the mutants are used in this paper (BRIDGES and BREHME 1944): *ma-l*=maroon-like eye color; *ry*=rosy eye color; *ma*=maroon eye color; *st*=scarlet eye color).

METHODS AND MATERIALS

The 2-amino-4-hydroxypteridine was synthesized by the method of CAIN *et al.* (1946). The impure product was dissolved in hot formic acid, treated with charcoal, and precipitated by chilling. It was then dissolved in alkali and precipitated by stepwise addition of small amounts of acid until the precipitated product was chromatographically pure. The isoxanthopterin and the 2-amino-4-hydroxypteridine were kindly donated by DR. H. S. FORREST. Other chemicals were of commercial origin.

Flies were raised on a standard medium containing corn meal (ten percent), agar (0.5 percent), dextrose (six percent), sucrose (three percent), brewers yeast (two percent), propionic acid (0.5 percent), and seeded with live yeast when cool. Adults were collected when less than three days old since the activity of the enzyme declines in older flies. Larvae and pupae were collected as previously described (GLASSMAN 1957b); they were also obtained by washing the

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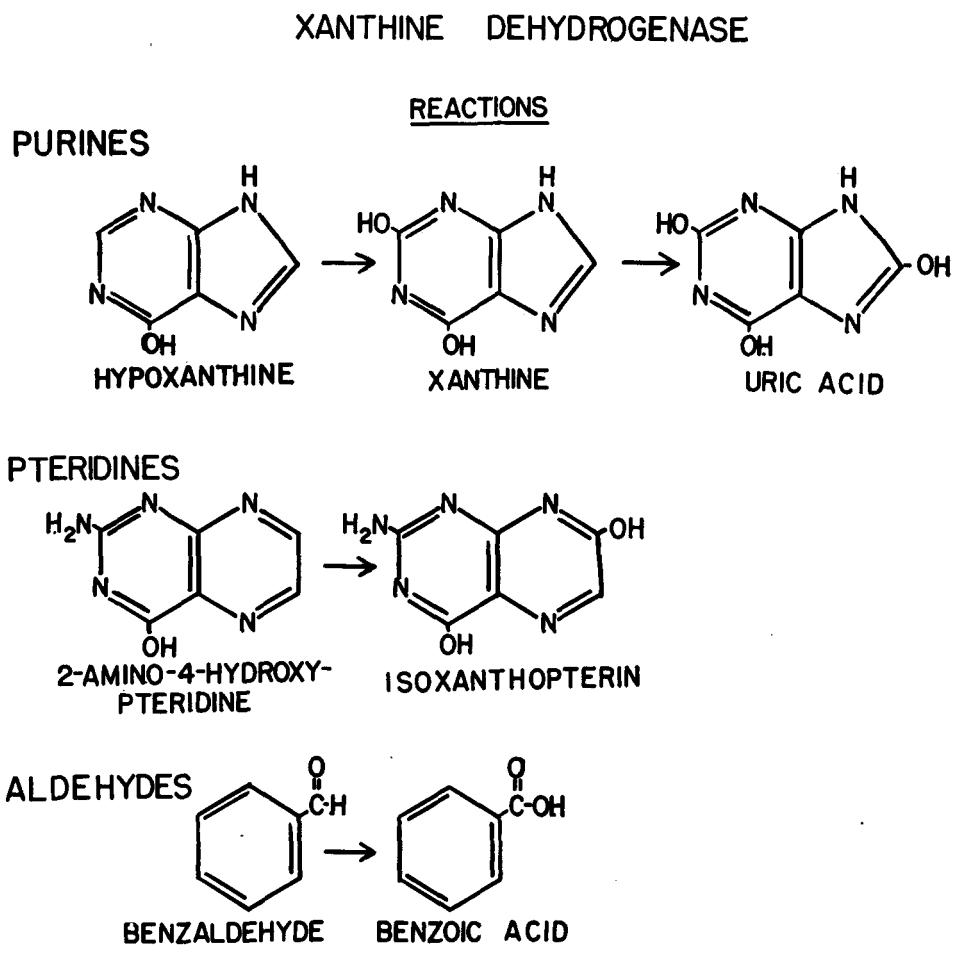

FIGURE 1.—Reactions of xanthine dehydrogenase.

top layer of food out of the bottles and then floating the larvae by addition of sodium chloride to saturation.

Flies were chromatographed by crushing individual adults directly on the paper as described by HADORN and MITCHELL (1951), or by homogenizing them in one percent ammonia and spotting the extract after centrifugation. The solvent system most used was propanol: one percent ammonia (2:1), but butanol:acetic acid:H_2O (4:1:5) and propanol:one percent acetic acid (2:1) were also used to help identify various substances. Isoxanthopterin and 2-amino-4-hydroxypteridine were detected on the chromatograms by their fluorescence using a U.V. lamp emitting strongly at 360 mμ, while uric acid and hypoxanthine were detected by their absorption with a U.V. lamp emitting at 260 mμ.

The most convenient assay of the enzyme is based on the change in fluorescence when 2-amino-4-hydroxypteridine is oxidized to isoxanthopterin (BURCH *et al.*

1956). (The following chemical abbreviations are used in this report: Tris=tris (hydroxymethyl) aminomethane; DPN=diphosphopyridine nucleotide; FAD= flavinadenine dinucleotide.) The reaction was carried out directly in the fluorimeter tube to which were added one ml of 0.1 M Tris buffer at pH 8.0; 0.01 to 0.1 ml of enzyme; 0.02 ml of 10^{-3} M 2-amino-4-hydroxypteridine; and either 0.02 ml of 10^{-2} M DPN or 0.01 ml of 10^{-3} M methylene blue. A #5860 Corning glass was used as a primary filter and a combination of a Corning #5113, a Coleman PC3, and a Klett #44 filter was used as the secondary filter. A quinine standard in 0.1 M H_2SO_4 which fluoresced the same as 2.5×10^{-6} M isoxanthopterin in 0.1 M Tris buffer, pH 8, was used. With this solution in place, the galvanometer scale of the Farrand fluorimeter (model A) was set at 30. The rate of the reaction was proportional to the enzyme concentration, and was linear for 5 to 20 minutes provided it did not exceed 10 to 15 galvanometer units per minute. A unit of enzyme activity is defined as that amount of enzyme which will cause a change of one galvanometer unit per minute in the system described above. This is equivalent to the production of 0.08 mμ moles of isoxanthopterin per minute.

When the reaction could not be run directly in the fluorimeter, aliquots of 0.04 ml were taken from a reaction mixture containing 0.03 ml of 5×10^{-3} M 2-amino-4-hydroxypteridine; 0.03 ml of 5×10^{-2} M DPN; 0.01 to 0.1 ml of enzyme; and enough buffer to make the volume 0.3 ml. These aliquots were taken at 10–20 minute intervals and diluted to one ml with 0.01 M Tris buffer, pH 8, containing 0.02 γ per ml of 2-amino-4-hydroxy-6-formylpteridine, which stopped the reaction. The extent of the reaction was measured by determining the fluorescence of this mixture as described above.

The rate of oxidation of hypoxanthine and xanthine was measured directly in the cuvette of a Beckman DU spectrophotometer by determining the increase in reduced DPN at 340 mμ. The relative rates of oxidation of the various substrates were determined by using 2,6-dichlorophenol indophenol as the electron acceptor, and following its reduction at 600 mμ in a modification of the method of Avis *et al.* (1956).

A rapid and relatively mild method of protein purification was developed to remove inhibitory substances (purines, pteridines, aldehydes, etc.) from the mutant extracts without denaturing a possibly unstable mutant enzyme. All steps were carried out below 5°C. The enzyme extract was prepared by homogenizing flies in two volumes (w/v) of 0.1 M Tris buffer, pH 8, and centrifuging the resulting extract at 80,000 × g for 40 minutes in a Spinco model L centrifuge. The resulting supernatant solution was treated with 100 mg of Norite-A per ml, and after ten minutes, it was centrifuged at 20,000 × g for 15 minutes. To the clear, colorless supernate was added a saturated solution of $(NH_4)_2SO_4$ to 30 percent saturation. The solution was centrifuged after 20 minutes and the supernate was brought to 50 percent saturation with the $(NH_4)_2SO_4$ solution. After 30 minutes, this was centrifuged at 20,000 × g for 15 minutes and the precipitate was redissolved in a volume of buffer one third that of the charcoal treated homogenate.

This extract will be referred to as AMM-I. When the flies were originally homogenized in a buffer other than Tris (phosphate, veronal, borate and 2-amino-2-methyl-1,3-propandiol were tried), large losses of enzyme activity were observed during the $(NH_4)_2SO_4$ precipitation. This loss could not be restored with FAD, Mo^{2+}, Fe^{3+}, or boiled enzyme.

AMM-I was treated with an equal volume of distilled water and a similar amount of calcium phosphate gel (SINGER and KEARNY 1950) containing 25 to 30 mg of gel per ml. After one hour the gel was centrifuged, and washed successively with 2 M Tris, pH 8.0; 0.1 M $(NH_4)_2SO_4$, pH 8.0; and distilled water. The gel was then eluted with 0.02 M potassium pyrophosphate in 0.1 M Tris, pH 8.5, and the eluates were concentrated to one half the volume of AMMS-I by repeating the 30–50 percent $(NH_4)_2SO_4$ precipitation. This final extract will be referred to as AMM-II. This procedure achieved purifications ranging from 10– to 50–fold with a recovery of enzyme activity between 50 to 75 percent. The purified enzyme was usually stable for several weeks when stored at −15°C, but some preparations lost activity after several days. Protein was assayed by a modification of the method of LOWRY *et al.* (1951).

Antibodies to the enzyme were prepared by injecting 1 to 3 ml of AMM-I or AMM-II into a rabbit three times a week for three weeks. Blood was taken five to nine days later, but increased titers were achieved by booster shots one month later. The antisera were heated at 54°C for 20 minutes to inactivate complement, and then treated with 50 mg of Norite-A per ml to remove aromatic inhibitors. The antibodies were then precipitated with 60 percent saturated $(NH_4)_2SO_{40}$, redissolved in Tris buffer and dialyzed.

Description of the mutants

Stocks of Oregon-R wild type, *ma-l* and ry^1 were obtained from the Drosophila collection at the California Institute of Technology. The strain of ry^2 was obtained from PROF. HADORN. No differences between ry^1 and ry^2 have been observed. Both *ma-l* and *ry* lack most of the red eye pigments and exhibit a dark reddish-brown eye color, which is similar in the two mutants and the double mutant, *ma-l; ry*. The combination *ma-l;st*, *st* ry^2, or *ma-l;st* ry^2 produces yellowish-orange eyes.

ma-l is an X-ray induced mutant discovered by OLIVER (see BRIDGES and BREHME 1944), who reported that it was a sex-linked recessive near vermillion, which is at locus 33. Our own studies indicate that *ma-l* is located closer to Beadex, which is at 57. Close location is hindered by the fact that *ma-l*$^+$ exerts a maternal effect, and thus, the genetically *ma-l* offspring of a female heterozygous for *ma-l* will have wild type eye color. This phenomenon is also evident when *ma-l; st* males are mated to attached-X; *st* females; their patroclinous sons have scarlet eyes and not the yellowish-orange associated with *ma-l; st* flies. However, genetic tests show that they are *ma-l*. Details of the experiments will be published elsewhere.

ry occurred spontaneously and is located at locus 51± of the third chromosome

(BRIDGES and BREHME 1944). The wild type allele of *ry* does not have a maternal effect. HADORN and SCHWINK (1956) have shown that this mutant is non-autonomous when whole tissues are transplanted, but recent attempts to change the eye color with cell-free extracts have not been successful (MITCHELL *et al.* 1958). We have also been able to show that *ma-l* is nonautonomous in gynandromorphs (GLASSMAN 1957a).

RESULTS

Enzyme properties: Large losses in enzyme activity were noted following treatment with charcoal. This loss could be restored with boiled enzyme extracts, or with the electron acceptors, DPN, methylene blue, or 2,6-dichlorophenol indophenol. This indicates that the enzyme from Drosophila resembles xanthine dehydrogenase of chicken liver (REMY *et al.* 1955), rather than the oxidase of milk. The rate of oxidation without addition of one of these electron acceptors is 1/10 to 1/20 the rate in their presence. As yet we have not been able to demonstrate a requirement for other co-factors, (FAD, Fe^{3+}, Mo^{2+}) usually associated with this enzyme when isolated from other sources. The rate of the reaction appears to be independent of pH between 7 and 8.2 when Tris buffer is used, but there does appear to be a slight optima as pH 7.8 in 0.1 M potassium phosphate buffer.

The enzyme oxidizes purines (hypoxanthine, xanthine), pteridines (xanthopterin, 2-amino-4-hydroxypteridine), and aldehydes (benzaldehyde). There is some doubt, however, whether acetaldehyde serves as a substrate. The Michaelis-Menten constant for 2-amino-4-hydroxypteridine is 6.7×10^{-6} M; for xanthine and hypoxanthine it is 2.5×10^{-5} M and 2.1×10^{-5} M, respectively. The latter two compounds are oxidized about 2½ times faster than 2-amino-4-hydroxypteridine. More detailed examination of the properties of the enzyme will be reported elsewhere.

The enzyme is present in extracts of larvae and pupae prepared as described for adults. No differences have been observed in the enzyme isolated from these three stages of Drosophila.

The Mutants: Extracts of ry^1, ry^2 and *ma-l* have been purified as described for the wild type enzyme. In no fraction was any activity observed using 2-amino-4-hydroxypteridine (Figure 2) or hypoxanthine as substrate, nor was it possible to elicit any activity in any of the mutant fractions by adding boiled enzyme, FAD, FMN, MoO_3, Na_2MoO_4, or $FeCl_3$. Mixing the extracts of the two mutants was also ineffective. It should be pointed out that crude extracts of the mutants, as well as wild type, partially inhibit the activity of the enzyme. Since charcoal can remove the inhibition it is presumably due to the presence of purines and pteridines normally found in this organism. It is of interest that the heterozygote $ry^2/+$ flies have less enzyme activity than the wild type.

As a consequence of the enzyme deficiency, uric acid and isoxanthopterin cannot be detected on paper chromatograms of extracts of the mutants; instead, there are increased amounts of their precursors, hypoxanthine and 2-amino-4-

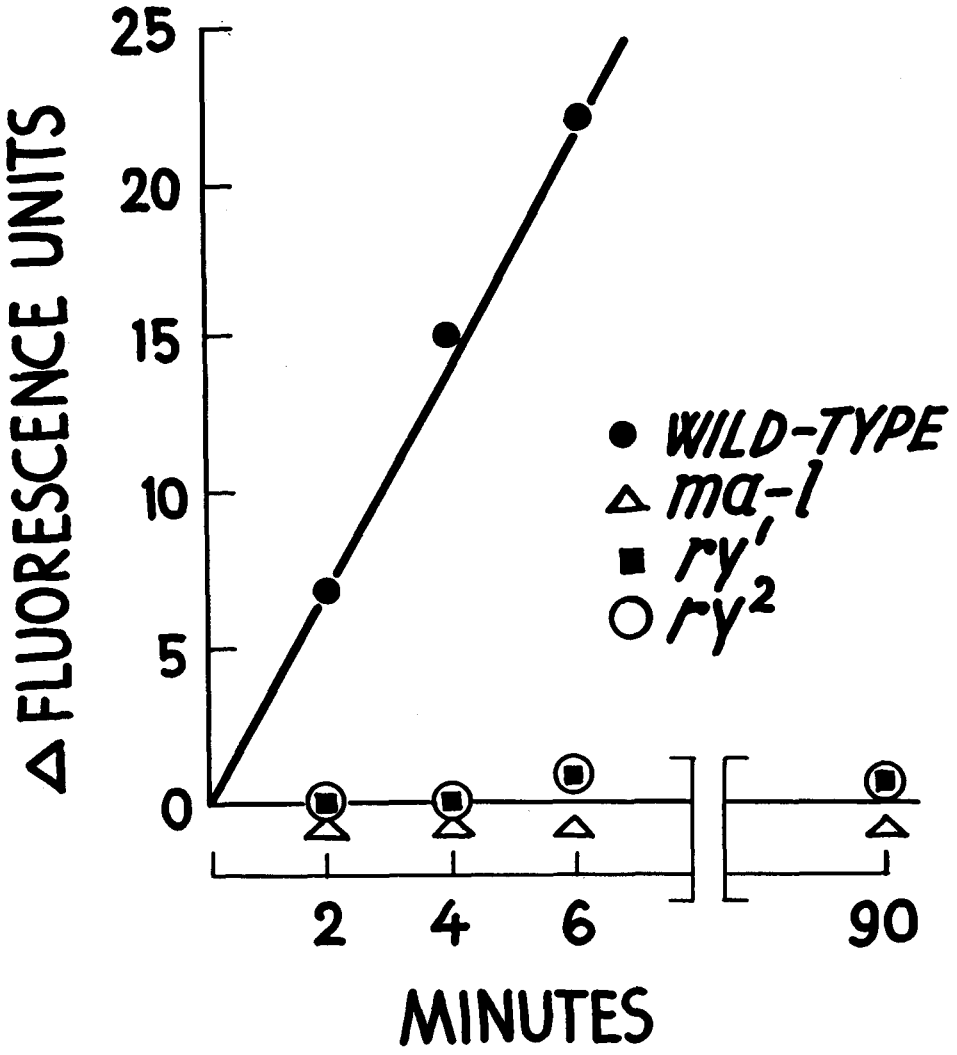

FIGURE 2.—Enzyme activity in wild type and mutant extracts. Each fluorimeter tube contained 0.1 ml of purified extract (AMM-II) made from flies homogenized the same day. The other constituents of the assay are described under Methods. Similar data were also obtained using the charcoaled treated preparations and AMM-I.

hydroxypteridine. Furthermore, increased amounts of 2-amino-4-hydroxypteridine in wild type and mutant pupae and adults could be induced by adding it to the food (1 mg/ml), but only the wild type showed an increase in isoxanthopterin. The mutants still had no detectable amounts of isoxanthopterin. These data indicate that the enzyme is physiologically important in these reactions, and that the enzyme is not operating in *ma-l* and *ry* in vivo as well as in vitro.

Antibodies: In order to ascertain whether the mutants produce an inactive pro-

tein, antibodies to the wild type enzyme were prepared as described under Methods. When testing for cross-reacting substances the concentration of the enzyme and the antisera were adjusted so that 65 to 85 percent inhibition of the enzyme occurred. The antisera was first added to the mutant extract, and after ten hours this mixture was added to the enzyme. Twelve hours later, the mixtures were centrifuged, and the supernate was assayed for enzymatic acivity. The extracts of *ma-l* and *ry* were always prepared at the same time, and had similar protein concentration.

Typical data are presented in Table 1. In this table, the rate of reaction of untreated enzyme is expressed as 100. When the enzyme was treated with antisera the rate was reduced to 14. However, when the antisera was first treated with ex-

TABLE 1

Cross reacting antigenic substances to xanthine dehydrogenase in mutant extracts

	Relative Rate (E = 100)	% Reversal of anti-sera inhibition
E	100	-
E + A-S	14	
E + [A-S + $\underline{ry}^2$ (AMM-I)]	17	3
E + [A-S + $\underline{ry}^2$ (AMM-II)]	31	20
E + [A-S + <u>ma-l</u> (AMM-I)]	66	46
E + [A-S + <u>ma-l</u> (AMM-II)]	95	92

In this experiment 0.1 ml of purified antisera were mixed with 0.1 ml of the mutant extract. This was allowed to stand at 4°C for ten hours. To this mixture was then added 0.2 ml of wild type enzyme (AMM–II). After 12 hours the mixtures were centrifuged, and 0.2 ml of the supernate was assayed for enzyme activity as described under Methods. Control mixtures were diluted with 0.1 M Tris buffer, pH 8. It should be noted that there was no enzyme inhibition with normal rabbit serum or with the mutant extracts.

E=enzyme; A–S=Antisera; AMM–I=1st $(NH_4)_2SO_4$ fraction; AMM–II=2nd $(NH_4)_2SO_4$ fraction.

tracts of *ma-l*, the inhibition of the enzyme by the antisera was suppressed (46 percent by AMM-I, 92 percent by AMM-II), whereas, ry^2 was much less effective in reversing enzyme inhibition (only three percent by AMM-I and 20 percent by AMM-II). These data indicate that both mutants contain a substance capable of reacting with the antibody to the enzyme, but *ma-l* has much greater amounts of these substances than ry^2. The increased effectiveness of AMM-II when compared with AMM-I for both mutants is probably due to the increased concentration of AMM-II.

DISCUSSION

The data presented above indicate that in *Drosophila melanogaster* either of two loci (*ma-l*$^+$ and ry^+) can mutate to produce a deficiency of xanthine dehydrogenase. Extracts of these mutants have been partially purified and all fractions lack detectable enzyme activity, indicating that the loss cannot be ascribed to the presence of a simple inhibitor.

The similarities between *ma-l* and *ry* are striking. Both have similar eye-colors, and because of the enzyme deficiency, the same biochemical excesses (hypoxanthine and 2-amino-4-hydroxypteridine) and deficiencies (uric acid and isoxanthopterin) occur. In addition, both mutants seem to be nonautonomous. However, we have noted differences between these mutants. The first is the maternal effect of *ma-l*$^+$ mentioned above, which is not exhibited by ry^+. The other difference is in the amounts of the substance (termed CRX) which reacts with the antibody to xanthine dehydrogenase. The data indicate that it is present in both mutants, but *ma-l* contains much greater amounts than ry^2. Its significance is obscure at present; it may represent the inactive enzyme protein. However, LERNER and YANOFSKY (1957) have shown that CRM, a material which cross reacts with the antibody to tryptophan synthetase in *E. coli* is the enzyme which catalyzes the preceding reaction in the sequence, i.e., the formation of indole. Whether CRX in Drosophila represents an enzyme whose substrate is closely related to the substrate of xanthine dehydrogenase remains to be shown. Unfortunately, very little is known about the enzymes involved in pteridine metabolism and tests for uricase have proved negative. Further work is contemplated on this point.

Certain tentative conclusions concerning gene action can be made. In order to fit these data into a unitary gene-enzyme relationship, it may be argued that only one of these genes is *directly* involved in the production of the enzyme; however, it would be difficult to determine which one on the basis of the present data. A number of cases have now been reported in various organisms (LANDMAN 1950; LEDERBERG 1951; MARKERT, 1950; HOROWITZ and FLING 1957; KURASHASHI 1957) in which more than one gene seems to play a role in the production of a single enzyme. Indeed, the presence of adenylsuccinase in a heterocaryon of Neurospora made from two mutants which lack this enzyme (GILES *et al.* 1957) might be interpreted in this way.

One can also visualize a situation where a single gene product can contribute

to the specificity of more than one enzyme, particularly if the enzymes catalyze the reactions of compounds of similar structure. This would most likely occur for enzymes in sequential reactions, and indeed, such a case has been reported by LERNER and YANOFSKY (1957) for tryptophan synthesis in *E. coli*, as mentioned above. It is most significant that the two enzymes which are deficient in the single mutant are antigenically alike, indicating that the same gene may have conferred similar specificity on these molecules. Another situation also suggestive of this phenomenon is the lac_3 mutation in *E. coli* (LEDERBERG 1951). It would be interesting to see if the enzymes hexokinase, amylomaltase, and lactase which are deficient in this strain (DOUDEROFF *et al.* 1949) are antigenically related. At any rate, it is clear from the data in this paper that the relationship between genes and enzymes (proteins) may be more complicated than a simple, unitary relationship.

After this paper was submitted, CRAWFORD and YANOFSKY (Proc. Nat. Acad. Sci. (Wash.), **44**: 1161, 1958) presented new evidence showing that, in *E. coli*, a complex of two proteins is necessary for normal tryptophan synthetase activity. CRM now seems to be one of the proteins in this complex.

SUMMARY

The enzyme xanthine dehydrogenase has been partially purified from extracts of wild type *Drosophila melanogaster* and some of its properties have been described. Two mutant loci, *ry* and *ma-l*, have been shown to produce a deficiency of this enzyme. Purified extracts of these mutants do not exhibit any detectable enzymatic activity indicating that simple inhibitors are not involved. Antibodies to the enzyme were prepared by injecting purified wild type extracts into rabbits. The analysis indicates that *ma-l* has much greater amounts of a substance capable of cross reacting with this antibody than ry^2. It is suggested that these data do not easily fit into a unitary gene-enzyme relationship.

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