PURINE CATABOLISM IN *DROSOPHILA MELANOGASTER*.
II. GUANINE DEAMINASE, INOSINE PHOSPHORYLASE AND
ADENOSINE DEAMINASE ACTIVITIES IN MUTANTS WITH
ALTERED XANTHINE DEHYDROGENASE ACTIVITIES¹

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A variety of mutants affect purine metabolism in *Drosophila melanogaster*. GEER (1963, 1964) has reported a strain with a polygenic nutritional requirement for yeast RNA. DANNEEL and ESCHRICH-ZIMMERMANN (1957) report that *bw* (brown) and *w* (white) mutants excrete excess uric acid. The most extensively studied are those mutants that control xanthine dehydrogenase (XDH) (GLASSMAN 1965): *lxd* (low xanthine dehydrogenase) at locus 33± on the third chromosome (KELLER and GLASSMAN 1964), *ry* (rosy eye color) at locus 52± on the third chromosome (BRIDGES and BREHME 1944) and *ma-l* (maroon-like eye color) at locus 64± on the X chromosome (HUBBY and FORREST 1960). Various mutant alleles of *ry* and *ma-l* have no detectable XDH activity (FORREST, GLASSMAN and MITCHELL 1956), while *lxd* has approximately 20 to 25% normal XDH activity (KELLER and GLASSMAN 1964). The *ry* locus is a structural gene for XDH (KARAM and GLASSMAN 1967; YEN and GLASSMAN 1965). It would be of interest to determine if mutants affecting XDH have any effect on the remainder of the pathway of purine catabolism. This could come about in various ways. For example, the genes coding for these enzymes might be coordinately controlled by a regulatory gene. Since the enzymes of this pathway react with purines, and if *ma-l*⁺, *ry*⁺, or *lxd*⁺ control a polypeptide common to other enzymes of purine catabolism, coordinate control might be present. Three enzymes close to XDH in the pathway of purine catabolism were chosen for this study; adenosine deaminase, inosine phosphorylase, and guanine deaminase (HODGE and GLASSMAN 1967). Some of their properties have been elucidated and the levels of their activities in these mutants have been ascertained.

MATERIALS AND METHODS

Chemicals: Crystalline bovine plasma albumin, the protein standard solution (10 mg protein nitrogen per ml) was purchased from the Armour Pharmaceutical Company. Guanine and milk xanthine oxidase were from the California Corporation for Biochemical Research. Norite A and

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Folin-Ciocalteu Reagent were from the Fisher Scientific Company. Adenosine, deoxyadenosine, inosine and deoxyinosine were from Schwarz BioResearch, Inc. Tris(hydroxymethyl)aminomethane was from the Sigma Chemical Company. The following abbreviations appear in the text: Tris = Tris(hydroxymethyl)aminomethane-HCL; Tris-albumin = Tris containing 1 mg per ml of crystalline bovine plasma albumin.

D. melanogaster strains: The following were used (BRIDGES and BREHME 1944): *Pac*, Pacific wild-type; *CS*, Canton-S wild type containing scarlet eye ($3.44 \pm$); *w ec* containing white eye ($1.15 \pm$) and echinus ($1.5 \pm$); *ru lxd by*, containing roughoid eye ($3.0 \pm$), low xanthine dehydrogenase ($3.33 \pm$) and blister wing ($3.48 \pm$); *st ry* containing scarlet eye ($3.44 \pm$) and rosy eye ($3.52 \pm$); *v f Bx³ mal-l* containing vermilion eye ($1.33 \pm$), forked bristles ($1.56 \pm$), Bead wing ($1.59 \pm$) and maroon-like eye ($1.64 \pm$); *bw*, brown eye ($2.104 \pm$). The Canton-S wild-type into which *st* had been introduced was a gift of Dr. B. W. GEER. All larvae were raised at $25 \pm 1^\circ\text{C}$ on a standard media (GLASSMAN and McLEAN 1962).

Preparation of Extracts: All extracts were prepared at temperatures below 5°C . Third-instar larvae were homogenized in a Kontes Duall glass tissue grinder for approximately one minute using 1.5 g of larvae per ml of buffer solution. Homogenates were centrifuged at $34,000 g$ for 20 minutes in a Sorvall RC-2 centrifuge. The supernatant solution was removed from beneath the overlying fatty layer and filtered through glasswool. Using the methods described elsewhere (HODGE and GLASSMAN 1967), this extract was either treated with charcoal (CHAR-crude) or used to make a pH5 supernatant solution which then was treated with charcoal (CHAR-pH5).

Enzyme assays: Assays are modifications of previously published procedures (PRICE, OTEY and PLESNER 1956; KAPLAN 1956; SHUSTER 1956) and were performed in triplicate in a Beckman DB Spectrophotometer. The change in optical density of a reaction mixture lacking substrate was used as a control. Assays were performed at 30°C using buffers and substrate solution kept at this temperature. Each enzyme was assayed at the optimal pH, at a concentration of the substrate which saturated the enzyme, in the range where reaction rate is proportional to enzyme concentration, and with amounts of enzyme that gave linear reaction rates for the entire time of the assay. The assays are based upon measurements of the initial rate of reaction such that the kinetics approached zero order. Enzymatic units and specific activities are defined according to the recommendations of the Commission on Enzymes (1961).

To assay guanine deaminase, larvae were homogenized in cold 0.1 M Tris, pH 7.0 and CHAR-pH5 was prepared. 2.8 ml of 0.1 M Tris-albumin, pH 7.3; 0.15 ml of $3 \times 10^{-3} \text{ M}$ guanine, and 0.1 ml of freshly prepared CHAR-pH5 were mixed rapidly. The decrease in optical density at $245 \text{ m}\mu$ was followed for 10 minutes. The optimum pH was 7.3 and a 50% reduction in activity occurred at pH 6.6 and at 8.4. The reaction rate was proportional to the amount of protein added between 0.075 to 0.60 mg. This preparation did not deaminate guanosine, deoxyguanosine, GMP or GTP. A unit of guanine deaminase is the amount of enzyme that catalyzes one micromole of guanine per minute.

To assay inosine phosphorylase, larvae were homogenized in 0.1 M sodium phosphate buffer, pH 7.5, and CHAR-crude was prepared. 1.8 ml of 0.05 M phosphate buffer, pH 6.8, 1.0 ml of 10^{-3} M inosine, 0.1 ml of milk xanthine oxidase (0.02 units), and 0.1 ml of extract were mixed rapidly. The increase in optical density at $290 \text{ m}\mu$ was followed for 90 minutes using inosine as substrate and 60 minutes using deoxyinosine as substrate. Deoxyinosine was split at a slightly higher rate than inosine in all strains, especially by extracts of the *Pac* strain. The reaction rate was proportional to the amount of protein added between 0.2 and 0.8 mg. 0.1 M or 0.005 M sodium phosphate buffer gave a maximum of activity suggesting that this enzyme is a phosphorylase. No activity was obtained with Tris buffer. The use of milk xanthine oxidase avoided difficulties when assaying activity in the XDH deficient mutants, *ma-l*, *ry* and *lxd*. A unit of inosine phosphorylase is the amount of enzyme that catalyses one micromole of inosine per minute.

To assay adenosine deaminase larvae were homogenized in cold 0.05 M succinate buffer, pH 6.8, and CHAR-pH5 prepared. 2.8 ml of 0.05 M succinate buffer, pH 6.8, 0.3 ml of $1.5 \times 10^{-3} \text{ M}$ adenosine or deoxyadenosine solution, and 0.8 ml of extract were rapidly mixed. The decrease in optical density at $265 \text{ m}\mu$ was recorded for 20 minutes. The activity exhibited a broad, flat

maximum at pH 6.9 with a 15% reduction in activity at pH 6.1 and 34% reduction at pH 8.6. The reaction rate was proportional to added protein between 0.1 to 0.6 mg. Adenine, AMP and ATP were not deaminated. Deoxyadenosine was deaminated at approximately half the rate as adenosine by all strains. Trace activity noted with AMP was probably due to adenosine derived from a breakdown of the AMP. A unit of adenosine deaminase is the amount of enzyme that catalyzes one micromole of adenosine per minute.

Protein determination: Protein was determined by a modification of the technique of LOWRY *et al.* (1951).

RESULTS

Table 1 shows the averages of replicate determination of the activities of guanine deaminase in seven strains. All extracts possessed guanine deaminase activity. Three strains, *ma-l*, *bw* and *w ec*, possess lower amounts of guanine deaminase activity, particularly the extracts of the *bw* strain which has only 50% of the activity of the Pacific wild type (Pac). The *lxd* and *ry* genes appear to have no effect on the levels of activity of this enzyme. The Canton-S (CS) strain which previously appeared by autoradiographic assay methods to be unable to convert guanine to xanthine (HODGE and GLASSMAN 1967) had guanine deaminase activity equal to Pac.

In an attempt to understand the differences between previous results (HODGE and GLASSMAN 1967) which indicated that extracts of the CS strain had little guanine deaminase and the present results, extracts of Pac and CS were incubated for various times at 30°C with occasional shaking. Under these conditions the CS extract showed a progressive loss of guanine deaminase activity of approximately 80% by 60 minutes, but the Pac extracts lost only about 25% in the same time (Figure 1, left). Mixtures of Pac and CS extracts are inactivated to the same degree as CS extracts suggesting that an inactivator of guanine deaminase activity is present in the CS extracts. Figure 1, right, shows that boiling inactivates this factor. It was noted that extracts of the CS strain became black during the incubation, suggesting that tyrosinase activity is very high. When sodium diethyldithiocarbamate, an inhibitor of tyrosinase, is added to the extracts of the CS strain, the loss of guanine deaminase activity is prevented.

TABLE 1
Activity of guanine deaminase in strains of D. melanogaster

Strain	Specific activity $\pm$ sd	Percent of Pacific
Pacific	38.7 $\pm$ 2.84	100
Canton-S	41.7 $\pm$ 2.21	108
<i>ry</i>	37.6 $\pm$ 2.20	97
<i>lxd</i>	32.6 $\pm$ 2.96	84
<i>w ec</i>	28.0 $\pm$ 1.34	72
<i>ma-l</i>	26.1 $\pm$ 1.47	67
<i>bw</i>	19.3 $\pm$ 1.40	50

These data are the average of the specific activity of six different extracts of each strain, except *bw* which is the average of nine different extracts. Specific activity is the micromicromoles of guanine converted per minute per milligram of protein. The average specific activity of the strains is compared to Pacific which is arbitrarily set at 100%.

FIGURE 1.—Inactivation of guanine deaminase. *Left:* Inactivation in Pacific and Canton-S extracts. 1.0 ml of various CHAR-pH5 were incubated in a water bath at 30°C. At the indicated times, 0.1 ml was removed and assayed for guanine deaminase activity. Percent of original activity is plotted against the time of incubation; open circles (O) Pac extract, open triangles (Δ) CS extract and crosses ($\times$) a mixture of 0.55 ml Pac and 0.45 ml CS extracts.

Right: Inactivation in mixtures of boiled Pacific and Canton-S. 1.0 ml of Pac or CS CHAR-pH5 was placed in boiling water for 1 minute and then centrifuged at 770g for 10 minutes. 0.5 ml of the boiled extract was added to 0.5 ml of CS CHAR-pH5. These mixtures were incubated in a water bath at 30°C. At appropriate times 0.1 ml was removed and assayed for guanine deaminase activity. Percent of original activity is plotted against time of preincubation; open circles (O) Pac CHAR-pH5 plus boiled CS extract; open triangles (Δ) CS CHAR-pH5 plus boiled Pac extract. — 1.0 ml of CS CHAR-pH5 was incubated with 0.1 ml of 10⁻² M sodium diethyldithiocarbamate at 30°C in a water bath. After appropriate times 0.1 ml was removed and assayed for enzymatic activity: crosses ($\times$).

Table 2 shows the activity of inosine phosphorylase in six strains, using inosine and deoxyinosine as substrate. CS and *w ec* have approximately the same activity as the Pac strain while the mutants that affect XDH show an increase in activity. Extracts of *ry* had 78% more enzymatic activity than Pac while extracts of *ma-l* had 86% more activity than Pac using inosine as substrate, but not using deoxy-

TABLE 2
Activity of the inosine phosphorylase in various strains

Strain	Inosine		Deoxyinosine	
	Specific activity $\pm$ sd	Percent of Pacific	Specific activity $\pm$ sd	Percent of Pacific
Pacific	6.1 $\pm$ 0.56	100	12.5 $\pm$ 1.01	100
Canton-S	5.8 $\pm$ 0.44	95	8.5 $\pm$ 1.06	68
<i>lxd</i>	7.5 $\pm$ 0.68	123	10.9 $\pm$ 0.84	87
<i>w ec</i>	7.7 $\pm$ 0.71	126	9.8 $\pm$ 0.58	78
<i>ry</i>	10.8 $\pm$ 0.81	177	11.5 $\pm$ 0.69	92
<i>ma-l</i>	11.3 $\pm$ 0.93	186	11.6 $\pm$ 0.76	93

These data are the average of the specific activities of six different extracts in each strain using inosine or deoxyinosine as substrate. Specific activity is the micromicromoles of inosine or deoxyinosine converted per minute per milligram of protein. The average specific activity of the strains is compared to the Pac strain, which is set at 100%.

TABLE 3

Activity of adenosine deaminase in various strains

Strain	Adenosine		Deoxyadenosine	
	Specific activity $\pm$ sd	Percent of Pacific	Specific activity $\pm$ sd	Percent of Pacific
Pacific	11.9 $\pm$ 0.51	100	6.8 $\pm$ 0.47	100
Canton-S	10.2 $\pm$ 1.30	86	4.7 $\pm$ 0.58	69
<i>bw</i>	12.4 $\pm$ 0.63	104	6.4 $\pm$ 0.52	94
<i>lxd</i>	14.3 $\pm$ 0.78	120	7.5 $\pm$ 0.71	110
<i>w ec</i>	15.5 $\pm$ 0.86	130	8.0 $\pm$ 0.61	118
<i>ma-l</i>	15.8 $\pm$ 1.10	132	8.1 $\pm$ 0.89	119
<i>ry</i>	16.9 $\pm$ 1.15	142	8.5 $\pm$ 0.95	125

These data are the average of the specific activities of six different extracts in each strain, except *ry* and Canton-S strains which are the average of nine separate extracts. Specific activity is the micromicromoles of adenosine or deoxyadenosine converted per minute per milligram of protein. The average specific activity of the strains is compared to Pacific, which is set at 100%.

inosine. Although strain differences do occur, no evidence for the coordinate control of the purine pathway is evident.

Table 3 shows the average of several determinations of adenosine deaminase activity. Once again, strain differences are present but the mutants that affect xanthine dehydrogenase (*ma-l*, *ry*, and *lxd*) do not have reduced levels of this enzyme. Nor do the other strains (CS, *bw* and *w ec*) exhibit major reductions in activity. *ma-l* has 30%, *ry* has 39%, and *w ec* has 60% more activity than the standard strain. Extracts of the CS strain have the lowest activity which is only 50% of the activity noted with extracts of *w ec*.

DISCUSSION

The purpose of these investigations was to ascertain whether there is coordinate expression between four enzymes of purine catabolism, xanthine dehydrogenase, guanine deaminase, inosine phosphorylase, and adenosine deaminase in *D. melanogaster*. These enzymes are involved in the conversion of purines to uric acid. They were chosen because mutants at three loci, *ma-l*, *ry*, and *lxd* decrease the level of XDH, and we wanted to determine whether any of these were regulator genes that also affect the rest of the pathway. A comparison of the level of these enzymes in *lxd*, *ma-l*, and *ry* strains clearly indicate that there is no coordinate expression between the activities of XDH, adenosine deaminase, guanine deaminase and inosine phosphorylase. This finding indicates that the genes coding for these enzymes are not coordinately controlled; nor is there any evidence for any common regulatory mechanism. This is not unexpected since operons as found in bacteria do not seem to exist in animals, and the regulation of enzyme synthesis probably occurs by different mechanisms. Variations in the levels of these enzymatic activities in various strains were found, however. Thus, *ma-l* and *ry* had 77% and 86% more inosine phosphorylase using inosine as substrate than Pac, while a *bw* strain had 50% less guanine deaminase than Pac. Whether these differences are due to transitory environmental influences, to the known

mutant genes, or to unsuspected genes present in these stocks must await further study.

Previous data indicated that the CS strain which contained the mutant gene scarlet eye was deficient in guanine deaminase. The present study has shown that this is due to an inactivator of the enzyme present in extracts of this strain. The inactivation appears to be linear with time of incubation, which could mean that the inactivation is an enzymatic process. It seems likely that tyrosinase is responsible for the loss in activity. Tyrosinase has been shown to oxidase the tyrosyl groups of crystalline pepsin, trypsin, chymotrypsin and insulin, with the resulting loss in trypsin and chymotrypsin activity (SIZER 1947). This interpretation is strengthened by the fact that sodium diethyldithiocarbamate, a copper inhibitor (ROTHCHILD 1951), prevents the loss of guanine deaminase activity in the Canton-S extracts.

SUMMARY

The mutant genes which affect xanthine dehydrogenase, *ma-l*, *ry*, and *lxd*, do not exhibit reduced levels of three other enzymes in the pathway of purine catabolism. This lack of coordinate expression makes it unlikely that there is pathway control by any of these genes. A Canton-S strain containing the mutant gene scarlet eye previously thought to be deficient in guanine deaminase, is not a guanine deaminase mutant, but extracts contain an inactivator of this enzyme. Differences in enzymatic levels were noted among strains, but further tests are necessary to determine the cause of these differences.

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