BIOCHEMICAL DIFFERENCES BETWEEN THE MUTANTS ROSY-2 AND MAROON-LIKE OF DROSOPHILA MELANOGASTER[1]

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THE conversion of 2-amino-4-hydroxypteridine into isoxanthopterin in wild-type *Drosophila melanogaster* has been demonstrated and investigated by several groups (FORREST, GLASSMAN and MITCHELL 1956; HADORN and SCHWINCK 1956; NAWA, TAIRA and SAKAGUCHI 1958). This reaction is catalyzed by an enzyme which will also catalyze the oxidation of hypoxanthine through xanthine into uric acid, xanthopterin into leucopterin (Figure 1), and benzaldehyde into benzoic acid, all of these being typical xanthine oxidase catalyzed reactions. However, the enzyme from Drosophila requires an electron acceptor, presumed to be diphosphopyridine nucleotide (DPN), in the living organism and has therefore been called a xanthine dehydrogenase (GLASSMAN and MITCHELL 1959a).

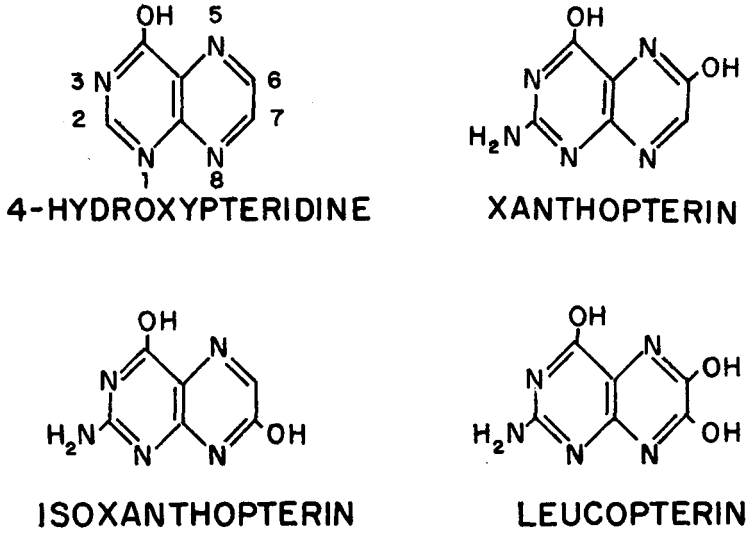

FIGURE 1.—Numbering system for the pteridine ring and chemical formulae of some of the compounds described in the text.

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Interest concerning this enzyme arises from the fact that two mutant sites are known which affect its activity. The mutants rosy (*ry*) (HADORN and SCHWINCK 1956) and maroon-like (*ma-l*) (FORREST, GLASSMAN and MITCHELL 1956) cannot effect any of the above conversions and therefore exhibit a typical and similar phenotype (dull red eyes) and chemotype [accumulations and deficiencies of various pteridines and purines (Table 3)] (HUBBY and FORREST 1960). The only known differences between the two mutants are (1) their genetic location, (2) a "maternal effect" exhibited by young *ma-l* flies under certain breeding conditions (GLASSMAN, HUBBY and MITCHELL 1958; GLASSMAN and MITCHELL 1959b) and (3) the production of a "cross-reacting material" by the *ma-l* mutant which presumably bears some close relationship to the active enzyme (GLASSMAN and MITCHELL 1959a). The rosy mutant does not exhibit a maternal effect and produces little or no cross-reacting material. It is the purpose of this paper to present evidence for a number of additional biochemical differences between the mutants, some of which may shed light on the relationship between the mutants and on the mode of action and properties of the enzyme under their control.

In this study an allele of rosy, rosy-2 (ry^2), was used. The two alleles are identical phenotypically and chemotypically; however, it has recently been reported that they can be separated by crossing over (SCHALET and CHOVNICK 1960). This observation is unlikely to affect the results described in this paper in any major aspect, although obviously an investigation of both alleles in the light of the results reported herein would be of considerable interest.

This investigation arose from a parallel study of the possibility that 4-hydroxypteridine is the first pteridine product in a postulated purine → pteridine transformation. In contrast to the statements in the literature regarding the action of xanthine oxidase on simple pteridines (BERGMANN and KWIETNY 1959), it was found that in extracts of the bacterium *Azotomonas insolita* 4-hydroxypteridine was converted into 2, 4-dihydroxypteridine in a reaction catalyzed by a xanthine oxidase-type enzyme (i.e., oxygen acts as an electron acceptor) (FORREST, HANLY and LAGOWSKI 1961). The same conversion was also found to occur in wild-type Drosophila extracts, and, furthermore, unlike all of the oxidative reactions mentioned above, the reaction did not require the presence of an electron acceptor such as DPN and is therefore a xanthine oxidase-type reaction. Of even greater significance is the fact that extracts of the mutant ry^2 can catalyze the conversion of 4-hydroxypteridine into 2, 4-dihydroxypteridine, but extracts of *ma-l* cannot. Further investigation led to the discovery of other differences between the two mutants ry^2 and *ma-l* (*vide infra*), several of which are probably dependent on the activity of the "oxidase" enzyme in ry^2 flies and its absence in *ma-l* flies.

METHODS AND MATERIALS

Enzyme preparations were made by grinding four-day-old adult flies (0.1 g) in a Ten Broek homogenizer with 0.02 M phosphate buffer, *p*H 7.6 (1 ml). The resultant homogenate was treated with 0.05 g activated carbon for five minutes at 4°C and then centrifuged for 20 minutes in a refrigerated Spinco, Model L,

at 20,000 rpm (Rotor No. 40). The supernatant was recentrifuged at the same speed for an additional 20 minutes. After removal of the fatty layer this final supernatant was dialyzed for three hours against 0.02 M phosphate buffer, *p*H 7.6, at 4°C. The charcoal treatment and dialysis removed endogenous pteridines, preventing their interference with assay procedures. In each preparation the protein concentration was measured using the Folin phenol reagent (LOWRY, ROSENBROUGH, FARR and RANDALL 1951) and was then adjusted to 5–7 mg/ml by dilution with 0.02 M phosphate buffer, *p*H 7.6. Reaction tubes generally contained 0.1 ml enzyme preparation, 0.1 ml substrate solution, 0.05 ml of a 1 mg/ml aqueous DPN solution (if used), and sufficient phosphate buffer (0.1 M at *p*H 7.5) to bring the final volume to 0.3 ml. Pyridoxal hydrochloride was used as a substrate at a concentration of 1 mg/ml water, while purine and pteridine substrates were made up as saturated aqueous solutions.

Reaction mixtures were allowed to incubate for two hours without agitation at 25°C and spotted directly on Whatman's No. 3 mm chromatographic paper, appropriate controls being used throughout. Chromatograms were developed in an ascending direction for 10–14 hours using a solvent composed of two parts 1-propanol:one part 6% aqueous ammonia. Compounds were located on the chromatograms by observing their fluorescence and/or absorption under ultraviolet light (principal emission 260 and/or 360 mμ). Control spots of authentic compounds provided a preliminary identification of the reaction products. After elution of these products from the chromatogram using 1% aqueous ammonia, their complete identification was achieved by comparison with authentic specimens, spectrophotometrically at three different *p*H values and paper chromatographically using several different solvent systems. The difficulties encountered in the paper chromatography of some of the compounds are discussed elsewhere (FORREST, HANLY and LAGOWSKI 1961).

RESULTS

Pteridine oxidations: 4-Hydroxypteridine was converted into 2, 4-dihydroxypteridine by wild-type Drosophila extracts purified by the procedure described above; however, conversion into 2, 4, 7-trihydroxypteridine did not occur until DPN was added to the preparation. In the presence of DPN, only a very small amount of 2, 4-dihydroxypteridine accumulated during the incubation period, the major product being 2, 4, 7-trihydroxypteridine. The addition of a cofactor (DPN) was also necessary for the conversions of hypoxanthine into uric acid and 2-amino-4-hydroxypteridine into isoxanthopterin by the wild-type extract. Extracts prepared from the mutant ry^2 did not effect xanthine dehydrogenase catalyzed reactions (in the presence of DPN) but did catalyze the oxidation of 4-hydroxypteridine into 2, 4-dihydroxypteridine without the addition of a cofactor. All of the substrates mentioned above were unchanged when added to and incubated with *ma-l* extracts.

Oxidation of pyridoxal: Pyridoxal (added originally with the intention of supplying a possible cofactor requirement) was converted by wild-type extracts

into a blue fluorescent compound. A quantity of this compound sufficient for identification was obtained by larger scale paper chromatography. Its ultraviolet absorption spectrum at different *p*H values was measured and compared with the corresponding spectrum of a synthetic sample of pyridoxic acid (HARRIS, HEYL and FOLKERS 1944; HUFF and PERLZWEIG 1944), revealing their close similarity (Table 1). A direct paper chromatographic comparison of the authentic and experimental materials in different solvent systems showed them to be identical (Table 2); further confirmatory evidence was obtained by paper electrophoresis and by comparison of the variation of the intensity of their fluorescences at different *p*H values (HUFF and PERLZWEIG 1944). Pyridoxamine, pyridoxine and pyridoxal phosphate were not converted into pyridoxic acid by the wild-type enzyme extract.

Extracts of the mutant ry^2 brought about this oxidation, but extracts from *ma-l* flies had no effect on pyridoxal.

Miscellaneous biochemical differences: A number of other differences between wild-type and mutant extracts were observed. In these cases, because of their complexity, the underlying reasons for the differences are obscure.

1. While investigating the possibility of using the increased absorption of DPNH over DPN at 340 mμ as a means of quantitatively studying the oxidation reactions listed above, it was found that the absorption at 340 mμ of wild-type enzyme extract itself increased with time. This extract contained 1 mg protein/ml, and, therefore, this observation does not necessarily contradict the statement made by HUBBY and FORREST (1960) that there was no increase in absorption at 340 mμ in a similar preparation, since in the latter case the protein concentra-

TABLE 1

Ultraviolet absorption spectra in aqueous solution

Compound	*p*H	λ_{max}(mμ)	λ_{min}(mμ)
Pyridoxic acid	1	315	264
	7	314	260
	14	306, 247	272, 237
Product from action of enzyme preparation on pyridoxal	1	312	269
	7	312	268
	14	306, 247 (inflection)	273

TABLE 2

Rf values in various solvents

Compound	1-propanol: 6% aqueous ammonia (2:1)	1-propanol: water (3:2)	1-butanol: acetic acid:water (4:1:1)
Pyridoxic acid	0.87	0.89	0.58
Product from action of enzyme preparation on pyridoxal	0.87	0.89	0.58

tion was 1/10 that used in these experiments. The increase in absorption was sensitive to changes in *p*H, protein concentration, and temperature and can therefore probably be attributed to an enzymatic process which may be releasing materials capable of reducing bound DPN or may be effecting release and oxidation of fully reduced, bound pteridines. The formation of such oxidation products would be expected to cause increased absorption at 340 mμ. With regard to the mutants, ry^2 extracts exhibited the same effect; *ma-l* extracts did not.

2. In experiments designed to investigate the occurrence of oxidase and dehydrogenase activities in larvae and pupae, it was noted that wild-type larval extracts and ry^2 larval extracts very rapidly darkened (2–3 minutes), becoming quite black, presumably due to melanin formation. Larval extracts of *ma-l* flies, on the other hand, remained colorless for at least 30 hours.

3. Another interesting difference between the mutants was observed while checking for the possible presence of a xanthine oxidase-type enzyme in these flies. Addition of xanthine to ry^2 preparations caused the formation of hypoxanthine; addition of uric acid unexpectedly caused the production of both hypoxanthine and xanthine. However, addition of uric acid to *ma-l* preparations led only to the production of hypoxanthine. On the other hand, the addition of uric acid to wild-type preparations caused the formation of neither compound. Using C^{14}-labeled uric acid (uric acid-6-C^{14}, Isotope Specialties Co., Inc.), it was established that the hypoxanthine and xanthine produced in these reactions came from endogenous sources and not from the added uric acid. Since the conversion of hypoxanthine into uric acid is blocked in both of these mutants, different pathways for the production of both xanthine and hypoxanthine must be assumed. The reason why uric acid affects these pathways, and does so only in the mutants, remains obscure.

Table 3 summarizes the differences outlined above and those reported by other investigators among wild type, ry^2 and *ma-l D. melanogaster*.

DISCUSSION

The reactions described in this paper probably do not have physiological significance, except possibly the conversion of 4-hydroxypteridine into 2, 4-dihydroxypteridine. This conversion may have some significance for pteridine biosynthesis, although this still remains to be proved. The point to be emphasized, however, is that the ry^2 mutant is capable of carrying on oxidative reactions and the *ma-l* mutant is not. It is possible, of course, that this oxidative activity has no connection with the effect of the rosy gene on the enzyme xanthine dehydrogenase. However, in view of the similarity of the substrates and the nature of the oxidation, it seems reasonable to presume that oxidation is due to residual activity in the enzyme affected by this gene.

Two theories may be advanced to explain the differences observed between the mutants. (1) The wild-type allele of *ma-l* could control formation or incorporation of a cofactor necessary for complete activity of the enzyme xanthine dehydrogenase. In the absence of this cofactor the enzyme would be completely

TABLE 3

The differences and similarities between ma-l *and* ry²

	Wild type	*ry²*	*ma-l*
2, 4-Di- → 2, 4, 7-tri-hydroxypteridine (DPN)	+	—	—
2-Amino-4-hydroxypteridine → isoxanthopterin (DPN)	+	—	—
Xanthopterin → 2-amino-4, 6, 7-trihydroxypteridine (DPN)	+	—	—
Hypoxanthine → xanthine (DPN)	+	—	—
Xanthine → uric acid (DPN)	+	—	—
4-Hydroxy- → 2, 4-dihydroxy-pteridine	+	+	—
Pyridoxal → pyridoxic acid	+	+	—
"Fast melanin production"	+	+	—
"340 mμ optical density increase"	+	+	—
Maternal effect	—	—	+
Endogenous hypoxanthine*	—	+	+
Endogenous hypoxanthine and xanthine*	—	+	—
Lack of isoxanthopterin	—	+	+
Reduced amounts of red pigments	—	+	+
Biopterin accumulated	—	+	+
2-Amino-4-hydroxypteridine accumulated	—	+	+
Sepia pteridines accumulated	—	+	+
CRX Activity	+++	±	++

* Hypoxanthine and/or xanthine produced from endogenous sources when uric acid is added to enzyme preparation.

inactive. The *ry²* allele, on the other hand, might be concerned with the synthesis of the apoenzyme, and, in the mutant condition, it would produce a modified enzyme still active in an oxidative capacity. A disadvantage of this theory is that it has been shown that *ma-l* extracts contain a protein capable of reacting with specific antibodies for xanthine dehydrogenase [cross-reacting (CRX) material]; *ry²* extracts contain little or no material of this nature. (GLASSMAN and MITCHELL 1959a). It seems rather unlikely that the alteration of an enzyme molecule (e.g., by the mutant *ry²* allele) which still leaves the enzyme oxidatively "active" would abolish its cross-reacting activity. On the basis of the presence or absence of cross-reacting material in the mutants, and the maternal effect exhibited by the *ma-l*⁺ allele, GLASSMAN and MITCHELL (1959b) postulated the gene sequence shown in Figure 2a as the most likely arrangement. This scheme would scarcely be tenable on the above "cofactor" theory.

It should also be pointed out that added cofactors [flavinadenine dinucleotide, flavin mononucleotide, molybdenum or iron, some of which are known cofactors for milk xanthine oxidase (*cf.* DE RENZO 1956)] caused no observable effects in extracts of either mutant, nor have mixing experiments between mutant extracts produced any enzyme activity (GLASSMAN and MITCHELL 1959a). Although none of these experiments can be considered to be conclusive—for example, the cofactors might be tightly bound and not exchangeable with exogenous material —they provide cogent arguments against the "cofactor" theory.

(2) Xanthine dehydrogenase could have two sites of enzyme activity, each

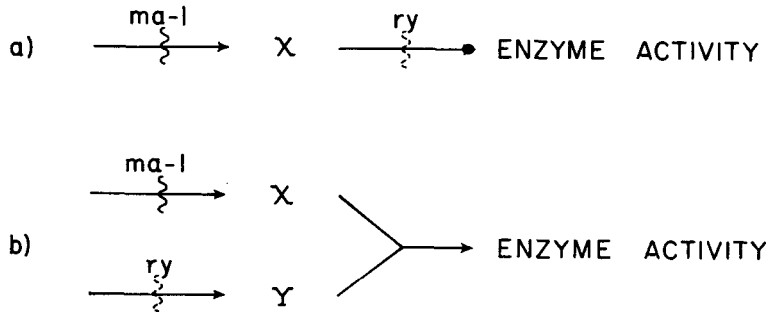

FIGURE 2.—Schemes proposed by GLASSMAN and MITCHELL (1959b) for the action of the two genes in the production of xanthine dehydrogenase.

controlled by a different gene. [Dual functions for the classical xanthine oxidase and chicken liver xanthine dehydrogenase, where purines, pteridines and aldehydes are oxidized at one site and reduced DPN is oxidized at another site, have been suggested by other data (*cf.* DE RENZO 1956)]. Thus, the mutant ry^2 would affect the dehydrogenase site (perhaps the site where the cofactor is bound), leaving the site of oxidation unaffected but probably with a markedly reduced efficiency. On the other hand, the mutant *ma-l* would necessarily affect the site of substrate binding, consequently interrupting both oxidase and dehydrogenase activities. Alteration of the substrate site would be assumed to have little or no effect on the protein's ability to react with specific antibodies.

This explanation would be more in accord with the second of GLASSMAN and MITCHELL's schemes for the action of the two genes on xanthine dehydrogenase activity (Figure 2b). Thus, *ma-l* flies would contain the product of the wild-type *ry* allele, y, having all of the cross-reacting ability but no enzyme activity. In contrast, ry^2 flies would contain the product of the wild-type *ma-l* allele, x, having no cross-reacting ability, but which would be enzymatically active with an efficiency, however, less than that of wild-type flies. In fact, by an extension of this scheme, it becomes somewhat analogous to the system in *Escherichia coli* controlling tryptophan synthetase (CRAWFORD and YANOFSKY 1958), where two proteins, A and B, are necessary to make a fully functional enzyme, although each, by itself, has some enzyme activity in the half reactions involved in the production of tryptophan. Cross-reacting activity, in contrast to this, is confined to one of the proteins (LERNER and YANOFSKY 1957). The analogy with the scheme proposed in this paper for the mutants ry^2 and *ma-l* is at once apparent.

To test these hypotheses purification of the proteins involved in the different enzyme activities is contemplated.

SUMMARY

In addition to the known differences between the mutants *ma-l* and *ry*, both of which lack a functional xanthine dehydrogenase, a number of new biochemical differences between the two have been discovered. The most significant of these

is the ability of extracts of the ry^2 mutant, like those of wild type, to catalyze the conversion of 4-hydroxypteridine into 2, 4-dihydroxypteridine and of pyridoxal into pyridoxic acid without the addition of DPN. Extracts from *ma-l* flies are unable to catalyze these reactions. Two theories have been advanced to explain these observations: (1) the "cofactor" theory in which it is assumed that the *ma-l* mutant is lacking an essential cofactor and the ry^2 mutant has an altered apoenzyme; (2) the "dual site" theory in which it is assumed that each mutant allele affects a different site on the enzyme molecule, the *ma-l* mutant having a lesion at the site of substrate binding and the ry^2 mutant at the site of dehydrogenase activity. An extension of this latter theory, to bring it into line with previous work on these mutants, suggests that these sites may be on different protein molecules, both of which are necessary for complete enzyme activity; one of the protein molecules contains cross-reacting activity, and the other contains residual oxidase activity.

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ADDENDUM

A referee has criticized the work reported herein on the basis that the mutants studied are not from coisogenic stocks. Admitting the validity of the criticism, we would like to point out the very great difficulties involved experimentally in attempting to carry out enzyme work on true coisogenic stocks of *Drosophila melanogaster*. We have now approached the problem from the opposite viewpoint in the following way. Two genetically different alleles of rosy, and one of maroon-like present in quite different genetic backgrounds have been shown to behave in exactly the same way as rosy-2 and maroon-like respectively. Thus, from random sources, the mutants all show their characteristic biochemical properties as described in this paper. It seems reasonable to assume, therefore, that their biochemical activities are, indeed, connected with their known genetic defects.

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