ANNOTATIONES ZOOLOGICAE JAPONENSES
Volume 27, No. 1—March 1954

Published by the Zoological Society of Japan
Zoological Institute, Tokyo University

Tyrosinase in *Drosophila virilis*

With 1 Text-figure

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(*Communicated by* T. FUJII)

During recent years, there has been much interest in the nature of the process of hardening and darkening, which takes place in the cuticles of msot insects after moulting and pupation*. In puparium formation of the blowfly, it has been pointed out that the hardening and a part of the pigmentation are due to the tanning action of o-quinone produced by oxidation of a phenolic substance (Pryor *et al.* '40, '47). According to the view of Fraenkel and Rudall ('47) and Dennell ('47a), the phenolic substance responsible for the tanning action is produced from blood tyrosine under the action of tyrosinase, both blood tyrosine and tyrosinase increasing in amount before pupation. In the author's previous paper ('53) it was shown that blood tyrosinase in *Drosophila melanogaster* increases abruptly at the time of puparium formation, and that tyrosinase occurs as an inert proenzyme which can be activated by incubating with tissue brei.

In the present paper, after confirming the above relations in the related species, *D. virilis*, some properties of the tyrosinase are presented. Furthermore, tyrosinase activity is compared among some mutants which differ in the degree of puparium pigmentation. A brief description of oxidase activity in the cuticles is also presented.

MATERIALS AND METHODS

Strains and culture method. The wild strain 'Pasadena' was used throughout this study. For the mutants, 'ebony' and 'yellow^{40a}' were

* For the most recent references, readers are referred to Hackman ('53a, b, c).

compared. The former has a darker colour both in the puparium and in the imaginal integument than the wild type, and the latter is lighter in both respects. To avoid the probable effect of modifiers, the following cross was repeated twice: the mutant strains were crossed to the wild strain 'Pasadena' and selected again from the F_2 flies. The materials were reared at 25°C on a standard medium of agar, molasses and cornmeal, which was seeded with yeast and reinforced daily with small blocks of live yeast.

Preparation of enzyme solution. The method of enzyme preparation was essentially the same as that described previously as 'dissection method accompanied by 1 hour incubation'. Larvae or pupae were weighed and dissected in a watch-glass containing distilled water, thus setting free the body fluid into the medium. After decanting the diluted body fluid, the body tissues were ground in a small glass mortar. Diluted body fluid, which contains protyrosinase, was added to the brei of body tissues, which has an activator, and the mixture was incubated for one hour at 25°C. After centrifuging, an aliquot of the supernatant was used as the enzyme solution.

Measurement of tyrosinase activity. The method of measurement and calculation of Q_{O_2} was the same as that described previously. Catechol was used as the substrate unless otherwise indicated.

Histochemical examinations. Nadi-reagent was made after the Graff method and the peroxidase activity was detected after the method of McJunkin (cited by Glick '49).

Preparation of acetone powder of cuticle. White prepupae were immersed in distilled water of 60°C for 5 min., and the cuticles were isolated by dissecting and scraping away the adherent tissues with needles. (Since heat treatment did not affect the oxidase activity, it was undertaken to facilitate the scraping by coagulating the tissues). Cuticles thus obtained were put into acetone and left for 24 hours, then dried in air. These were ground finely in a glass mortar.

RESULTS

a) *Nature of blood tyrosinase.*

After confirming the fact that the tyrosinase activity of *D. virilis* is mostly located in the body fluid and that tyrosinase occurs as an inert proenzyme which can be readily activated by incubating with tissue brei, the following experiments were carried out to clarify the nature of the enzyme. These experiments were made with the enzyme preparation obtained from the white prepupae.

The oxidation rates of various substrates were compared with that

of catechol, using the same enzyme preparation. The results are given in Table 1. Hydroquinone was not oxidized directly, but was oxidized

Table 1.

Tyrosinase activity towards different substrates, taking catechol as the basis of comparison

substrate	activity (a)	activity on catechol (b)	ratio (a/b×100)	induction period
	Q_{O_2}	Q_{O_2}	%	
p-cresol 5 mg	16.3	60.9	27	+
〃 〃	13.9	63.8	22	+
tyrosine 0.5 mg	19.5	73.5	27	+
〃 〃	11.4	54.4	21	+
dopa 5 mg	59.5	60.8	98	−
〃 〃	59.5	60.7	98	−
hydroquinone 5 mg	0		0	
hydroquinone 5 mg + catechol 0.1 mg	47.3	60.7	78	−
pyrogallol 5 mg	24.9	60.7	41	−

indirectly in the presence of catechol. Monophenols, i.e., *p*-cresol and tyrosine, were also attacked and an induction period of about 15 min. was observed in these cases.

It may be seen from Table 2 that the activity was suppressed by several compounds which combine with heavy metals, especially with

Table 2.

Effect of inhibitors

compound	concentration	activity	% inhibition
Na diethyldithiocarbamate	10^{-3}M	0	100
thiourea	10^{-3}M	4.7	93
KCN	10^{-3}M	3.4	95
CO	(CO/air 1/3)	0	100
p-nitrophenol	1.4×10^{-2}M	0	100

copper. *p*-Nitrophenol was not oxidized, but acted as a competitive inhibitor on catechol oxidation.

Table 3 shows the effect of pH on tyrosinase activity. Owing to the fact that aqueous solutions of catechol buffered above pH 7.0 are subjected to extensive autoxidation, it is not feasible to investigate the activity on the alkaline side. As is shown in Table 3, there appears to be no significant difference in activity between pH 5.5 and 7.0.

Danneel reported that the enzyme of *D. melanogaster* capable of oxidizing dopa to red substance was bound to some cellular structure

and could be mostly thrown down by centrifuging at 5,000 and 15,000 r.p.m. for 10 min.

An enzyme preparation of *D. virilis* was centrifuged at 22,300*g* (16,000 r.p.m.) for 15 min. and the activity of the supernatant fluid was compared with that of uncentrifuged stock solution. About 76 per cent of the activity remained in the supernatant solution (Table 4). It was concluded, therefore, that tyrosinase in *D. virilis* is for the most part in a soluble state.

Table 3.

Effect of pH

pH	5.5	6.0	6.5	7.0
Q_{O_2}	52.8	59.0	59.0	55.9

Table 4.

Effect of centrifugal force (22,300*g* for 15 min.)

supernatant fluid (a)	non-centrifuged (b)	ratio (a/b×100)
Q_{O_2} 44.7	Q_{O_2} 58.9	75.9

b) *Physiological roles of blood tyrosinase.*

Figure 1 shows the change in tyrosinase activity during the developmental period. It is clear that the activity increases rapidly at the

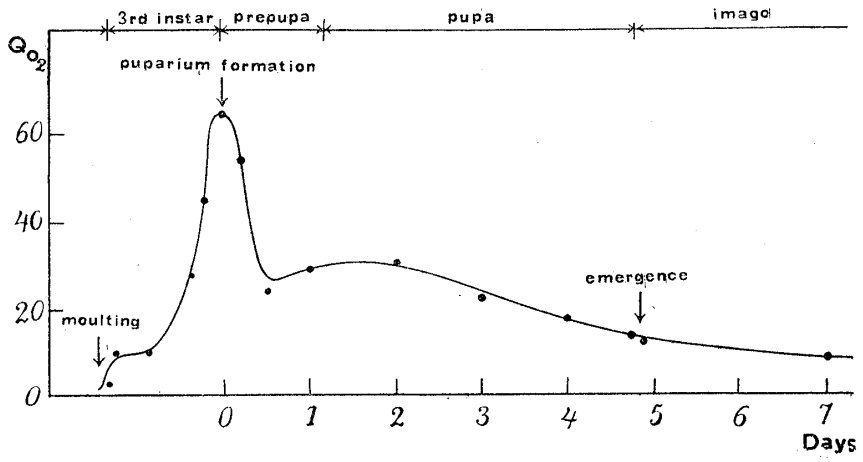

Fig. 1. Change in tyrosinase activity during development.
Abscissae: Time in days after puparium formation.
Ordinates: Tyrosinase activity expressed in Q_{O_2} (O_2 uptake/mg body weight/hour)
Each point represents the mean value of 3–7 measurements.

time of pigmentation of the puparium, as in the case of *D. melanogaster*. But in contrast to the latter species, which shows a relatively low value except for a sharp peak at the time of puparium formation, the tyrosinase activity of *D. virilis* maintains a considerably higher level during the

3rd instar and the whole pupal life. After emergence, it diminishes gradually and becomes almost nil in about a week. No rise in the activity was detected at the time of imaginal pigmentation.

The above measurements suggest an intimate relation between tyrosinase activity and puparium formation. It will be interesting, in this connection, to compare the tyrosinase activity at the time of puparium formation among the wild type 'Pasadena' and the mutants 'yellow' and 'ebony', which differ in degree of puparium pigmentation. The result shown in Table 5 indicates that the activity of 'ebony' is higher and that of 'yellow' is lower than that of the wild type. These differences, although not very large, are statistically significant. This indicates that the mutant which has a darker puparium possesses higher tyrosinase activity.

Table 5.

Comparison of tyrosinase activity among wild type, 'yellow' and 'ebony' at the time of puparium formation

genotype	activity mean	standard deviation	No. of determinations	$t'\left(\frac{\bar{x}-\bar{y}}{\sqrt{\frac{S_1^2}{n'}+\frac{S_2^2}{n''}}}\right)$
	Q_{O_2}			
Pasadena	63.84	±7.49	27	
ebony	71.03	±4.09	26	(between+and eb) 4.3
yellow^{40a}	53.57	±6.26	30	(between+and y^{40a}) 5.6

c) *Oxidase activity of cuticle.*

As the site of pigmentation at puparium formation is the cuticle, it is necessary to examine the oxidase activity responsible for quinone tanning in the cuticle itself. Dennell ('47b), Blower ('51) and Krishnan ('51) observed that the cuticles of some insects and crustaceans show a positive reaction with nadi-reagent and they attributed this fact to the presence of polyphenol oxidase.

When white prepupae of *D. virilis* were dissected in nadi-reagent, the cuticle, mid-gut, hind-gut, muscle and Malpighian tubes were stained. But if prepupae were heated at 60°C for 5 min. beforehand, the reactivity was mostly lost except in the cuticle. The reactivity of the cuticle is retained even after the treatment at 60°C for 40 min., although it is lost by exposure to 100°C for 5 min. The reaction was also positive in acetone powder of cuticle.

It was suppressed by cyanide of relatively high concentration (10^{-1}M) and by warm solution of diethyldithiocarbamate (10^{-1}M, 60°C). Cuticle, fresh as well as acetone dried, was also positive with the peroxidase test of McJunkin.

An experiment was designed to determine the relation between the oxidase in the cuticle and the tyrosinase in the blood. The oxygen uptake of acetone powder of cuticle was followed manometrically, using catechol and dimethyl-*p*-phenylenediamine as substrates. As shown in Table 6, the oxidase in the cuticle differs from the tyrosinase in the body fluid in that it can oxidize dimethyl-*p*-phenylenediamine without the participation of catechol.

Table 6.

Oxidation rate on catechol and dimethyl-*p*-phenylenediamine by acetone powder of cuticle and blood tyrosinase

substrate / material	catechol 5 mg (a)	dimethyl-*p*-phenylene-diamine 5 mg (b)	dimethyl-*p*-phenylenediamine 5 mg, catechol 0.1 mg (c)	ratio
	Q_{O_2}	Q_{O_2}	Q_{O_2}	
acetone powder of cuticle 10 mg	1.26	1.53		(b/a) 1.2
blood tyrosinase	64.7	0	8.5	(c/a) 0.13

DISCUSSION

As was mentioned previously, it is generally accepted that free tyrosine occurring in the body fluid is a source of a phenolic substance which is responsible for quinone-tanning and melanin formation. An experiment of Yabe (unpublished) in *D. virilis* also supports the view that the pigment in the puparium is mainly derived from tyrosine or phenylalanine. Although the details are not yet clear, it is assumed that tyrosine is oxidized in the body fluid to 3, 4-dihydroxyphenylalanine (dopa) and then deaminated and degradated to 3, 4-dihydroxybenzoic acid (protocatechuic acid), which is transferred to the cuticle through the hypodermis (Dennell '47a, '49, Pryor *et al.* '47, Fraenkel and Rudall '47, Hackman '53c, etc.). The fact that tyrosinase activity in the blood rises abruptly at the time of puparium pigmentation suggests that it plays some significant role in the hardening and darkening of the puparium, and this is probably the oxidizing of tyrosine to dopa in these processes. It is interesting that 'ebony' has higher, and 'yellow' has lower, tyrosinase activity than the wild type at the time of puparium formation, and this relation holds in the case of 'ebony' of *D. melanogaster*, which has less tyrosinase activity and a lighter puparium than the wild type (unpublished data*).

All the properties of *Drosophila* tyrosinase activity, so far as they have been examined, are in good agreement with those of mushroom

* The connection between the present experiments and those of Graubard ('33) and Danneel ('43) will be discussed in another paper.

tyrosinase (Nelson and Dawson '44). It may, therefore, be concluded that the activity is derived from a single enzyme, belonging to tyrosinase or polyphenol oxidase.

The oxidase activity of the cuticle in *D. virilis* is different from that of typical tyrosinase or polyphenol oxidase, which has no action on dimethyl-*p*-phenylenediamine (Keilin '29). The fact that it resists acetone and dryness and is relatively stable under heat treatment excludes the possibility that it is cytochrome oxidase (Keilin '33).

Summary

1. The tyrosinase activity of *D. virilis* shows the same properties as mushroom tyrosinase in its specificity, its inhibitors and pH effect.

2. Tyrosinase activity increases rapidly at the time of puparium formation, as in the case of *D. melanogaster*. But in contrast to the latter species, the enzyme maintains a relatively high activity throughout the 3rd instar and pupal periods.

3. The mutant 'ebony', which has a darker puparium, has higher tyrosinase activity and the mutant 'yellow', in which the puparium is lighter in colour, shows lower tyrosinase activity than the wild type 'Pasadena'.

4. The oxidase in the cuticle differs from the tyrosinase of the blood in being able to oxidize dimethyl-*p*-phenylenediamine directly.

The writer is indebted to Prof. Y. Akita for his constant guidance and encouragement and to Prof. T. Fujii of Tokyo University for his kind advice and valuable criticism. Thanks are also due to Prof. K. Dan for reading the manuscript.

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