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Studies on the Tyrosinase System in Lepidopterous Insects
II. Reducing Power of the Body Fluid of *Philosamia cynthia ricini*

By

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In the epidermis of *Bombyx mori*, it was established that the white part contains larger amount of reducing substances such as SH compound and ascorbic acid than the black part does (INAGAMI, 1956). This seems to imply that the rate of the melanosis which occurs in a given part of the *Bombyx* epidermis is determined by the amount of reducing substances that are present there together with tryosinase and phenolic substrates; that is, the more the reducing substances are, the lower the rate of the melanosis is. According to the author's experiments, it is apparent that the melanosis of the body fluid of *Philosamia cynthia ricini* and *Samia cynthia* is suppressed by SH compound naturally occurring in it (HARADA and KATO, in press; HARADA, in press). But, the following points remain unsettled that (1) what is the principal reducing substance in the body fluid of these insects and (2) whether SH compound inhibits the tyrosinase activity by virture of keeping the reducing power of the body fluid strong enough to prevent the oxidation of phenols.

In order to get some informations concerning these questions, the present work was undertaken. Firstly, the contribution of SH compound to the reducing power of the body fluid was examined, and secondly, the content of ascorbic acid was measured.

Materials and Methods

The body fluid of *Philosamia cynthia ricini* was used as material. It was collected into tubules previously chilled with ice-water. (1) The reducing power of the body fluid was estimated according to the decolorizing rate of the solutions of redox dyes, such as methylene blue and toluylene blue, in THUMBERG's tube. Into the tube were poured 2.0 ml of 1/15 M phosphate buffer (pH 6.8), 0.5 ml of the body fluid and 0.5 ml of either distilled water or the solution of p-chloromercuribenzoic acid. Each solution of the redox dyes was poured into the side arm of the tube. After 3 minutes for evacuation and 10 minutes for the temperature equilibrium at 37°C, the redox dye solution in the side arm

was mixed with the liquid in the tube and then the time required for the dye-decolorization was measured. (2) The content of ascorbic acid in the body fluid was estimated with MITSUDA and SHIKANAI's method (1955) of using the decolorizing rate of 2,6-dichlorophenolindophenol butanol solution.

In order to remove protein, 10 ml of the body fluid was mixed with 10.0 ml of 5% metaphosphoric acid, prepared just prior to use, and the mixture was centrifuged at 3.000 r.p.m. for 10 minutes. One ml of the supernatant was treated with 0.25 ml of saturated solution of p-chloromercuribenzoic acid (PCMB) to take away SH compound and was mixed with 5.0 ml of 2,6-dichlorophenol-indophenol solution by shaking the tube vigorously for 3 minutes. The extinction of the butanol fraction separated by centrifugation was measured at 530 mμ by means of BECKMANN's spectrophotometer.

Experimental Results

1. *Change of the reducing power of the body fluid estimated as the bleaching rate of methylene blue during metamorphosis*: As is given in Fig. 1, considerably rapid decolorization of methylene blue (final concentration 1/13×0.01%) is observed in the body fluid taken from the larvae at the 4th sleeping stage.

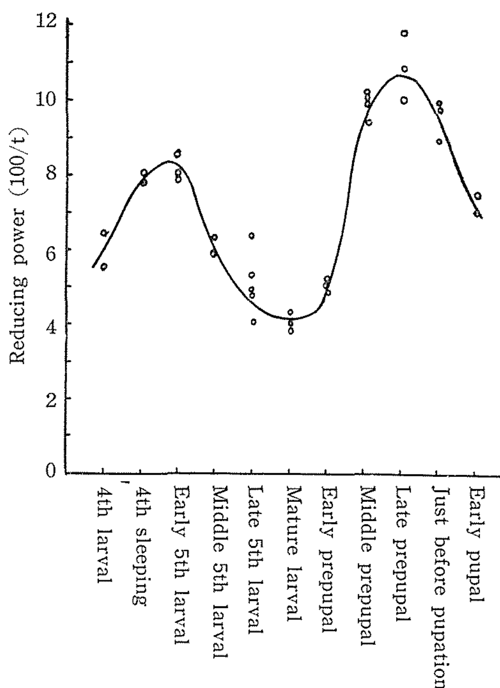

Fig. 1. Fluctuation of the reducing power for methylene blue.

However, the reducing power decreases gradually as the development proceeds, and it reaches the minimal rate at the mature and the early prepual stages. At the middle and the late prepupal stages in which the worms shrink remarkably for pupation, it increases again considerably, succeeded with a little decrease at the time of pupation.

2. *Change of the reducing power estimated as the bleaching rate of toluylene blue*: In this series, the side arm was filled with 1.0 ml of toluylene blue solution (final concentration $1/4 \times 0.01\%$). As is shown in Fig. 2, fluctuation of the reducing power during metamorphosis indicates the quite similar pattern to that revealed in the previous examination using methylene blue. Generally speaking, decolorization of toluylene blue proceeds more rapidly than that of methylene blue. This seems a matter of course, since the former has a higher redox potential than the latter.

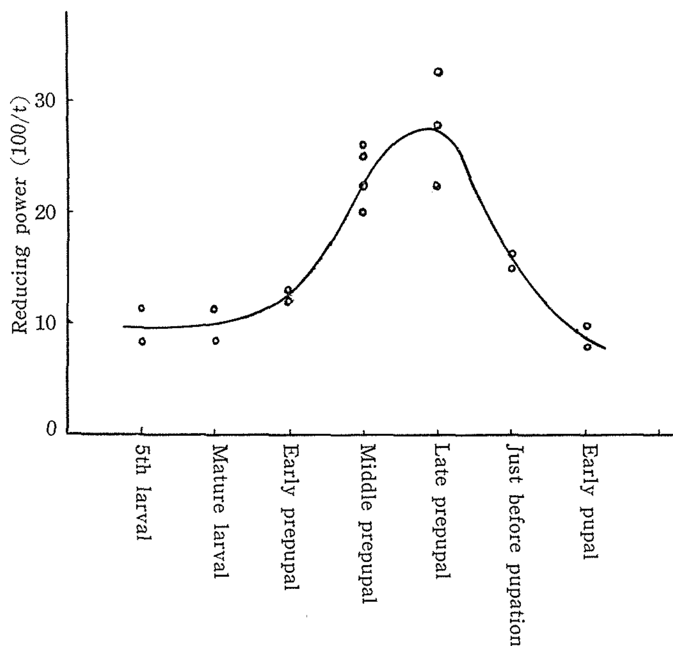

Fig. 2. Fluctuation of the reducing power for toluylene blue.

3. *Effect of PCMB on the reducing power of the body fluid*: To make clear how much the endogenous SH compound contributes to the reducing power of the body fluid, the effect of PCMB on the reducing power of the body fluid was next examined. The final concentration of PCMB was quite enough to cause an enormous increase of the tyrosinase activity in the body fluid (HARADA and KATO, in press). As seen in Table 1, retardation of the reductive bleach-

Table 1. Effect of PCMB on the reducing power of the *Philosamia* body fluid (methylene blue was used as the redox dye).

Developmental stage	In the absence of PCMB (control)	In the presence of PCMB	
		2×10^{-5}M*	1/6 saturated*
4th larval	6.5	6.5	
	5.2		5.2
4th sleeping	8.0	7.9	
	8.0		8.0
Early 5th larval	9.1	9.1	
	7.9		7.5
Mature larval	4.0		4.1
	4.6		5.0
Early prepupal	5.0		5.2
	5.3	5.8	
Middle prepupal	10.0	9.5	9.5
	9.5	10.0	10.0
Late prepupal	10.0		9.7
	11.7		12.2
Early pupal	10.0		10.0

* final concentration

ing of methylene blue by PCMB is not observed at any stage of the development. A similar result was obtained when toluylene blue was used as the redox dye, so far as the author has examined.

Therefore, it seems safe to state that SH compound hardly relates to the reducing power of the body fluid of *Philosamia*. This seems to suggest that the established fact that the melanosis is inhibited by the endogenous SH compound may not be brought about through the mechanism that SH compound naturally occurring in the body fluid keeps the reducing power strong enough to prevent the oxidation of phenolic substances.

Then, it arises the question what substance occupies the principal part of the reducing power of the body fluid. Ascorbic acid or reducing sugars may be surmised to be such a principal reducing substance. In the present work, the content of ascorbic acid, which is known to be in a close relation to the tyrosinase system (LERNER, 1953; KRUEGER, 1950, 1958), was measured at various stages of metamorphosis.

4. *Ascorbic acid content of the body fluid*: Fluctuation of the content of ascorbic acid in the body fluid during the course of metamorphosis is shown in Fig. 3.

The content is relatively low at the larval and early prepupal stages, high at the middle and late prepupal stages and it becomes again slightly low near the onset of pupation.

This type of change is quite identical with that of the reducing power of

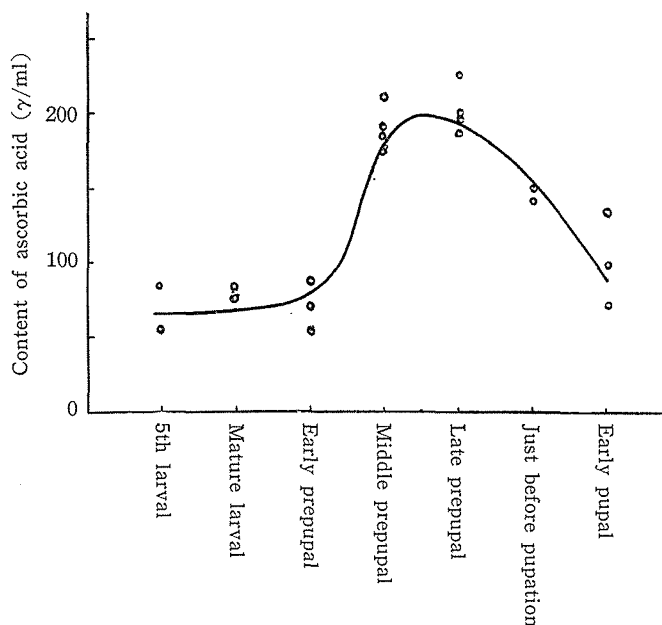

Fig. 3. Fluctuation of ascorbic acid content.

the body fluid estimated as the bleaching rate of methylene blue and toluylene blue. Moreover, contrary to the extremely low content of SH compound, large amount of ascorbic acid was estimated from the larval stage to the early pupal stage.

Discussion

As above described, addition of PCMB exerts no noticeable effect on the reducing power of the body fluid estimated as the bleaching rate of methylene blue and toluylene blue. This fact seems to imply that SH compound contributes nothing, or at least not appreciable amount, to the reducing power of the body fluid. This view is supported by the finding that amount of SH compound in the body fluid of *Philosamia* is negligible (KATO and MIURA, 1959). Therefore, as to the mechanism of the inhibition of the melanosis by SH compound, it may be reasonable to deny the possibility that SH compound in the body fluid operates chiefly upon the maintenance of such a low redox-potential that the oxidation of phenols fails to occur. Contrary to SH compound, it was demonstrated that a large amount of ascorbic acid is involved in the body fluid at every stage of the development, and that the fluctuation of its content from the larval stage to the early pupal stage is quite similar to that of the reducing power of the body fluid. This finding appears to indicate that ascorbic acid is

the principal reducing substance in the body fluid. Moreover, from the facts that the melanosis of the body fluid does not take place before most part of the endogenous ascorbic acid is oxidized and that addition of ascorbic acid to the body fluid delays the occurrence of the melanosis remarkably (HARADA, in press), it may be assumed that it is not SH compound but ascorbic acid that keeps the reducing power of the body fluid strong enough to prevent the oxidation of phenolic substances. On this standpoint of view, it may be concluded that the inhibitory action of SH compound to the melanosis must be brought about through some specific relationship between them.

Here, the following two possibilities propose themselves as the explanation on the mechanism of the inhibition of the melanosis by SH compound. That is to say, one is the possibility that SH compound may be nothing but some dehydrogenase which requires SH group for its action and that o-quinone produced by tyrosinase may be reduced into o-diphenol continuously by the action of the dehydrogenase. Another is that the inhibition of the melanosis may be induced through the block of copper in the tyrosinase molecule with sulphur of SH compound.

But it is to be noticed that the reducing power of the body fluid estimated as the bleaching rate of such redox dyes as methylene blue and toluylene blue was never decreased by addition of PCMB, while the melanosis of the body fluid can be enhanced very markedly by the same reagents (HARADA and KATO, in press). In view of this fact, the first possibility is considered not to be of much importance for the following reason. If the conspicuous acceleration of the melanosis of the body fluid by PCMB and other SH reagents, established in the body fluid of mature larvae, prepupae and pupae, is due to their inhibitory effect to the action of the dehydrogenase, the bleaching rate of those dyes would also be decreased more or less by the same reagent since they are known as the electron acceptor from most dehydrogenase systems.

Accordingly, at the present stage of the investigation, the second possibility may be accepted more easily, though further studies must be directed to clarify the validity of this view.

Summary

1. Fluctuation of the reducing power in the body fluid of *Philosamia cynthia ricini* during ecdysis and metamorphosing period was determined.

2. The reducing power expressed in terms of the decolorizing rate of redox dyes is weak in the body fluid of the larval and early-prepupal stages, but powerful at the middle and late prepupal stages. It becomes slightly less powerful near the onset of pupation.

3. SH compound in the body fluid appears to contribute nothing to the dye-decolorization, since PCMB exerts no appreciable effect on it. Therefore, it will be safe to state that SH compound is not the essential reducing substance

and that inhibition of tyrosinase by SH compound may be due to the specific relationship between them.

4. In the body fluid ascorbic acid exists in a relatively large amount at every stage of the development, and its fluctuation during the metamorphosing period is quite identical with that of reducing power of the body fluid. Consequently, it is evident that ascorbic acid is the chief reducing substance in the body fluid of *Philosamia*.

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