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Studies on the Tyrosinase System in Lepidopterous Insects
V. Tyrosinase Activity of the Body Fluid in Various Concentrations of Saline or Sucrose Solution

By

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NITTONO *et al.* (1953, 1954) established that the tyrosinase activity of the egg extract of *Bombyx mori* varies markedly according to the saline concentration in the medium in which the eggs are ground; that is, the tyrosinase activity decreases as the saline concentration rises higher. Moreover, OHNISHI (1954) demonstrated that the activation of protyrosinase in the body fluid of *Drosophila virilis* can be inhibited in the media of higher concentrations of the saline solution (>M/12). But, little has been known on the mechanism of these phenomena.

On the other hand, the author has carried out a series of experiments with the aim of clarifying the nature of tyrosinase of the Lepidopterous body fluid *in vivo*, and so far he has demonstrated how the tyrosinase activity varies under various conditions *in vitro*. This paper is concerned firstly with the pattern of variation of the tyrosinase activity which is induced by means of diluting the body fluid with various media and secondly with the discussion about the possible mechanism of the variation.

Materials and Methods

The body fluid of *Bombyx mori* (race: J122×C115) and *Philosamia cynthia ricini* and the suspension of the acetone powder prepared from their body fluid were used as materials. The tyrosinase activity was estimated by the oxygen uptake and the darkening rate of the reacted mixture.

The measurement of the oxygen uptake was made by means of the Warburg apparatus as follows. The main cup of the manometer flask was poured with 2.0 ml of the saline or sucrose solution of various concentrations as the medium. The side arm of the flask was filled with 0.5 ml of the body fluid or of the suspension of the acetone powder, and the center well was occupied by 0.2 ml of 10% KOH solution. After the temperature equilibrium at 25°C, the

fluid in the side arm was mixed with the medium in the main cup and then the measurement of the oxygen uptake was performed.

The colours of the reacted mixtures were compared with each other by means of the Duboscq colorimeter and the darkening rate was calculated.

The acetone powder of the body fluid was prepared as follows. One volume of the body fluid taken from the 5th instar larvae of *Bombyx mori* or from the prepupae of *Philosamia cynthia ricini* was treated with ten volumes of chilled acetone. The resulted precipitate was washed 5 times with chilled acetone and finally with ethyl-ether. Then it was dried by evacuation. The white powder thus obtained was preserved in a deep-freezer at −20°C and in case of need 0.1 g of it was suspended in 5.0 ml of the distilled water.

Experimental Results

1. *Tyrosinase activity of the body fluid in the various concentrations of the saline solution*: The solution of NaCl (M/200, M/100, M/50) and the distilled water were used as the experimental media. As given in Fig. 1, the oxygen uptake of the body fluid decreases gradually as the concentration of NaCl increases. This change of the tyrosinase activity runs parallel with the darkening rate of the reacted mixtures: that is to say, the darkening rate of the reacted mixture decreases against the increase of the NaCl concentration. Since the oxygen uptake and the darkening rate are inhibited almost completely by addition of phenylthiocarbamide (final concentration, $1/6\times10^{-3}$ M), it is evident that both phenomena are caused by the action of tyrosinase.

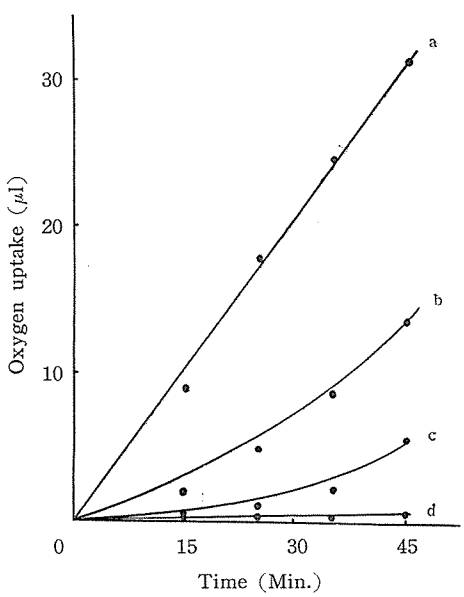

Fig. 1. Effect of varying the concentration of NaCl in the medium on the tyrosinase activity of the body fluid of the mature larvae of *Philosamia*. a, M/200 NaCl; b, M/100 NaCl; c, M/50 NaCl; d, M/200 NaCl with phenylthiocarbamide.

The similar variation is observed when the plasma of the body fluid, the supernatant centrifuged at 3000 r.p.m. for 7 minutes, is used as materials, or when RINGER'S solution of various dilutions is used as medium.

Table 1. Dilution effect of inorganic salts in the medium on the darkening rate of the body fluid and of the plasma during the first 5 minutes of reaction.

	Materials	RINGER's solution	1/2 RINGER's solution	1/4 RINGER's solution
Body fluid	Prepupal (*Philosamia*)	No darkening	1.00	1.32
		No darkening	1.00	1.45
	5th larval (*Bombyx*)	1.00	1.29	1.37
		1.00	1.22	1.33
		1.00	1.19	1.54
Plasma	5th larval (*Bombyx*)	1.00	1.20	1.34
		1.00	1.06	1.27
		1.00	1.11	1.40
	Prepupal (*Bombyx*)	1.00	1.41	1.50
		1.00	1.09	1.24

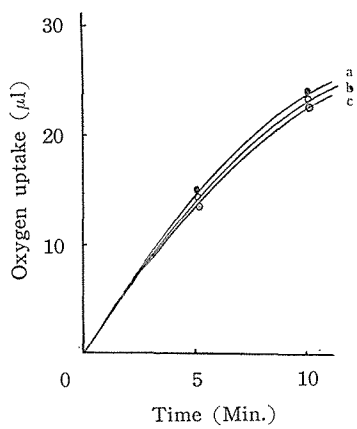

Fig. 2. Effect of varying the concentration of NaCl in the medium on the tyrosinase activity of the acetone powder of the body fluid of *Philosamia* prepupae. 0.5 ml of M/100 *p*-cresol solution was added as the substrate. a, M/200 NaCl; b, M/100 NaCl; c, M/50 NaCl.

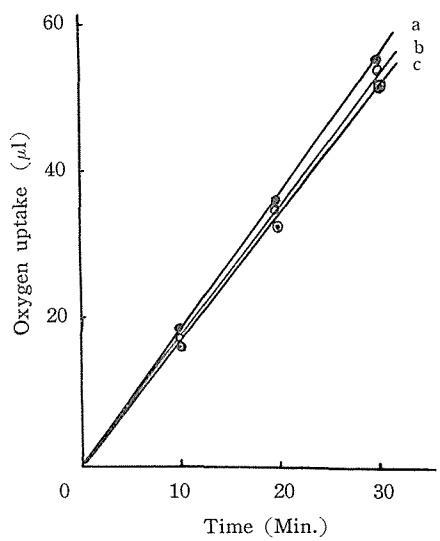

Fig 3. Effect of varying the concentration of RINGER's solution on the tyrosinase activity of the acetone powder of the body fluid of *Philosamia* prepupae. 0.5 ml of 0.04% *l*-tyrosine solution was added as the substrate. a, Undiluted RINGER's solution; b, 1/2 RINGER's solution; c, 1/4 RINGER's solution.

2. *Tyrosinase activity of the suspension of the acetone powder prepared from the body fluid in the various concentrations of the saline solution*: Five ml of the distilled water was suspended with 0.1 g of the acetone powder. In this series of experiments 0.5 ml of the solution of *l*-tyrosine (0.04%) or of *p*-cresol (M/100) was added as the substrate.

Figs. 2 and 3 illustrate the results with the acetone powder of the prepupal body fluid of *Philosamia*. Contrary to the previous results using the body fluid, the change of the tyrosinase activity does not occur practically in spite of the change of the saline concentration in the medium.

This is true of the acetone powder of the *Bombyx* body fluid.

3. *Tyrosinase activity of the body fluid in the various concentrations of the sucrose solution*: Next, as a solution of non-electrolyte, the sucrose solution of various concentrations (M/100, M/50, M/25, M/2 and 1M) was employed as the experimental medium. In this series, the body fluid of *Philosamia cynthia ricini* served exclusively as materials.

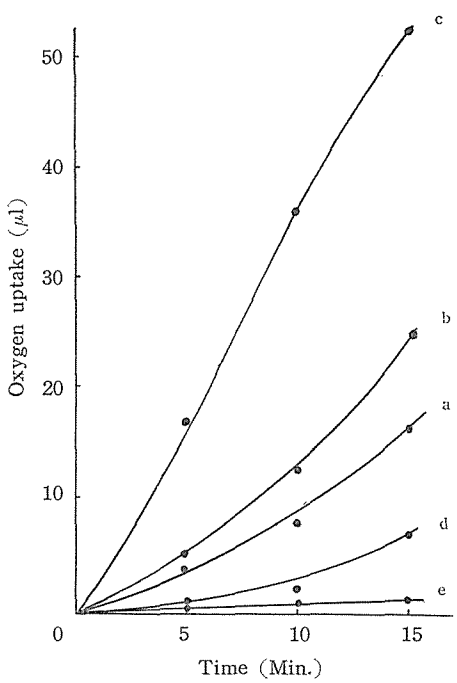

Fig. 4. A representative case of the effect of varying the concentration of sucrose in the medium on the tyrosinase activity of the body fluid of the mature larvae of *Philosamia*. a, distilled water; b, M/50 sucrose; c, M/2 sucrose; d, 1M sucrose; e, M/2 sucrose with phenylthiocarbamide.

Fig. 4 illustrates the representative case of the results obtained in the body fluid taken from the mature larvae of *Philosamia*. The tyrosinase activity varies markedly according to the sucrose concentration in the medium. But, it must be noted that the pattern of variation is quite different from that in the series of the saline solution: that is, the activity becomes more and more intense as the concentration of sucrose increases up to M/2. However, in the solution of 1M, it is much more feeble than in the medium of the distilled water. This type of variation is also observed when the plasma is used as materials.

In this connection, it seems to be interesting that dilution of the body fluid with sucrose solution of higher concentrations such as 1M, M/2 and M/25 yields a transparent mixture, while dilution with the

distilled water or sucrose solution of lower concentrations results in the opaque suspension. The reason for this phenomenon is yet uncertain, but the opaque suspension seems to occur according to the decrease of the solubility of proteins dissolved in the body fluid.

In the prepupal body fluid, such variation of the tyrosinase activity is not observed so clearly as in the case of the mature larvae. That is, the tyrosinase activity in this stage is so high even in the medium of the distilled water, that the variation of the activity due to media is detectable only in the early period of reaction (2 or 3 minutes) except in 1M solution. In 1M solution of sucrose, however, the tyrosinase activity is conspicuously lower than in the distilled water and this difference lasts for approximately 20 minutes as in the case of the body fluid of the mature larvae.

4. *Tyrosinase activity of the suspension of the acetone powder prepared from the body fluid in the various concentrations of the sucrose solution*: As is seen in Fig. 5, the tyrosinase activity of the acetone powder of the *Philosamia* body fluid does not vary at all notwithstanding the change in the concentration of sucrose. This is in accord with the result obtained in the medium of the saline solution. Therefore, it may be safe to state that the tyrosinase activity of the acetone powder is kept quite constant regardless of the change of concentration of the solute in the medium.

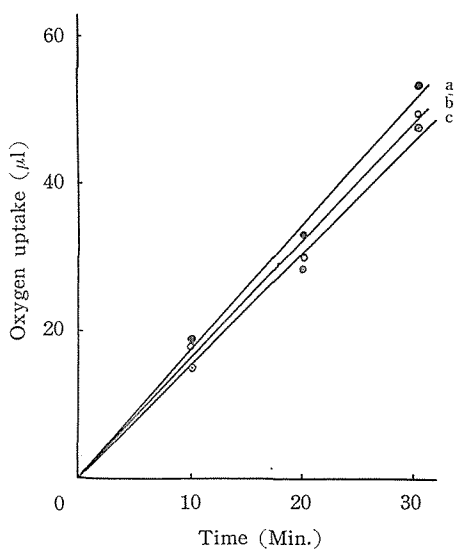

Fig. 5. Effect of varying the concentration of sucrose in the medium on the tyrosinase activity of the acetone powder of the body fluid of *Philosamia* prepupae. 0.5 ml of 0.04% *l*-tyrosine solution was added as the substrate. a, distilled water; b, M/50 sucrose; c, M/25 sucrose.

Discussion

As mentioned above, the tyrosinase activity in the body fluid varies generally according to the various concentrations of saline or sucrose in the medium with which the body fluid is diluted, but in the suspension of the acetone powder such variation of the tyrosinase activity does not occur. This seems to suggest that the nature or the state of tyrosinase in the body fluid is different from that in the suspension of the acetone powder. To make clear the reason for the difference between in the body fluid and in the suspension of the acetone powder, further investi-

gation must be required. But, two possibilities may be proposed as the tentative explanation: one is that the variation of the tyrosinase activity seen in the body fluid may be caused by the degree of osmotic destruction of the haemocytes, while these cells are absent as such in the suspension of the acetone powder. This view stands on an assumption that most of tyrosinase molecules are localized upon the haemocytes. However, the results described in this paper cannot favour this view, for the similar variation of the tyrosinase activity is observed in the plasma of the body fluid. But, there still remains an excuse for this view, if we assume that tyrosinase molecules have their loci upon such cellular particles as microsomes and mitochondria, which are liberated into plasma from the haemocytes at the time of centrifugation of the body fluid. Granted that the assumption is right, the degrees of the osmotic destruction of these cellular particles may also induce a marked variation of the tyrosinase activity, because the membrane of these particles is considered to be of the same nature as the protoplasmic membrane.

Concerning the intracellular localization of tyrosinase molecules, however, the comments so far available are different. For instance, LERNER *et al.* (1949) reported that the enzyme is present in the cellular structure such as microsomes and mitochondria in the melanoma tissue of mammals. Contrary to this, FOX and BURNETT (1959) have opined that in the cells of *Neurospora crassa* tyrosinase is not in association with the particles of mitochondrial and microsomal size but it exists in the soluble state. Under these considerations, the further investigation must be directed to disclose the localization of tyrosinase in the body fluid.

The other possibility is that the variation of the tyrosinase activity in the body fluid may be brought about by the change of the chemical properties of tyrosinase molecule itself, which is caused by treating the body fluid with the saline or sucrose solution. The author has established that tyrosinase is inhibited by SH compound naturally occurring in the body fluid of *Philosamia cynthia ricini* and *Samia cynthia*, but not in that of *Bombyx mori* and he has expressed his opinion that the inhibition by SH compound can be considered to occur through the chemical association between sulfur of SH compound and copper in the active center of the tyrosinase molecule (HARADA and KATO, 1960; HARADA, 1960). However, the variation of the tyrosinase activity caused by varying the media seems not to be induced by means of the change in the dissociation constant of the chemical bond between copper and sulfur. For, the variation in the saline solution is observed very clearly in the *Bombyx* body fluid in which tyrosinase appears not to be inhibited by the endogenous SH compound. Moreover, the deeply frozen body fluid (at $-20°C$) of the mature larvae of *Philosamia*, in spite of the fact that addition of *o*-iodosobenzoic acid failed to cause an increase in the tyrosinase activity, is diluted with the sucrose solution, with the resultant marked variation of the tyrosinase activity (HARADA, unpublished data). This seems to indicate that freezing treatment

of the body fluid removes the inhibition by the endogenous SH compound, but it can not exert any noticeable effect on the susceptibility of tyrosinase to the change of the sucrose concentration.

Under these considerations, the author is inclined to opine that the variation of the tyrosinase activity will be caused by the change of the protein part of the enzyme. It is well known that the protein part of phenolases is apt to change with a slight modification of treatments (HONDA and MIURA, 1954). In connection with this, the following observation is instructive that dilution of the body fluid with saline or sucrose solution yields a transparent mixture, while dilution with the distilled water results in the production of a cloudy suspension. As mentioned above, difference may be brought about by the difference of the solubility of the protein in the body fluid. If so, it may not be unreasonable to infer that some delicate change would also be induced in the protein part of tyrosinase by the saline or sucrose solution of various concentrations. The fact that variation of the tyrosinase activity does not occur in the suspension of the acetone powder will be interpreted from this point of view as follows: the treatment of the body fluid with acetone will cause some denaturation in the protein part of the enzyme so as to eliminate the ability to react with the inorganic ions or sucrose molecules.

It must be discussed how the difference in the pattern of variation of the tyrosinase activity does occur between in the sucrose solution and in the saline solution. Since, however, the contribution of the protein part to the mode of action of tyrosinase is scarcely known, it is impossible to say about the mechanism of the effect of inorganic ions and sucrose molecules.

Summary

1. The pattern of the change of the tyrosinase activity in the medium of various concentrations of saline or sucrose solution was investigated, using the materials of both body fluid and acetone powder prepared from it.

2. The tyrosinase activity of the body fluid of *Philosamia cynthia ricini* and *Bombyx mori* decreases as the concentration of saline in the medium increases.

3. The tyrosinase activity of the body fluid taken from the mature larvae of *Philosamia* increases in parallel with the increase of the sucrose concentration in the medium, at least up to M/2. But, it becomes much lower in the medium of 1M solution than in the distilled water.

4. No appreciable variation of the tyrosinase activity of the acetone powder occurs in the medium of saline or sucrose solution.

5. Therefore, it is highly probable that the property or the state of tyrosinase is different in the body fluid and in the acetone powder.

Acknowledgment

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