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Beta-Alanine and Ketocatechol in the Hydrolysate of the Puparial Case of *Drosophila melanogaster*

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Abstract The puparial cases of three kinds of body color mutants, *yellow*, *black*, and *ebony* of *D. melanogaster* were hydrolyzed and examined for neutral ketocatechol and β-alanine. The quantity of neutral ketocatechol released was largest in *ebony*, followed by *black*, and smallest in *yellow*. On the contrary, the quantity of β-alanine was largest in *yellow*, small in *black*, and zero in *ebony*.

When *black* was fed on a β-alanine-containing medium, β-alanine in the hydrolysate of puparial case increased, while the neutral ketocatechol decreased.

These results suggest that the quantity of β-alanine in puparial case and the extent of β-sclerotization may be inversely related to each other.

Introduction

Biochemistry of the sclerotization of insect cuticle is now making a considerable progress. It may be summarized as follows. (1) There are two kinds of mechanisms of sclerotization, namely, quinone tanning and β-sclerotization (Andersen, 1976, 1977, 1979). (2) Just at the time of sclerotization, β-alanine is incorporated into cuticle (Bodnaryk and Levenbook, 1969; Bodnaryk, 1971a; Hodgetts, 1972; Ross and Morroe, 1972). (3) In insects, there exists N-β-alanyldopamine (Umebachi, 1975a and 1980). (4) N-β-alanyldopamine may act as a sclerotizing agent in addition to N-acetyldopamine (Hopkins *et al.*, 1982). And (5) in some insects, the cuticle of black mutant strains lacks β-alanine, while the cuticle of wild type strains contains the amino acid (Seki, 1962; Fukushi and Seki, 1965; Fukushi, 1967; Jacobs and Brubaker, 1963; Jacobs, 1966). Umebachi (1958, 1961, 1975b), Umebachi and Yoshida (1970), and Umebachi and Yamashita (1976, 1977) have investigated the structure of the pale yellow pigment (Papiliochrome II) of *papilio* butterflies and found that the pigment consists of kynurenine and N-β-alanyldopamine derivative. Rembold *et al.* (1978) and Rembold and Umebachi (1983) have shown the structure of papiliochrome II to be N^{ar}-[α-(3-aminopropionylaminomethyl)-3', 4'-dihydroxybenzyl]-L-kynurenine. In the course of these investigations, Umebachi and Aburano (1978, 1979)

have examined the presence or absence of β-alanine in the hydrolysate of the wing-scales of various butterflies and found that, in general, there is an inverse relation between quantities of β-alanine and ketocatechol released on hydrolysis. This suggests that there may be an inverse relationship between the mechanism of β-sclerotization and the quantity of β-alanine in scales. In other words, β-alanine may depress the β-sclerotization.

The purpose of the present paper is to see whether the inverse relationship between β-alanine and ketocatechol is true also of cuticle. For this purpose, three kinds of body color mutant strains (*yellow, black,* and *ebony*) of *D. melanogaster* were used. The puparial cases of these mutants were hydrolyzed, and the β-alanine and ketocatechol released were determined. Feeding-experiments of β-alanine using *black* were also performed.

Materials and Methods

Materials

Three kinds of body color mutant strains of *Drosophila melanogaster, yellow, black,* and *ebony* were used. *Ebony* is a mutant which can synthesize β-alanine but can not incorporate (or fix) the amino acid into cuticle (Fukushi, 1967; Jacobs and Brubaker, 1963; Jacobs, 1966; Hodgetts, 1972). As a result, the cuticle lacks β-alanine. The body color of adult is black, whearas the puparial case is paler than that of the wild type strain. *Black* is a mutant in which the synthesis of β-alanine is completely or partially blocked but the incorporation of the amino acid into cuticle is normal (Hodgetts, 1972; Hodgetts and Konopka, 1973). Consequently, the cuticle contains little β-alanine. The body color of adult is black, whearas the puparial case is paler than that of the wild type strain. *Yellow* is a mutant of which the cuticle contains a larger quantity of β-alanine than that of the wild type strain (Fukushi, 1967; Umebachi *et al*., 1983). The color of puparial case is more yellowish brown than that of the wild type starin. All these mutant strains had been backcrossed to the wild type starin, Oregon R, at least five times.

The flies were raised in the culture medium containing malted rice 125g, sugar 50g, agar 12.5g, water 1000ml, KH_2PO_4 900mg, Oxiben M (10% methyl-*p*-hydroxybenzoate in 95% ethanol), and raw yeast.

For the feeding-experiments of β-alanine, the last instar larvae of *black* were transferred to the following culture medium and were allowed to pupate: agar, 500mg; Ebios (powder of dead beer yeast), 15g; the Oxiben M solution, 2ml; and 1 M β-alanine in water, 10ml. For the control medium, 10ml of water was added instead of the β-alanine solution.

The culture were all performed at 25±1°C.

Cuticle powder

After the emergence of flies, the puparial cases (exuviae) were gathered, and washed first with 70% ethanol at room temperature several times and then with diethyl ether three times. The exuviae were dried and pulverized in a mortar. The powder thus obtained was kept at 4°C until use and is below called the starting material.

Hydrolyses

For the determination of ketocatechol, the above-mentioned starting material was refluxed in 1 N HCl at 100°C for 5 hr. The hydrolysate was centrifuged at 1400 *g* for 10 min, and the supernatant was filtered through Centriflow (Amicon, CF-25). The filtrate was submitted to the Biogel P-2 column mentioned later.

For the determination of β-alanine, the starting material was refluxed in 6N HCl at 100°C for 24 hr.

The hydrolysate was centrifuged at 1400 *g* for 10 min, and the supernatant was evaporated to dryness in a rotary evaporator at 60°C. After being kept over NaOH in a vacuum desiccator, the residue was dissolved in 70% ethanol or water and was again brought to dryness in a vacuum dessicator. After the residue was further dissolved in a little water and centrifuged at 1400*g* for 10 min, the supernatant was submitted to the isotachophoresis mentioned below.

Biogel P-2 column

The above-mentioned filtrate through Amicon was applied to the Biogel P-2 column (1.5×60cm or 2×60cm) and eluted with 0.2N acetic acid. The effluent was automatically monitored at 280nm, and then fracions of 5.9ml were collected.

Thin-layer chromatography

Fractions from the Biogel P-2 column were pooled for each peak, lyophilyzed, dissolved in 70% ethanol, and submitted to one-or two-dimensional thin-layer chromatography with cellulose sheet (Merck, No. 5552). The solvent for one-dimensional chromatography was 70% methanol (MeOH) or a mixture of *n*-butanol, acetic acid, and water (12:3:5) (BAW). For two-dimensional chromatography, 70% MeOH was used for the first direction, and BAW, for the second direction. After development, the phosphomolybdic acid-NH_3test (Umebachi and Yoshida, 1970) or the ninhydrin reaction was carried out on the thin-layer sheet.

Estimation of neutral ketocatechol

This was performed by a slight modification of the method of Andersen (1980). The fractions of neutral ketocatechol from the Biogel P-2 column were combined, and its volume was measured. Then, absorbance of the solution was measured at 310nm. The relative value of neutral ketocatechol was represented by the absorbance multiplied by the volume.

Estimation of β-alanine

This was performed with isotachophoresis (Umebachi et al., 1983). The above-mentioned sample obtained from the hydrolysate in 6N HCl was submitted to a capillary tube isotachophoresis, Shimazu IP-1B. Leading electrolyte consisted of 0.01M HCl, 0.02M Ammediol (2-amino-2-methyl-1,3-propandiol), and 0.1% Triton X-100. The pH was 8.9. Terminal electrolyte contained 0.01M ε-aminocaproic acid and 0.01M Ammediol. The pH was adjusted to 10.8 with $Ba(OH)_2$. The capillary tube was 26cm in lengh and 0.57mm in diameter. The migration current was 100 to 150 μampere. The temperature was 20°C. The quantity of β-alanine was calculated using the calibration curve which had been prepared with authentic β-alanine.

Synthetic neutral ketocatechol

In some experiments, 2-hydroxy-3', 4'-dihydroxyacetophenone was used as a standard of ketocatechol. This compound was synthesized by the method of Voswinckel (1909).

Results

Elution pattern from Bigel P-2 column

When the hydrolysate of the starting material in 1N HCl was submitted to the Biogel P-2 column, the elution pattern as shown in Fig. 1 was obtained. The elution pattern was common to *yellow, black,* and *ebony.* The result was not substantially influenced by the diameter of column and the mesh of Biogel.

Peaks of Ketocatechol

Absorption spectra of the peaks A, B, C, D, and E of Fig. 1 were taken over the range

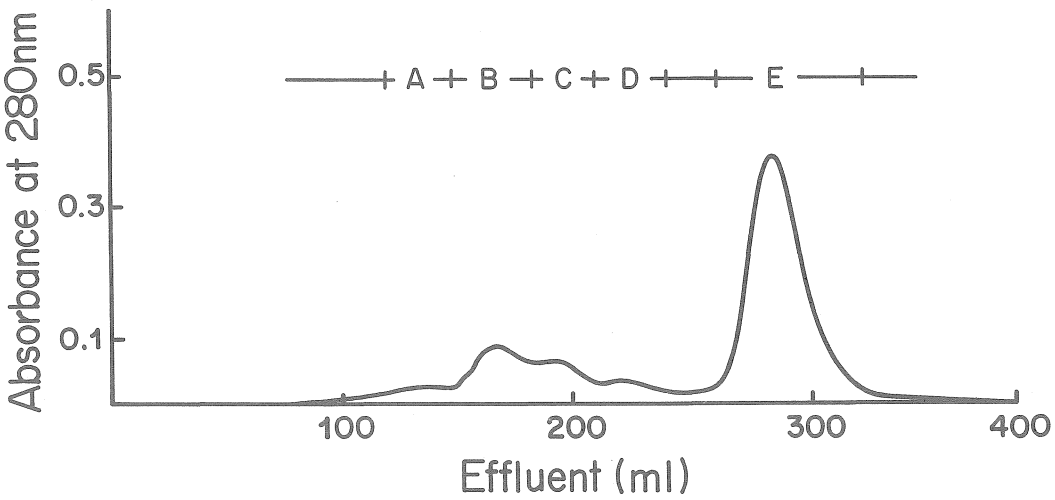

Fig. 1. Biogel P-2 gel filtration of the hydrolysate of puparial case, in 0.2N acetic acid.

of 230 to 400nm in 0.2N acetic acid. The spectra of the peaks D and E showed absorption maxima around 280 and 310nm (Fig. 2). This indicates that these two peaks D and E contain ketocatechols. For the purpose of comparison, the absorption spectrum of 2-hydroxy-3', 4'-dihydroxyacetophenone is also given in Fig. 2.

The fractions of the peaks D and E of Fig. 1 were pooled respectively, lyophilyzed, dissolved in 70% ethanol, and submitted to two-dimensional thin-layer chromatography. The results are given in Fig. 3. The spot 1 from the peak D fraction was positive to both the ninhydrin reaction and the phosphomolybdic acid-NH_3test. The spots 2 and 3 from the peak E were negative to the ninhydrin reaction but positive to the phosphomolybdic acid-NH_3test. The former spot (spot 2 in Fig 3) coincided with 2-hydroxy-3', 4'-dihydroxyacetophenone in both thin-layer chromatographic behaviors and color tests.

All these results show that the peak E of Fig. 1 contain neutral ketocatechols as reported by Andersen (1971, 1980). According to Andersen's reports, the spot 3 of Fig. 3 may be dihydroxyphenyl ketoaldehyde. There is a possibility that the peak D of Fig. 1 may contain a ketocatechol containing NH_2-group in its side-chain. But, as the amount of the peak D fraction was small, the quantitative determination of ketocatechols in the cuticle hydrolysate was made with the peak E.

In some cases, a faint peak appeared immediately before the peak E. The substance was similar to uric acid in thin-layer chromatographic behaviors, color tests, and absorption spectrum. But the quantity was small.

Estimation of neutral ketocatechols in the hydrolysate

The relative amounts of the peak E are given in Table 1, which shows that the amount of neutral ketocatechols in the hydrolysate is largest in *ebony*, followed by *black*, and less in *yellow*. The differences between *ebony* and *black* and between *black* and *yellow* were significant.

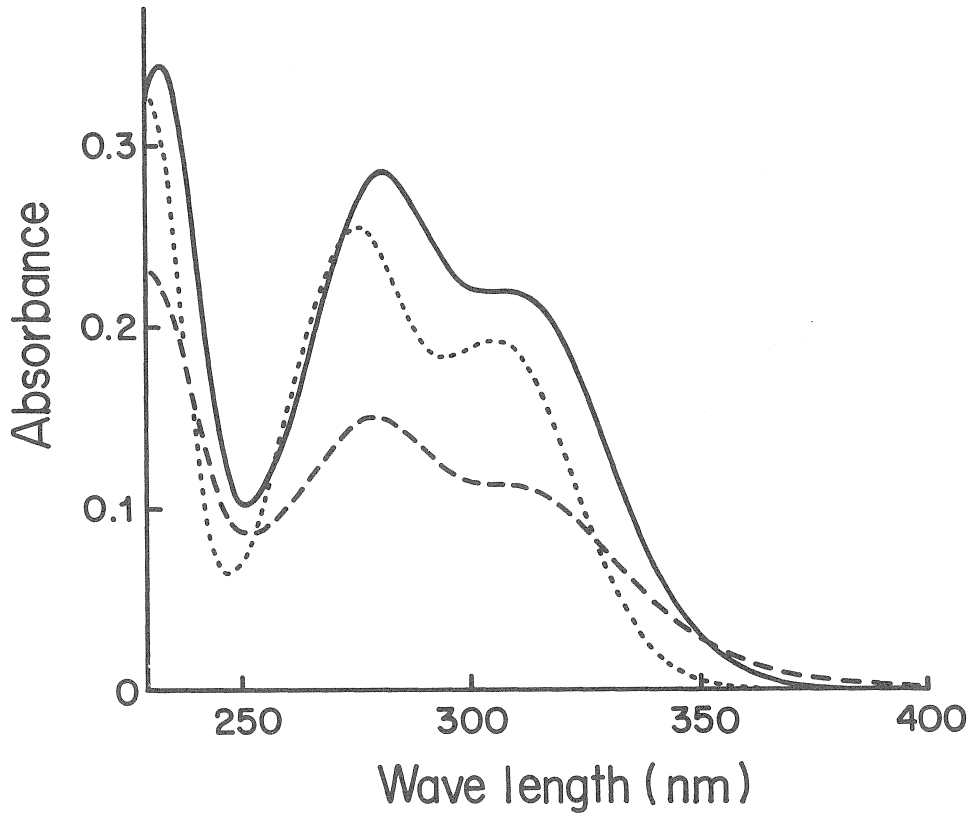

Fig. 2. Absorption spectra of the peaks D and E and a synthetic ketocatechol. Dashed, unbroken, and dotted lines; peak D, peak E, and 2-hydroxy-3', 4'-dihydroxy-acetophenone, respectively.

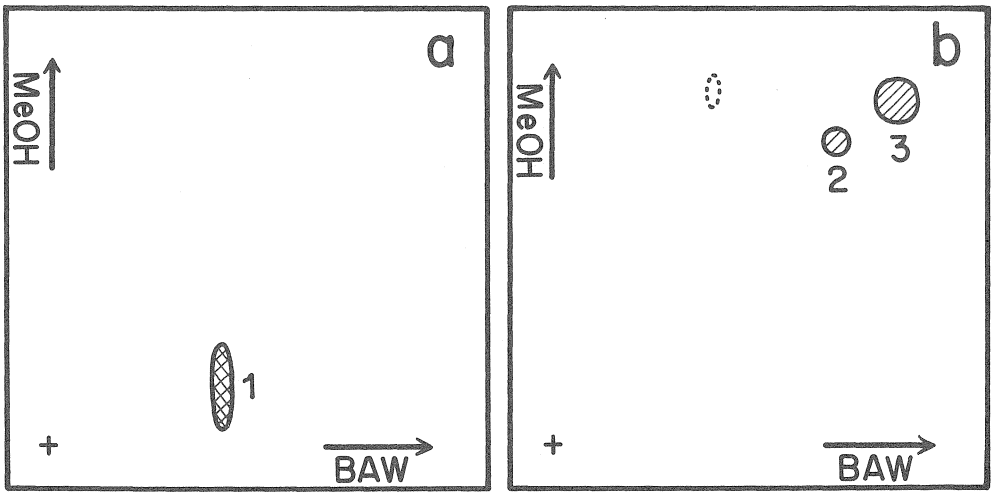

Fig. 3. Thin-layer chromatograms of the fractions of peaks (a) D and (b) E. Oblique line from left to right, positive to ninhydrin; oblique line from right to left, positive to phosphomolybdic acid-NH_3.

Table 1. Relative value of neutral ketocatechol in the hydrolysate of puparial case

Mutant	Neutral ketocatechol (relative value)	
Yellow	17.7±0.2*	P<0.001
Black	25.7±0.4	P<0.01
Ebony	32.5±1.9	

*Mean of four experiments±S. E.

Estimation of β-alanine in the hydrolysate

The results are given in Table 2, which shows that *yellow* contains a large quantity of β-alanine and that *black* contains a small quantity of it. On the other hand, *ebony* did not show the amino acid. The differences between *yellow* and *black* and between *black* and *ebony* were significant.

Table 2. Beta-alanine in the hydrolysate of puparial case

Mutant	β-Alanine (μg/100mg of puparial case*)	
Yellow	625.9±25.8**	P<0.001
Black	65.5±9.9	P<0.01
Ebony	0	

* This means the starting material mentioned in the section of methods.
** Mean of three experiments±S. E.

Feeding-experiments of β-alanine in black

The puparial case of *black* is pale but that of the *black* which was fed with β-alanine turned yellowish brown, that is, the color of the puparial case came near that of the wild type strain.

The amounts of neutral ketocatechol and β-alanine in the hydrolysate of the puparial case of the β-alanine-fed *black* are given in Table 3, which shows that β-alanine increased in comparison with the control animal, while neutral ketocatechols decreased. This indicates that *black* incorporated the fed-β-alanine into the puparial cuticle and that, in response to the incorporation of β-alanine, ketocatechols decreased.

Table 3. Neutral Ketocatechol and β-alanine in the hydrolysate of the puparial case from the β-alanine-fed-*black*

Experiment	Neutral ketocatechol (relative value)		β-Alanine (μg/100mg of puparial case*)	
Beta-alanine-fed	85.5±0.5** (4)†	P<0.01	187.7±12.4 (4)	P<0.001
Control	97.1±2.3 (3)		70.0±0.8 (3)	

* See the footnote in Table 2.
** Mean±S. E.
† The numbers of experiments are indicated in parentheses.

Discussion

The presence of β-alanine in insect cuticle is well known. What role does the β-alanine play in cuticle? To what compound in cuticle is the β-alanine bonded? The answer to these problems must explain the following two facts. (1) Beta-alanine is incorporated into cuticle just at the time of sclerotization. The incorporation of β-alanine into cuticle requires the presence of dopamine (Bodnaryk, 1970, 1971b; Hodgetts, 1972; Hodgetts and Konopka, 1973). (2) The cuticle of black body color mutants lacks β-alanine, while that of wild type strain contains β-alanine (Seki, 1962; Fukushi and Seki, 1965; Fukushi, 1967; Jacobs and Brubaker, 1963; Jacobs, 1976; Hodgetts, 1972; Hodgetts and Konopka, 1973). In other words, the presence of β-alanine seems to depress the melanin formation. As a compound which meets these two requirements, N-β-alanyldopamine, which was found for the first time by Umebachi (1975a, b, 1980) and Rembold et al. (1978), may be considered. Recently, Hopkins et al. (1982) have reported that N-β-alanyldopamine may act as a sclerotizing agent in cuticle. More recently, Yago et al. (1984) has reported the presence of N-(N-acetyl-β-alanyl) dopamine in the colleterial gland of *Mantis religiosa*.

Umebachi and Aburano (1978, 1979) have investigated the presence or absence of β-alanine in scales of butterflies and have found that β-alanine and ketocatechol released on acid hydrolysis are inversely related to each other. The results of the present paper show that the inverse relationship between β-alanine and ketocatechol is also true of the hydrolysate of the puparial case of *D. melanogaster*. It is especially worthy of remark that this inverse relationship has been confirmed by the feeding-experiment of β-alanine using *black*. All these results suggest that the presence of β-alanine in cuticle may be inversely related to the extent of β-sclerotization. What does this mean? There may be a possibility that the ratio of quinone-tanning to β-sclerotization is controlled by β-alanine. And the key substance in this connection may be N-β-alanyldopamine. It is tempting to speculate that although N-β-alanyldopamine acts as a sclerotizing agent for quinone-tanning, it does not act as a sclerotizing agent for β-sclerotization and that even if N-β-alanyldopamine can form indole ring, the compound can not polymerize to form melanin.

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