

## CATECHOLAMINES AND $\beta$ -ALANINE IN THE RED FLOUR BEETLE, *TRIBOLIUM CASTANEUM*

### ROLES IN CUTICLE SCLEROTIZATION AND MELANIZATION\*

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**Abstract**—Catecholamines and  $\beta$ -alanine titres were measured in a rust-red wild strain of the red flour beetle, *Tribolium castaneum* Herbst and in a mutant strain which has a black cuticle. During the first three days after adult eclosion,  $\beta$ -alanine and *N*- $\beta$ -alanyldopamine (NBAD) are present in the former strain, but they are absent or very low in the latter. Dopamine is approx. 3-fold higher in the black than in the rust-red strain while *N*-acetyldopamine, the major catecholamine in both strains, is about two times more abundant in the black. Fully sclerotized rust-red elytra contain nearly ten times more NBAD than black elytra, while the latter have about ten times more dopamine. Injection of  $\beta$ -alanine into newly eclosed mutant adults rescues the rust-red phenotype. One-day and two-week-old adults of the black strain exhibit 2 to 3-fold higher levels of catechol oxidase activity than wild-type adults. Cuticle pigmentation is probably determined by the relative concentrations of catecholamines,  $\beta$ -alanine and catechol oxidase which are utilized for production of sclerotin and melanin. High dopamine titres appear to cause the black pigmentation through melanin synthesis while  $\beta$ -alanine and its dopamine conjugate NBAD are associated with the rust-red colouration.

**Key Word Index:** Catecholamines,  $\beta$ -alanine, red flour beetle, *Tribolium castaneum*, cuticle, sclerotization, melanization, dopamine, *N*-acetyldopamine, *N*- $\beta$ -alanyldopamine, mutant, catechol oxidase, metabolism

### INTRODUCTION

Catecholamines are important metabolites for the sclerotization and melanization of insect cuticle (Brunet, 1980; Hopkins *et al.*, 1982; Kramer *et al.*, 1983b; Aso *et al.*, 1983). They are converted into cross-linking and polymerizing agents for sclerotin and melanin, respectively. In addition to linkage of acetate or  $\beta$ -alanine to the amino group, dopamine, *N*-acetyldopamine (NADA) and *N*- $\beta$ -alanyldopamine (NBAD) may also be conjugated on the aromatic ring with glucose, phosphate or sulphate (Brunet, 1980). Apparently, the titre and distribution of cuticular tanning substrates are determined by a complex interplay of enzymes which catalyze catecholamine biosynthesis, conjugation, oxidation, degradation, transport, utilization and excretion. The

hardness and pigmentation of cuticle appear to depend in part on which catecholamine is produced and subsequently utilized for the formation of sclerotin or melanin. For example, NADA may be used for the formation of a colourless sclerotin, NBAD for a brown sclerotin and free dopamine for melanin (Brunet, 1980; Hopkins *et al.*, 1982).

$\beta$ -Alanine also plays an important role in cuticle sclerotization, apparently due to its conjugation with dopamine as NBAD (Hopkins *et al.*, 1982). This amino acid is incorporated into cuticle which becomes brown and is usually absent in black cuticle (Seki, 1962; Fukushi, 1967; Brunet, 1980). Indeed, NBAD is a major catecholamine in several species of insects that form brown cuticle (Hopkins *et al.*, 1982).

Several mutants of the red flour beetle, *Tribolium castaneum* Herbst, exhibit varying degrees of adult body colouration (Sokoloff *et al.*, 1960). To determine whether a correlation exists between body colour, catecholamines,  $\beta$ -alanine and oxidase levels, we have compared metabolite and enzyme levels of the black mutant with those of a wild-type strain. Preliminary reports of certain portions of this study have appeared elsewhere (Kramer *et al.*, 1983a; Roseland *et al.*, 1983).

### MATERIALS AND METHODS

#### Insects

The wild-type strain of *Tribolium castaneum* was from the culture maintained at the U.S. Grain Marketing Research

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Laboratory. The mutant strain (black) was obtained from Dr Alexander Sokoloff, California State College, San Bernardino, CA 92407. The black mutation is semi-dominant on linkage group 3 (Sokoloff *et al.*, 1960). All insects were reared on whole wheat flour containing 5% (w/w) brewer's yeast.

#### Tissue extraction

Larvae were removed from the rearing medium and animals estimated to be third and fifth instars taken for analysis. Pupae were collected at the black-eye stage and adults were obtained either 1–12, 48–72 or >72 hr after eclosion and frozen immediately. Ten to twenty insects were washed, pooled and homogenized in 0.1 M HCl (20 mg wet weight/ml) using a Tekmar Tissueemizer<sup>®</sup>. After centrifugation at 3000 g and 4°C for 10 min, the supernatants were filtered through a 0.8  $\mu$  cellulose filter and lyophilized to dryness. The residues were redissolved in 1.2 M HCl for catechol analysis or in water for amino acid analysis. Elytra were removed from adults at about four days post-eclosion, washed with water, blotted dry and homogenized in 1.2 M HCl (1 mg/10.1 ml) using a ground glass tissue grinder.

#### Catecholamine and amino acid analyses

Catecholamines from crude extracts were adsorbed on alumina at pH 8.8, released by lowering the pH to 2.4 and subsequently analyzed by reverse-phase liquid chromatography with electrochemical detection (LCEC) (Hopkins *et al.*, 1982). To release conjugated catecholamines before alumina adsorption the dried supernatants were hydrolyzed in 1.2 M HCl at 100°C for 10 min. The LCEC mobile phase consisted of 0.1 M formic acid, 15 mM trichloroacetic acid, 0.1 mM (ethylenedinitrilo)tetraacetic acid disodium salt, 22% methanol (v/v), pH 3.8. The retention times at 1 ml/min were 3,4-dihydroxybenzylamine (internal standard), 5.2 min; dopamine, 6.5 min; 3,4-dihydroxyphenylacetic acid (DOPAC), 8.8 min; *N*-acetyldopamine, 10.2 min and *N*- $\beta$ -alanyldopamine, 11.6 min. The limit of detection for the catecholamines was 1 pmol/mg body wt.

Amino acids were analyzed by liquid chromatography of their fluorescent *o*-phthalaldehyde derivatives (Hill *et al.*, 1979) as modified by Lookhart *et al.* (1982). The retention times at a flow rate of 1 ml/min for aspartic acid and  $\beta$ -alanine were 9.3 and 28.0 min, respectively. The limit of detection for amino acids were 100 pmol/mg body wt.

#### Catechol oxidase assay

Adult beetles (0.1 g wet weight) were washed and homogenized in 1 ml 0.1 M sodium potassium phosphate

buffer, pH 6 at 4°C. The filtrate obtained after passage through a 0.8  $\mu$  Millex-PF filter was assayed for catechol oxidase activity by monitoring absorbance change at 470 nm of a 15 mM DL-DOPA solution in the same buffer (Kramer *et al.*, 1983a). Units were defined as  $\Delta A_{470} \text{ min}^{-1} \text{ mg protein}^{-1}$  and data reported were the means from three determinations  $\pm$  SD. Protein concentration was determined by the dye binding procedure of Bradford (1976).

#### Microinjection of amino acids

Needles were prepared from 1.2 mm o.d. glass capillaries drawn out with a standard microelectrode puller. The diameter of the tip was  $5 \times 10^{-2}$  mm and the optimal beveled shape was formed by breaking the tip with a "minuten" pin. A foot-controlled Isco microapplicator was calibrated to deliver an injection volume of 0.1  $\mu$ l using a microlitre syringe. Larger injection volumes could not be reproducibly introduced into the body cavity. Before injection, newly eclosed adult beetles were immobilized by chilling on ice. Each insect was positioned and immobilized with a watchmaking forceps. The needle was inserted through the thin membrane between the third and fourth abdominal sternites near the pleurite at a flat angle and directed towards the thorax. Pigmentation of cuticle was determined four days post-injection.

## RESULTS

### Catecholamine levels

The most abundant catecholamines in whole body extracts of the wild-type and black mutant strains of *T. castaneum* were dopamine, NBAD and NADA. NADA accounted for 50–85%, dopamine 14–40% and NBAD <1–20% of the total catecholamines (Table 1). The titres of these compounds were generally similar during the larval and pupal stages in the two strains, but they were quite different in the adult stage (Fig. 1). The highest total titre of catecholamines was found in the adult between 1 and 72 hr after eclosion with the titre in the black mutant (1.1–1.4 nmol/mg, Table 1) much higher than in the wild-type ( $\sim$ 0.5 nmol/mg). These results suggest that one cause of the darker body pigmentation is the higher overall titre of catecholamines in the black mutant strain. The total titre in the adult fell precipitously 72 hr post-eclosion. Since the beetles require about four days to become fully pigmented and

Table 1. Percentage distribution and total concentration of major catecholamines in wild type and black mutant strains of *Tribolium castaneum*\*

Strain	Stage	Percent			Total concentration (nmol/mg body weight)
		NADA	Dopamine	NBAD	
Wild	Larva†				
	third stadium	84 $\pm$ 2	14	2	0.18
	fifth stadium	85 $\pm$ 8	13 $\pm$ 3	2 $\pm$ 0.4	0.20
	Pupa	78	20	2	0.30
	Adult				
	1–12 hr	74 $\pm$ 12	22 $\pm$ 5	4 $\pm$ 1	0.53
48–72 hr	48 $\pm$ 7	31 $\pm$ 5	21 $\pm$ 1	0.54	
> 72 hr	63 $\pm$ 21	16 $\pm$ 2	21 $\pm$ 7	0.09	
Black	Larva				
	third stadium	83 $\pm$ 13	14 $\pm$ 4	3 $\pm$ 2	0.21
	fifth stadium	75 $\pm$ 3	23 $\pm$ 2	2 $\pm$ 1	0.32
	Pupa	78	20	2	0.39
	Adult				
	1–12 hr	67 $\pm$ 8	32 $\pm$ 3	1	1.14
48–72 hr	60 $\pm$ 1	40 $\pm$ 1	<1	1.40	
> 72 hr	65 $\pm$ 15	25 $\pm$ 5	10 $\pm$ 2	0.06	

\*Mean values of 2 to 4 determinations  $\pm$  SD.

†Larvae were sampled during feeding stage.

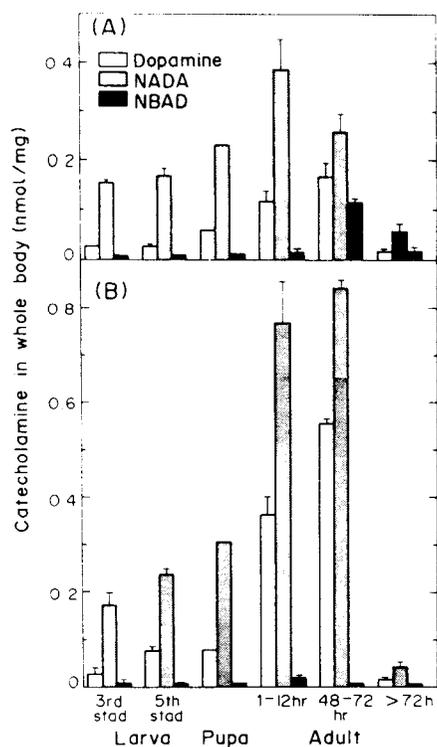


Fig. 1. Major catecholamines in larval, pupal and adult stages of *Tribolium castaneum*. (A) Wild-type strain. (B) Black mutant strain. Mean values from 2 to 4 determinations  $\pm$  SD.

sclerotized, the probable reason that catecholamines decrease after three days is that tanning metabolism has converted them to other compounds and there is no further synthesis.

Significant differences are readily apparent in the levels of individual catecholamines during the adult stage. At 1–12 hr post-eclosion dopamine was three times (0.4 nmol/mg) and NADA about two times higher (0.8 nmol/mg) in the black mutant than in the wild-type adult (Fig. 1). Between 1 and 12 hr, and 48 and 72 hr, the dopamine titre increased by 43% in the rust-red adult and by 53% in the black adult. At the same time NADA declined by 34% and NBAD increased by more than 500% in the wild-type adult while the former increased by 10% and the relatively low NBAD titre declined in the black mutant. These data show that a failure to accumulate NBAD is associated with elevated levels of both dopamine and NADA in the black mutant. Exceptionally low titres of NBAD and abnormally high titres of dopamine and NADA occur during sclerotization and melanization of the mutant.

We also examined fully tanned cuticle, the elytra, which were collected about four days post-eclosion

for the presence of catechols (Table 2). Wild-type elytra contained about ten times more NBAD than black elytra but only one-tenth as much dopamine. The titre of dopamine in the black elytra was three times higher than the highest titre detected in whole body extracts of adults. Similarly, the titre of NBAD in wild-type elytra was about seven times higher than the highest titre detected in whole body extracts. Very large quantities of DOPAC were found in wild-type elytra (110 nmol/mg) while black elytra contained less than half that amount. High titres of DOPAC (>5 nmol/mg body weight) were also observed in whole body extracts of the adults of both strains which were more than 72 hr post-eclosion.

#### Catechol oxidase levels

Catechol oxidase activity was measured in the red flour beetle strains using DL-DOPA as the substrate. The specific activity in the wild-type adult was  $0.15 \pm 0.01$  units at 1–24 hr post-eclosion and  $0.25 \pm 0.05$  at 15 days. The adult of the black mutant exhibited 2 to 3-fold higher levels ( $0.35 \pm 0.02$  at 1–24 hr and  $0.84 \pm 0.17$  at 15 days). Thus, another difference between the wild-type and black mutant strains was the total enzyme activity which catalyzes the oxidation of various catechols to quinones during the period of cuticular hardening and pigmentation. In the older adult (>30 days post-eclosion), however, the two strains had about the same catechol oxidase activity ( $0.21 \pm 0.01$  in the wild type and  $0.25 \pm 0.01$  in the black mutant).

#### $\beta$ -Alanine levels

Because NBAD titre differed greatly in brown and black cuticles and whole body extracts of *T. castaneum*, we examined whether the black mutant was deficient in NBAD because it lacked one of its precursors,  $\beta$ -alanine. The levels of  $\beta$ -alanine and aspartic acid, the latter a possible precursor of  $\beta$ -alanine in insects (Nakai, 1971; Ross and Monroe, 1972), were determined in extracts of larvae, pupae and adults by amino acid analysis (Table 3).  $\beta$ -Alanine was present in larvae (0.6–1.6 nmol/mg body weight) but absent or very low (<0.1 nmol/mg) in pupae of both strains. In the wild-type adult a greater than 20-fold increase in  $\beta$ -alanine occurred after ecdysis where the concentration (2.3 nmol/mg) was more than four times higher than the total concentration of the catecholamines (0.5 nmol/mg). No  $\beta$ -alanine (<0.1 nmol/mg) was detected in the black mutant during the three-day period following adult eclosion. However, the titre increased to the wild-type level thereafter (1.7 nmol/mg). These data indicate that the low titre of NBAD in the black mutant post-ecdysis is due to a deficiency of  $\beta$ -alanine.

Table 2. Catechols in elytra of wild and black mutant strains of *Tribolium castaneum* four days after adult eclosion\*

Strain	Dopamine	N-acetyldopamine	N- $\beta$ -alanyldopamine	3,4-Dihydroxyphenyl acetic acid
Wild	$0.17 \pm 0.03$	<0.06	$0.86 \pm 0.05$	$109.9 \pm 10.2$
Black	$1.73 \pm 0.45$	<0.06	$0.09 \pm 0.07$	$41.9 \pm 14.0$

\*Unit = nmole of unconjugated catechol/mg. Means of three samples  $\pm$  SD.

Catechols extracted from the elytra were almost totally unconjugated. NADA values are estimated due to interference from tailing of DOPAC in elution profile.

Table 3.  $\beta$ -Alanine and aspartic acid levels in wild-type and black mutant strains of *Tribolium castaneum*\*

Strain	Stage	$\beta$ -Alanine	Aspartic acid
Wild	Larval†		
	third stadium	1.67 $\pm$ 0.05	+
	fifth stadium	0.76 $\pm$ 0.27	+
	Pupa	<0.10	0.39 $\pm$ 0.03
	Adult		
	1-12 hr	2.26 $\pm$ 0.86	0.21 $\pm$ 0.13
Black	Larva		
	third stadium	1.03 $\pm$ 0.08	+
	fifth stadium	0.59 $\pm$ 0.03	+
	Pupa	<0.10	0.44 $\pm$ 0.18
	Adult		
	1-12 hr	<0.10	0.51 $\pm$ 0.07
48-72 hr	<0.10	0.22 $\pm$ 0.02	
> 72 hr	1.70 $\pm$ 0.05	+	

\*Unit = nmol/mg body weight. + Indicates aspartic acid present but actual concentration not determined. Mean values from 2 to 5 determinations  $\pm$  SD.

Aspartic acid was present during all stages of flour beetle development at 0.2–0.5 nmol/mg levels (Table 3). The young mutant adult exhibited a slightly higher level of aspartic acid (0.5 nmol/mg) than did the wild strain (0.2–0.3 nmol/mg). If aspartic acid is not decarboxylated to  $\beta$ -alanine in the mutant strain, its titre might be expected to be higher.

We first attempted to rescue the rust-red pigmentation of the black mutant by culturing larvae on ground wheat medium supplemented with  $\beta$ -alanine (>1000 ppm on a weight basis). This treatment was unsuccessful. Perhaps  $\beta$ -alanine supplied by the larval diet is not absorbed or utilized for adult cuticle tanning in *T. castaneum*. Both the wild-type and black mutant strain exhibited similar  $\beta$ -alanine levels in the larval stage.

A successful rescue of the black phenotype was accomplished by microinjection of  $\beta$ -alanine into untanned black mutant adults. Of 74 teneral b/b adults injected with 0.4  $\mu$ mol of  $\beta$ -alanine, 92% developed the wild-type rust-red body colour while 8% darkened to the black phenotype colour. In contrast, 87% of 63 control animals injected with either saline or 2 M lysine darkened as the black phenotype. The remaining control insects either died or remained unpigmented probably as a result of the injury of injection. The quantity of  $\beta$ -alanine injected was more than 170-fold greater than the endogenous level detected in whole body extracts. Lower levels were less effective.

#### DISCUSSION

In the red flour beetle, *T. castaneum*, synthesis of  $\beta$ -alanine and *N*- $\beta$ -alanyldopamine appears to be associated with red-brown pigmentation while black pigmentation is associated with low amounts of these compounds and large amounts of dopamine and *N*-acetyldopamine. In *Calliphora erythrocephala*, isolated larval cuticle becomes brown or black when incubated with *N*- $\beta$ -alanyldopamine or free dopamine, respectively (Andersen, 1977). In *Manduca sexta*, NBAD accumulates during the formation of brown pupal cuticle or dark brown larval man-

dibles, while NADA accumulates during the hardening of colourless cuticle in larvae and adults (Hopkins *et al.*, 1982 and unpublished data). In *T. castaneum*, NADA is the most abundant catecholamine in whole body extracts of the light coloured larvae and pupae as well as the heavily pigmented adults. It seems likely in *T. castaneum* that NADA is responsible for much of the hardness of the cuticle but little, if any, of the colour. The high titre of NADA in the adults of the black mutant may be related to the unusually large amount of dopamine which was available for acetylation.

The black pigmentation of the mutant probably results from the availability of unconjugated dopamine as a substrate for the cuticular catechol oxidase. One cause of the high titre of dopamine in the young adult of the mutant may be a high rate of dopamine synthesis. At 1–12 hr post-eclosion, the dopamine titre is about three times higher in the mutant than it is in the wild-type strain even though the titre of NBAD is still low in both strains. Since relatively little pigmentation has occurred by this time, the turnover rate of NBAD is probably not very high. It appears that the mutant's lack of capacity for NBAD synthesis does not account for the amount of dopamine which has already accumulated. However, the lack of NBAD synthesis at 48–72 hr post-eclosion in the mutant must contribute to the accumulation of dopamine (and NADA) at that time.

A causal relationship between the lack of  $\beta$ -alanine for NBAD synthesis and black pigmentation is suggested by the ability of  $\beta$ -alanine injections to rescue the normal rust-red phenotype. The quantity of  $\beta$ -alanine that was used for the rescue had to be more than 170-fold greater than the normal level to be completely effective. This is not too surprising however. The black colour resulting from the polymerization of dopamine could mask the normal red-brown colour. A very high proportion of dopamine must be conjugated with either  $\beta$ -alanine or acetate before it is transported into the cuticle if normal pigmentation is to occur. Further work is in progress to determine the effect of  $\beta$ -alanine injections on catecholamine titres in the cuticle.

Although a  $\beta$ -alanine deficiency exists in the young adult of the black mutant, the titre of  $\beta$ -alanine returns to normal after three days. A protein which modulates the activity of one of the biosynthetic enzymes may be defective in the mutant. The slightly higher titre of aspartic acid in the young adult of the mutant may indicate that the rate of decarboxylation of aspartic acid to  $\beta$ -alanine is abnormally low. Besides aspartic acid, however, the potential precursors of  $\beta$ -alanine include uracil, pantothenate, propionate and malonate (Jacobs, 1968; Ross and Monroe, 1972).

The relationship of  $\beta$ -alanine to cuticular pigmentation has been previously compared in brown and black phenotypes of *Drosophila melanogaster* and *Musca domestica*. Like black *T. castaneum*, the ebony and black mutants of *Drosophila* are also abnormal in  $\beta$ -alanine synthesis or utilization (Jacobs and Brubaker, 1963; Hodgetts, 1972; Hodgetts and Konopka, 1973; Hodgetts and Choi, 1974). Black *Drosophila* contains much less  $\beta$ -alanine than does the wild-type. Ebony *Drosophila* has normal

$\beta$ -alanine levels, but is unable to incorporate it into the cuticle, perhaps because the  $\beta$ -amino acid is not conjugated with dopamine. In *M. domestica* dietary  $\beta$ -alanine rescues puparial colour (Fukushi, 1967), where a dietary concentration of 0.5 M transformed the *bp* strain (black) into the phenocopy of the wild-type (brown). These observations are consistent with the hypothesis that failure to conjugate dopamine with  $\beta$ -alanine can prevent the normal formation of red or brown colour during sclerotization and cause the formation of black melanin.

Dopamine, NADA and NBAD all serve as substrates *in vitro* for cuticular tyrosinase (Barrett and Andersen, 1981; Hopkins *et al.*, 1982; Kramer *et al.*, 1983b; Aso *et al.*, 1983). In *Drosophila*, the activity of catechol oxidase was similar in black and wild-type strains while the lighter coloured blond strain exhibited a higher activity (Mitchell *et al.*, 1967). The high activity of catechol oxidase in whole body homogenates of the black mutant of *T. castaneum* may not be causally related to the formation of black melanin. The relative concentrations of substrates which are transported into the cuticle are more likely to be the primary factor which determines cuticular pigmentation. High catechol oxidase activity, however, may be appropriate in the black mutant since much higher titres of total catecholamines are used up during cuticular stabilization and pigmentation than is observed in the wild-type.

These enzyme data are difficult to interpret in terms of cuticular tanning and pigmentation because of the multiple roles performed by phenol and catechol oxidases in haemolymph and other tissues for wound healing and defence against pathogens and parasites (Crossley, 1975). The oxidases are usually activated during tissue homogenization. Thus, additional experimentation will be required to determine if any of the enzymes with catechol oxidase activity are directly correlated with the black phenotype and melanin production.

High levels of DOPAC are present in older adults of both strains of *T. castaneum*. The titre of DOPAC in black elytra at four days post-eclosion was less than one-half of the level found in rust-red elytra. DOPAC has been detected previously in whole body extracts of the red and confused flour beetles (Hackman *et al.*, 1948) and in exocuticle of *Tenebrio molitor*, *Cetonia aurata*, *Potosia cupreca*, *Melolontha hippocastani* and *Pachynoda epphipiata* (Schmalfuss, 1937; Schmalfuss *et al.*, 1933; Anderson, 1975). DOPAC may be an end product of degradation of surplus catecholamines. The importance of this pathway may be less in the black mutant of *T. castaneum* than in the wild-type strain.

The pigmentation of red flour beetle cuticle is apparently determined by the enzyme-regulated production of precise levels of catecholamines and their oxidized metabolites. *N*- $\beta$ -alanyldopamine, *N*-acetyldopamine and dopamine are all available for production of reddish-brown cuticle. However, the formation of black cuticle is correlated with an abnormally low titre of NBAD together with more elevated titres of dopamine and NADA. The young adult of the black mutant has a higher catechol oxidase activity than the wild-type adult. At the present time it is unclear whether all of these

differences are causally related to differences in cuticle pigmentation, structure and mechanical properties. Nevertheless, it does seem very likely that the formation of reddish-brown colour requires NBAD while black requires unconjugated dopamine. The higher titres of dopamine in the mutant strain are at least partially caused by a deficiency of  $\beta$ -alanine which prevents synthesis of NBAD. The mechanism by which these compounds are utilized for cuticle stabilization is currently under investigation including genetic testing to verify linkage of colour genes to biochemical differences.

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#### REFERENCES

- Andersen S. O. (1975) Cuticular sclerotization in the beetle *Pachynoda epphipiata* and *Tenebrio molitor*. *J. Insect Physiol.* **21**, 1225–1232.
- Andersen S. O. (1977) Arthropod cuticles: their composition, properties and functions. *Symp. zool. Soc. Lond.* **39**, 7–32.
- Aso Y., Kramer K. J., Hopkins T. L. and Whetzel S. F. (1984) Properties of tyrosinase and dopa quinone imine conversion factor from pharate pupal cuticle of *Manduca sexta* L. *Insect Biochem.* In press.
- Barrett F. M. and Andersen S. O. (1981) Phenoloxidases in larval cuticle of the blowfly, *Calliphora vicina*. *Insect Biochem.* **11**, 17–23.
- Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72**, 248–252.
- Brunet P. C. J. (1980) The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. *Insect Biochem.* **10**, 467–500.
- Crossley A. C. (1975) The cytophysiology of insect blood. *Adv. Insect. Physiol.* **11**, 117–221.
- Fukushi Y. (1967) Genetic and biochemical studies on amino acid composition and color manifestation in pupal sheaths of insects. *Jap. J. Genet.* **42**, 11–21.
- Hackman R. H., Pryor M. G. M. and Todd A. R. (1948) The occurrence of phenolic substances in arthropods. *Biochem. J.* **43**, 474–477.
- Hill D. W., Walters F. H., Wilson T. D. and Stuart S. D. (1979) High performance liquid chromatographic determination of amino acids in the picomole range. *Analyt. Chem.* **51**, 1338–1341.
- Hodgetts R. B. (1972) Biochemical characterization of mutants affecting the metabolism of  $\beta$ -alanine in *Drosophila*. *J. Insect Physiol.* **18**, 937–947.
- Hodgetts R. B. and Choi A. (1974)  $\beta$ -Alanine and cuticle maturation in *Drosophila*. *Nature* **252**, 710–711.
- Hodgetts R. B. and Konopka R. J. (1973) Tyrosine and catecholamine metabolism in wild-type *Drosophila melanogaster* and a mutant ebony. *J. Insect Physiol.* **19**, 1211–1220.
- Hopkins T. L., Morgan T. D., Aso Y. and Kramer K. J. (1982) *N*- $\beta$ -Alanyldopamine: major role in insect cuticle tanning. *Science* **217**, 364–366.
- Jacobs M. E. and Brubaker K. K. (1963)  $\beta$ -Alanine utilization in ebony and nonebony *Drosophila melanogaster*. *Science* **139**, 1282–1283.
- Jacobs M. G. (1968) Beta-alanine use by ebony and normal *Drosophila melanogaster* with notes on glucose, uracil, dopa and dopamine. *Biochem. Genet.* **1**, 267–275.

- Kramer K. J., Beeman R. W., Morgan T. D., Hopkins T. L., Lookhart G. L., Aso Y. and Roseland C. R. (1983a) The biochemical basis for the black mutation in *Tribolium castaneum*. *Tribolium Informat. Bull.* **23**, 86–88.
- Kramer K. J., Nuntnarumit C., Aso Y., Hawley M. D. and Hopkins T. L. (1983b) Electrochemical and enzymatic oxidation of catecholamines involved in sclerotization and melanization of insect cuticle. *Insect Biochem.* **13**, 475–479.
- Lookhart G. L., Jones B. L., Cooper D. B. and Hall S. B. (1982) A method for hydrolyzing and determining the amino acid compositions of picomole quantities of proteins in less than 3 hours. *J. biochem. biophys. Meth.* **7**, 15–23.
- Mitchell H. K., Weber U. M. and Schaar G. (1967) Phenol oxidase characteristics in mutants of *Drosophila melanogaster*. *Genetics* **57**, 357–368.
- Nakai S. (1971) Genetic and biochemical studies on the  $\beta$ -alanine metabolism in the housefly, *Musca domestica* L. *Jap. J. Genet.* **46**, 53–60.
- Roseland C. R., Beeman R. W., Kramer K. J. and Hopkins T. L. (1983) Microinjection of amino acids in *Tribolium*. *Tribolium Informat. Bull.* **23**, 132.
- Ross R. H. and Monroe R. E. (1972)  $\beta$ -Alanine metabolism in the housefly, *Musca domestica*: studies on anabolism in the early puparium. *J. Insect Physiol.* **18**, 1593–1597.
- Schmalfuss H. (1937) Über das Vorkommen und den Nachweis von 3, 4-Dihydroxyphenyleisigsäure in den Rosenkafern *Cetonia aurata* und *Potosia cuprea* und im Maikafer *Melolontha hippocastani*. *Biochem. Z.* **294**, 112–119.
- Schmalfuss H., Heider A. and Winkelmann K. (1933) 3,4-Dihydroxyphenyleisigsäure, Farbvorstufe der Flügeldecken des Mehlkäfers, *Tebebrio molitor*. *Biochem. Z.* **257**, 188–193.
- Seki T. (1962) Absence of beta-alanine in hydrolyzate of the pupal sheaths of ebony mutant of *Drosophila virilis*. *Drosophila Informat. Service* **36**, 115.
- Sokoloff A., Slatis H. M. and Stanley T. (1960) The black mutation in *Tribolium castaneum*. *J. Hered.* **51**, 131–135.