

ROLE OF β -ALANINE IN PUPAL CUTICLE COLORATION IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

EFFECTS OF β -ALANINE ANALOGUES ON MELANIZATION AND CATECHOLAMINE LEVELS

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Abstract—Hydrazino and aminoxy derivatives of β -alanine were found to cause blackening of *Manduca sexta* pupal cuticle when they were injected into pharate pupae at the onset of pre-ecdysial tanning. One of these compounds, ethyl hydrazinoacetate (EHA), was used for further study. It was effective if injected up to about 4 hr before pupal ecdysis. These melanized cuticles contained excessive amounts of dopamine and decreased amounts of *N*- β -alanyldopamine (NBAD) and *N*-acetyldopamine (NADA). Furthermore, EHA induced elevated dopamine and lowered β -alanine levels in the hemolymph. Similar blackening occurred when 20 mg/animal dopamine was injected. Injection of excess β -alanine rescued the normal brown color, irrespective of the concentration of EHA. Also, EHA caused melanization *in vitro* in the presence of dopamine, whereas the addition of β -alanine and NBAD allowed normal pupal coloration *in vitro*. These hydrazino and aminoxy compounds likely interfere with β -alanine synthesis or mobilization and thus with *N*-acylation of the catecholamines to form NBAD and *N*- β -alanyl norepinephrine.

Key Word Index: β -Alanine, role in cuticle color, melanization, inhibition by β -alanine, β -alanine, hydrazino derivatives, *Manduca sexta*

INTRODUCTION

After ecdysis and the consequent cuticular expansion the insect cuticle must harden (sclerotize) in order to function as the exoskeleton. At the same time it may also incorporate melanin-related polymers and thus darken. Sclerotization is thought to depend on various catecholamine derivatives, particularly dopamine, *N*-acetyldopamine (NADA) and *N*- β -alanyldopamine (NBAD; Andersen, 1979; Brunet, 1980; Hopkins *et al.*, 1982, 1984; Lipke *et al.*, 1983; Fig. 1). Whether the sclerotized cuticle is brown or black depends on the relative levels of β -alanine, dopamine or their conjugated derivative *N*- β -alanyldopamine (NBAD; Fig. 1). Exogenous β -alanine will cause the formation of a normal tan cuticle in black cuticular mutants of *Musca* (Fukushi, 1976), *Drosophila* (Hodgetts and Choi, 1974), and *Tribolium* (Kramer *et al.*, 1984). *Tribolium* also has elevated dopamine levels. The *ebony* mutant of *Drosophila* has sufficient β -alanine but cannot incorporate it into the cuticle (Hodgetts, 1972; Jacobs, 1976) and thus also has a black instead of the normal brown adult cuticle.

Both NADA and NBAD are incorporated into the

sclerotized pupal cuticle of *Manduca sexta* (Grün and Peter, 1984), but NBAD appears to be the major catecholamine component of this cuticle (Hopkins *et al.*, 1984). Moreover, the presence of NBAD or *N*- β -alanyl norepinephrine (NBANE) is necessary for normal tanning of *Manduca* pupal cuticle *in vitro* (Roseland *et al.*, 1986). Since little NADA is present, the β -alanyl derivative of dopamine has been proposed to be a precursor of the sclerotizing agent in this cuticle (Hopkins *et al.*, 1982, 1984). Supporting this hypothesis is the observation of an abnormal lamellar structure and a physical weakness of the cuticle from the *black Drosophila* mutant, both of which were corrected when β -alanine is given (Jacobs, 1978, 1985).

The biosynthesis of β -alanine can occur via several pathways (Cheldelin and Baich, 1963; Meister, 1965; Drey, 1977; Fig. 1): (1) uracil degradation; (2) decarboxylation of L-aspartic acid by an α -decarboxylase containing a pyruvoyl prosthetic group (Williamson and Brown, 1979); (3) transamination of malonic semialdehyde with glutamic acid. In the housefly *Musca domestica* uracil was found to contribute 56% and aspartic acid 24% of the β -alanine found in the puparial cuticle (Ross and Monroe, 1972), confirming earlier findings of Hijikuro (1968) and Nakai (1971).

Although the biosynthetic or degradative pathway of β -alanine in *Manduca* has not been studied, we screened on pharate *Manduca* pupae a series of

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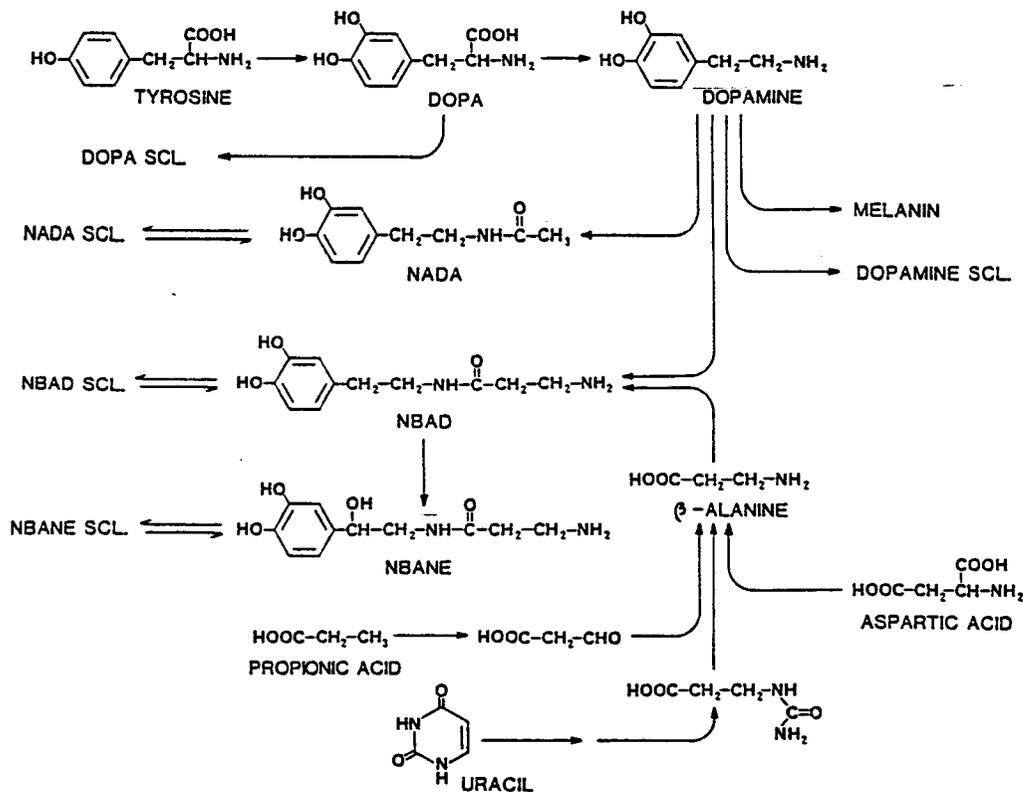


Fig. 1. Hypothetical pathways for catecholamine and β -alanine metabolism in insects. SCL = sclerotin.

compounds that might interfere with the biosynthesis of β -alanine to determine whether β -alanine or NBAD is critical for sclerotization as well as for coloration of the pupal cuticle. Hydrazinoacetic acid and (aminoxy) acetic acid and some of their derivatives were found to cause the formation of black rather than brown pupae which was prevented by coinjection of β -alanine. The black pupae appeared to sclerotize normally although the cuticular content of NBAD was substantially decreased and that of dopamine increased.

MATERIALS AND METHODS

Animals

Manduca sexta larvae were reared on artificial diet according to the methods of Bell and Joachim (1976) under a 12 hr light:12 hr dark photoperiod at 25.5°C. Wandering larvae were collected each morning and placed in wooden blocks in the same room. Three days later the prepupae were removed from the blocks and observed for the onset of tanning of the dorsal metathoracic bars which usually occurred around the time of lights-off. Pupal ecdysis was 18 hr after the onset of bar tanning.

The compound to be assayed was dissolved in water and 0.025 ml was routinely injected into the abdomen of the CO₂-anesthetized pharate pupae at the onset of metathoracic bar tanning. Since dopamine was relatively insoluble in water, up to 0.3 ml of the solution was injected. Water-insoluble compounds were dissolved in acetone, and 0.01 ml was applied topically along the dorsal midline. The coloration of the pupa was scored one day after pupal ecdysis as outlined in Table 1.

Chemicals

2-Aminoethanephosphonic acid, D-cycloserine, L-cysteic acid were from Aldrich. β -Alanine, (aminoxy) acetic acid hemihydrochloride, 6-azauracil, dopamine hydrochloride, hyalazine hydrochloride, isoniazid, and taurine were from Sigma. 2,2-Dichloropropionic acid was from Dow Chemical Co. Hydrazine sulfate, hydroxylamine hydrochloride, and semicarbazide hydrochloride were from J. T. Baker Chemical Co.

(\pm) α -Methylaspartic acid was prepared according to the procedure described by Pfeiffer and Heinrich (1936). The hydrazino derivatives of acetic and propionic acids were synthesized by the method of Carmi *et al.* (1960). Hydrazinoacetamide hydrochloride was prepared according to Niedrich (1985). (Benzamidoxy) acetic acid (benzadox) was prepared from benzohydroxamic acid and chloroacetic acid according to the procedure of McHale *et al.* (1960). Benzohydroxamic acid was prepared according to Jones and Hurd (1921). The ethyl ester of benzadox was synthesized from benzohydroxamic acid and ethyl chloroacetate. Ethyl(1-methylhydrazino) acetate hydrochloride was synthesized as described by Moffett *et al.* (1977). Acetohydrazide was prepared from ethyl acetate and hydrazine hydrate in a conventional manner (Vogel, 1978) [Z -(α -

Table 1. Scoring system for *Manduca* pupal cuticular melanization assay

Score	Description
0	Normal tan pupa
1	Tan pupa with black intersegmental membranes
2	Brownish black pupa with black intersegmental membranes
3	Blackish brown pupa with black intersegmental membranes
4	Black pupa

Phenylbenzylidene)hydrazino] acetic acid and [(α -methylbenzylidene)aminoxy acetic acid were synthesized by the method of van Dijk and Zwagemakers (1977). 3,6-Bis(aminooxymethyl)-2,5-piperazinedione was prepared from D-cycloserine according to the procedure of Ongania (1979).

Analysis of catecholamines

The hemolymph was collected from the tip of the abdomen of the pharate or ecdysed pupa into a tube in an ice bath. It was then lyophilized and stored at -20°C . The cuticle was cleaned of epidermis, rinsed and lyophilized to dryness. Dried samples were homogenized in 1 N HCl in a ground glass homogenizer. Aliquots of the supernatant were heated at 100°C for 10 min to release catechols from glucoside conjugates. Catecholamines were isolated by adsorption to alumina, then released in 1 M acetic acid and analyzed by reverse phase HPLC with an electrochemical detector (Hopkins *et al.*, 1984). The free β -alanine content of the hemolymph was analyzed by the method of Kramer *et al.* (1984).

Culture of pharate pupal cuticle

The pharate pupal integument was explanted to 0.5 ml of either complete Grace's medium or Grace's medium lacking 2.4 mM β -alanine and cultured on a glass wool support for 24 hr at 26°C under a 95% O_2 -5% CO_2 atmosphere as previously described by Roseland *et al.* (1986). The coloration of the pupal cuticle was then assessed after the various treatments.

RESULTS

Assay of compounds interfering with β -alanine metabolism

Our strategy to inhibit β -alanine or NBAD metabolism was to use structural analogues of β -alanine to determine if they would interfere with cuticle sclerotization and pigmentation. Analogues of either β -alanine or of its possible precursors, aspartic acid or uracil, were selected according to the isosteric modification principle (Fig. 2). Table 2 shows that when injected into pharate *Manduca* pupae, both the sulfonyl (18, taurine) and phosphonyl (19) analogues of β -alanine and the sulfonyl analogue of aspartic acid [17, cysteic acid which is an inhibitor of β -alanine biosynthesis in micro-organisms (Ravel and Shive, 1946)] had no effect on pupal tanning. Also, α -methylaspartic acid (16) was inactive.

When (aminoxy) acetic acid (5) and ethyl hydrazinoacetate (2, EHA) were injected, the pupal cuticle became black rather than brown (Table 3 and Fig. 3). A similar blackening also occurred with pupae given (benzamidooxy) acetic acid (benzadox, 1) or the ethyl ester of benzadox (3; Table 3). Benzadox is readily hydrolyzed to compound 5 (Nakamoto *et al.*, 1982) and it is likely that compound 3 is also hydrolyzed to compound 5. Interestingly, 6-azauracil (6) had little activity when given at the onset of tanning. When given 1.5 days earlier (2 days after wandering), 6-azauracil was quite effective, indicating that the compound had likely undergone a slow degradation to hydrazinoacetic acid *in vivo* as it does in the test tube (Lišmane *et al.*, 1977). None of the compounds prevented hardening of the pupal cuticle.

Modification of the aliphatic chain of compounds 2 and 5 resulted in a decline in biological activity

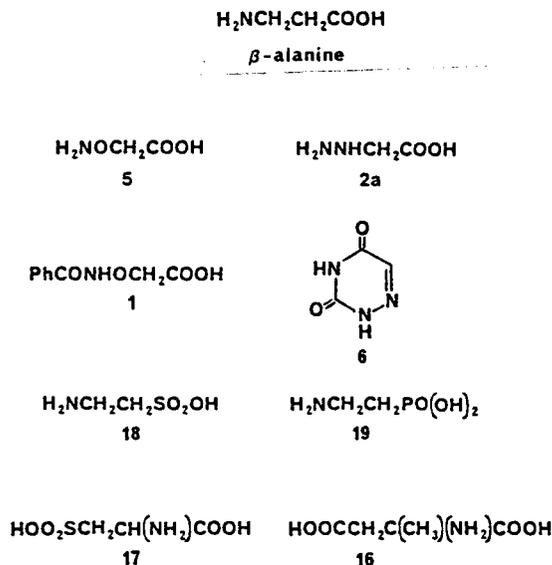


Fig. 2. Structural analogues of β -alanine and its possible biosynthetic precursors: (aminoxy) acetic acid (5), hydrazinoacetic acid (2a), (benzamidooxy) acetic acid (1), 6-azauracil (6), 2-aminoethanesulfonic acid (18), 2-aminoethanephosphonic acid (19), cysteic acid (17) and α -methylaspartic acid (16).

(Table 3). Compounds 4, 8 and 9 in which the hydrazino or aminoxy groups are attached to the α -carbon of propionic acid, possessed some activity as did the phthalimido compound (7), a diacyl derivative of 9. The amide derivative 10 of hydrazinoacetic acid (2a) was slightly active. The introduction of an additional methylene group between the hydrazino and carbonyl groups to form ethyl 3-hydrazinopropionate (12) resulted in a substantial drop in activity. Two additional hydrazine derivatives, compounds 11 and 13, had marginal activities. Compounds 14 and 15, in which the reactive heteroatom-bound amino groups of the parent compound 3 and 5 are "protected" as stable hydrazino or oxime derivatives of the corresponding carbonyl compounds, were inactive (Table 2).

Table 2. Inactive compounds causing no visible coloration anomalies (score 0) at a dose of 200 μg per pharate pupa of *M. sexta* ($N = 3$)

Compound number	Compound name
14	[2-(α -Phenylbenzylidene)hydrazino] acetic acid
15	[(α -Methylbenzylidene)aminoxy] acetic acid
16	(\pm)- α -Methylaspartic acid
17	L-Cysteic acid
18	2-Aminoethanesulfonic acid (taurine)
19	2-Aminoethanephosphonic acid
20	2,2-Dichloropropionic acid (dalapon)
21	Propionic acid
22	Hydrazine sulfate
23	Hydroxylamine HCl
24	Semicarbazide HCl
25	Phenylhydrazine HCl*
26	D-Cycloserine
27	3,6-Bis(aminooxymethyl)-2,5-piperazinedione
28	Isoniazid
29	Hydralazine HCl
30	Iproniazid phosphate

*Inactive also at a dose of 500 μg per pupa.

Table 3. Effects of aminoxy- and hydrazinoacetic acids and related compounds on the melanization of the pupal cuticle of *M. sexta*

Compound number	Compound name	ED ₅₀ (μ g/pupa)	ED ₅₀ (nmol/pupa)
1	(Benzamidoxy) acetic acid	2.7	13.8
2	Ethyl hydrazinoacetate HCl	3.2	20.7
3	Ethyl (benzamidoxy)acetate†	7.4	33.2
4	(\pm)Ethyl 2-hydrazinopropionate HCl	9.6	56.9
5	(Aminoxy) acetic acid 1/2 HCl	6.3	57.6
6	6-Azauracil‡	7.8	69.0
7	(\pm)Ethyl 2-(phthalimidooxy)propionate†	28.0	106
8	(\pm)Ethyl 2-(benzamidoxy)propionate†	25.5	107
9	(\pm)-2-(Aminoxy)propionic acid HCl	29.0	205
10	Hydrazinoacetamide HCl	87.0	693
11	Ethyl(1-methylhydrazino)acetate HCl	235	1394
12	Ethyl 3-hydrazinopropionate HCl	240	1423
13	Acetohydrazide	155	2092

*Determined from a linear plot of melanization score vs log dose of the compounds using 5-7 doses ($N = 5-15$).

†Applied topically in acetone solution.

‡Applied in water at W2 stage larvae.

Two reported antimetabolites of β -alanine, 2,2-dichloropropionic acid (20) and propionic acid (21; Van Oorschot and Hilton, 1963), were totally inactive (Table 2). Also, no simple carbonyl reagents (22-24) or other inhibitors (25-30) of pyridoxal phosphate or pyruvyl prosthetic group-containing amino acid decarboxylases (Clark, 1963; Boeker and Snell, 1972) and amino group transferases (Braunstein, 1973) were effective.

Effects of ethylhydrazinoacetate (EHA) on pupal coloration

For a detailed study of the effects of the β -alanine antagonists on pupal tanning, we used ethyl hydrazinoacetate (EHA). A dose-response curve (Fig. 4) showed that 7-8 μ g per pharate pupa was sufficient to cause the formation of black pupae. The ED₅₀ was 3.2 μ g when injected at the onset of tanning of the metathoracic bars. When 10 μ g EHA was given at different times from the initiation of wandering to pupal ecdysis, the compound was found to be effective until shortly after the onset of tanning (Fig. 5). The decline in effectiveness was rapid so that by the time of pupation EHA had no effect. All of these pupae appeared to be normally sclerotized. Injection of 10 μ g EHA into pharate pupae reared under a

17 hr light: 7 hr dark photoperiod had no adverse effect on adult development or eclosion ($N = 10$).

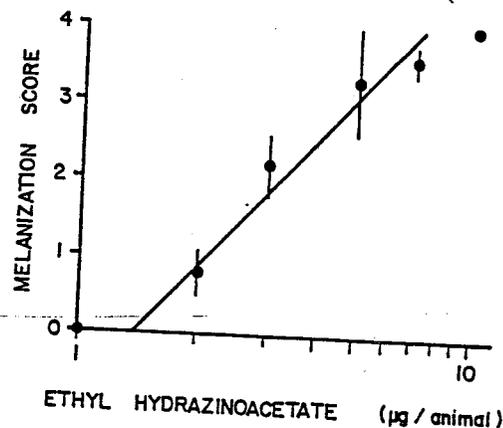


Fig. 4. Dose-response curve for ethyl hydrazinoacetate (EHA) in melanization of the pupal cuticle of *Manduca*. EHA was injected into pharate pupae at the onset of tanning of the metathoracic bars. The degree of melanization of the subsequent pupa 1 day after ecdysis was visually scored as outlined in Materials and Methods. Each point represents the average \pm SD for 20 animals. Average weight of the animals at the time of injection was 6.8 ± 0.4 g ($N = 28$).

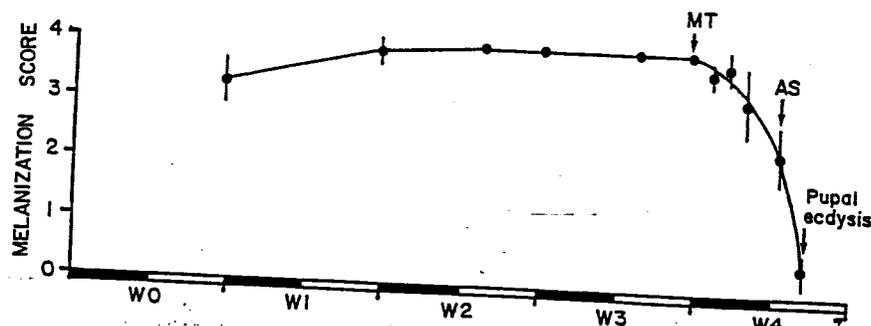


Fig. 5. Time of effectiveness of 10 μ g ethyl hydrazinoacetate (EHA) in causing melanization of *Manduca* pupal cuticle. The pupae were scored as in Table 1. Each point represents the average \pm SD for 10 animals. The X-axis indicates the days after the onset of wandering behavior. MT, onset of tanning of metathoracic bars; AS, beginning of resorption of molting fluid in anterior regions.

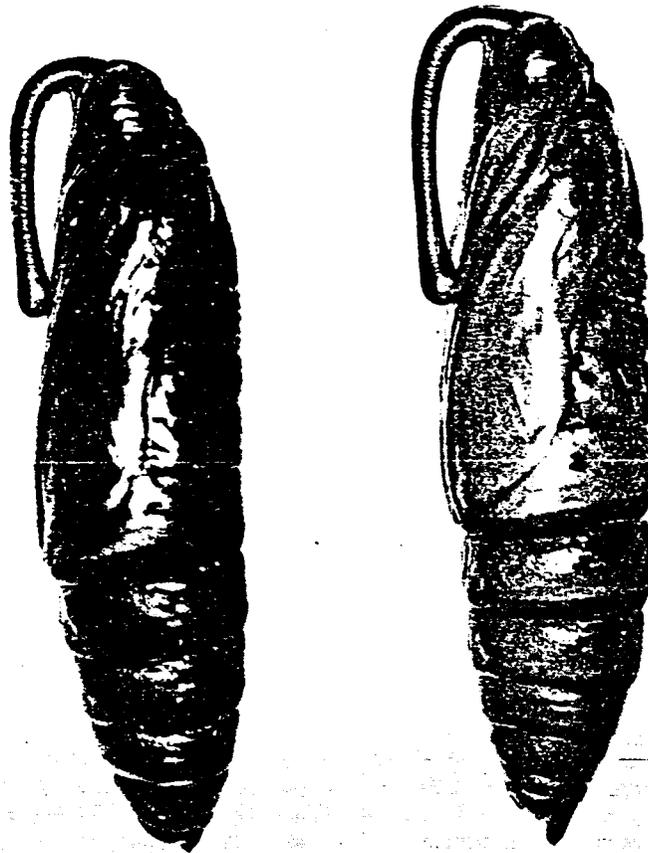


Fig. 3. Left: *Manduca* pupa that received 10 µg ethyl hydrazinoacetate (EHA) at the onset of metathoracic bar tanning 18 hr before pupal ecdysis. Right: normal pupa.

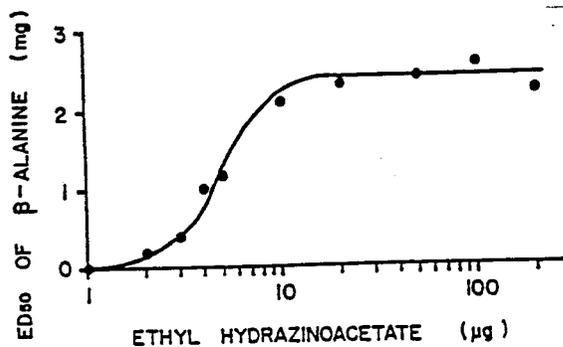


Fig. 6. The amount of β -alanine required to prevent blackening of 50% of *Manduca* pupae that also received the designated dose of ethyl hydrazinoacetate (EHA) at the onset of metathoracic bar tanning. Each ED₅₀ was determined by injecting 10–15 animals each with 6–8 different doses of β -alanine ranging from 0.01 to 6 mg.

When 6 mg β -alanine was injected together with a supramaximal dose of EHA (10 μg) at the onset of metathoracic bar tanning, the normal tan coloration was seen. The ED₅₀ dose of β -alanine to prevent blackening was 2.4 mg [equivalent to 10 mM, assuming an hemolymph volume of ca 40% of the body weight as in the 5th instar larva (Beckage and Riddiford, 1982); Fig. 6]. Importantly, this dose of β -alanine was sufficient to prevent blackening, irrespective of the dose of EHA given up to 200 μg . In molar doses, about 750-fold excess β -alanine is required to counteract the effect of EHA.

The black pigment in *Manduca* larval cuticle is composed primarily of dopamine melanin (Hori *et al.*, 1984; Hiruma *et al.*, 1985). Acid hydrolysis of the black pupal cuticle (Hackman and Goldberg, 1971) formed after EHA treatment produced a black residue, 91% of which was soluble in 0.1 N NaOH at room temperature, and therefore is likely dopamine melanin (Swan, 1974). To determine whether excess dopamine could cause pupal cuticle blackening, we injected dopamine into pharate pupae at the onset of pupal tanning. As seen in Fig. 7, 20–80 mg dopamine

per pupa caused blackening. When compared on a molar basis, apparently 4000 times more dopamine than EHA is required for a melanization score of 2. Moreover, when 1 μg EHA was given in addition to dopamine, the dose of the latter required for black cuticle formation was reduced by one-half (Fig. 7), indicating a synergistic effect between dopamine and EHA. Thus, the blackening of the pupal cuticle of EHA-treated animals is probably due to excess dopamine.

Effect of EHA on catecholamines in hemolymph and cuticle

To determine the effects of EHA on catecholamine levels, pharate pupae were given 10 μg EHA just before the onset of tanning of the metathoracic bars, then the catecholamine content of the hemolymph and cuticle was analyzed at selected times thereafter. Figure 8A shows that the NBAD levels in the hemolymph were unaffected by EHA up to 12 hr after ecdysis. Hemolymph of both control and EHA-treated animals exhibited a biphasic fluctuation in NBAD titer with elevated levels up to 3 hr after the onset of pre-ecdysial tanning followed by a decrease in titer during the rapid post-ecdysial tanning. The NADA level remained low in both the control and EHA-treated animals (Fig. 8A). By contrast, dopamine in the hemolymph of EHA-treated animals began to increase at pupal ecdysis and by 6 hr thereafter was 40-fold higher than the control hemolymph level (Fig. 8B). Presumably some of this excessive dopamine is incorporated into cuticular melanin causing black pupae. β -Alanine in the hemolymph at the time of ecdysis was 3 times lower in EHA-treated pupae than in untreated pupae (0.79 ± 0.62 mM for EHA-treated vs 2.48 ± 0.05 mM for the control), indicating that EHA may interfere with β -alanine synthesis or mobilization.

Analysis of the pupal cuticle of EHA-treated animals showed an elevated dopamine content and depressed NBAD and NADA levels (Fig. 9). After ecdysis increasing amounts of dopamine were incorporated into the cuticle as compared to control pupae

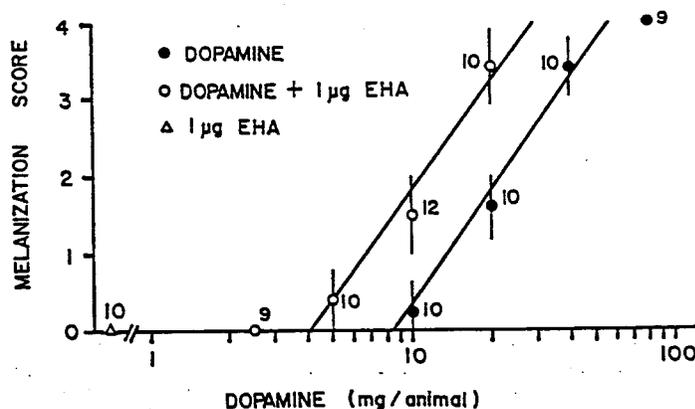


Fig. 7. Dose-response curve for melanization of *Manduca* pupae given dopamine (●) or dopamine and 1 μg EHA (○) or 1 μg EHA (△) at the onset of metathoracic bar tanning. The increasing doses of dopamine beginning with 10 mg were injected in 0.041, 0.083, 0.165, and 0.33 ml water respectively due to its relative insolubility in water. Each point represents the average \pm SD for *N* larvae assayed.

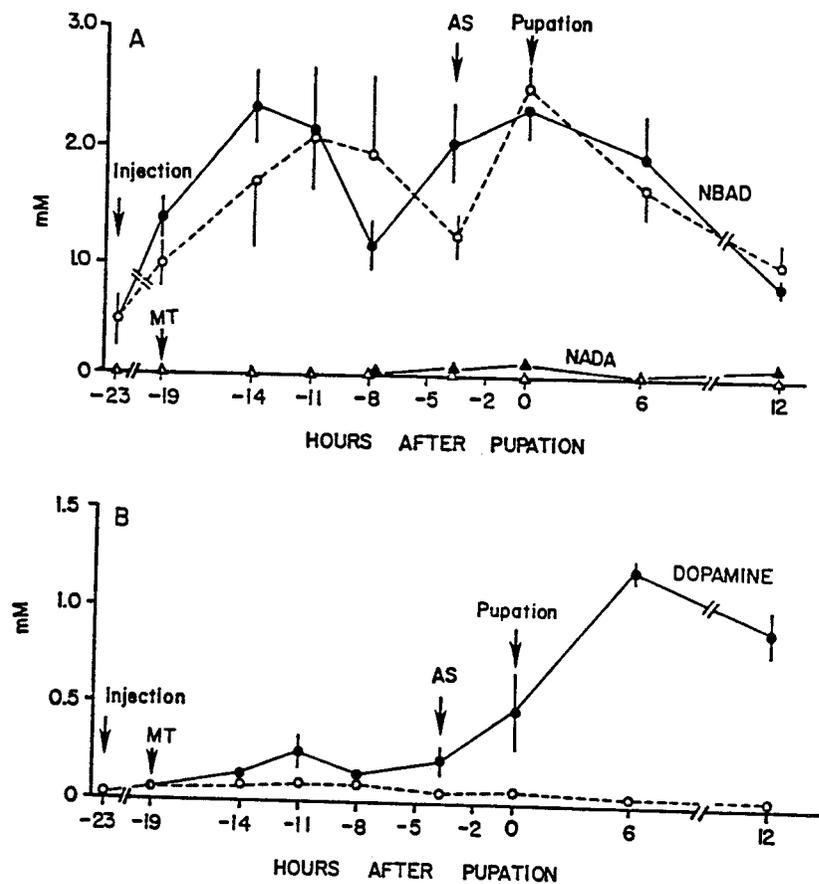


Fig. 8. (A) Titer of NBAD and NADA in the hemolymph of control (O, Δ) and EHA-treated (●, ▲) *Manduca* prepupae and pupae. (B) Dopamine levels in the hemolymph of control (O) and EHA-treated (●) prepupae and pupae. The EHA was injected 23 hr before expected pupal ecdysis. Points represent the average \pm SD for three replicates. MT, onset of tanning of metathoracic bars; AS, beginning of resorption of molting fluid in anterior regions.

so that by 6 hr the level was nearly 50-fold higher. By contrast, NBAD incorporation was decreased so that by 12 hr its level was 4-fold lower. Also, NADA incorporation was somewhat depressed. In fully sclerotized pupal cuticle (2 weeks after ecdysis; diapausing pupae), all of the catecholamine levels were greatly diminished with dopamine only two-fold higher and the *N*-acylated catecholamines about four-fold lower in EHA-treated animals (Table 4). These results indicate that EHA by interfering with β -alanine metabolism suppresses catecholamine acylation which allows dopamine levels to increase.

Effects of EHA in vitro

When untanned pharate pupal abdominal integument was explanted 6 hr before the onset of metathoracic bar tanning and cultured in Grace's medium

Table 4. Effect of EHA on catecholamine level (nmol/g, mean \pm SE) in 2-week-old pupal cuticle

Sample	N	Dopamine	NBAD	NADA
Control	5	13 \pm 2	159 \pm 30	20 \pm 7
EHA	3	26 \pm 3	38 \pm 12	4 \pm 1

Table 5. Effect of EHA, β -alanine and NBAD on the coloration of *Manduca* pharate pupal cuticle after 24 hr in Grace's medium containing 10 mM dopamine

Dopamine-supplemented Grace's medium	N	Coloration of pupal cuticle
Control	8	Normal: reddish-brown background with dark red-brown to dark brown pocks
0.3 mM EHA	8	Normal: reddish-brown background with dark red-brown to dark brown pocks
Minus β -alanine	8	Normal: reddish-brown background with dark red-brown to dark brown pocks
Minus β -alanine + 0.3 mM EHA	8	Brownish-black background with black pocks
Minus β -alanine + 0.3 mM EHA + 4 mM NBAD	6	Normal: reddish-brown background with dark red-brown to dark brown pocks

Grace's medium contains 2.24 mM β -alanine.

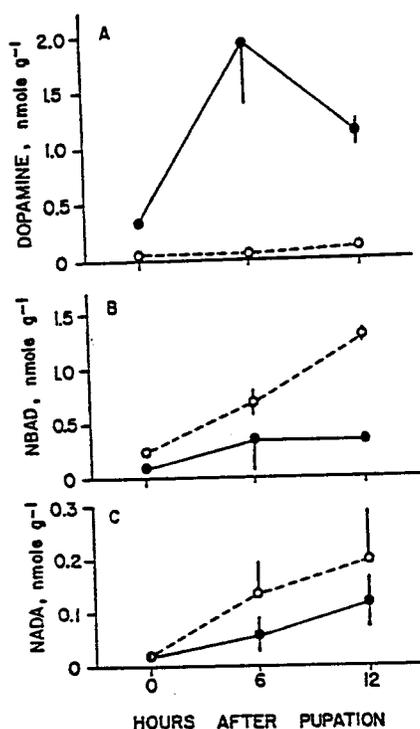


Fig. 9. Effect of EHA on catecholamines in pupal cuticle of *Manduca* after ecdysis. (○---○) Control; (●—●) EHA-treated. A—Dopamine; B—NBAD; C—NADA. Mean \pm SE, $N = 2-3$.

(which contains 2.24 mM β -alanine) with 10 mM dopamine for 24 hr, the pupal cuticle became a deep reddish-brown with dark pocks confirming earlier results (Roseland *et al.*, 1986). The addition of 0.3 mM EHA to the medium had no effect on the subsequent coloration (Table 5). When the untanned integument was cultured in the absence of β -alanine in the medium, tanning also occurred normally, except when EHA was present. In the latter case, the cuticle blackened. Importantly, when 4 mM NBAD was also present, the blackening was prevented. These results suggest that sufficient β -alanine is present in the epidermis and/or new cuticle at the time of explantation to allow NBAD formation and subsequent tanning of the cuticle. EHA apparently inhibits this reaction so that only dopamine is incorporated into the cuticle unless β -alanine is also present in the medium.

DISCUSSION

These data show that α -aminoxy or α -hydrazino derivatives of acetic acid cause blackening of *Manduca* pupal cuticle if injected into pharate pupae before the onset of tanning. This blackened cuticle contained an excess of dopamine which was incorporated into melanin as in β -alanine-deficient mutants of flies (Fukushi, 1967; Hodgetts, 1972; Hodgetts and Choi, 1974) and beetles (Kramer *et al.*, 1984). This blackening could be prevented by the addition of excess β -alanine, either *in vivo* or *in vitro*, indicating that these compounds likely interfere with β -alanine synthesis or mobilization and thus with the continued *N*-acylation of the catecholamines to form all the

NBAD and NBANE necessary for tan coloration of *Manduca* pupal cuticle (Hopkins *et al.*, 1984; Roseland *et al.*, 1985). Such a competitive inhibition would lead to the observed increased level of dopamine and lower concentrations of β -alanine, NBAD and NADA. In spite of the decreased amount of NBAD and NADA, the pupal cuticle appeared to stabilize normally, indicating that the amount of catecholamines present was sufficient for hardening in they are the only sclerotizing agents.

An analysis of the structure-activity relationships among the compounds tested shows that close structural analogy with β -alanine as well as the presence of a reactive NH_2 group in both the hydrazino and aminoxy derivatives is essential for inhibition of β -alanine metabolism. Possibly these reactive groups form stable hydrazones or oximes with enzymes containing carbonyl groups in their active sites. The inactivity of the hydrazino and oxime compounds 14 and 15 supports this idea since they are stable and cannot easily hydrolyze or react with carbonyl compounds. By contrast, the *N*-acylated derivatives are active because they can be hydrolyzed under physiological conditions in the pharate pupa to release free NH_2 -containing hydrazino or aminoxy derivatives.

The active compounds assayed should inhibit all enzymes containing active carbonyl groups and consequently would be expected to inhibit both decarboxylases and transaminases (Oehme *et al.*, 1969; Braunstein, 1973; John *et al.*, 1978; Williamson and Brown, 1979). Further studies are required to assess the precise mode of action of these compounds on β -alanine biosynthesis or catecholamine acylation.

The effectiveness of EHA in causing black coloration irrespective of the time that it is given up to the onset of tanning shows that the compound or an active derivative is relatively stable in the prepupa. Once pre-ecdysial tanning begins, EHA rapidly becomes ineffective, showing that it does not interfere with tanning *per se* and that β -alanine and/or NBAD synthesis must be complete by this time. The finding that NBAD levels in the hemolymph are generally unaffected by EHA both before and after ecdysis also indicates that sufficient β -alanine and/or NBAD are already present at the time of injection. Later NBAD synthesis during cuticular tanning however apparently is blocked so that dopamine accumulates in the hemolymph. Dopamine then is incorporated into the cuticle as melanin causing blackening.

The site of β -alanine or NBAD synthesis in *Manduca* prepupa is unknown. Since both compounds are present in increasing amounts in the hemolymph, one assumes that the fat body is the site of synthesis. Our finding that EHA prevents normal tanning of pharate pupal cuticle *in vitro* and causes the same type of blackening seen *in vivo* suggests that the epidermis may also contribute to the synthesis of NBAD as it does to the synthesis of cuticular melanin in the black or allatectomized larva (Hiruma and Riddiford, 1984, 1985, 1986; Hiruma *et al.*, 1985). Further studies using labeled precursors are necessary to substantiate this hypothesis.

These compounds effectively change the pupal coloration but apparently do not interfere with sclerotization. Since some β -alanyl derivatives are still found in the hemolymph and cuticle of these

treated pupae, these compounds apparently cannot quantitatively inhibit β -alanine synthesis or catecholamine acylation. Therefore, further work is necessary to find a compound that will completely block the metabolism of β -alanine or its derivatives before their role in cuticular hardening can be directly assessed.

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