

## N- $\beta$ -ALANYLDOPAMINE LEVELS AND SYNTHESIS IN INTEGUMENT AND OTHER TISSUES OF *MANDUCA SEXTA* (L.) DURING THE LARVAL-PUPAL TRANSFORMATION

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**Abstract**—N- $\beta$ -Alanyldopamine (NBAD) and other diphenols in tissues of the fifth larval instar of the tobacco hornworm, *Manduca sexta* (L.), were analyzed by HPLC with electrochemical detection. NBAD accumulated in the integument during the intermolt feeding period, with maximal levels in the wandering stage (6 mmol/g). It then declined to a low level during apolysis and endocuticle digestion, while hemolymph NBAD increased during the same interval to a peak concentration (3 mM) shortly before pupal ecdysis. Trachea and foregut contained lesser amounts of NBAD (0.5 mmol/g), perhaps associated with cuticle, whereas fat body, muscle, midgut and hindgut had 0.1 mmol/g or less. Dopamine (DA), N-acetyldopamine and 3,4-dihydroxyphenylalanine (DOPA) were at least 10-fold less abundant than NBAD in the integument. NBAD synthase, which catalyzes the formation of NBAD from DA and  $\beta$ -alanine, was assayed in both integument and fat body. Highest activity was detected in the integument, where two peaks were observed, one at day 3 near the end of larval feeding and the other at day 9 as pupal cuticle tanning was initiated. Fat body enzyme was substantially less and was detected only in the pharate pupa. Maximal NBAD synthesis by integument cultured *in vitro* was dependent upon DA supplementation of at least 1.4 mM. 20-Hydroxyecdysone did not alter NBAD synthesis *in vitro* in either the integument or the fat body, even though injection of this hormone into isolated larval abdomens induced synthesis and/or transport of integumental NBAD back into the hemolymph. The rate-limiting steps in the NBAD biosynthetic pathway appear to be the production of DOPA and DA, because  $\beta$ -alanine occurs in the hemolymph at relatively high levels throughout larval-pupal development.

**Key Word Index:** diphenol, catecholamine, N- $\beta$ -alanyldopamine,  $\beta$ -alanine, dopamine, 20-hydroxyecdysone, integument, fat body, hemolymph, cuticle, sclerotization, *Manduca sexta*

### INTRODUCTION

Insect cuticle is secreted by the underlying epidermis in response to a specific hormonal regimen (reviewed by Riddiford, 1985). It is then stabilized by a complex and variable process involving both noncovalent and covalent interactions between protein and chitin polymers, dehydration and mineralization depending upon the functional requirements of the structure (reviewed by Brunet, 1980; Andersen, 1979; Hepburn, 1985; Kramer and Hopkins, 1987). In cuticles of different developmental stages of the tobacco hornworm, *Manduca sexta* (L.), two N-acylated dopamine derivatives, N- $\beta$ -alanyldopamine (NBAD) and N-acetyldopamine (NADA), are major tyrosine metabolites that apparently serve as precursors for cross-linking and stabilizing agents (Hopkins *et al.*, 1982, 1984; Morgan *et al.*, 1987). The formation of

these derivatives is catalyzed by the N-acyltransferase enzymes, NBAD synthase or NADA synthase.

Little is known about tissue localization of the enzymes involved in N-acylated catecholamine synthesis during sclerotization. Substantial NADA synthase activity has been found in neural tissue, fat body, gut, Malpighian tubules, skeletal muscles of several insect species (reviewed by Brown and Nestler, 1985), and perhaps oenocytes (Maranda and Hodgetts, 1977). Other studies with *M. sexta* (Krueger *et al.*, unpublished data) demonstrated that NBAD and NBAD synthase are found in the nervous system of *M. sexta*, but no data are available on the localization of the enzyme in other tissues. Thus, our goals were to determine the abundance of NBAD in tissues of *M. sexta* during the larval-pupal transformation, localize its site of synthesis, and also study the hormonal regulation of NBAD synthesis. In addition, tissue levels of other catecholamines, in particular NADA, were determined.

Abbreviations used are NBAD, N- $\beta$ -alanyldopamine; DOPA, 3,4-dihydroxyphenylalanine; DA, dopamine; NADA, N-acetyldopamine; AMD,  $\alpha$ -methyl DOPA; 20-HE, 20-hydroxyecdysone; W, wandering stage; OR, ocellar retraction stage; BB, metathoracic brown bar stage; E, ecdysis; LCEC, liquid chromatography with electrochemical detection.

### MATERIALS AND METHODS

#### Animals

*M. sexta* were reared as described by Bell and Joachim (1976) with a nondiapausing photoperiod of 16L:8D.

Insects were developmentally staged by selecting larvae undergoing ecdysis to the fourth instar to obtain a synchronous population (Riddiford *et al.*, 1979). Larvae then were selected again on the day of ecdysis (E) to the fifth instar (day 0). By the end of day 3, larvae had attained a weight of 7–9 g. Wandering stage (W), which was identified as the day of dorsal heart exposure and wandering behavior, was initiated on day 5 of the fifth instar. On day 7, ocellar retraction (OR) began when the larval head capsule was slipped, marking the beginning of a large increase in hemolymph 20-hydroxyecdysone titer (Mitsui and Riddiford, 1976). Day 9 of the fifth instar was marked by the appearance of tanned, metathoracic brown bars (BB) in new pharate pupal cuticle.

#### Tissue collection

Catecholamine concentrations were measured in several tissues of *M. sexta*. After the insects were chilled on ice, integument was excised from segments 5 and 6 of the dorsal abdomen and dissected free of muscle, fat body and trachea. Samples of fat body, foregut, midgut, hindgut and trachea were collected after slitting the dorsal abdomen. Tissues were dissected immediately and rinsed briefly in deionized water. All tissues were then blotted on absorbent paper and rapidly weighed. Tissues were homogenized in ground glass grinders at 4°C in 1.2 M HCl containing 10 µg/ml  $\alpha$ -methyl dopa (AMD) as an internal standard. Catecholamines were extracted and analyzed as indicated below.

#### Catecholamine and $\beta$ -alanine analyses

Catecholamines in tissue homogenates or culture medium (0.1 ml) were extracted with 1.2 M HCl according to the procedure of Hopkins *et al.* (1982, 1984) and analyzed by HPLC with electrochemical detection (LCEC) at a potential of +0.72 V. A reversed-phase C-18 column (25 cm  $\times$  4.6 mm, particle size = 5 µm) was used with a mobile phase of 10–12% methanol, 0.8%  $H_3PO_4$  and 0.06–0.08 g/l 1-octane sulfonate at pH 2.6 and 30°C. A second mobile phase of 20% acetonitrile, 1–2%  $H_3PO_4$  and 0.16 g/l sodium lauryl sulfate at pH 3 also was used to further verify the identity of compounds. The retention times of extracted catecholamines were compared to standard compounds and quantitated by comparison of integrated areas to that of the internal standard AMD. For  $\beta$ -alanine analysis, 0.05 ml of hemolymph was mixed with 1 ml of 10% (w/v) trichloroacetic acid. After centrifugation, the supernatant was extracted twice with 1 ml of ethyl ether and lyophilized.  $\beta$ -Alanine was analyzed by cation-exchange liquid chromatography (Dionex amino acid analyzer). Statistical tests of significant differences between stages and statistical differences from zero were performed using the SAS least-squares means procedure with  $P < 0.05$  (Statistical Analysis Systems Institute Inc., Cary, North Carolina).

#### Tissue culture

Tissues cultured *in vitro* were integument, fat body and hemocytes. Insects were rinsed in deionized water and surface sterilized in 70% ethanol for 2 min followed by two rinses in sterile deionized water. Integument from segments 5 and 6 was excised, cleaned of muscle, trachea, and fat body, and cut into halves. Fat body tissues were removed from the hemocoel and cleaned of contaminating tissues. Approximately equal quantities of each tissue were placed in the wells of a 24-well tissue culture plate. Hemocytes were obtained by clipping a proleg and collecting hemolymph in ice-cold Grace's insect culture medium in sterile 1.5 ml centrifuge tubes. After centrifugation for 5 min at 1000 g at 4°C, the supernatant was discarded, and the pellet was collected and used for cell culture. All tissues were cultured for 2 days in 0.5 ml of Grace's medium containing

35 units of streptomycin sulfate and 80 units of penicillin (Riddiford *et al.*, 1979). In addition, the culture medium was saturated with phenylthiourea (PTU), and cultures were maintained at 26°C in an atmosphere of 95%  $O_2$ –5%  $CO_2$ . Some cultures were supplemented with dopamine. At the end of the culture period, tissues were weighed or counts of viable hemocytes were made using trypan blue as an indicator. Catecholamines in the culture medium were extracted and analyzed by LCEC.

#### 20-Hydroxyecdysone treatment

Larvae in the wandering stage were double-ligated with thread between the first and second abdominal segments, and the head and thorax were removed. After 2 days, abdomens were injected with 20-hydroxyecdysone (20-HE) dissolved in 10% isopropanol at doses ranging from 0 to 15 µg/g of abdomen weight, while control abdomens received only the solvent. After 2 additional days, 0.05 ml of hemolymph was collected as above for analysis of catecholamines.

Tissue cultures of fat body and integument also were treated with 20-HE to observe hormone effects *in vitro*. Tissues were excised, as above, except half of the tissues were cultured in medium supplemented with 5 µg/ml ecdysone ( $n = 5-7$ ). After 2 days, the media from all cultures were collected for catecholamine analysis, as previously described. Tissues were rinsed briefly in Grace's insect tissue culture medium. All tissues then were cultured for 2 additional days in media containing DA and PTU, as usual, but no ecdysone. On day 4, samples of media were collected and analyzed for catecholamines. Thus, two culture time points were used for catecholamine analysis, 0–2 days and 2–4 days, for both ecdysone-treated and control tissues.

## RESULTS

#### Tissue catecholamine levels

NBAD in fifth instar larval integument accumulated to high levels during the feeding period, followed by depletion during the wandering stage and the pharate pupal period (Fig. 1A). A maximal

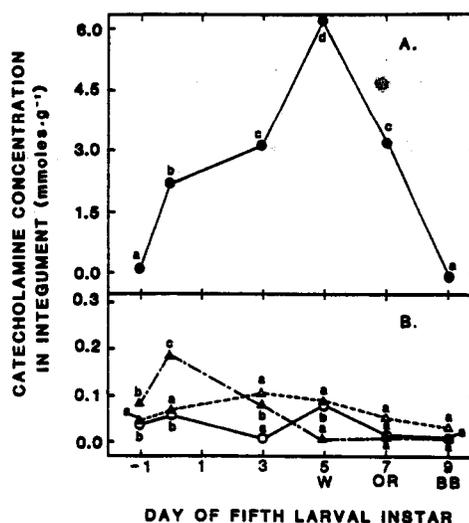


Fig. 1. Catecholamines in the integument of *Manduca sexta* during fifth instar larval and pupal development. For individual compounds means with the same letter are not significantly different ( $P = 0.05$ ,  $n = 3-5$ ). Stages are as indicated in "Materials and Methods". NBAD, ●; DOPA, △; DA, ▲; NADA, ○.

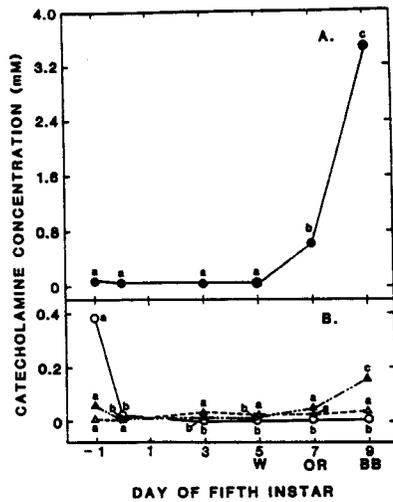


Fig. 2. Catecholamines in the hemolymph of *Manduca sexta* during fifth instar larval and pupal development. For individual compounds means with the same letter are not significantly different ( $P = 0.05$ ,  $n = 3-5$ ). Stages are as indicated in "Materials and Methods". NBAD, ●; NADA, ○; DOPA, △; DA, ▲.

NBAD concentration of 6 mmol/g wet weight of integument occurred on day 5, at the initiation of wandering behavior. After day 5, NBAD diminished until day 9, when the NBAD concentration of the combined larval exuvia and new pupal cuticle was not significantly different from that of day 0 integument ( $<0.1$  mmol/g). The concentration of NBAD in the late feeding fourth instar integument also was measured and found to be  $3.0 \pm 0.3$  mmol/g, a value similar to that of day 3 integument from the feeding fifth instar larvae. Thus, high levels of extractable NBAD accumulate in larval integument during the intermolt period of both instars, suggesting that NBAD may play a role other than as a tanning precursor for stiff brown cuticle during pupal development.

The distribution of NBAD in day 5 integument was determined by separating the epidermis from the cuticular portion, extracting each with cold acid and analyzing by LCEC. Epidermis contained  $0.72 \pm 0.21$  mmol/g NBAD, while cuticle had  $4.75 \pm 0.52$  mmol/g. Approximately 85% of the NBAD in day 5 integument is localized in the cuticle and only about 15% in the epidermis.

Unlike NBAD, other diphenols in the integument showed little variation during the last larval stadium (Fig. 1B). The highest concentration of DA occurred early in the fifth instar at days 0-3. However, during this time, NBAD was more than 10 times as abundant than DA. NADA and DOPA were also low in concentration throughout the sampling period.

Hemolymph NBAD is primarily conjugated with glucose on a phenolic oxygen (Hopkins *et al.*, 1984). Its titer appears to be inversely related to that of the integument during fifth larval instar and pharate pupal development (Fig. 2A). NBAD in the hemolymph of day 1 to day 5 insects was detected only at trace levels, but on day 7 at the start of larval-pupal

apolysis, its concentration increased to c. 0.6 mM and finally reached nearly 3.5 mM on day 9 a few hours before pupal ecdysis. DA showed a similar temporal pattern, although it was much lower in concentration (Fig. 2B). NADA was highest in early instar hemolymph, but it declined to insignificant levels by day 1. DOPA was not present in hemolymph at concentrations greater than 0.05 mM throughout development.

Some of the other tissues examined also accumulated diphenols. The trachea contained significant levels of NBAD primarily on day 5 (0.5 mmol/g, Fig. 3A), whereas tissues such as the muscle, fat body, hindgut and midgut contained lesser quantities (Fig. 3A, B). However, the foregut contained nearly 0.5 mmol/g on day 3. This tissue is lined with a cuticular intima and NBAD may be involved in maintenance of this structure during feeding. When feeding ceased and wandering behavior commenced (day 5), NBAD in the foregut declined nearly 5-fold, perhaps in synchrony with the onset of molting.

The concentrations of NBAD and other diphenols also were measured in molting fluid, midgut fluid, and Malpighian tubules obtained from pharate pupae. NBAD and DA were present in the molting fluid at concentrations of  $110 \pm 70$   $\mu$ M and  $7 \pm 4$   $\mu$ M, respectively. In midgut fluid, the NBAD concentration was  $0.33 \pm 0.21$  mM, whereas the DA concentration was  $4.15 \pm 0.71$  mM. Midgut fluid was the greatest source of DA in *M. sexta*. The Malpighian tubules contained 0.012 mmol/g DA, whereas NBAD was not detected.

#### NBAD synthesis in vitro

Integument, fat body and hemocytes were cultured to determine the importance of these tissues as sites for NBAD and NADA synthesis. Since Grace's tissue culture medium contains  $\beta$ -alanine but no DA, the synthesis of NBAD as a function of increasing DA supplementation in cultures of day 9 integument was determined (Fig. 4). With no supplemental DA,

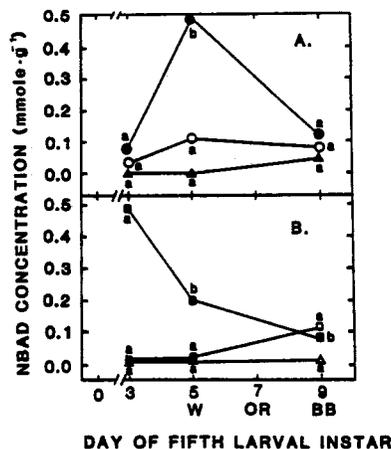


Fig. 3. *N*- $\beta$ -Alanyldopamine (NBAD) in tissues of *Manduca sexta* during fifth instar larval and pupal development. For individual tissues means with the same letter are not significantly different ( $P = 0.05$ ,  $n = 3$ ). Trachea, ●; muscle, ○; fat body, ▲; foregut, ▲; hindgut, ■; midgut, □.

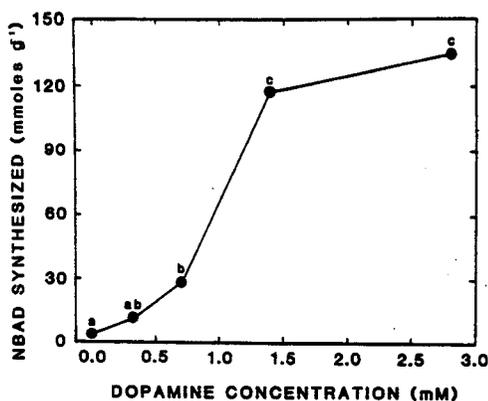


Fig. 4. *N*- $\beta$ -Alanyldopamine (NBAD) synthesis as a function of dopamine concentration in cultures of *Manduca sexta* integument. Integument excised from pharate pupae was cultured for 2 days in Grace's insect tissue culture medium at 25°C in 95% O<sub>2</sub>-5% CO<sub>2</sub>. Means with the same letter are not significantly different ( $P = 0.05$ ,  $n = 3-5$ ).

only minor amounts of NBAD were found in the culture medium. Increasing concentrations of DA significantly increased NBAD formation. Maximal NBAD synthesis of *c.* 120–140 mmol/g occurred after 2 days with DA supplementation of at least 1.4 mM. No conjugated form of NBAD was detected *in vitro* when samples were subjected to acid hydrolysis. NBAD was the major catecholamine synthesized from DA by integument. Less than 0.01 mmol/g of NADA was formed in these cultures, and only minor amounts of DOPA were present (<0.01 mmol/g). In the absence of DA supplementation, total DA synthesis was only  $0.7 \pm 0.2$  mmol/g.

The integument of *M. sexta* was capable of synthesizing different quantities of NBAD, depending upon the day of excision during larval development (Fig. 5A). When excised on the day prior to ecdysis, the pharate fifth instar integument produced about 30 mmol NBAD/g in the 2-day culture period (day = -1 of Fig. 5). Tissue excised on day 0 formed about half this amount. By day 3, NBAD synthesis had increased to nearly 75 mmol/g. After this time, a sharp decline in rate of synthesis occurred at days 5 and 7, followed by an increase to its maximal level at day 9 in the pharate pupal stage (nearly 100 mmol/g). NADA was produced only to a limited extent (9 mmol/g) in pharate larvae prior to ecdysis and then declined to trace levels (Fig. 5A). The NBAD synthetic capacity of late feeding fourth instar integument ( $42.5 \pm 4.5$  mmol/g) was similar to that of day 2 or day 3 of the fifth instar integument. NADA production in fourth instar larval integument was similar to that of the day -1 tissue, as shown in Fig. 5A.

Fat body and hemocytes also were cultured to determine whether NBAD synthesis occurred in these tissues. The fat body produced NBAD in the later stages of the instar (days 7, 9), with a synthetic rate of approximately 10 mmol/g after 2 days (Fig. 5B). NADA was produced by fat body only in early stage tissue (day<sup>-1</sup>), as in the integument, with less than 10 mmol/g synthesized.

A low level of NBAD synthesis ( $0.7-1.3$  nmol/10<sup>5</sup> cells) occurred in hemocytes collected from day 4 wandering larvae and BB pharate pupae when the culture medium was supplemented with 1.4 mM DA. Without exogenous DA, NBAD synthesis ranged from  $0.03-0.26$  nmol/10<sup>5</sup> cells. A low level of DOPA production also occurred in the hemocyte cultures. In day 3 cultured hemocytes, DOPA synthesis was  $0.20 \pm 0.25$  nmol/10<sup>5</sup> cells after 2 days. In cultures of day 5 and day 7 hemocytes, DOPA synthesis increased significantly ( $P \leq 0.05$ ) to  $3.4 \pm 1.3$  and  $4.3 \pm 1.1$  nmol/10<sup>5</sup> cells, respectively. No significant quantities of DA were extracted from hemocyte cultures, indicating that either decarboxylation does not occur or the DA formed is rapidly *N*-acylated or metabolized by another pathway.

#### $\beta$ -Alanine concentration in hemolymph

Free  $\beta$ -alanine concentrations in hemolymph were determined during larval and pupal development to establish whether  $\beta$ -alanine availability might limit NBAD synthesis *in vitro*.  $\beta$ -Alanine levels were not significantly different during the fifth larval stadium, remaining between 0.6 and 1.0 mM (Fig. 6). However, a significant increase in the hemolymph concentration occurred after the time of larval-pupal apolysis, reaching a peak titer of 1.6 mM as tanning of pupal cuticle was visible (BB). Thus,  $\beta$ -alanine concentrations appeared to be sufficient for NBAD biosynthesis throughout larval and pupal development. The significant increase in hemolymph  $\beta$ -alanine also correlated with increased NBAD synthesis and accumulation (Figs 2 and 5). Uptake of  $\beta$ -alanine from the hemolymph by the epidermis should occur readily *in vivo*, since this process occurs

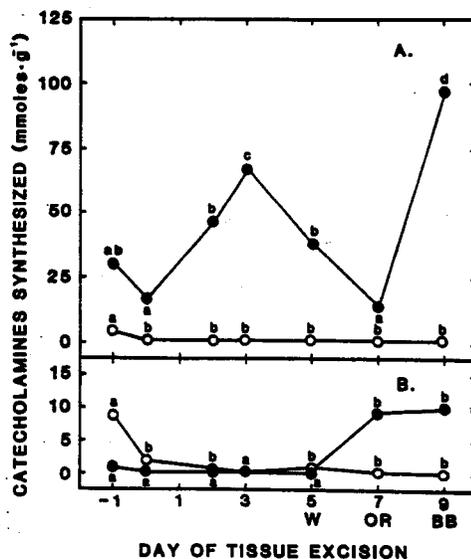


Fig. 5. *N*- $\beta$ -Alanyldopamine (NBAD) and *N*-acetyldopamine (NADA) synthesis by (A) integument and (B) fat body excised from *Manduca sexta* at different times during fifth instar larval and pupal development. Grace's culture medium supplemented with 1.4 mM DA. For individual compounds means with the same letter are not significantly different ( $P = 0.05$ ,  $n = 4-7$ ). Stages are as indicated in "Materials and Methods". NBAD, ●; NADA, ○.

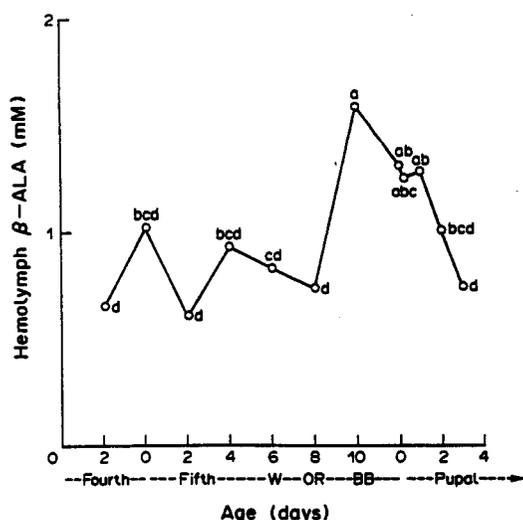


Fig. 6.  $\beta$ -Alanine ( $\beta$ -ala) concentrations in the hemolymph of *Manduca sexta* during fifth instar larval and pupal development. Means with the same letter are not significantly different ( $P = 0.05$ ,  $n = 3$ ).

in cultured tissues. The concentration of  $\beta$ -alanine in the Grace's culture medium used in the NBAD synthase experiments was 2.2 mM, which is at least 40% higher than the hemolymph level.

#### Effect of 20-hydroxyecdysone (20-HE) on catecholamine levels

Ligated abdomens of fifth instar day 5 *M. sexta* were injected with 20-HE to determine if ecdysteroids regulate the increase in NBAD observed in the hemolymph. Since 20-HE stimulates apolysis and new cuticle secretion, it also may directly or indirectly initiate catecholamine synthesis for cuticle tanning. When at least 5  $\mu$ g 20-HE/g abdomen was injected into ligated abdomens, a significant increase in NBAD in the hemolymph occurred (Fig. 7A). Maximal effect was observed with an injection of 15  $\mu$ g/g, which caused a 15-fold increase in hemolymph NBAD to >1 mM levels. There was a smaller effect on the concentrations of the other hemolymph catecholamines (Fig. 7B).

*In vitro* 20-HE supplementation (5  $\mu$ g/ml) for 2 days, followed by 2 days in Grace's medium without 20-HE of day 0, day 5, or day 7 integument cultures, did not affect catecholamine synthesis; only 19, 15 and 17 nmol NBAD/g, respectively, were found at these stages. Also, fat body showed no increase in NBAD after 20-HE treatment using identical conditions.

#### DISCUSSION

NBAD was the most abundant catecholamine in both fourth and fifth instar integument of *M. sexta* during the intermolt periods, whereas trachea and foregut had the next highest levels of NBAD, perhaps associated with the cuticle of those tissues. Most of the NBAD in the integument was localized in the cuticle. NBAD also occurred in high concentration in the hemolymph of pharate pupae, primarily as a

glucose conjugate (Hopkins *et al.*, 1984). Moderate concentrations of NBAD were also detected in the nervous system of *M. sexta* larvae during the last stadium (Krueger *et al.*, unpublished data).

Our data suggest that the larval integument may serve as a storage site for NBAD during the intermolt feeding period for later use in tanning the new pupal cuticle. Peak concentrations of NBAD rapidly disappear from the integument after wandering and apolysis have occurred. The hemolymph of the pharate pupa then becomes the main storage site, as NBAD concentrations increase to peak levels shortly before ecdysis. Although it has not been demonstrated that NBAD in the integument is translocated to the hemolymph at a later time, the reciprocal nature of NBAD disappearance from the integument and appearance in the hemolymph suggests this to be the case.

Using total tissue weights for *M. sexta* provided by Williams-Boyce and Jungreis (1980), the experimentally measured concentration of NBAD in the integument would account for a 3 mM concentration of NBAD in the hemolymph, assuming all of the NBAD is transported. Cornell and Pan (1983) have shown that *M. sexta* transports molting fluid to the midgut, so that the dissolved components may be absorbed. This is supported by our finding that both DA and NBAD occur in the molting and midgut fluids. The high DA concentration in the midgut contents suggests that hydrolysis of NBAD occurs, perhaps via the action of a specific hydrolase in molting fluid, since DA concentrations in the integument and other tissues are relatively low compared to those of NBAD. Another possibility is that NBAD is transported to the hemolymph prior to

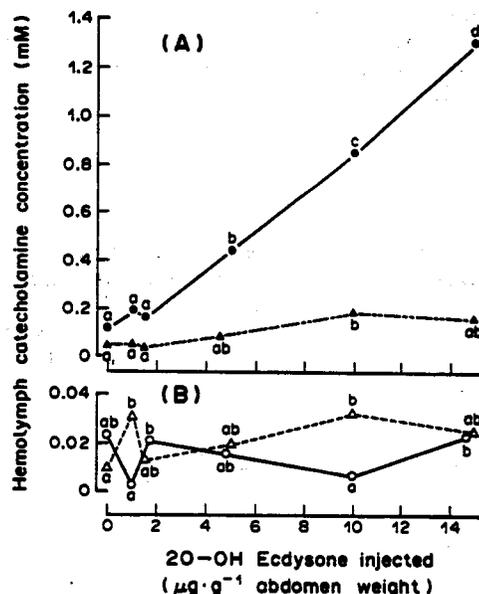


Fig. 7. The catecholamine concentration in hemolymph of ligated abdomens of fifth instar *Manduca sexta* as a function of 20-hydroxyecdysone injected. Abdomens were from wandering stage animals. For individual compounds means with the same letter are not significantly different ( $P = 0.05$ ,  $n = 3$ ). NBAD,  $\bullet$ ; DA,  $\blacktriangle$ ; DOPA,  $\triangle$ ; NADA,  $\circ$ .

apolysis by movement directly into hemolymph from the epidermis. Approximately half of the integumental NBAD disappears before ocellar retraction, supporting this conclusion.

Although larval synthesis and storage of NBAD may be important for pupal cuticle tanning, NBAD synthesis during pharate pupal development must also play an important role. The enzyme NBAD synthase occurs at relatively high levels of activity during the fifth larval stadium and pharate pupal period when NBAD is observed to accumulate in the integument and hemolymph, respectively. *In vitro* tissue culture experiments demonstrated that NBAD synthase is mostly localized in the integument and fat body, with the integument being the primary source. Kiely and Riddiford (1985a,b) have demonstrated that several epidermal proteins are synthesized until pupal commitment (day 5 in our study) and then are not synthesized again until the pharate pupal stages (day 9). The NBAD synthase activity profile appears to fit this bimodal pattern of protein synthesis during larval and pupal development. NBAD synthase is likely one of the proteins synthesized by both larval and pupal epidermis, and synthesis is switched on and off by hormonal events that regulate new cuticle formation and molting cycle events. This hypothesis is supported by the experiments in which NBAD synthesis was stimulated by injection of 20-HE into ligated larval abdomens.

The exact function of NBAD in the integument is still speculative. Hopkins *et al.* (1982, 1984) presented evidence that NBAD is associated with the production of stiff, brown cuticle and NADA is linked to formation of stiff clear cuticle. Roseland *et al.* (1987) have correlated  $\beta$ -alanine and NBAD with rust red versus black coloration and more rapid sclerotization in strains of *Tribolium castaneum*. However, the current study has shown that free NBAD predominates not only in stiff, brown cuticle, but also in the clear, flexible larval cuticle. Wolfgang and Riddiford (1987) found that extensibility of larval cuticle is highest during the first 3 days of the fifth larval stadium and then decreases dramatically at wandering, as intrinsic stiffness of the cuticle increases. NBAD increased during this same interval to a peak concentration at the start of wandering. Therefore, the highest NBAD concentration in the integument correlates with the period when the larval cuticle shows maximum stiffness, but whether NBAD plays a role in stabilization resulting in this property is unknown. NBAD also may serve as a precursor for quinonoid metabolites used for wound healing and pigmentation.

NBAD synthase in cultured fat body occurred at measurable levels only on day 7 and 9 in pharate pupae. The temporal pattern of synthesis suggests that NBAD at this time is especially critical for subsequent sclerotization and may be regulated by 20-HE in the absence of juvenile hormone. Fat body enzyme may function to acylate with  $\beta$ -alanine excess DA in the hemolymph before it is transported to the epidermis. Taking into consideration total tissue weights in the *M. sexta* body (Williams-Boyce and Jungreis, 1980), the integument is capable of synthesizing 160 mmol NBAD/g tissue, while in the same time period, the fat body synthetic capacity is only

7 mmol NBAD/g. Thus, synthesis by the integument exceeds that of the fat body by more than 20-fold.

Since NBAD synthesis declined sharply in the integument at days 5–7 and then increased substantially at day 9, it appears that hormonal regulation may be operative. In addition, the onset of NBAD synthesis in the fat body must be regulated. We found that 20-HE increased hemolymph titers of NBAD *in vivo*, but did not alter NBAD synthesis *in vitro* in either the integument or the fat body. Apparently 20-HE is not directly inducing the epidermal or fat body cells to synthesize catecholamines. 20-HE may play a role in regulating NBAD production by tissues other than integument or fat body, or may have an indirect effect on the synthesis of NBAD. Its action may be mediated by a secondary effector.

Several possible effectors have been screened. In addition to ecdysone sensitivity, initial experiments demonstrated that heat stable extracts of abdominal nerve cords from day 5 *M. sexta* injected into post-wandering larvae (day 8–9) can increase NBAD in hemolymph 2-fold. However, cAMP, cGMP, or phosphodiesterase inhibitors, as well as brain tissue extracts, could not mimic this response (Krueger *et al.*, unpublished data). It is also possible that hemolymph NBAD may be a product of recycled NBAD from the cuticle and that 20-HE acts only to initiate the recycling process.

Another factor that may regulate NBAD synthesis, other than enzyme levels, is substrate availability. The availability of DA and  $\beta$ -alanine, as well as their precursors, is essential for the synthesis of NBAD. Our results showed that substantial concentrations of  $\beta$ -alanine (0.6–1.6 mM) are present in hemolymph throughout the fourth and fifth larval stadia. In *Papilio xuthus* hemolymph,  $\beta$ -alanine fluctuated from 1 to 6 mM during the pupal stage (Ishizaki and Umebachi, 1988). Thus,  $\beta$ -alanine is probably not a limiting factor for NBAD synthesis. Instead, NBAD synthesis is probably limited by the availability of DA or its precursors. It has been suggested that DA is the limiting substrate for puparial tanning in certain Diptera and that DOPA decarboxylase catalyzes the rate-limiting step of the sclerotization pathway (reviewed by Sekeris and Fragoulis, 1985). However, hydroxylation of tyrosine to DOPA may also be a regulated step in the biosynthetic pathway for catecholamines involved in cuticle tanning (Seligman *et al.*, 1977; Hopkins 1982). The conversion of tyrosine to DOPA and DOPA to DA are suggested to be the rate-limiting steps for the production of NBAD in *M. sexta*. Integument cultured *in vitro* in Grace's medium containing tyrosine produced very little DOPA, DA or NBAD, suggesting that the hydroxylating step was lacking. Although the experiment of adding DOPA to the culture medium has not yet been done to monitor the activity of DOPA decarboxylase, supplementation with DA showed NBAD synthase to be present at the critical time preceding tanning.

Roseland *et al.* (1986) have shown that both DA and  $\beta$ -alanine enhance pupal cuticle tanning of *M. sexta* integument cultured *in vitro*, suggesting the presence of NBAD synthase activity in the epidermis. NBAD supplemented into the medium was even more effective than DA and  $\beta$ -alanine in stimulating cuticle tanning. The presence of substantially more

NBAD synthetic activity in intermolt epidermis relative to epidermis from other stages is consistent with the observation that intermolt fifth instar larval epidermis is able to tan pupal cuticle formed *in vitro* much better than wandering stage epidermis (L. M. Riddiford, personal communication).

20-HE, which increases hemolymph titers of NBAD *in vivo*, did not affect NBAD synthase activity *in vitro* in fat body or integument. The clearance of ecdysteroids may be required before the synthesis and/or activation of the hydroxylase or decarboxylase enzyme can occur. Further research on the hormonal regulation of NBAD synthesis is in progress, as well as efforts to isolate and further characterize NBAD synthase.

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