

## ***N*- $\beta$ -Alanyldopamine: Major Role in Insect Cuticle Tanning**

**Abstract.** *N*- $\beta$ -Alanyldopamine is the major tyrosine metabolite in the hemolymph and cuticle during pupal tanning in the tobacco hornworm, *Manduca sexta* L. Its concentration in hemolymph increases over 800-fold above larval levels by the start of tanning and decreases as the pupal cuticle darkens and hardens. It is a major catechol in species representing several insect orders and is the preferred substrate for pupal cuticular *o*-diphenol oxidase. In insects, *N*- $\beta$ -alanyldopamine appears to be the main precursor for tanning chemicals at certain developmental stages.

*N*-Acetyldopamine has been considered the principal catecholamine metabolite in sclerotization or tanning of insect cuticle (1). We now report that *N*- $\beta$ -alanyldopamine (NBAD) is the major catecholamine metabolite in hemolymph and cuticle during tanning of pupal cuticle in the tobacco hornworm, *Manduca sexta*, and that *N*-acetyldopamine is a relatively minor component.

Hemolymph was extracted from *M. sexta*, and the catechols were analyzed by liquid chromatography with electrochemical detection (LCEC) (2). The major oxidizable substance in hemolymph extracts from pharate or newly ecdysed pupae was an unknown compound with a longer retention time than dopa, dopamine, or *N*-acetyldopamine (compound V in Fig. 1). Labeled compound V was

Table 1. Tyrosine metabolite concentrations in hemolymph of *Manduca sexta* during pupal development. Data are the means of three to four insects  $\pm$  standard error. The percentage conjugated is shown in parentheses.

Stage*	Tyrosine metabolite concentration ( $\mu$ M)			
	Dopa	Dopamine	N-Acetyl-dopamine	N- $\beta$ -Alanyl-dopamine
Wandering larva				
Heart exposed	2.8 $\pm$ 0.4 (43)	9.2 $\pm$ 3.9 (63)	20.5 $\pm$ 11.3 (96)	5.2 $\pm$ 1.6 (21)
Pharate pupa				
Ocellar retraction	9.5 $\pm$ 1.0 (33)	77.2 $\pm$ 21.9 (88)	14.5 $\pm$ 5.0 (100)	180.1 $\pm$ 47.4 (52)
Tanned patches	31.5 $\pm$ 12.4 (17)	721.6 $\pm$ 148.9 (99)	48.6 $\pm$ 19.0 (94)	4166.4 $\pm$ 581.5 (97)
Pupa				
Newly ecdysed	25.6 $\pm$ 8.1 (16)	630.8 $\pm$ 75.2 (97)	48.9 $\pm$ 15.2 (94)	3356.5 $\pm$ 643.1 (79)
1 hour	32.8 $\pm$ 4.4 (14)	659.0 $\pm$ 132.8 (96)	39.3 $\pm$ 7.3 (93)	4119.6 $\pm$ 502.4 (70)
6 hours	28.7 $\pm$ 5.6 (20)	478.0 $\pm$ 84.0 (91)	18.4 $\pm$ 7.1 (65)	2984.0 $\pm$ 235.7 (39)
24 hours	34.2 $\pm$ 9.1 (18)	161.0 $\pm$ 19.0 (41)	18.7 $\pm$ 3.8 (0)	561.8 $\pm$ 45.7 (2)
48 hours	33.5 $\pm$ 5.9 (20)	85.3 $\pm$ 33.6 (43)	7.5 $\pm$ 2.2 (79)	214.3 $\pm$ 82.1 (2)

\*See (12) for description of developmental events.

isolated after wandering larvae or pharate pupae were injected with [ $U$ - $^{14}$ C]tyrosine (50  $\mu$ Ci). The metabolite showed an ultraviolet absorption spectrum at pH 3 identical to that of dopamine (maximum wavelength, 278 nm). Products resulting from hydrolysis with HCl were dopamine and  $\beta$ -alanine, as indicated by LCEC and by an amino acid analyzer, respectively (3). When the hydrolyzate was silylated and analyzed by gas-liquid chromatography, two peaks with retention times identical to those of  $\beta$ -alanine and dopamine were observed (4). Synthetic NBAD, when subjected to LCEC and ultraviolet spectroscopy, exhibited a retention time and absorption spectrum identical to those of the unknown metabolite (5). This evidence demonstrated that the major catechol metabolite of tyrosine during pupal tanning of *M. sexta* was NBAD.

N- $\beta$ -Alanyldopamine was present at low concentrations in hemolymph of last-stadium larvae of *M. sexta* during the feeding and wandering phases. Higher levels occurred in early pharate pupae and peaked with an increase of about 800-fold shortly before pupal ecdysis (Table 1). During the accumulation of NBAD in pharate pupae, much of the substance was present as a conjugate, probably as a  $\beta$ -glucoside. Mild acid hydrolysis of extracts (1.2N HCl for 10 minutes at 100°C) resulted in a large increase in NBAD, as did incubation with  $\beta$ -glucosidase (Sigma) (acetate buffer, pH 5.6, for 1 hour at 37°C). We have also found that tyrosine is conjugated with glucose in this species (2), and this modification may protect phenol and di-phenol substituents from oxidative enzymes until tanning is initiated (1). Dopamine was the second most abundant catechol, while N-acetyldopamine and dopa were present at relatively low concentrations. Throughout the first hour after

ecdysis NBAD remained at high levels in hemolymph; then, as the cuticle tanned over the next 24 to 48 hours, NBAD levels, and the percentage conjugated, declined (Table 1).

N- $\beta$ -Alanyldopamine was also the major catechol in cuticle from tanning pupae. Scraped abdominal tergites from 3-hour pupae contained 145 nmole/g, whereas wing cuticle had 35 nmole/g. No N-acetyldopamine was detected in either extract. These data suggested that at least a portion of the large pool of NBAD

in hemolymph is transported to cuticle, where it probably serves as the primary precursor for sclerotizing compounds.

In contrast, N-acetyldopamine was the primary catecholamine in hemolymph from newly ecdysed fifth-stadium larvae (708  $\mu$ M); dopamine and NBAD were less abundant (172 and 140  $\mu$ M, respectively). N-Acetyldopamine was also the predominant catechol in hemolymph of pharate adults close to ecdysis (2800  $\mu$ M), whereas NBAD was a minor constituent (20  $\mu$ M). Larval and adult cuticle of *M. sexta* may be sclerotized by compounds derived from N-acetyldopamine and pupal cuticle by metabolites of NBAD.

We used LCEC to survey seven other insect species from four different orders for the occurrence of NBAD (6). In all cases, NBAD was either the predominant catechol or a major component during tanning of pupal, puparial, or adult cuticles that formed brown sclerotins.

Phenoloxidases are enzymes that catalyze the formation of cross-linking agents for cuticle sclerotization (1). A phenol oxidase from blow fly cuticle has been reported to oxidize synthetic NBAD faster than it oxidizes N-acetyldopamine (7). We found a similar enzyme in pharate pupal integument of *M. sexta* (8). The relative turnover numbers (the ratio of the maximum velocity to the Michaelis constant) for NBAD, N-acetyldopamine, and dopamine were 0.87, 0.54, and 0.11 per minute, respectively. Thus NBAD was the preferred substrate for the hornworm enzyme also.

Our study demonstrates that NBAD is a naturally occurring metabolite in insects.  $\beta$ -Alanine, which has been isolated from the cuticles of many insect species, had previously been associated with the formation of brown cuticles (1). Its uptake and use during puparial tanning of Diptera apparently depends on

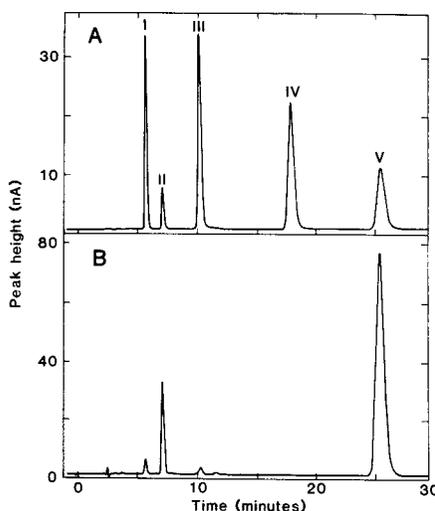


Fig. 1. (A) LCEC chromatogram of catechol standards. Internal standard, 30 ng; other catechols, 60 ng. I, dopa; II, 3,4-dihydroxybenzylamine (internal standard); III, dopamine; IV, N-acetyldopamine; V, N- $\beta$ -alanyldopamine. (B) LCEC chromatogram of an unhydrolyzed extract of hemolymph from a newly ecdysed pupa of *Manduca sexta*. Liquid chromatography buffer was 5 percent methanol and 95 percent 0.1 M potassium phosphate monobasic (pH 3), 0.2 mM sodium octyl sulfate, and 0.1 mM sodium EDTA. Flow rate of 1 ml/min at ambient temperature through a reverse phase octadecylsilane 5- $\mu$ m spherical particle 25-cm column [Bioanalytical Systems LC 304 and electrochemical detector LC 4A (+0.72 V)].

dopamine incorporation (9). Synthetic  $^{14}\text{C}$ -labeled NBAD was incorporated into isolated larval cuticle of *Calliphora erythrocephala*, which became light brown (10). Another  $\beta$ -alanyl catechol, *N*- $\beta$ -alanyl noradrenaline, has been identified from the yellow wing pigments of the swallowtail butterfly, *Papilio xuthus* (11).

Our evidence points toward a central role for NBAD in the pupal tanning of *M. sexta* and in other insects that form brown cuticle in pupae, puparia, or adults. Its occurrence as a major catecholamine metabolite in several insect orders suggests that conjugation of dopamine with  $\beta$ -alanine may be as important as acetylation for sclerotization processes in insects. The type of cuticle formed and its coloration, however, may depend on which form of dopamine is produced.

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#### References and Notes

1. P. C. J. Brunet, *Insect Biochem.* **10**, 467 (1980).
2. *Manduca sexta* were reared and tissues were collected as described [K. J. Kramer, T. L. Hopkins, R. F. Ahmed, D. Mueller, G. Lockhart, *Arch. Biochem. Biophys.* **205**, 146 (1980)]. Hemolymph was homogenized in ice-cold 1.2M HCl, 1:9 (by volume), containing 4.8 mM sodium bisulfite and the internal standard, dihydroxybenzylamine, and centrifuged at 6500g for 10 minutes. The catechols were adsorbed from the supernatant on alumina and analyzed by LCEC [L. L. Murdock and D. Omar, *Insect Biochem.* **11**, 161 (1981)]. Mobile phase and operating conditions are described in Fig. 1. Portions of the supernatants were also hydrolyzed in 1.2N HCl at 100°C for 10 minutes to release conjugated catechols before alumina adsorption and LCEC.
3. Samples of compound V isolated by liquid chromatography and desalted on a Biogel P-2 column were hydrolyzed at reduced pressure in 6N HCl for 2 hours and 24 hours. Analysis of the constituent amino acids by liquid chromatography as their fluorescent *o*-phthalaldehyde derivatives [D. W. Hill, *et al.*, *Anal. Biochem.* **51**, 1338 (1979)] gave a single peak eluting at the retention time of  $\beta$ -alanine (30.5 minutes). Analysis of the 6N HCl hydrolyzate by LCEC showed the disappearance of compound V and the appearance of dopamine.
4. Samples were hydrolyzed at reduced pressure in 2N HCl for 2 hours, silylated, and analyzed by gas chromatography [R. A. Wirtz and T. L. Hopkins, *J. Insect Physiol.* **20**, 1143 (1974)]. The retention times for silylated  $\beta$ -alanine and dopamine were 2 and 23 minutes, respectively, on a 5 percent OV-1 column with a column temperature of 200°C and a carrier gas flow rate of 84 ml/min.
5. Synthetic NBAD was prepared by coupling dopamine with *N*- $\alpha$ -*t*-butyloxycarbonyl- $\beta$ -alanine-*N*-hydroxysuccinimide ester in potassium tetraborate buffer and deblocking the conjugate in 0.1N HCl. Identity was confirmed by LCEC and ultraviolet spectroscopy. Purity was > 99 percent.
6. Concentrations of hemolymph NBAD and *N*-acetyldopamine, respectively, for A, white adults; L, mature larvae; and P, white pupae or puparia were: *Periplaneta americana* (A), 91

- and 63  $\mu\text{M}$ ; *Sarcophaga bullata* (P), 254 and 53  $\mu\text{M}$ ; *Diatraea grandiosella* (P), 310 and 10  $\mu\text{M}$ ; and *Thyridopteryx ephemeraeformis* (L), 80 and 22  $\mu\text{M}$ . Whole body concentrations were *Plodia interpunctella* (P), 147 and 168  $\mu\text{mole/g}$ ; *Ephesia cautella* (P), 87 and 8  $\mu\text{mole/g}$ ; and *Tenebrio molitor* (P), 41 and 77  $\mu\text{mole/g}$ .
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  8. *o*-Diphenol oxidase was extracted from pharate pupal cuticle, precipitated with 30 percent ammonium sulfate and chromatographed on Sephadryl S-300 in 0.1M ammonium bicarbonate, pH 8.5. Activity was determined spectrophotometrically at 470 nm (dopamine) and 390 nm (*N*-acetyldopamine and NBAD). Velocity data were treated by Lineweaver-Burk and nonlinear least-squares analyses.
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13. We thank R. F. Ahmed for gas-liquid chromatography analysis and G. Lookhart for  $\beta$ -alanine analysis by liquid chromatography of some hydrolyzates. This is contribution No. 82-157-j from the Departments of Entomology and Biochemistry, Kansas Agricultural Experiment Station, Manhattan 66506. Cooperative investigations of Agriculture Research Service, Department of Agriculture, and the Kansas Agricultural Experiment Station. Supported in part by research grant PCM-8003859 from the National Science Foundation.

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