

CATECHOLAMINES IN HAEMOLYMPH AND CUTICLE DURING LARVAL, PUPAL AND ADULT DEVELOPMENT OF *MANDUCA SEXTA* (L.)

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Abstract—Catecholamines in haemolymph and cuticle of the tobacco hornworm, *Manduca sexta* (L.), were analyzed by liquid chromatography with electrochemical detection. During pupal tanning, *N*- β -alanyldopamine (NBAD) was the major dopamine derivative in haemolymph. It increased to maximal titres by the time of larval-pupal ecdysis, and subsequently decreased as pupal cuticle tanned. NBAD progressively increased in cuticle for several days after ecdysis. *N*-acetyldopamine (NADA), although present in only minor amounts in pupae, was the primary catecholamine in haemolymph of both larvae and adults shortly before and after ecdysis. NADA predominated in the hard colourless cuticle of the larval head capsule, and in the wings and abdominal tergites of the adult. NBAD, however, was predominant in the dark cuticle of larval mandibles, and in the thin soft larval abdominal cuticle after stabilization. Dopamine, the precursor of NBAD and NADA, was usually the second or third most abundant catecholamine in both haemolymph and cuticle. The kinds of sclerotins produced may depend in part upon the relative abundance of the different catecholamines which are selectively incorporated into the various regions of the exoskeleton. The catecholamines in haemolymph occurred mainly as ring hydroxyl conjugates that were probably β -glucosides. The relative percentage of free or unconjugated compounds increased after ecdysis and accumulated in cuticle. Conjugation, therefore, appears to be important for sequestration of tanning precursors in haemolymph, followed by hydrolysis and uptake of free catecholamines for stabilization and pigmentation of new cuticle.

Key Word Index: *N*- β -alanyldopamine, *N*-acetyldopamine, dopamine, *Manduca sexta*, tobacco hornworm, sclerotization, tanning, pigmentation, catecholamines, haemolymph, cuticle

INTRODUCTION

Tanning or sclerotization of insect cuticle is a complex process of stabilization that involves the dehydration and crosslinking of the chitin-protein matrix with catechols and quinones derived from tyrosine (reviewed by Andersen, 1979; Brunet, 1980). A number of catecholic compounds have been identified in insects. Their involvement in cuticular tanning is suggested by changes in concentration which correlate with hardening and darkening of new cuticle, and by incorporation of radiolabelled precursors into tanned cuticle. *N*-acetyldopamine (NADA) has been considered to be the primary tanning agent precursor for insect cuticle. Recently we demonstrated that *N*- β -alanyldopamine (NBAD) is the major dopamine metabolite in haemolymph during pupal tanning of the tobacco hornworm, *Manduca sexta* (L.), and certain other Lepidoptera (Hopkins *et al.*, 1982). It increases to maximal titre primarily as a conjugate before pupal ecdysis while NADA is a minor constituent. However, we found NADA in the form of a conjugate to be the primary catecholamine in haemolymph during both larval and adult ecdysis of *M. sexta*. These results suggest that the dopamine derivative produced may be related to the type of sclerotization and pigmentation occurring in different developmental stages. To investigate this hypothesis further, we have analyzed the catecholamines in

haemolymph and cuticle during development of larval, pupal and adult stages of *M. sexta*. The distribution and importance of NBAD and NADA conjugates were also studied as to their role in cuticular sclerotization.

MATERIALS AND METHODS

Haemolymph and cuticle extraction

M. sexta was reared as described by Bell and Joachim (1976) at $27 \pm 1^\circ\text{C}$ and a non-diapausing photoperiod of 16 hr light-8 hr dark. Haemolymph was collected from larvae by cutting the anal horn and bleeding into an ice cold spot plate containing a few crystals of phenylthiourea. Pupae were bled by cutting the proboscis, and pharate and emerged adults by making a small incision through an abdominal tergite. Aliquots (0.05 ml) were drawn into calibrated capillary pipettes and transferred into 0.45 ml of 1.2 M HCl containing ascorbic acid (5 mM) and the internal standard dihydroxybenzylamine (DHBA; $\mu\text{g}/0.02\text{ ml}$) in 1.5 ml centrifuge tubes. The samples were mixed by vortexing, centrifuged at 6500 *g* for 10 min at 4°C , and the supernatant removed for analysis.

Cuticle samples were dissected, scraped clean of adhering cells and tissues, and rinsed with distilled water. Adult cuticle had the scales removed by scraping and rinsing. The pieces of cuticle were blotted on absorbent tissue, weighted and then homogenized in the extraction solvent used for haemolymph (1-10 mg cuticle/0.25 ml) in a ground glass tissue grinder. The homogenate was centrifuged at 6500 *g* for 10 min at 4°C and the supernatant removed for analysis.

Aliquots of haemolymph and cuticle supernatants were heated at 100°C for 10 min under nitrogen to hydrolyze glucosides or other relatively labile conjugates of the catechols. The weakness of the glycosidic bond relative to the amide bond of NADA and NBAD allowed the use of acid hydrolysis for estimation of the titre of conjugated catecholamines. Hydrolysis of conjugates was essentially complete under these conditions as determined by analysis of sample aliquots hydrolyzed for variable lengths of time. Losses of NADA and NBAD were held to 10% or less as determined by hydrolysis of the synthetic standards followed by catecholamine analysis. The concentration data presented are those actually observed and are uncorrected for the small NBAD and NADA losses due to hydrolysis or for corresponding increases in dopamine. Recovery of catecholamines or their conjugates as determined by sequential extraction of haemolymph or cuticular homogenates was 96 ± 0.8 (SEM)%, $n = 10$.

Liquid chromatography and electrochemical analysis of catechols

Samples of the hydrolyzed and unhydrolyzed extracts were prepared for analysis by liquid chromatography with electrochemical detection (LCEC) essentially as described by Murdock and Omar (1981). The catechols extracted from the alumina with 0.1 ml of 1 M acetic acid were analyzed (0.02 ml) on a Bioanalytical Systems LC 304 chromatograph with an LC 4A electrochemical detector operated at +0.72 V and a reverse phase ODS 5 $\mu\text{m} \times 25$ cm spherical particle column. The most commonly used mobile phase consisted of 0.1 M phosphoric acid, 0.28 mM sodium octyl sulphate, 0.1 mM sodium EDTA, and 15% (v/v) methanol adjusted to pH 3.2 with NaOH. Retention times were 5.2 min for DHBA, 6.7 min for dopamine, 12.1 min for NADA and 13.2 min for NBAD. The resolution of these compounds from interfering peaks usually appeared to be adequate. Several of the samples from each type of extract were chromatographed in more than one mobile phase to confirm identification of compounds and check for interfering substances. One of the mobile phases used for this purpose

consisted of 0.1 M formic acid, 4.8 μM sodium lauryl sulphate, 0.1 mM sodium EDTA, and 25% (v/v) methanol adjusted to pH 3.8 with NaOH. Retention times were 5.3 min for DHBA, 5.7 min for NADA, 6.4 min for dopamine, and 9.6 min for NBAD. The different ion pair strength, methanol concentration and pH of this mobile phase altered the relative retention times of many of the compounds present in the insect samples. The quantities of individual catecholamines were calculated based on the peak height response of known amounts of standard compounds. The percentage recovery of the internal standard in each extract and the mean percentage recovery of standard compounds (greater than 70%) was used to correct for the losses during recovery from alumina.

To determine if certain electroactive metabolites observed by LCEC were derived from tyrosine, the animals were injected with [^{14}C]tyrosine and the metabolites were isolated and counted according to the procedures of Hopkins *et al.* (1982).

RESULTS

Catecholamines in larval haemolymph

The three major catecholamines found in larval haemolymph during the fourth and fifth stadia of *M. sexta* were *N*-acetyldopamine, *N*- β -alanyldopamine and dopamine (Fig. 1). NADA predominated in the newly ecdysed larvae of both instars, reaching 0.8 mM. Dopamine and NBAD were individually less than one fourth the concentration of NADA. Total catecholamines declined to very low concentrations (~ 0.05 mM) as the larval cuticle tanned and pigmented during the first 24 hr after ecdysis. In the fifth instar this decrease occurred between 3 and 12 hr post-ecdysis and titres remained low through the wandering phase in the last part of the stadium.

Biosynthesis of catecholamines for larval tanning and pigmentation probably begins shortly after apo-

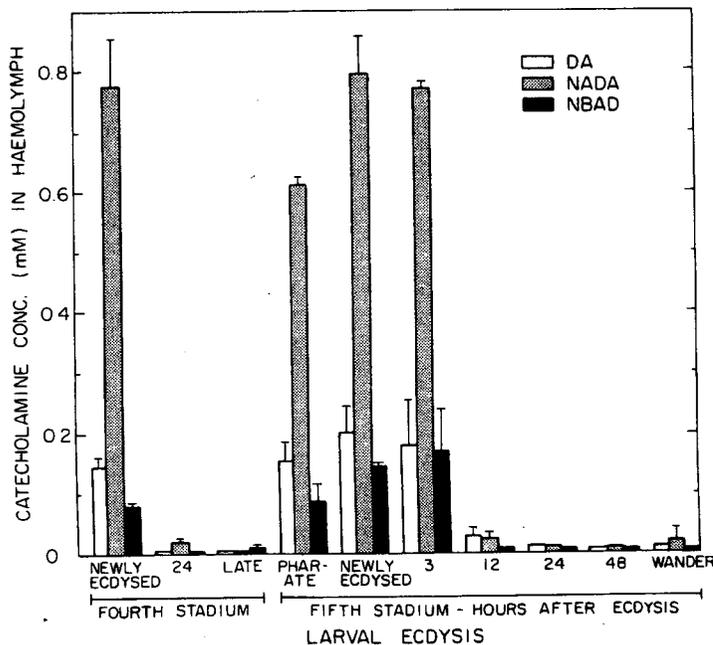


Fig. 1. Catecholamine concentrations (conjugated plus free) in haemolymph during larval development of *M. sexta*. Data are the means of 3-4 animals \pm standard error. DA—dopamine, NADA—*N*-acetyldopamine, NBAD—*N*- β -alanyldopamine.

Table 1. Percentage catecholamines conjugated in haemolymph during larval development of *M. sexta**

Instar	Hours after ecdysis	<i>N</i> -acetyl-dopamine	<i>N</i> - β -alanyl-dopamine
Fourth	0	97.2 \pm 0.2	83.1 \pm 3.1
	24	33.1 \pm 17.3	14.5 \pm 14.5
	late	50.2 \pm 11.4	23.7 \pm 3.7
Fifth	pharate	98.8 \pm 0.4	60.2 \pm 0.6
	0	97.4 \pm 0.4	66.1 \pm 5.9
	3	98.0 \pm 0.2	79.0 \pm 9.8
	12	70.0 \pm 0.4	39.1 \pm 1.6
	24	49.6 \pm 10.0	1.6 \pm 1.1
	48	42.7 \pm 9.3	0
	wandering, early	93.2 \pm 2.4	19.4 \pm 3.4
24	100	57.9 \pm 19.1	

*Means of 3 animals \pm standard error.

lysis. In late fourth instars the total concentrations were very low (0.02 mM). About one day after slippage of the fourth instar head capsule and 10 hr before ecdysis when darkening of the new mandibles of the pharate fifth instar begins, catecholamine titres were nearly at the same level as in newly ecdysed larvae.

NADA and NBAD were stored in the haemolymph primarily as conjugates (hydrolyzed by 1.2 M HCl at 100°C for 10 min) in pharate and newly ecdysed larvae (Table 1). NBAD was less conjugated (60–83%) than NADA (97–99%) in pharate and recently ecdysed larvae. The percentage of catecholamines bound as conjugates generally decreased during the period of cuticular stabilization as did the actual titres. Free catecholamine titres never exceeded 0.05 mM. The identities of the conjugates in larval haemolymph are not known, but preliminary evidence from enzyme susceptibility studies suggests that they are β -D-glucosides of catechols linked via one of the aromatic ring oxygens (Hopkins, Morgan and Kramer, unpublished).

Catecholamines in larval cuticle

Three types of larval cuticle were sampled from fifth instars; hard colourless cuticle (head capsule), hard dark brown cuticle (mandible), and soft colourless cuticle with black markings (abdominal dorsum—first seven segments). The unhardened head capsule contained relatively low concentrations of catecholamines at ecdysis (Table 2). By 3 hr after ecdysis, NADA had increased over 260-fold while dopamine and NBAD showed little change. About 90% of NADA was bound as a conjugate which was acid labile. NADA continued to accumulate in the hardened head capsule cuticle reaching over 3 μ mol/g by 24 hr. Almost 50% of this was the free catechol.

The total titre of catecholamines in dorsal abdominal cuticle at ecdysis (83 nmol/g) was about six times higher than that in the unsclerotized head capsule. However, 24 hr later after cuticular hardening and stabilization, the head capsule had five times the concentration of catecholamines as did abdominal cuticle, most of which was NADA. NADA titres in abdominal tergites showed very little increase during the first 24 hr after ecdysis in contrast to the head capsule. NBAD increased over 30-fold during the same period which was surprising since this cuticle remains relatively soft and colourless except for the black markings. A light brown colouration does occur in the larval exuvium after ecdysis. Dopamine titre increased 5-fold during the first 3 hr and then decreased to near the ecdysial level by 24 hr. Melanization of specific areas of the cuticle may be related to availability of free dopamine. The dorsal thoracic cuticle which lacks the black markings had less than one-tenth as much dopamine at ecdysis as did abdominal cuticle. Although the black stripes are visible in abdominal cuticle at ecdysis, melanization may continue for a short interval after moulting.

The hard dark brown cuticle of larval mandibles had the highest total concentration of catecholamines compared to hard or soft clear cuticle. At 3 hr after ecdysis, NBAD was the major catecholamine, with a concentration more than twice that of NADA. It was present totally as a free catechol while NADA was 70% bound as a conjugate.

Catecholamines in pupal haemolymph

The three major catecholamines increased significantly in concentration after larval-pupal apolysis, as indicated by ocellar retraction (Fig. 2). In late pharate pupae approx. 12 hr after the start of cuticular tanning as first observed by the brown meta-thoracic bars, NBAD concentrations in haemolymph were maximal (4.2 mM), having increased more than 800-fold over levels in wandering larvae. Based on the haemolymph volumes reported by Williams-Boyce and Jungreis (1980), total NBAD was estimated to be nearly 2 mg/pupa or about 400 times the total in wandering larvae. By 24 hr post-ecdysis NBAD had declined to 0.6 mM and a further decrease to 0.2 mM was observed at 48 hr. The dramatic increase and the subsequent decrease in the concentration of NBAD strongly suggests its importance as the major tanning precursor in pupae. The fluctuation of NADA titres was about 5-fold with a maximal concentration of only 0.05 mM.

The percentage of bound or conjugated NBAD in haemolymph during pupal development is shown in

Table 2. Catecholamines (nmol/g) in larval cuticle of *M. sexta* (L.)*

Cuticle	Hours after ecdysis	Dopamine	<i>N</i> -acetyl-dopamine	<i>N</i> - β -alanyldopamine
Head capsule	0	< 2	10 \pm 3 (34)	3 \pm 1 (42)
	3	12 \pm 7	2691 \pm 745 (91)	9 \pm 1 (14)
	24	10 \pm 1	3302 \pm 698 (54)	19 \pm 3 (12)
Abdominal dorsum	0	22 \pm 9	43 \pm 22 (49)	18 \pm 6 (35)
	3	107 \pm 21	69 \pm 30 (25)	64 \pm 18 (11)
	24	39 \pm 5	65 \pm 31 (12)	598 \pm 96 (1)
Mandibles	3	~ 50	1557 \pm 180 (70)	3828 \pm 655 (0)

*Means of 3–4 animals \pm standard error. Total NADA and NBAD titres are shown with percentage conjugated in parentheses. Dopamine was largely unconjugated so only free titres determined before hydrolysis of samples are shown.

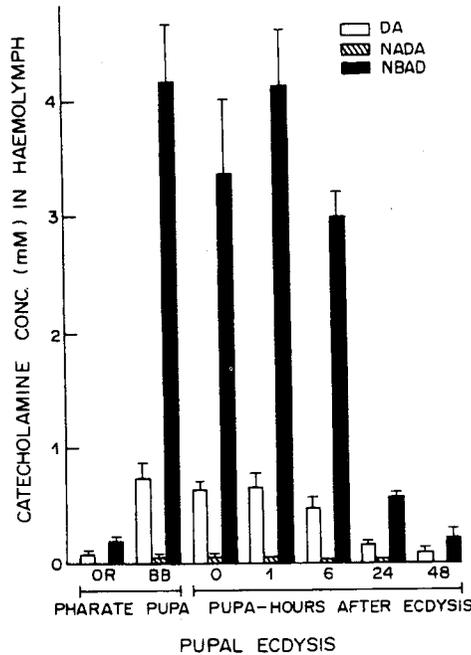


Fig. 2. Catecholamines concentrations (conjugated plus free) in haemolymph during pupal development of *M. sexta*. Data are the means of 3-4 animals \pm standard error. OR—ocellar retraction, BB—brown bar stage.

Table 3. During the main period of NBAD synthesis in pharate pupae, the amount conjugated rose from about 60% to almost 100%. Free NBAD increased at pupal ecdysis and continued to a peak concentration of 1.9 mM 6 hr later. This large amount of free NBAD suggests that much of the NBAD conjugate may be hydrolyzed before the catecholamine is trans-

ported into the integument. The small amounts of NADA in pupal haemolymph remained almost totally conjugated through ecdysis as was observed in larval haemolymph. Preliminary evidence indicates that the conjugates of NBAD and NADA are β -glucosides. The conjugates were hydrolyzed by β -glucosidases and radiolabelled glucose was incorporated into the NBAD conjugate (Hopkins *et al.*, 1982; Hopkins, Morgan and Kramer, unpublished).

Catecholamines in pupal cuticle

NBAD was found in much higher concentrations in pupal wing and the third and fourth abdominal tergite cuticle than NADA or dopamine, and progressively increased for several days after ecdysis (Table 4). NBAD accumulated less rapidly in the dorsal cuticle of the forewing than in abdominal tergites. Visible tanning begins in the wing at about 3 hr after ecdysis, while the abdominal tergites begin to tan a few hours before ecdysis. The highest titres of NBAD were observed in abdominal tergites at 76 hr after ecdysis, with about half this concentration remaining in the exuvium after adult eclosion. NADA was not detected in either cuticle until several hours after the start of tanning and remained at relatively low concentrations compared to NBAD. Both NBAD and NADA were largely unconjugated in pupal cuticle.

Catecholamines in adult haemolymph

Haemolymph was sampled beginning approx. two days before adult eclosion and one day after the start of melanization of the dorsal scales of the forewing. NADA was the predominant catecholamine and increased 5-fold to reach a level of 1 mM a few hours before eclosion (Fig. 3). High levels persisted through adult ecdysis and decreased as the adult cuticle

Table 3. Percentage catecholamines conjugated in haemolymph during pupal development of *M. sexta**

Stage	Hours after ecdysis	<i>N</i> -acetyldopamine	<i>N</i> - β -alanyldopamine
Pharate pupa	apolysis (ocellar retraction)	100	59.1 \pm 14.6
	tanning (brown bar)	90.5 \pm 6.0	96.8 \pm 0.8
Pupa	0	95.8 \pm 2.6	77.7 \pm 3.1
	1	93.5 \pm 3.3	69.6 \pm 3.0
	6	61.4 \pm 4.7	42.6 \pm 5.8
	24	0	2.4 \pm 2.4

*Means of 3 animals \pm standard error

Table 4. Catecholamines (nmol/g) in pupal cuticle of *M. sexta**

Cuticle	Hours after ecdysis	Dopamine	<i>N</i> -acetyldopamine	<i>N</i> - β -alanyldopamine
Wing	3	ND	0	60 \pm 24 (0)
	6	85 \pm 25	~5	592 \pm 68 (5)
	24	ND	39 \pm 10	1031 \pm 196
	48	ND	23 \pm 7	1260 \pm 143
Abdominal tergites	0	~3	0	153 \pm 13 (17)
	6	61 \pm 45	28 \pm 10 (15)	1198 \pm 205 (2)
	24	31 \pm 11	131 \pm 31 (3)	880 \pm 70 (2)
	48	ND	61 \pm 37	970 \pm 220
	76	7 \pm 2	115 \pm 85 (39)	2228 \pm 211 (0)
	exuvium	68 \pm 9	113 \pm 35 (31)	1142 \pm 281 (5)

*Mean of 3-4 animals \pm standard error. Total NADA and NBAD titres are shown with percentage conjugated in parentheses. Dopamine was largely unconjugated so only free titres determined before hydrolysis of samples are shown. ND—not determined.

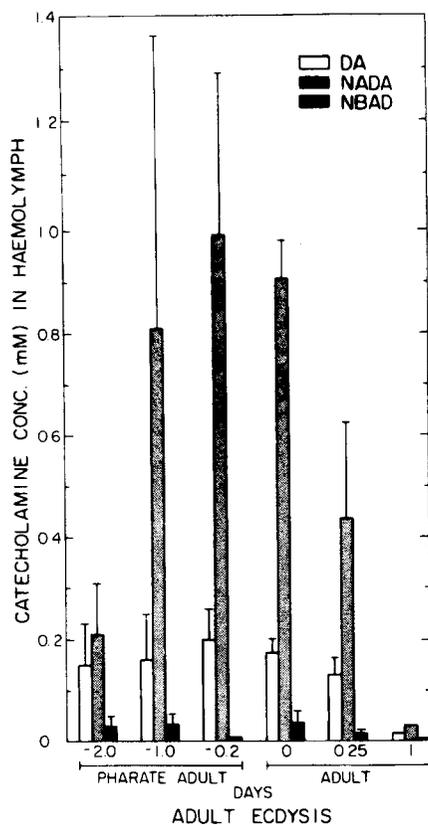


Fig. 3. Catecholamine concentrations (conjugated plus free) in haemolymph during adult development of *M. sexta*. Data are the means of 3-4 animals \pm standard error.

tanned during the next 6 hr. Very low concentrations (~ 0.05 mM) were present 24 hr after eclosion. NBAD concentrations were consistently low (~ 0.05 mM) throughout the late period of pharate adult development and for 24 hr after eclosion. NADA was almost totally conjugated in haemolymph both before and after adult eclosion (Table

5). Titres of free NADA, NBAD or dopamine were less than 0.04 mM.

Catecholamines in adult cuticle

The analysis of hindwing and third and fourth abdominal tergites with scales from both cuticles removed showed that NADA was the most abundant catecholamine (Table 6). Moderate titres were present even 24 hr before eclosion and accumulated as much as ten times in the tergites by 24 hr after eclosion. Free dopamine in wings was unusually high compared to other cuticles and equaled those of NADA at the later interval. Since wing samples include epidermis and haemolymph as well as cuticle, it is not known where dopamine may have been localized. NBAD accumulated from relatively low titres at eclosion to moderate titres in the stabilized cuticle. The abdominal tergites remain nearly colourless after sclerotization as do the membranous areas of the wing with the scales removed. The wing veins become brown which may account for higher NBAD in wings than in tergites.

Other catechols in haemolymph and cuticle

Unidentified electroactive compounds that became labelled after injection of [$U-^{14}C$]tyrosine and compounds having identical retention times to standard catechols by different chromatographic conditions were encountered in both haemolymph and cuticle extracts from different developmental stages of *M. sexta*. Minor quantities of compounds with the retention times of 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxybenzoic acid were observed in pupal cuticle extracts. Three major unknown electroactive metabolites derived from tyrosine were extracted from cuticle samples during sclerotization of the three developmental stages. One of these is a derivative of norepinephrine which we have tentatively identified as *N*- β -alanyl norepinephrine. It was a major catecholamine in pupal cuticle during tanning ($1.9 \mu\text{mol/g}$ of the abdominal tergites at 6 hr after eclosion).

Table 5. Percentage catecholamines conjugated in haemolymph during adult development of *M. sexta**

Stage	Hours before or after eclosion	<i>N</i> -acetyldopamine	<i>N</i> - β -alanyldopamine
Pharate adult	-48	92.2 \pm 4.8	48.6 \pm 24.4
	-24	98.4 \pm 0.1	100
Adult	0	98.1 \pm 0.6	69.6 \pm 13.6
	0.25	95.0 \pm 1.1	—

*Means of 3 animals \pm standard error

Table 6. Catecholamines (nmol/g) in adult cuticle of *M. sexta**

Cuticle	Hours before or after eclosion	Dopamine	<i>N</i> -acetyldopamine	<i>N</i> - β -alanyldopamine
Wing	-24	226 \pm 45	475 \pm 4 (90)	20 \pm 4 (0)
	0	60 \pm 10	448 \pm 50 (32)	14 \pm 2 (0)
	24	1274 \pm 156	1161 \pm 666 (45)	455 \pm 117 (2)
Abdominal tergites	-24	60 \pm 27	162 \pm 39 (20)	96 \pm 20 (0)
	0	28 \pm 6	220 \pm 102 (11)	13 \pm 0 (8)
	24	~ 21	2678 \pm 935 (45)	201 \pm 68 (44)

*Means of 2-3 animals \pm standard error. Total NADA and NBAD titres are shown with percentage conjugated in parentheses. Dopamine was largely unconjugated so only free titres determined before hydrolysis of samples are shown.

DISCUSSION

We had previously shown that NBAD is the major catecholamine in pupal haemolymph of *M. sexta* during cuticle tanning, whereas NADA is present in very minor concentrations (Hopkins *et al.*, 1982). The accumulation of high NBAD levels in pupal cuticle further supports its suggested role as the primary substrate for tanning of heavily sclerotized brown cuticle. In contrast, NADA was the major dopamine derivative in haemolymph from moulting larvae and adults, and appears to be the main precursor for sclerotization of the hard clear cuticle in these stages. Specific catecholamines and their relative abundance appear to be related to different types of sclerotization of cuticle. We have found a general correspondence of catecholamine profiles in haemolymph and cuticle in pupae and adults. However, in larvae localized areas of cuticle exhibiting varying degrees of hardness and darkness differ considerably in catecholamine composition. The hard colourless cuticle of the head capsule was most closely correlated with the haemolymph profile. The titre of NADA was over 100 times higher than NBAD or dopamine. In the soft thin abdominal cuticle the catecholamines were less abundant than in the hardened head capsule. The absence of a large build up of crosslinking precursors in the abdominal cuticle, which remains largely unsclerotized, might be expected. NBAD titres did increase to moderate levels about ten times higher than NADA by 24 hr after ecdysis. This was a somewhat surprising result since the cuticle remains colourless except for black markings. Free dopamine was more abundant in abdominal cuticle than in thoracic cuticle of the larva, perhaps because the latter lacks black pigmentation. Dopamine is very likely a precursor for melanin in *M. sexta* (Aso *et al.*, 1984). We have previously found a black strain of the flour beetle, *Tribolium castaneum*, to have higher amounts of dopamine than the normal rust-red strain (Kramer *et al.*, 1984).

The dark hard cuticle of the larval mandibles showed a much different profile of catecholamines than head capsule cuticle. NBAD was more than double the concentration of NADA. Total catecholamine concentration in mandibular cuticle at 3 hr post-ecdysis (13 hr after the onset of mandibular tanning) was twice that of head capsule cuticle at 24 hr post-ecdysis. The proportion of the unreacted catechols that eventually became crosslinked with cuticular proteins or polymerized to insoluble pigments is unknown, but they tend to accumulate as the cuticle stabilizes. In general hard cuticle had much higher levels of catecholamines than soft cuticle. Andersen (1974) has shown that the more highly sclerotized mandibles and the mesothoracic cuticle of the locust, *Schistocerca gregaria*, also contain larger amounts of covalently bound catechols when compared to the less sclerotized abdominal tergite.

Catecholamine composition of pupal haemolymph and cuticle from wing and abdominal tergite were directly correlated during tanning. NBAD titre in the cuticle was much higher than NADA or dopamine and accumulation continued for several days after ecdysis. Pupal cuticle eventually sclerotizes to a uniformly dark brown structure without appreciable black or hard clear cuticle, and therefore probably

lacks the wide variation of sclerotins found in the larval exoskeleton. Pupal wing and abdominal tergite cuticle showed similar trends in accumulation of NBAD. Although the pupal cuticle becomes highly sclerotized, some of the catechols that continue to be deposited are easily extracted and, therefore, are not tightly bound to protein or other components. Free NBAD decreased to intermediate levels in the pupal exuvium. A derivative of norepinephrine which we have tentatively identified as *N*- β -alanyl norepinephrine was also very abundant in pupal cuticle during and following tanning. This compound was previously identified in the yellow wing pigments of the butterfly, *Papilio xuthus* (Rembold *et al.*, 1978). *N*- β -alanyl norepinephrine may be synthesized directly from NBAD, since the hydroxylation of the β -carbon of exogenous NADA has been shown to occur in pupal cuticle of *M. sexta* (Peter, 1980) and in the puparial cuticle of *Sarcophaga bullata* (Sugumaran and Lipke, 1983).

The essential role of β -alanine in the formation of hard brown cuticles of several insect species (reviewed by Brunet, 1980; Kramer *et al.*, 1984) can be explained by high titres of NBAD which are generated for tanning pupal cuticle in *M. sexta*. β -Alanine was reported to exist in *S. bullata* cuticle at the amino-terminal position of proteins (Bodnaryk, 1971). However, it was bound weakly to sclerotin and was readily released by hydrolysis with dilute acid. We propose that β -alanine is not covalently linked to protein, but that it is taken up as a part of the crosslinking molecule derived from *N*- β -alanyldopamine, i.e. it probably occurs at the crosslinking sites of sclerotin and is not a part of the polypeptide chain itself. Acylation of the amino group of dopamine with β -alanine greatly slows cyclization of the side chain and consequently prevents indole formation and polymerization to melanins (Kramer *et al.*, 1983). Andersen (1977) points out that the free amino group of β -alanyl compounds may interact with quinones or β -carbon-activated dopamine derivatives, thereby increasing the polymer content of hard cuticle. Pryor (1962) and Hackman and Goldberg (1977) have suggested that stabilization and crosslinking between adjacent protein chains may be accomplished by polymers rather than by smaller molecules.

The progressive accumulation of unreacted catecholamines or their conjugates in different types of cuticle raises the question of their role in the sclerotization process and the proportion that eventually becomes a part of the sclerotin in exocuticle. The increase in cuticular titres is correlated with the decrease in haemolymph levels, suggesting transport and storage for later utilization either in wound healing or additional sclerotization of the exoskeleton. The pupal cuticle of *M. sexta* continues to darken visibly for several days after ecdysis, suggesting a prolonged period of sclerotization. In adult locusts, *S. gregaria*, sclerotization continues for at least two weeks after ecdysis (Andersen and Barrett, 1971; Andersen, 1974). An additional function of the unreacted catechols in hard cuticle may be stabilization of the chitin-protein matrix by dehydration, in which water molecules are displaced by diphenols (Vincent and Hillerton, 1979; Hillerton and Vincent, 1979).

We had previously found that NBAD and NADA in *M. sexta* haemolymph were conjugated almost totally during the period before and shortly after pupal ecdysis (Hopkins *et al.*, 1982). The percentage of free catecholamines, in particular NBAD, increased during tanning as haemolymph titres decreased, suggesting that hydrolysis occurs prior to transport into the cuticle. Only free NBAD accumulated in pupal cuticle. Preliminary evidence shows that the conjugate is hydrolyzed by β -glucosidase and that it is labelled by $[U-^{14}C]$ glucose injected into pharate pupae. Koeppe and Gilbert (1974) observed a radioactive conjugate in *M. sexta* after injection of radiolabelled dopamine and glucose in pharate and tanning pupae. The label from dopamine but not glucose became incorporated in the cuticle. This conjugate was quite likely NBAD glucoside since it is the major catecholamine metabolite in haemolymph during pupal tanning and is hydrolyzed for incorporation of free NBAD in the cuticle.

We observed a similar phenomenon in both larvae and adults of *M. sexta* in which the percentage of conjugates decreased after ecdysis or eclosion. However, a much lower concentration of free NADA is present in haemolymph during sclerotization of these stages than was the case with NBAD during pupal tanning. A substantial amount of conjugated NADA was also found in cuticle of larvae and adults. Glucosides and other conjugates of diphenols commonly occur in insects and usually are assigned a protective and possibly a transport role in the regulation of substrate availability for tanning (Brunet, 1980).

The pathways proposed for production of catecholamines for cuticular tanning of *M. sexta* are shown in Fig. 4. Tyrosine is largely sequestered as a glucoside in the last larval stage of *M. sexta* (Kramer *et al.*, 1980; Ahmed *et al.*, 1983a). Free tyrosine and glucose are later made available for pupal cuticle formation by a tyrosine glucoside hydrolase system regulated by 20-hydroxyecdysone (Ahmed *et al.*, 1983b). Tyrosine appears to be hydroxylated to DOPA by a tyrosinase in pupal cuticle (Aso *et al.*, 1984). Although the exact sites of hydroxylation and subsequent decarboxylation are unknown, the conjugates of dopamine, NADA and NBAD are mainly found in the haemolymph during the moulting cycle of each developmental stage. The ratio of free to conjugated catecholamines increase in haemolymph as total titres decrease during sclerotization. Since the catechols accumulating in cuticle are largely free, conjugation appears to be correlated with sequestration and build up of sclerotizing precursors. The nature and regulation of the enzyme systems responsible for synthesizing and hydrolyzing catechol conjugates involved in new cuticle formation are largely unknown.

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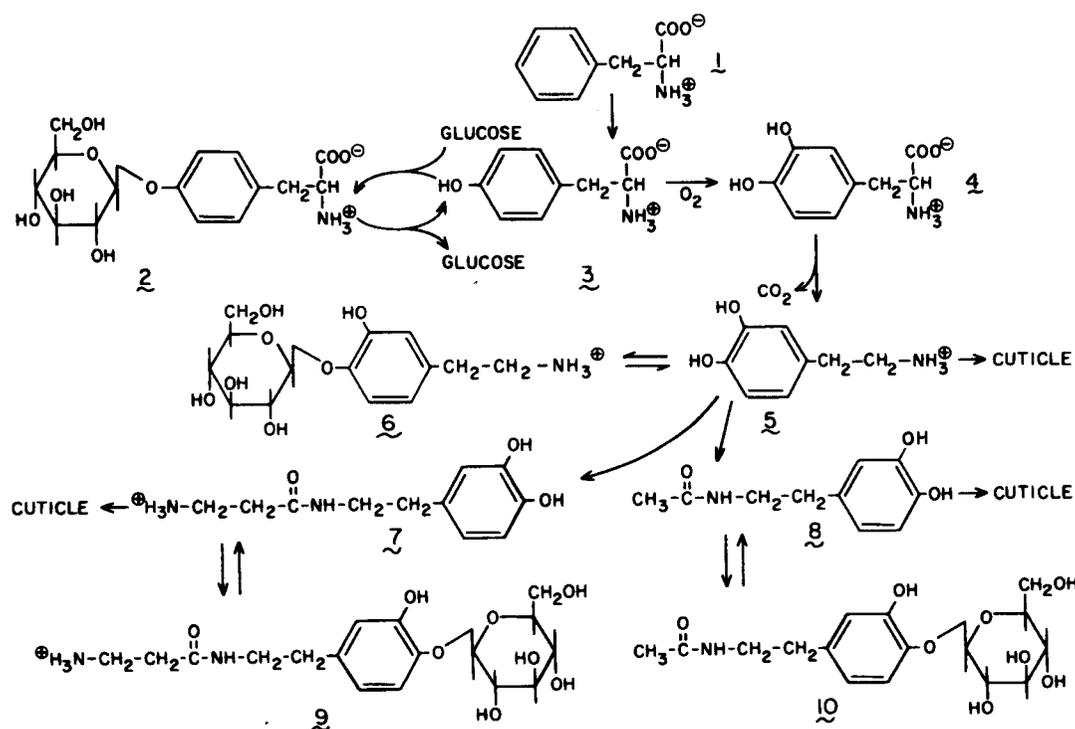


Fig. 4. Proposed catecholamine pathways for cuticle tanning in *M. sexta*. 1, Phenylalanine; 2, tyrosine glucoside; 3, tyrosine; 4, DOPA; 5, dopamine; 6, dopamine glucoside; 7, *N*- β -alanyldopamine; 8, *N*-acetyldopamine; 9, *N*- β -alanyldopamine glucoside; 10, *N*-acetyldopamine glucoside. The β -D-glycopyranosyl-4-O-catechol conjugates are shown but other conjugate structures are possible.

REFERENCES

- Ahmed R. F., Hopkins T. L. and Kramer K. J. (1983a) Tyrosine and tyrosine glucoside titres in whole animals and tissues during development of the tobacco hornworm *Manduca sexta* (L.). *Insect Biochem.* **13**, 369–374.
- Ahmed R. F., Hopkins T. L. and Kramer K. J. (1983b) Tyrosine glucoside hydrolase activity in tissues of *Manduca sexta* (L.): effect of 20-hydroxyecdysone. *Insect Biochem.* **13**, 641–645.
- Andersen S. O. (1974) Cuticular sclerotization in larval and adult locusts, *Schistocerca gregaria*. *J. Insect Physiol.* **20**, 1537–1552.
- Andersen S. O. (1977) Arthropod cuticles: their composition, properties and functions. *Symp zool. Soc. Lond.* **39**, 7–32.
- Andersen S. O. (1979) Biochemistry of insect cuticle. *A. Rev. Ent.* **24**, 29–61.
- Andersen S. O. and Barrett F. M. (1971) The isolation of ketocatechols from insect cuticle and their possible role in sclerotization. *J. Insect. Physiol.* **17**, 69–83.
- 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.* **14**, 463–472.
- Bell R. A. and Joachim F. G. (1976) Techniques for rearing laboratory colonies of tobacco hornworm and pink bollworm. *Ann. ent. Soc. Am.* **69**, 365–373.
- Bodnaryk R. P. (1981) *N*-terminal β -alanine in the puparium of the fly *Sarcophaga bullata*: evidence from kinetic studies of its release by partial acid hydrolysis. *Insect Biochem.* **1**, 228–236.
- 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.
- Hackman R. H. and Goldberg M. (1977) Molecular cross-links in cuticles. *Insect Biochem.* **7**, 175–184.
- Hillerton J. E. and Vincent J. F. V. (1979) The stabilization of insect cuticles. *J. Insect Physiol.* **25**, 957–963.
- Hopkins T. L., Morgan T. D., Aso Y. and Kramer K. J. (1982) *N*- β -Alanyldopamine: major role in insect cuticle tanning. *Science* **217**, 364–366.
- Koeppel J. K. and Gilbert L. I. (1974) Metabolism and protein transport of a possible pupal cuticle tanning agent in *Manduca sexta*. *J. Insect Physiol.* **20**, 981–992.
- Kramer K. J., Hopkins T. L., Ahmed R. F., Mueller D. and Lookhart G. (1980) Tyrosine metabolism for cuticle tanning in the tobacco hornworm, *Manduca sexta* (L.) and other Lepidoptera: identification of β -D-glucopyranosyl-*O*-L-tryosine and other metabolites. *Archs Biochem. Biophys.* **205**, 146–155.
- Kramer K. J., Morgan T. D., Beeman R. W., Hopkins T. L., Roseland C. R., Aso Y. and Lookhart G. L. (1984) Catecholamines and β -alanine in the red flour beetle, *Tribolium castaneum*. Roles in cuticle sclerotization and melanization. *Insect Biochem.* **14**, 293–298.
- Kramer K. J., Nuntnarumit C., Aso Y., Hawley M. D. and Hopkins T. L. (1983) Electrochemical and enzymatic oxidation of catecholamines involved in sclerotization and melanization of insect cuticle. *Insect Biochem.* **13**, 475–479.
- Murdock L. L. and Omar D. (1981) *N*-Acetyldopamine in insect nervous tissue. *Insect Biochem.* **11**, 161–166.
- Peter M. G. (1980) Products of *in vitro* oxidation of *N*-acetyldopamine as possible components in the sclerotization of insect cuticle. *Insect Biochem.* **10**, 221–227.
- Pryor M. G. M. (1962) Sclerotization. In *Comparative Biochemistry* (Edited by Florkin M. and Mason H. S.), Vol. 4, Part B, p. 373. Academic Press, New York.
- Rembold H., Rascher J., Eder J. and Umebachi Y. (1978) Partial structure of papiliochrome, the yellow wing pigment of the papilionid butterflies. *Z. Naturforsch.* **33c**, 498–503.
- Sugumaran M. and Lipke H. (1983) Quinone methide formation from 4-alkylcatechols: a novel reaction catalyzed by cuticular polyphenol oxidase. *FEBS Lett.* **155**, 65–68.
- Vincent J. F. V. and Hillerton J. E. (1979) The tanning of insect cuticle—a critical review and a revised mechanism. *J. Insect Physiol.* **25**, 653–658.
- Williams-Boyce P. K. and Jungreis A. M. (1980) The larval-pupal transformation of *Manduca sexta*: changes in body tissue and tissue weights and amount of haemolymph contaminating the tissues. *Ann. ent. Soc. Am.* **73**, 602–608.