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N-*β*-ALANYLNOREPINEPHRINE: BIOSYNTHESIS IN INSECT CUTICLE AND POSSIBLE ROLE IN SCLEROTIZATION

T. D. MORGAN,[1] T. L. HOPKINS,[1]* K. J. KRAMER,[2,3] C. R. ROSELAND,[1]† T. H. CZAPLA,[1] K. B. TOMER[4] and F. W. CROW[4]‡

[1]Departments of Entomolgy and [2]Biochemistry, Kansas State University, Manhattan, KS 66506, [3]U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, KS 66502 and [4]Department of Chemistry, University of Nebraska, Lincoln, NE 68598, U.S.A.

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Abstract—*N*-*β*-alanylnorepinephrine (NBANE), a metabolite of *N*-*β*-alanyldopamine (NBAD) in pupal cuticle of the tobacco hornworm, *Manduca sexta*, was identified by analysis of hydrolytic products, by comparison of HPLC retention times with a synthetic standard and by fast atom bombardment mass spectrometry. NBANE and NBAD were abundant in the brown cuticles of *M. sexta* pupae, *Periplaneta americana* adults, *Tribolium castaneum* adults and *Musca domestica* puparia. Side chain modification of NBAD was rapid during the first few hours of sclerotization as evidenced by a dramatic increase in NBANE extracted from *M. sexta* pupal cuticle with cold 1.2 M HCl. In later stages of sclerotization less NBANE was recovered indicating further metabolism and possible involvement in the formation of crosslinks to cuticular components. Although similar amounts of the two catecholamines were extracted by 80% methanol from pupal cuticle which was just starting to tan, over 15-fold more NBANE than NBAD was recovered with cold dilute HCl. Low pH may be necessary to remove physically entrapped NBANE from the insoluble polymeric matrix of the cuticle, or NBANE released by the acid treatment may be a hydrolytic product from NBAD covalently linked by its *β*-carbon to cuticular components. NBANE was synthesized from NBAD by an insoluble enzyme in tanning pupal cuticle that also catalyzed to a lesser extent the *β*-hydroxylation of *N*-acetyldopamine (NADA). However, NADA was a better precursor than NBAD for compounds that yield 3,4-dihydroxphenylketoethanol after heating in 1 M HCl. Therefore, two pathways were observed in tanning cuticle for side chain metabolism of the *N*-acylated dopamines, and the type of acyl group influenced the relative amounts of different sclerotizing agents produced.

Key Word Index: *N*-*β*-alanyldopamine, *N*-*β*-alanylnorepinephrine, *N*-acetyldopamine, dopamine, *β*-alanine, *N*-acetylnorepinephrine, norepinephrine, 3,4-dihydroxyphenylethanol, 3,4-dihydroxyphenylketoethanol, catecholamines, *Manduca sexta*, *Tribolium castaneum*, *Musca domestica*, *Periplaneta americana*, cuticle, sclerotization, tanning

INTRODUCTION

Catecholamines are oxidized to electrophilic intermediates that may covalently bond with the chitin-protein matrix of insect cuticle during sclerotization (reviewed by Andersen, 1979a, 1985; Brunet, 1980; Lipke *et al.*, 1983). The diphenol moiety of the catecholamine is readily oxidized to a benzoquinone by cuticular phenoloxidases, but the side chain also undergoes important reactions that apparently lead to sclerotizing agents. *N*-acetyldopamine (NADA)§ is oxidized to 1,2-dehydro-NADA by an insoluble cuticular enzyme in *Locusta migratoria* (Andersen and Roepstorff, 1982). The unsaturated metabolite in the presence of a phenoloxidase can then react via its side chain with a catechol or it may form bonds with cuticular proteins. Hydroxylation of the side chain *β*-carbon also occurs when NADA is incubated with cuticular homogenates of *Manduca sexta* pupae (Peter, 1980) or with puparial cuticle from *Sarcophaga bullata* (Sugumaran and Lipke, 1983a). Instead of direct *β*-hydroxylation, formation of a quinone methide was proposed which then reacts spontaneously with water to form the *β*-hydroxylated derivative, *N*-acetylnorepinephrine (NANE). Quinone methides have also been proposed as intermediates involved in side chain cross linking reactions or *β*-sclerotization (Lipke *et al.*, 1983).

Sclerotization through catecholamine side chain linkages to proteins is thought to predominate in light colored cuticle while darker cuticle is more associated with phenolic crosslinks or quinone tanning (Andersen, 1974; Andersen and Roepstorff, 1982). However, the ratio of side chain to phenolic crosslinking may vary greatly even in those species with dark brown cuticles (Anderson, 1974; Sugumaran and Lipke, 1983b). The relative importance of ring or side chain crosslinks and the resulting light and dark

*Reprint requests to T. L. Hopkins.
†Present address: Department of Entomology, North Dakota State University, Fargo, ND 68015, U.S.A.
‡Present address: UpJohn Company, Kalamazoo, Michigan, U.S.A.
§See Table 1 for names and structures of catechols.

colored cuticles may be dependent upon the kinds and amounts of N-acyldopamines available to the oxidative enzymes in cuticle (Hopkins *et al.*, 1982, 1984). N-β-alanyldopamine (NBAD) is a major catecholamine in developmental stages of insects that form dark brown cuticles (Hopkins *et al.*, 1982, 1984; Kramer *et al.*, 1984; Roseland *et al.*, 1985). Individual sclerites or areas of cuticle can vary greatly in types of N-acylcatecholamines and show correlations with pigmentation. NADA predominated in the hard clear cuticle of the larval head capsule of *M. sexta* while NBAD was highest in concentration in the hard brown mandible (Hopkins *et al.*, 1984).

Although NBAD is oxidized by cuticular enzymes, little is known about the structure of resulting metabolites (Andersen, 1977; Barrett and Andersen, 1981; Aso *et al.*, 1984). In an earlier paper we tentatively identified N-β-alanylnorepinephrine (NBANE) as a major catecholamine in addition to NBAD in brown pupal cuticle of *M. sexta* (Hopkins *et al.*, 1984). We have now confirmed its identity and report its occurrence in brown cuticle of species from several orders as well as its synthesis from NBAD by an insoluble cuticular enzyme from *M. sexta*. The significance of NBANE in relation to cuticle sclerotization is discussed.

MATERIALS AND METHODS

Insects

M. sexta was reared at 27°C 16L:8D as described by Bell and Joachim (1976) from eggs supplied by the Metabolism and Radiation Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota, U.S.A. House flies, *Musca domestica*, were reared on CSMA medium (Chemical Specialties Manufacturers Association, Ralston Purina, St Louis, Missouri, U.S.A.). Red flour beetles, *Tribolium castaneum*, were cultured on whole wheat flour containing 5% (w/w) brewer's yeast. Cockroaches, *Periplaneta americana*, were reared on lab animal pellets and water.

Synthetic standards

α-Methyldopa (AMD), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), NADA and norepinephrine (NE) were from commercial sources. α-Hydroxy-(3′,4′ dihydroxy)-acetophenone (3,4-dihydroxyphenylketoethanol, DOPKET) and 3,4-dihydroxyphenylethanol (DOPET) were gifts from Dr S. O. Andersen, Copenhagen, Denmark, and Hoffmann–LaRoche Inc., Nutley, New Jersey respectively. NANE was synthesized by methods described by Andersen (1980). NBAD and NBANE were synthesized by coupling N-α-butyloxycarbonyl-β-alanine-N-hydroxysuccinimide ester with DA or NE, respectively, in 10% potassium tetraborate, pH 9.8. The conjugate was deblocked in 0.1 M HCl, and the N-acylated catecholamines were separated from the starting materials by gel filtration on BioGel P-2 in 5% acetic acid (Hopkins *et al.*, 1982; Kramer *et al.*, 1983).

Mass spectrometry

Full-scan fast atom bombardment (FAB) mass spectra were obtained on a Kratos MS-50 triple analyzer mass spectrometer equipped with a DS-55 data system at the Midwest Center for Mass Spectrometry, University of Nebraksa (Gross *et al.*, 1982). The sample of unknown compound was prepared by dissolving a small amount in glycerol and placing a drop of the resulting solution on the copper target of a direct-insertion probe. Bombardment was with 7 KeV argon atoms and the ions produced were accelerated through 8 KeV. The high-resolution accurate mass measurement was determined by peak matching at a resolution of 30,000, making use of the m/z 241.12873 ion of the glycerol matrix ion, $(\text{glycerol})_3H^+\text{-}2H_2O$, as a reference.

Isolation and LCEC analysis of catecholamines

Cuticle or hemolymph samples were extracted in 1.2 M HCl containing 5 mM ascorbic acid and an internal standard, AMD (0.6 μg/0.1 ml; Hopkins *et al.*, 1984). Free catechols were recovered from alumina and were quantified by reverse-phase high performance liquid chromatography with electrochemical detection (LCEC; Hopkins *et al.*, 1984). Several mobile phases were used, but chromatograms obtained with the two most commonly used mobile phases are depicted in Fig. 1. Mobile phase A consisted of 0.1 M phosphoric acid, 1.1 mM soldium lauryl sulfate, 0.05 mM disodium EDTA, and 26% (v/v) acetonitrile adjusted to pH 3.3 with NaOH. Retention times for positively charged catecholamines were AMD(I), 6.2 min; NBANE(II), 7.6 min; NE(III), 8.7 min; DA(IV), 16.6 min and NBAD (V), 17.4 min. Positively charged catecholamines were well resolved by this mobile phase due to the influence of lauryl sulfate, a relatively apolar ion pair, but non-positive catechols such as NANE, DOPKET, DOPET, DOPAC and NADA were eluted in 3 min or less. Mobile phase B which provided better resolution of the non-positive catechols consisted of 0.1 M phosphoric acid, 0.09 mM sodium octyl sulfate, 0.1 mM disodium EDTA, and 13% (v/v) methanol adjusted to pH 3.0 with NaOH. Retention times were NE, 3.9 min; NBANE, 5.4 min; NANE(VI), 6.5 min; DA, 7.5 min; AMD, 8.4 min; DOPKET(VII), 10.6 min; DOPET(VIII), 11.4 min; NBAD, 16.1 min and NADA(IX), 18.0 min. DOPAC although not shown in the chromatogram had a retention time of 14.8 min. Mobile phases were used at a flow rate of 1.0 ml/min through a 25 cm ODS 5 μm spherical particle column (Table 1).

Isolation of NBANE for analysis of chemical structure was done by making extracts from tanned pupal cuticle of *M. sexta* in 1.2 M HCl, collecting fractions by reverse-phase LC, desalting by adsorption on aluminia and recovery in 1 M acetic acid. Aliquots containing isolated compounds were hydrolyzed in 1.2 M HCl at 100°C under nitrogen for 10 min. Catechols were analyzed by LCEC and amino acids by cation-exchange chromatography followed by post column ninhydrin derivatization. Other aliquots were dried for FAB mass spectrometry.

Extraction experiments

Epidermis was removed from the cuticle by scraping under saline. Cleaned cuticle from *M. sexta* or *P. americana* was extracted soon after excision but cuticle from *M. domestica* and *T. castaneum* was stored at −20°C prior to homogenization. Each cuticle (3–6 mg) was extracted four times with 0.25 ml of solvent and the extracts combined prior to analysis. The solvents included 80% (v/v) aqueous methanol, 7 M urea with 0.13% sodium dodecylsulfate (SDS), and 0.1 M phosphate buffer adjusted to ph 6.0 and saturated with phenylthiourea to inhibit the tyrosinase found in cuticle (Aso *et al.*, 1984). All solvents contained 5 mM ascorbic acid as an antioxidant. For comparison, paired samples of cuticle from *M. sexta* or *P. americana* were also extracted in 1.2 M HCl. The pronotum of *P. americana* was cut in half to allow one half to be extracted in the test solvent and the other half in 1.2 M HCl. The outer cuticle of a forewing of *M. sexta* was extracted in the test solvent and cuticle from the opposite forewing from the same pupa was extracted in 1.2 M HCl. Since it was impractical to pair samples of the puparial cuticle of *M. domestica*, similar amounts of cuticle were extracted with the test solvent or 1.2 M HCl.

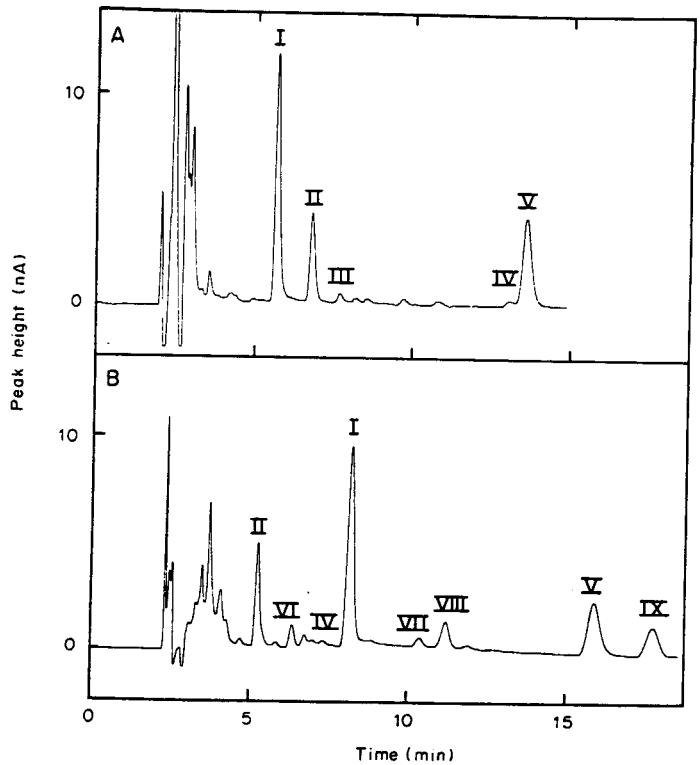
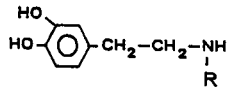
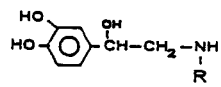
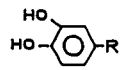


Fig. 1. Chromatograms obtained with two mobile phases by reverse-phase, ion-pairing LCEC of the diphenols present in a cold 1.2 M HCl extract from the pronotum of a 24-hr-old adult of *Periplaneta americana*. Mobile phase A resolved positively charged catecholamines well, but mobile phase B was useful for neutral and charged catechols. Peaks: **I**, AMD (internal standard); **II**, NBANE; **III**, NE; **IV**, DA; **V**, NBAD; **VI**, NANE; **VII**, DOPKET; **VIII**, DOPET; **IX**, NADA. See Materials and Methods for additional details.

Enzyme studies on NBANE synthesis

Paired forewings were severed from *M. sexta* pupae at about 2.4 hr after pupal ecdysis, and the outer integument was removed and washed in ice cold saline. For isolation of cuticle, the epidermis was carefully scraped away under saline and the cuticle rinsed, blotted on tissue and quickly weighed (averaging about 2.5 mg per forewing). The weight of cuticle in the integument sample from the opposite forewing of the same pupa was assumed to be the same, and the samples were compared on the basis of cuticle wet weight. Each piece of integument or cuticle was homogenized in 0.1 ml of 0.1 M phosphate buffer (pH 6.0) in a ground glass tissue grinder and stored at −20°C. An insoluble fraction of the cuticle homogenates was obtained by centrifuging the homogenate at 6500 *g* and then washing the precipitate with a second aliquot of buffer. For comparison of different substrates, 0.015 ml aliquots of the

Table 1. Structures and abbreviations of catechols extracted from insect cuticle

Compound	Abbreviation	Formula	R
Dopamine	DA		H
N-β-alanyldopamine	NBAD		O ‖ C—CH_2—CH_2—NH_2
N-acetyldopamine	NADA		O ‖ C—CH_3
Norepinephrine	NE		H
N-β-alanylnorepinephrine	NBANE		O ‖ C—CH_2—CH_2—NH_2
N-acetylnorepinephrine	NANE		O ‖ C—CH_3
3,4-Dihydroxyphenylketoethanol	DOPKET		O ‖ C—CH_2OH
3,4-Dihyroxphenylethanol	DOPET		CH_2—CH_2OH
3,4-Dihydroxyphenylacetic acid	DOPAC		CH_2—COOH

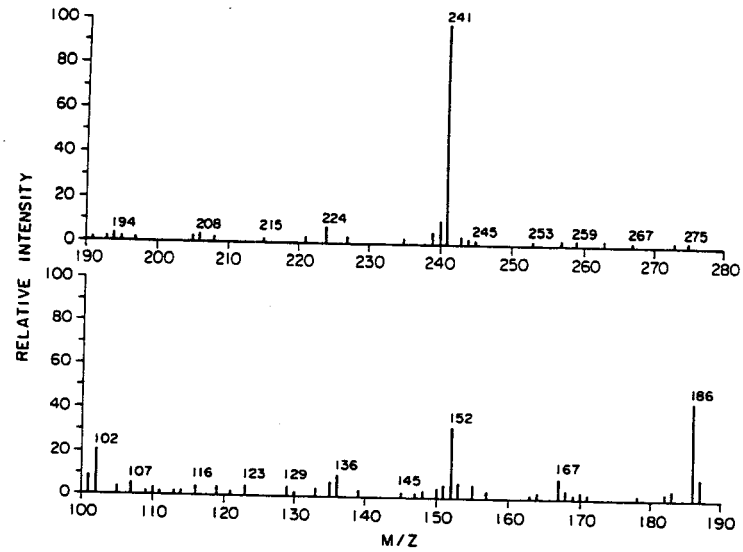


Fig. 2. Scanning fast atom bombardment mass spectrum of N-β-alanylnorepinephrine isolated from pupal cuticle of *Manduca sexta*. The m/z 241 corresponds to the molecular cation $(M + H)^+$ of the proposed structure of NBANE. The m/z 186 and 167 ions are from the glycerol matrix and matrix minus water background, while other background has been subtracted.

insoluble cuticle preparation were centrifuged and resuspended in 0.25 ml of buffer containing 1 mM substrate. The vessel used for incubation was a 1.5 ml disposable centrifuge tube which was placed at an acute angle for proper mixing on a horizontal shaker at 27°C. The reaction was stopped by adding 0.17 ml of 1.2 M HCl containing the internal standard.

RESULTS

Identification of N-β-*alanylnorepinephrine*

In addition to NBAD a major unknown catecholic compound was detected in acidic extracts of *M. sexta* pupal cuticle. The unknown compound was isolated by HPLC and tentatively identified as NBANE based on recovery of NE and β-alanine after incubation in 1.2 M HCl at 100°C for 10 min (Hopkins *et al.*, 1984). β-Alanine was present in an equimolar amount relative to NE. Purified NBANE had an ultraviolet absorption spectra with $\lambda_{max} = 278$ nm at pH 3 which was identical with that of NE. Synthetic NBANE had identical retention times with the natural compound during LCEC with different mobile phases.

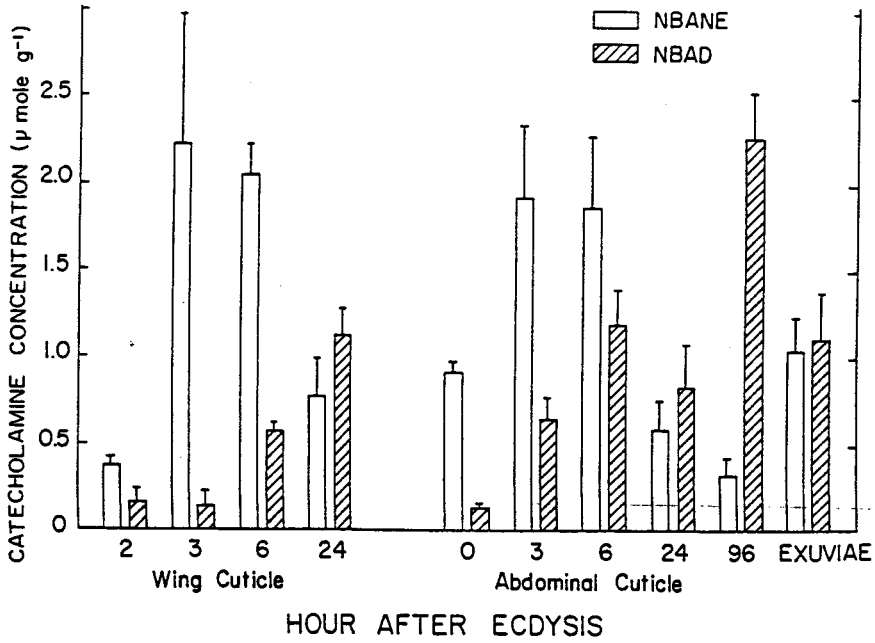


Fig. 3. N-β-Alanyldopamine (NBAD) and N-β-alanylnorepinephrine (NBANE) concentrations in the pupal cuticle of *Manduca sexta* during sclerotization. The cuticle samples were homogenized and extracted with cold 1.2 M HCl. See Materials and Methods for details. Means ± SEM, $n = 3$.

High resolution FAB mass spectrometry was used to determine the molecular weight of the unknown compound purified from pupal cuticle (Fig. 2). The most abundant high mass ion observed by FAB mass spectrometry was that at m/z 241. This ion has the m/z expected of the intact molecular cation $(M+H)^+$ corresponding to the proposed structure of NBANE. Synthetic NBANE was found to exhibit a spectrum identical to that of the natural compound. The measured high-resolution mass of 241.1188 matched the value calculated for $C_{11}H_{17}O_4N_2$ with less than 1 ppm error. Thus, high-resolution mass measurement confirmed the composition of NBANE.

The N-acylated derivatives of DA and NE exhibited distinct differences in the susceptibility of the amide bond to acid hydrolysis. Large amounts of NE were liberated under conditions of mild acid hydrolysis (1.2 M HCl at 100°C for 10 min) of cuticle extracts, while the compound later identified as NBANE disappeared. Synthetic NBANE and NANE were almost totally hydrolyzed (~95%) after 10 min at 100°C in 1.2 M HCl while less than 10% NBAD and NADA were lost under similar conditions. The lability of the amide bond should be considered when processing or storing samples of the N-acylnorepinephrine derivatives. NBANE and NANE are unstable in acidic solutions during exposure to elevated temperature or during freezing. These compounds can be stored, however, at 5°C or less in 1 M acetic acid (pH 2.4) for several months with no apparent hydrolysis.

N-β-*alanylcatecholamines in cuticle of* M. sexta

Catecholamines were extracted in 1.2 M HCl from the outer cuticle of the forewing and the dorsal cuticle of the third and fourth abdominal segments of *M. sexta* pupae and were analyzed by LCEC (Fig. 3). Abdominal cuticle shows extensive localized tanning at ecdysis, and it contained twice as much extractable NBANE as does untanned wing cuticle at 2 hr after ecdysis. When tanning of wing cuticle begins by 3 hr as evidenced by a light brown coloration, NBANE dramatically increased to peak levels similar to those reached by abdominal cuticle. NBANE concentrations remained high through 6 hr post ecdysis in both types of cuticle, but decreased over 50% by 24 hr when tanning appeared to be nearly complete. The accumulation of NBAD during cuticular tanning was delayed, when compared to NBANE, but both were nearly equivalent by 24 hr. The highest levels of NBAD were observed at 96 hr in abdominal cuticle, but wing cuticle was not sampled at that time (see also Hopkins *et al.*, 1984). Concentrations of both catecholamines approximately equal to those at 24 hr were present in acidic extracts of pupal abdominal exuviae after adult eclosion.

Larval cuticle was also analyzed for N-β-alanylcatecholamines. In 1-day-old fifth stage larvae of *M. sexta*, acidic extracts of the distal edge of the mandible contained NBANE ($2.0 \pm 0.6\ \mu mol\ g^{-1}$) and NBAD ($10.0 \pm 2.6\ \mu mol\ g^{-1}$). The dorsal cuticle of the larval abdomen had much lower concentrations of the N-β-alanylcatecholamines (NBANE, $0.06 \pm 0.01\ \mu mol\ g^{-1}$ and NBAD, $0.45 \pm 0.13\ \mu mol\ g^{-1}$).

NBANE and other cuticular catechols in different insect species

Comparative studies were made on the N-acylcatecholamines and other catechols in cuticle from species of insects representing three orders. Cuticular samples were homogenized and extracted with four successive aliquots of cold 1.2 M HCl instead of a single extraction. Otherwise the methods were the same as the standard procedure. The cuticles of *M. sexta*, *P. americana*, and *M. domestica* released large amounts of NBANE in the cold acidic solvent (Table 2). NBAD accumulated to higher levels than NBANE by 24 hr after ecdysis in *M. sexta*, as we had previously observed (Table 2, Fig. 3). NADA and NANE, the N-acetylcatecholamines, were barely detectable at the early intervals after pupation or pupariation in *M. sexta* and *M. domestica*, but small amounts were present in the cuticle of 24 hr old *M. sexta* pupae. The highest concentrations of NADA and NANE were found in the acidic extracts of the pronotum of 24 hr old adults of *P. americana*.

In all species examined substantial amounts of NE and DOPKET were present in 1.2 M HCl extracts after heating at 100°C for 10 min (Table 2). The amount of NBANE and NANE present before heating accounted for 75–99% of the total NE present in the hydrolysate, indicating that NE was primarily a hydrolytic product of free NBANE and NANE. Since DOPKET has been commonly found as a hydrolysis product of catecholamine dimers in insect cuticle (Andersen, 1985), NE and DOPKET are probably derived from the products of different

Table 2. N-Acylcatecholamines extracted from insect cuticle with cold 1.2 M HCl and catechols present after heating ($\mu mol\ g^{-1}$ wet weight of cuticle)*

Species (cuticle)	Hours after ecdysis or pupariation	NBAD	NBANE	NADA	NANE	After heating: NE	After heating: DOPKET	After heating: DOPET
Manduca sexta (pupal wing)	3	0.09 ± 0.03	1.57 ± 0.26	0.01	0.01	1.74 ± 0.29	1.14 ± 0.36	0.01
	6	0.35 ± 0.04	1.26 ± 0.12	0.01	0.01	1.47 ± 0.14	0.50 ± 0.04	0.04
	24	1.03 ± 0.41	0.58 ± 0.04	0.09 ± 0.02	0.07 ± 0.01	0.79 ± 0.06	0.48 ± 0.08	2.81 ± 0.42
Periplaneta americana (adult pronotum)	24	1.55 ± 0.21	1.10 ± 0.02	0.90 ± 0.11	0.14 ± 0.03	1.66 ± 0.03	4.77 ± 0.51	0.79 ± 0.40
Musca domestica (puparia)	1–2	0.14 ± 0.02	3.66 ± 0.35	0.01	0.01	3.72 ± 0.30	0.44 ± 0.04	0.01

*Data are means of three cuticle samples ± SEM. Cuticular homogenates were extracted with four aliquots of ice-cold 1.2 M HCl containing 5 mM ascorbate, centrifuged and the pooled supernatants were analyzed before and after heating (100°C for 10 min). See Materials and Methods for details.

Table 3. Extractability of *N*-β-alanylcatecholamines by different solvents from insect cuticle (μmol g^{-1} wet weight of cuticle)*

Species (cuticle)	Hours after ecdysis	Solvent	NBAD	NBANE
Manduca sexta (pupal wing)	3	80% MeOH	0.07 ± 0.02 (65)†	0.05 ± 0.01 (3)†
	6	80% MeOH	0.18 ± 0.03 (56)	0.02 ± 0.01 (2)
	24	80% MeOH	0.73 ± 0.19 (65)	0.04 ± 0.01 (7)
		Urea-SDS	0.54 ± 0.22 (62)	0.03 ± 0.01 (7)
		Phosphate buffer	1.05 ± 0.64 (84)	0.07 ± 0.02 (14)
Periplaneta americana (pronotum)	24	80% MeOH	1.01 ± 0.07 (67)	0.14 ± 0.02 (13)
Musca domestica (puparia)	1–2	80% MeOH	0.05 ± 0.02 (40)	0.10 ± 0.02 (3)
Tribolium castaneum (elytra)	7 days	80% MeOH	0.35 ± 0.03 (ND)	0.17 ± 0.02 (ND)

*Data are means of three cuticle samples ± SEM. The cuticle was finely ground and extracted with four aliquots of ice-cold solvent containing 5 mM ascorbate. The phosphate buffer (pH 6) was also saturated with phenylthiourea. The supernatants were pooled and analyzed by LCEC. See Materials and Methods for details. ND = not determined.

†Percent relative to the amount extracted with 1.2 M HCl.

pathways of side chain metabolism. The ratio of NE to DOPKET in our hydrolyzed extracts of cuticle was 8.4 in early puparia of *M. domestica*, 1.5–2.9 in pupal wing cuticle of *M. sexta* and 0.3 in 24-hr-old adults of *P. americana*. Large amounts of NE and DOPKET were present at 3 hr post ecdysis in *M. sexta* pupae, but by 24 hr these levels were reduced by over 50%. Some free DOPKET was usually present in the 1.2 M HCl extracts, but these amounts were always less than 15% of the amounts present after heating.

Another catechol, 3,4-dihydroxphenylethanol (DOPET), was detected in hydrolysates of some cuticular extracts (Table 2). In *M. sexta* DOPET was absent at 3 hr, but it increased to become the predominate catechol at 24 hr. Substantial amounts of DOPET were also present in hydrolyzed extracts from 24-hr-old adult cuticle of *P. americana*. The amount of free DOPET was 19 and 71% of the amount present in hydrolyzed extracts from 24 hr cuticle of *M. sexta* and *P. americana*, respectively, indicating substantial amounts of DOPET conjugates are present in cuticle. Approximately one-half of the DOPKET and all of the DOPET which was extracted by cold 1.2 M HCl from *P. americana* cuticle could also be extracted by a milder solvent, 80% methanol. The elytra from 7-day-old adults of *Tribolium castaneum* also contained free DOPET (21 ± 3 μmol g^{-1}), DOPKET (1.7 ± 0.4 μmol g^{-1}) and DOPAC (143 ± 9 μmol g^{-1}), as estimated by extraction with 80% methanol ($n = 3$).

Extraction of N-β-*alanylcatecholamines from cuticle*

The ability of different aqueous and methanolic solvents to extract NBAD and NBANE from insect cuticle was investigated (Table 3). For this experiment four successive aliquots of solvent were used to extract cuticular homogenates. For comparison, data for NBAD and NBANE extracted with 1.2 M HCl are shown in Table 2. When the amount extracted with cold acidic solvent is considered to be 100%, the mean percentage of NBAD extracted by 80% methanol, 7 M urea with 0.1% SDS or pH 6 phosphate buffer varied from 62% to 84%. However, those solvents always extracted much less NBANE than the amount extracted by the acidic solvent. Aqueous methanol removed only 3% of the the acid extractable NBANE from the relatively soft forewing cuticle of 3-hr-old pupae and 7% from tanned forewing cuticle of 24-hr-old pupae. Urea with SDS gave results similar to methanol while phosphate buffer extracted about twice more, but still substantially less NBANE was recovered than by acidic extraction. Similar results were obtained with aqueous methanol extraction of NBAD and NBANE from cockroach pronotal cuticle and house fly puparial cuticle. These results indicated that while large quantities of NBANE are tightly bound within the cuticular matrix, substantial amounts of free NBANE are present in the brown cuticles of *M. sexta*, *P. americana*, *M. domestica*, and *T. castaneum*. Methanol extractable NBANE remained relatively constant in the forewing cuticle of *M. sexta* pupae between 3 and 24 hr after ecdysis while acid extractable NBANE decreased by over 50% (Table 2). The concentration of NBAD, however, in both methanolic and acidic extracts increased dramatically during this same time period.

After extraction with cold 1.2 M HCl was completed, the residue from the wing cuticle from 6-hr-old pupae of *M. sexta* was washed, resuspended, and heated in 1.2 M HCl at 100°C for 10 min. This hot acid treatment liberated additional NBAD (0.61 ± 0.12 μmol g^{-1}) and NE (1.71 ± 0.20 μmol g^{-1}), suggesting that substantial amounts of catecholamines are strongly bound (perhaps covalently) within the cuticular matrix.

N-β-*Alanylcatecholamines in hemolymph of* M. sexta

At pupal ecdysis, the free NBAD hemolymph titer was 0.7 mM and the concentration of an acid labile conjugate of NBAD (probably a glucoside) was about 2.6 mM. However, the concentration of free

Table 4. Side chain metabolism of dopamine and N-acyldopamines by the insoluble enzyme fraction from pupal wing cuticle of *Manduca sexta* (μmol g^{-1} wet weight of cuticle)*

Substrate	β-Hydroxylated product	After heating†	
		NE	DOPKET
None	0.1	0.1	0.1
NBAD	22.0 ± 3.0 (NBANE)	20.0 ± 2.1	2.5 ± 0.2
NADA	3.8 ± 0.2 (NANE)	4.1 ± 0.4	12.1 ± 0.6
DA	0.9 ± 0.1 (NE)	1.0 ± 0.1	0.1

*The insoluble fraction of cuticle homogenates was incubated with 1 mM substrate (67 μmol g^{-1} original wet weight of cuticle) at pH 6 for 10 min at 27°C. Data represent means ± SEM (n = 3).
†An aliquot of the incubation mixture was heated at 100°C for 10 min in 1 M HCl. The small amounts of NBANE remaining after heat treatment were added to the total amount of NE.

NBANE in the hemolymph was only 4.2 ± 0.2 μM. After hydrolysis in 1.2 M HCl at 100°C for 10 min, total NE was 14 ± 1 μM. Apparently only low levels of NBANE were present in hemolymph.

Metabolism of catecholamines in pupal cuticle of M. sexta

The synthesis of β-hydroxylated catecholamines appears to occur mainly in cuticle. When NBAD (5 mM) was incubated with integumental homogenates, NBANE increased to 40 ± 4 μmol g^{-1} after 10 min, based on wet weight of cuticle, while in cuticular homogenates NBANE increased to 49 ± 9 μmol g^{-1}. Essentially the same amount of NBANE accumulated during incubation with either the whole homogenates or the residual fraction. Therefore, the residual fraction was used in all subsequent studies. Heating at 80°C for 10 min at pH 6 destroyed about 85% of the hydroxylating activity. The residual fraction of pupal wing cuticle was incubated with 1 mM NBAD, NADA or DA to compare the relative suitability of these substrates for enzymes which modify the catecholamine side chain (Table 4). After 10 min at 27°C, a large amount of β-hydroxylated catecholamine accumulated in the NBAD incubation, while much smaller amounts accumulated with NADA or DA as substrate. The reaction was stopped by acidification, but addition of methanol gave similar results indicating that cold acid does not release additional β-hydroxylated catecholamines from the cuticular residue. Heating the enzyme reaction mixtures at 100°C for 10 min in 1 M HCl did not significantly increase the β-hydroxylated catecholamines present.

Large amounts of DOPKET were produced by acid hydrolysis of the NADA incubation mixtures while NBAD incubations had five-fold less DOPKET, and DA incubations 100-fold less. DA was a poor substrate for the insoluble enzyme of pupal cuticle. Its concentration decreased by less than 10% during the incubation, while the concentration of NADA or NBAD decreased by 40–80%. The total amount of modified catechols present in hydrolysates of the DA incubation respresented only 2% of the original amount of DA. In contrast, 33% of the original amount of NBAD was recovered as NBANE and 4% as DOPKET, while 6% of the original NADA was recovered as NANE and 18% as DOPKET. These results indicated that both NADA and NBAD are relatively good substrates for modification of the side chain during cuticle sclerotization. NBAD appears to be a better substrate for the β-hydroxylation pathway while side chain metabolism of NADA leads mainly to adducts that yield DOPKET during acid hydrolysis.

DISCUSSION

NBANE was identified as a metabolite of NBAD in pupal cuticle of *M. sexta* and it was also extracted from the brown cuticles of cockroaches (*P. americana*), beetles (*T. castaneum*), and fly puparia (*M. domestica*). This compound cochromatographed with synthetic NBANE, and high-resolution FAB mass spectrometry further confirmed its identity. It had been previously found in acidic extracts of pupal cuticle of *M. sexta* (Hopkins *et al.*, 1984), and as an acid hydrolysis product from a wing pigment in *Papilio xuthus* (Rembold *et al.*, 1978; Rembold and Umebachi, 1984).

The N-β-alanylcatecholamines which were extractable with cold dilute HCl had different patterns of accumulation in pupal cuticle of *M. sexta* during sclerotization. NBANE increased rapidly to peak levels as brown pigment became visible in the wing cuticle by 3 hr post ecdysis. NBAD, its precursor, however, remained at relatively low concentrations until several hours later. A similar pattern of change occurred in abdominal cuticle except that tanning and NBANE accumulation began before ecdysis. These results indicated that a period of intensive side chain metabolism of NBAD occurs during the initial stages of sclerotization. Our *in vitro* studies showed that pupal wing cuticle at 2.4 hr after ecdysis had a high capacity for synthesis of NBANE from exogenous NBAD.

NBAD gradually accumulated in both wing and abdominal cuticle between 3 and 24 hr after pupal ecdysis, while NBANE decreased to levels approximating those of NBAD. Our previous work had shown that the accumulation of NBAD in the cuticle during tanning is associated with a gradual disappearance of the large reservoir of NBAD in hemolymph (Hopkins *et al.*, 1982, 1984). The rate of side chain or ring metabolism of NBAD in the cuticle may decrease during sclerotization allowing it to accumulate.

The decrease in acid extractable NBANE during the later stages of tanning may be due to formation of covalent bonds to cuticular components or conversion to other metabolites. Both NBANE and NBAD stimulated cuticle tanning *in vitro* to a much greater degree than other catecholamines as scored by brown coloration of pupal cuticle of *M. sexta* (Roseland *et al.*, 1986). Since NBANE was more stimulatory than NBAD, it is possible that much of the NBAD in cuticle is converted to NBANE before oxidation to a quinone that covalently bonds to cuticular proteins. Quinone tanning also appears to be an important process in the sclerotization of the pupal cuticle of *M. sexta* based on studies showing radiolabeled precursors such as NBAD or DA become bound to cuticular components by acid stable bonds (Grün and Peter, 1983, 1984; Sugumaran and Lipke, 1983).

The covalent and non-covalent interactions between N-β-alanylcatecholamines and the chitin-protein matrix have not been elucidated. However, the recoveries of NBANE and NBAD by extraction with non-acidic and cold or hot acidic solvents provide an indication of the complexity of the interactions. Acidic solvents were only twice as effective as non-acidic ones in the extraction of NBAD, but 7–60 times more effective for NBANE extraction. Much of the NBANE in the acidic extracts could be a hydrolysis product from NBAD weakly bonded through the β-carbon to cuticular components (Morgan, Kramer and Hopkins, unpublished data). In this regard, a butterfly wing pigment consisting of NBAD linked by its β-carbon to the aromatic amine of kynurenine released NBANE and kynurenine during mild acid hydrolysis (Rembold and Umebachi, 1984). It is also possible that more NBANE than NBAD is physically entrapped within the cuticle during sclerotization and that low pH is required to release it. Large amounts of NBAD and NE were released when the insoluble residue from cold acidic homogenates was resuspended in 1.2 M HCl and heated at 100°C for 10 min. The source of hot acid extracted NBAD is unknown but it may be released from NBAD conjugated with cuticular components. Since NBANE is the major β-hydroxylated catecholamine in this cuticle and is easily hydrolyzed under these conditions, NE is likely to be a product of entrapped or bound NBANE.

β-Hydroxylation of catecholamines occurred during *in vitro* incubations with the insoluble fraction of pupal cuticle homogenates from *M. sexta* with substrate preference being NBAD > NADA > DA. β-Hydroxylation of NBAD also occurs to some extent *in vivo* since free NBANE can be extracted from tanning cuticle by aqueous methanol. β-Hydroxylation of exogenous NADA has been observed previously during *in vitro* incubations of cuticle preparations from *M. sexta* pupae and *S. bullata* puparia (Peter, 1980; Sugumaran and Lipke, 1983a). It was suggested that NADA may be oxidized to a quinone methide which can then spontaneously hydrate to form NANE. If the quinone methide is formed, its β-carbon can be expected to react with a variety of nucleophiles in the cuticle (Lipke *et al.*, 1983). Cuticular enzymes which catalyze the synthesis of β-hydroxylated catecholamines have not been characterized, but our studies indicated that the enzyme in pupal cuticle is mostly insoluble and relatively heat stable. A cuticular enzyme in *L. migratoria* which releases tritium from the β carbon of NADA is also insoluble and relatively heat stable (Andersen, 1979b). This enzyme appears to introduce a double bond in the side chain of NADA and may also catalyze the oxidation of diphenols to quinones (Andersen and Roepstorff, 1982; Andersen, 1985).

Metabolism of the side chain of catecholamines may involve more than one pathway. When NBAD or NADA was incubated with the insoluble fraction of *M. sexta* cuticle, β-hydroxylated products accumulated and compounds that released DOPKET during hot acid treatment were formed. DOPKET has been found to be a hydrolysis product of NADA linked via the α- and β-carbons to another diphenol (Andersen *et al.*, 1980). For example, NE and DOPKET were the hydrolysis products of a compound which consisted of NANE linked by it phenolic oxygens to the α, β-carbons of NADA (Andersen and Roepstorff, 1981). Similar products of NBAD metabolism were not isolated (Andersen, 1985). NBAD and NBANE were the only catecholamines we detected in substantial amounts during initial tanning of cuticle from pupae of *M. sexta* and the puparia of *M. domestica*. Extracts of these cuticles also contained substantial amounts of DOPKET after mild acid hydrolysis. Therefore, the intensive side chain metabolism of NBAD which occurs during the early stages of sclerotization appears to include the synthesis of compounds which can be recovered as DOPKET.

The N-acylated dopamines were metabolized in *M. sexta* cuticle by at least two pathways *in vitro*, but preferences were exhibited according to the acyl substituent. The β-hydroxylation pathway was preferred when NBAD was the substrate, but NADA was predominantly metabolized to compounds that were released as DOPKET during acid hydrolysis. NBAD was abundant in the brown cuticle of *M. sexta*, *M. domestica* and *P. americana*, and the extracts contained large quantities of NBANE. NADA was more abundant in the brown cuticle of *P. americana* than in those of *M. sexta* and *M. domestica*, and the amount of DOPKET in hydrolyzed extracts from *P. americana* was at least 10-fold higher than the amount observed in the other species. The amount of N-acylated dopamine which possesses the N-β-alanyl group appears to have an inverse relationship to the concentration of metabolites which can be recovered as ketocatechols. The amounts of β-alanine and ketocatechols in cuticular hydrolysates of pupal cuticle from different mutant strains of *Drosophila melanogaster* were also inversely related (Umebachi and Kiyokawa, 1984). When a β-alanine deficient strain was supplied with β-alanine, the amount of ketocatechols recovered from the cuticle decreased. The relative suitability of NBAD and NADA as substrates for different pathways of side chain metabolism may ultimately determine the physical properties of the fully sclerotized cuticle.

High levels of catechols are often present in hard cuticle, prompting suggestions that they may play an important role as antioxidants or dehydrating agents (Atkinson *et al.*, 1973; Vincent and Hillerton, 1979). The highest concentration of any catechol observed in the present study was that of DOPAC which exceeded 100 μmol g^{-1} in 7-day-old elytra of *T. castaneum*. DOPAC accumulated in elytra after NBAD had already reached peak concentration (Roseland *et al.*, unpublished). A similar temporal sequence was observed with accumulation of DOPET in the pupal cuticle of *M. sexta*. Although little DOPET was present in the cuticle during tanning at 3 or 6 hr after ecdysis, substantial amounts accumulated by 24 hr after ecdysis. We have also found this catechol in adult cuticle of *T. castaneum and P. americana*. DOPET has been previously identified from exuviae of *P. americana* (Atkinson *et al.*, 1973), and DOPAC has been found in adult cuticle of several species of beetles (reviewed by Brunet, 1980).

N-β-Alanylcatecholamines are more abundant than N-acetylcatecholamines in the brown cuticles that we have so far examined. Side chain metabolism

of NBAD leading to the synthesis of NBANE or other sclerotizing precursors is an important process in the stabilization of hard brown cuticle. Further research is needed, however, to identify the reactive intermediates and the types of bonds formed within the chitin-protein matrix.

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