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Identification of Catecholamine β-Glucosides in the Hemolymph of the Tobacco Hornworm, *Manduca sexta* (L.), during Development

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Catecholamine conjugates were isolated from the hemolymph of larval, pupal and adult stages of the tobacco hornworm, *Manduca sexta*, and their identities were determined by synthesis from ^{14}C-labeled precursors, HPLC analysis, ^{1}H-NMR and FAB-mass spectrometry, and GLC analysis of the sugar moieties. β-Glucosides of N-acetyldopamine (NADA) and N-β-alanyldopamine (NBAD) were the primary conjugates identified and their relative abundance depended on the stage of development. NADA glucosides were the predominant conjugates in hemolymph of pharate or newly ecdysed fifth stadium larvae with 86% as the 3-*O*-glucoside and 14% as the 4-*O*-glucoside. Low concentrations of dopamine (DA) 3-*O*-glucoside and NBAD 3-*O*-glucoside also were found. In pharate or newly ecdysed pupal hemolymph, NBAD 3-*O*-glucoside was the major conjugate (98%) along with small amounts of NBAD 4-*O*-glucoside, DA 3-*O*-glucoside and NADA 3-*O*-glucoside. In hemolymph of pharate or newly ecdysed adults, NADA 4-*O*-glucoside (67%) was more abundant than NADA 3-*O*-glucoside. Only traces of NBAD 3-*O*-glucoside were found. Therefore, a preponderance of catecholamine 3-*O*-glucosides occurred in larval and pupal hemolymph as storage precursors for cuticle sclerotization agents, whereas the 4-*O*-glucoside of NADA predominated in adult hemolymph. The role of catecholamine glucosides in hemolymph in relation to cuticle sclerotization is discussed.

Catecholamines Dopamine β-Glucosides Conjugates Tobacco hornworm *Manduca sext* N-Acetyldopamine N-β-Alanyldopamine Hemolymph

INTRODUCTION

Catecholamines that serve as precursors for quinone sclerotizing agents of insect cuticle or tanned structures such as oothecae often are stored in hemolymph or colleterial glands, respectively, as glucose, sulfate or phosphate conjugates (Brunet, 1980; Kawasaki and Yago, 1983; Yago and Kawasaki, 1984; Hopkins *et al*, 1984; Hopkins and Kramer, 1991). In the tobacco hornworm, *Manduca sexta* (L.), conjugates of N-acetyldopamine (NADA) predominate in the hemolymph of pharate and newly ecdysed larvae and adults, whereas a conjugate of N-β-alanyldopamine (NBAD) is the major storage form in pharate and newly ecdysed pupae (Hopkins *et al.*, 1982, 1984). The catecholamines apparently are released from the conjugates by specific hydrolases, transported through the epidermis into the cuticle, and then oxidized to quinonoid sclerotizing agents (Hopkins and Kramer, 1992). A decrease in concentration of NBAD glucoside and an increase in free NBAD in hemolymph during the initial stages of pupal cuticle tanning are in accord with the mobilization of these storage molecules for stabilization and pigmentation of the exoskeleton. Previously, we identified β-D-glucopyranosyl-*O*-L-tyrosine (tyrosine glucoside) in the hemolymph of last stadium larvae of *M. sexta* and suggested its role as a precursor storage molecule for pupal cuticle tanning agents (Kramer *et al.*, 1980; Lu *et al.*, 1982; Ahmed *et al.*, 1983a). Its synthesis was found to be regulated by juvenile hormone in the last larval instar and its hydrolysis by 20-hydroxyecdysone in the pharate pupa (Ahmed *et al.*, 1983b; 1985).

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Early studies indicated that the catecholamine conjugates found in hemolymph of different developmental stages of M. sexta were β-glucosides (Hopkins *et al.*, 1982, 1984). Additional chemical studies on NBAD and DA conjugates purified from pupal hemolymph showed that they were mainly 3-*O*-glucosyl derivatives (Hopkins and Kramer, 1991; Mueller *et al.*, 1993). To further elucidate the structures of these conjugates, we isolated both major and minor catecholamine metabolites from larval, pupal and adult hemolymph. The concentrations of the different catecholamine glucosides that occur during development were also determined.

MATERIALS AND METHODS

Insects and chemicals

M. sexta was reared as described by Bell and Joachim (1976) at 27°C and a photoperiod of 16L:8D. Hemolymph was collected from larvae, pupae and adults according to Hopkins *et al.* (1984).

Catecholamine and carbohydrate standards were from Sigma, St Louis, MO or Aldrich, Milwaukee, WI, except for NBAD, which was synthesized according to Kramer *et al.* (1983). Glucose-U-^{14}C and tyrosine-U-^{14}C were obtained from ICN, Irvine, CA.

Radiolabeling and mapping of conjugates

Tyrosine metabolites and their glucosides occurring in pharate pupae during the early stages of cuticle tanning were isolated after injection of either tyrosine-U-^{14}C or glucose-U-^{14}C into fifth stadium larvae at the beginning of wandering behavior. The pharate pupae (brown metathoracic bar stage) were bled, and the hemolymph was deproteinated with 80% methanol. The extracts were concentrated and analyzed on cellulose thin layer sheets by two-dimensional electrophoresis and thin layer chromatography (TLC) (Kramer *et al.*, 1980; Czapla *et al.*, 1988). The ^{14}C-labeled metabolites were located by autoradiography (Kodak XAR, Eastman) and the radioactive zones were collected by scraping the cellulose powder from the sheet and extracting with 80% methanol. The conjugates labeled with either ^{14}C-glucose and ^{14}C-tyrosine precursors were analyzed on separate plates. Aliquots of these extracts were dried and heated in 1.2 M HCl at 100°C for 10 min to hydrolyze the glucoside conjugates. This interval is sufficient for near quantitative hydrolysis of the glucosidic bonds but less than 10% of the amide bonds are cleaved. The aglucones were analyzed by high performance liquid chromatography (HPLC) with electrochemical detection (Hopkins *et al.*, 1982, 1984).

Purification of catecholamine conjugates

Hemolymph from newly ecdysed larvae, pharate adults, or brown bar stage pharate pupae was heated at 100°C for 5–10 min and centrifuged to remove denatured proteins. The supernatant from the hemolymph of the pupae was loaded onto minicolumns of Bio-Rex 70 weak cation exchange resin (BioRad Laboratories, Richmond, CA) that were equilibrated in 0.1 M sodium phosphate buffer, pH 6.5. The columns were washed with water, then the amines were eluted with 2 M formic acid and further purified by gel filtration on Bio Gel P-2 in 5% acetic acid. The fractions of NBAD or dopamine conjugates, as determined by absorbance at 280 nm, were then pooled and subjected to semipreparative HPLC with a reversed phase ODS 5 μm column using a mobile phase of 3.5% acetonitrile and 0.05 M ammonium formate, pH 3.8, to isolate individual compounds.

The supernatant from deproteinated hemolymph of larvae or adults was acidified with acetic acid to 3 M and passed through minicolumns of Dowex-50 strong cation exchange resin (BioRad Laboratories) to remove free amines and amino acids. The effluent then was subjected to gel filtration on BioGel P-2 in 5% acetic acid. The pooled fractions of NADA conjugates then were subjected to semipreparative HPLC on an ODS 5 μm column using a mobile phase of 7% acetonitrile and 0.05 M ammonium formate, pH 3.8, for isolation of individual glucosides.

HPLC of conjugates

Hemolymph samples were deproteinated with perchloric acid (0.3 M final concentration) containing 6 ng/μl of the internal standard α-methyldopa (AMD), and the supernatant was injected onto a reversed phase ODS 5 μm sperical particle column (4.6 × 250 mm). A duplicate sample was collected in 1.2 M HCl and heated at 100°C for 10 min to hydrolyze the glucosidic bonds. The principal mobile phase consisted of 7.6% acetonitrile and 0.1 M sodium phosphate (pH 3.3) containing 1.5 mM sodium octyl sulfate (SOS) and 0.1 mM sodium EDTA. Acetonitrile sometimes was lowered to 3.8% to reverse the order of elution of the 3-*O* and 4-*O*-glucosides. The flow rate was 1 ml/min, and the catecholamines and their conjugates were quantified by passing first through a UV absorbance detector set at 280 nm and then an electrochemical detector set at 0.6 V.

GLC of sugars

Catecholamine glycosides isolated by chromatography on ion-exchange and Biogel P-2 columns and finally by HPLC, were hydrolyzed in 1.2 M HCl at 100°C for 10 min. Aliquots were dried under a stream of nitrogen, and the sugar moieties derivatized with equal parts of *bis*-trimethylsilyl trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (Regisil RC-2, Regis Chem. Co., Morton Grove, IL) and pyridine at 90°C for 30 min. The samples and derivatized standards of α- and β-D-glucose, D-galactose, and D-mannose were analyzed by gas liquid chromatography with flame ionization detection (GLC-FID) on a 3% OV 101 6 ft × 2 mm column with nitrogen as the carrier gas at 20 ml/min. The temperature was programmed from 150–250°C at 10°C/min.

Glucosidic linkage analysis

The pure conjugates were methylated with diazomethane in diethyl ether (Pau and Acheson, 1968). After removal of the solvent, they were dissolved in 1.2 M HCl and heated at 100°C for 10 h to hydrolyze the glycosidic and N-acyl substituents from the methylated dopamines (Kawasaki and Yago, 1983). The resulting 3-*O*- and 4-*O*-methyldopamines were analyzed by HPLC (Yago *et al.*, 1984). The 3-*O*-glucoside yielded 4-*O*-methyldopamine after hydrolysis, whereas the 4-*O*-glucoside yielded 3-*O*-methyldopamine.

Mass spectrometry

Fast atom bombardment mass spectrometry (FAB-MS) of the isolated catecholamine conjugates was conducted at the Midwest Center for Mass Spectrometry, University of Nebraska, on a Kratos MS-50 triple analyzer as previously described (Morgan *et al.*, 1987). Pure samples of the conjugates were dissolved in a small amount of a dithioerythritol:dithiothreitol mixture and bombarded 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, making use of cesium iodide as a standard reference. Collisionally activated decomposition (CAD) spectra were obtained by collision of the MS-I selected parent ion at 8 Kev with helium ions (50% beam reduction) to induce fragmentation with the fragment ions determined by scanning MS-II.

^{1}H-NMR

Samples of DA glucoside (0.32 mg) and NADA-glucoside (0.78 mg) were lyophilized twice from 99.8% and once from 99.98% ^2H_2O before being dissolved in 0.5 mL of 99.98% ^2H_2O in 5 mm NMR tubes for NMR analysis. Spectra were obtained with a 400 MHz Bruker WM spectrometer at about 30°C using 512 acquisitions, 16k data points, 50° pulses, 1.868 s acquisition times, and 1.952 s delays between pulses. The data were transformed after being zero-filled to 32k data points and 0.1 Hz exponential line broadening applied. Chemical shifts were referenced to sodium 3-(trimethylsilyl)propionate in ^2H_2O at 30°C taken as 0 ppm.

RESULTS

Radiolabeling of conjugates

Two metabolites labeled by both ^{14}C-tyrosine and ^{14}C-glucose were visualized on the autoradiograms from the hemolymph of different pharate pupae (Fig. 1). One compound (spot # 2) was identified as tyrosine glucoside as previously reported to occur in fifth stadium larvae of *M. sexta* (Kramer *et al.*, 1980). A second unidentified metabolite (spot # 3) that was labeled by both precursors, was located adjacent to tyrosine (spot # 1). This compound was extracted and hydrolyzed in 1.2 M HCl and the hydrolysate analyzed by HPLC. NBAD was identified as the aglucone of this conjugate. Another metabolite (spot # 4) was labeled only by ^{14}C-tyrosine and was identified as free NBAD. Other minor tyrosine metabolites (spots # 5 and 6) were not identified. Therefore, the major catecholamine conjugate in pharate pupal hemolymph was indicated to be a glucoside conjugate of NBAD.

HPLC of catecholamine conjugates

Comparison of the response ratios between electrochemical detection at 0.6 V and absorbance at 280 nm of unknown and standard compounds was useful for characterizing the catecholamine conjugates and monitoring their purity during HPLC. The response ratios (nanoamps/absorbance) were more than 10-fold higher for the free catecholamines than for the glucosides, and the ratio of the 3-*O*-glucosides was found to be more than three-fold higher than that of the 4-*O*-glucosides. This trend also occurred for 3-*O*-methyl and 4-*O*-methyl dopamines. The unidentified compounds resolved by ion exchange and gel filtration were separated by HPLC, the eluate fractions collected and hydrolyzed, and the free catechols again analyzed by HPLC. Disappearance of the parent compound and appearance of a known catecholamine after hydrolysis provided preliminary evidence for a conjugate. This technique identified the conjugates of DA, NADA, and NBAD on the chromatograms of hemolymph extracts. These fractions were collected for further structure elucidation.

Sugar analysis

The retention times of the derivatized sugar moieties were compared with those of silyated monosaccharide standards. After hydrolysis, NBAD, NADA, and DA conjugates and the synthetic glucosides all gave silylated monosaccharide retention times identical to the D-glu-

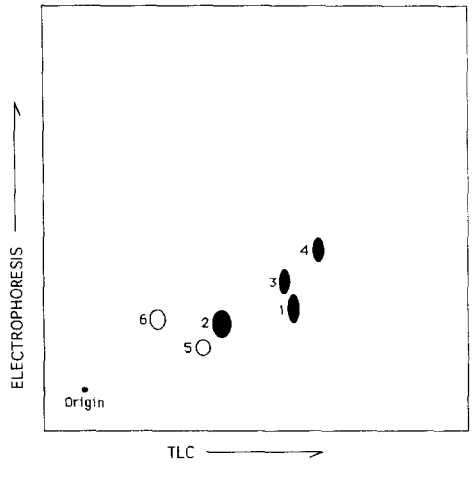


FIGURE 1. Reconstructed autoradiogram of a two-dimensional electrophoresis—TLC map of extracts from *M. sexta* pharate pupal hemolymph injected as wandering larvae with either ^{14}C-U-glucose or ^{14}C-U-tyrosine. The ^{14}C-labeled compounds extracted from the cellulose layer and analyzed by HPLC were: (1) tyrosine; (2) tyrosine glucoside; (3) NBAD glucoside; (4) NBAD; and (5, 6) minor unidentified tyrosine metabolites. Solid spots were labeled only by ^{14}C-tyrosine; cross-hatched spots were labeled by both ^{14}C-tyrosine and ^{14}C-glucose.

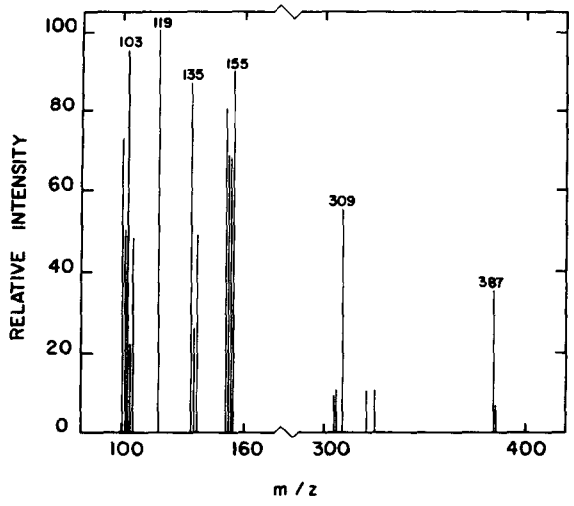


FIGURE 2. FAB mass spectrum of NBAD glucoside I (M/z 387 = M + H$^+$) isolated from hemolymph of newly ecdysed pupae of *M. sexta*.

cose standard (6.84, 7.74 min), but not to those of two other sugars, D-galactose (6.54, 6.98 min) and D-mannose (6.06, 7.04 min). Coinjection of the derivatized conjugate hydrolysate and the glucose standard gave only two symmetrical peaks corresponding to α- and β-D-glucose silylated derivatives. When the quantities of the catecholamine aglucones in the hydrolysates were analyzed and compared with the quantities of glucose released by hydrolysis, a molar ratio of approximately one was observed. Therefore, all catecholamine conjugates in *M. sexta* examined were confirmed to be glucosides.

Mass spectrometry

FAB-MS was used to determine the molecular masses of catecholamine conjugates isolated from hemolymph. The high mass ion m/z 387 corresponded to the molecular ion (M + H) of the proposed structure of NBAD glucoside (Fig. 2). Other ions in this spectrum arise from the FAB matrix. Dopamine and NADA conjugates exhibited ions at m/z 316 and 358, respectively, in their FAB-MS spectra (data not shown) that corresponded to M + H molecular ions of dopamine glucoside and NADA glucoside.

High resolution mass measurement of the NBAD conjugate also was determined by peak matching and using cesium iodide as a reference standard. Of all the combinations of C, H, N and O that yield masses within 10 ppm of the observed m/z (387.1774), the composition $C_{17}H_{27}N_2O_8$ was in closest agreement (within 2 ppm) and corresponds to the elemental composition of NBAD glucoside.

The CAD spectrum of the (M + H)$^+$ NBAD conjugate contained two major fragment ions of m/z 225 and 137 (Fig. 3A). The ion of m/z 225 can be accounted for by the loss of glucose (as $C_6H_{10}O_5$) from the intact cation and is typical of compounds containing glycosidic linkages (Crow *et al.*, 1986; Tomer, 1989). Further loss of β-alanylamide gives rise to the ion of m/z 137. The less abundant fragment ions of m/z 267 and 253 are due to cleavages across the glucose ring (Fig. 4A) (Crow *et al.*, 1984; Tomer, 1989).

The CAD spectrum of the (M + H$^+$) ion of the dopamine conjugate isolated from pupal hemolymph showed three fragments at m/z 299, 154 and 137 (Fig. 3B). The fragment at m/z 299 is attributable to the loss of NH_3 from the parent ion. The m/z 154 ion results from the loss of glucose (as $C_6H_{10}O_5$) from the intact cation. The combined loss of both glucose and NH_3 gives rise to the m/z 137 ion. Minor ions corresponding to cleavages across the glucose ring are observed at m/z 196 and 182 are also present (Fig. 4B). The CAD spectra are therefore consistent with the proposed structures of NBAD and dopamine glucosides.

Position of the glucosidic bonds and developmental profiles

Hemolymph collected from pharate or newly ecdysed pupae contained both major (peak 7, Fig. 5) and minor (peak 6) glucosides of NBAD as well as free NBAD (peak 9). The relative amounts of the two NBAD conjugates were approximately 98% and 2%, respectively (Table 1). To determine the position of glucosidic linkage on the catechol hydroxyl groups, samples of purified NBAD, NADA, and dopamine glucosides were methylated, hydrolyzed to release the *O*-methyl dopamine products, and analyzed by HPLC (Kawasaki and Yago, 1983; Yago *et al.*, 1984). The major NBAD glucoside in pharate or newly ecdysed pupal hemolymph released 4-*O*-methyl dopamine, indicating a 3-*O*-glucosidic linkage. The minor NBAD glucoside released 3-*O*-methyldopamine and, therefore, is a 4-*O*-glucoside. The dopamine glucoside from pupal hemolymph was shown to have the 3-*O*-glucosidic bond by the same procedure.

In similar extracts prepared from the hemolymph of newly ecdysed fifth stadium larvae, two conjugates of NADA predominated. The relative amounts of major and minor conjugates of NADA were 86 and 14%, respectively (Table 1). The major conjugate was determined to be NADA 3-*O*-glucoside, whereas the minor conjugate was identified as NADA 4-*O*-glucoside. A low concentration of NBAD conjugate also was detected in larval hemolymph. Conversely, NADA 4-*O*-glucoside predominated in the hemolymph of pharate and newly eclosed adults (~67%), with the remaining one third of NADA conjugated in the 3-*O*-position (Fig. 5, Table 1). Low to trace amounts of DA and NBAD 3-*O*-glucosides were also present in adult hemolymph.

^{1}H-NMR

The chemical shifts and observed coupling constants, where discernible, for the major ^{1}H-resonances of the NADA 3-*O*-glucoside fraction are compared to those for NADA and DA 3-*O*-glucoside (Table 2, Structure 1). The spectrum of DA 3-*O*-glucoside (not shown) indicated considerable distortion of aromatic ring coupling patterns apparently due to second order effects from the near overlap of the 5H and 6H resonances. In the

spectrum of NADA 3-*O*-glucoside, the overlap of those resonances appeared complete since the single resonance at 6.885 ppm integrated two hydrogens relative to the C(=O) CH_3 hydrogens and all ring coupling collapsed. In that case the aromatic ring hydrogens would form an ABX system with ν_A-$\nu_B = 0$ (ν_{5H}-$\nu_{6H} = 0$) similar to that described by Bovey (1988), in which essentially no coupling was observable. Given that situation, the overall agreement in chemical shifts and coupling constants among NADA, NADA 3-*O*-glucoside and DA 3-*O*-glucoside (Table 2) corroborate the HPLC and chemical evidence that the major glycoside conjugate isolated from *M. sexta* pharate larvae was the 3-*O*-glucoside derivative of NADA.

Some minor resonances were also apparent in the spectrum of the NADA 3-*O*-glucoside sample. The relative intensities of NADA 3-*O*-glucoside peaks compared with the total hydrogen intensity of the sample indicated that the metabolite represented about 75% of the sample. Comparison of the chemical shift and coupling constants most prominent of the minor peaks (~15% of the total intensity) to those of known

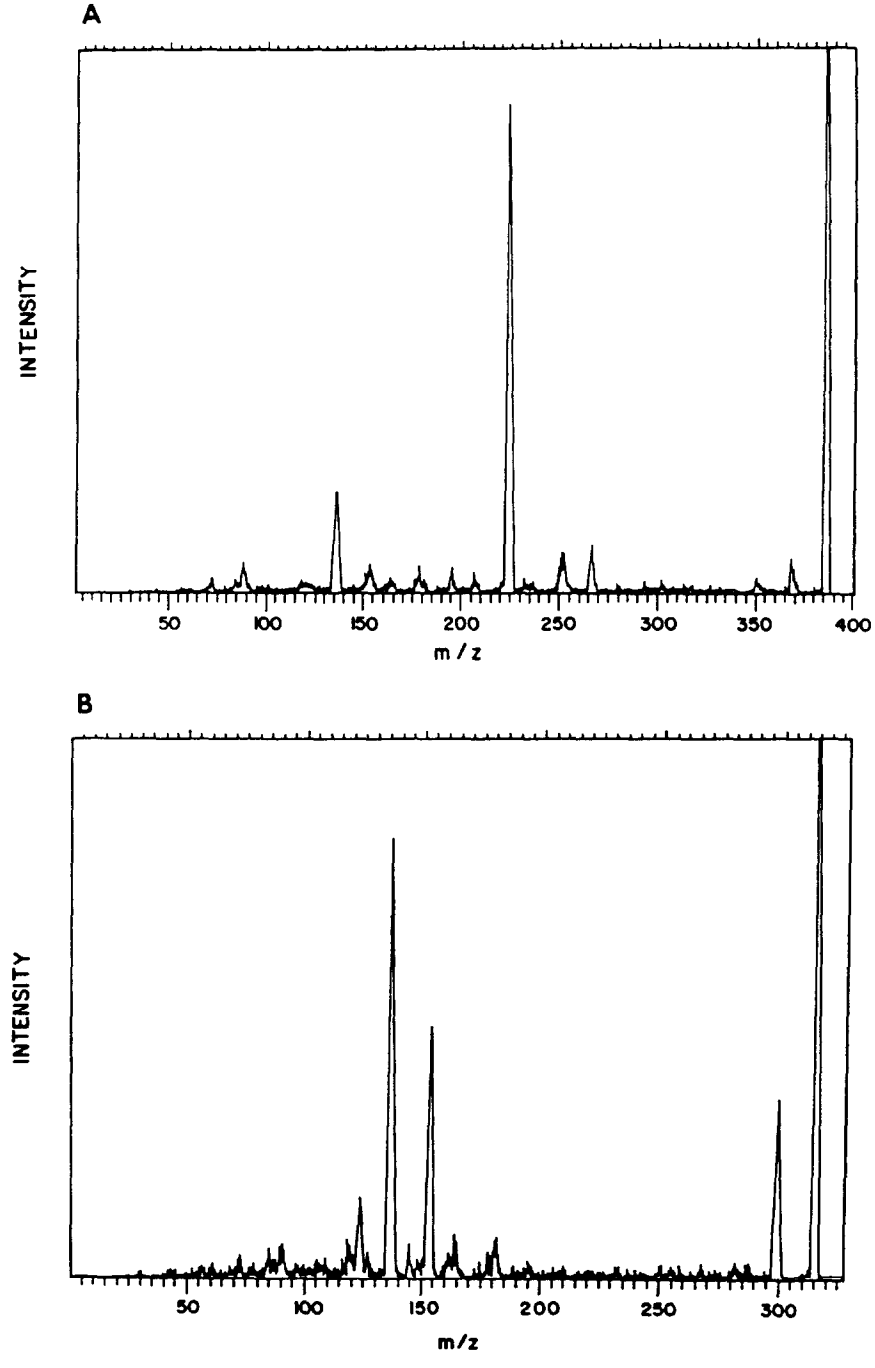


FIGURE 3. Collisionally activated decomposition (CAD) spectra of (A) NBAD glucoside and (B) DA glucoside isolated from the hemolymph of newly ecdysed pupae of *M. sexta*.

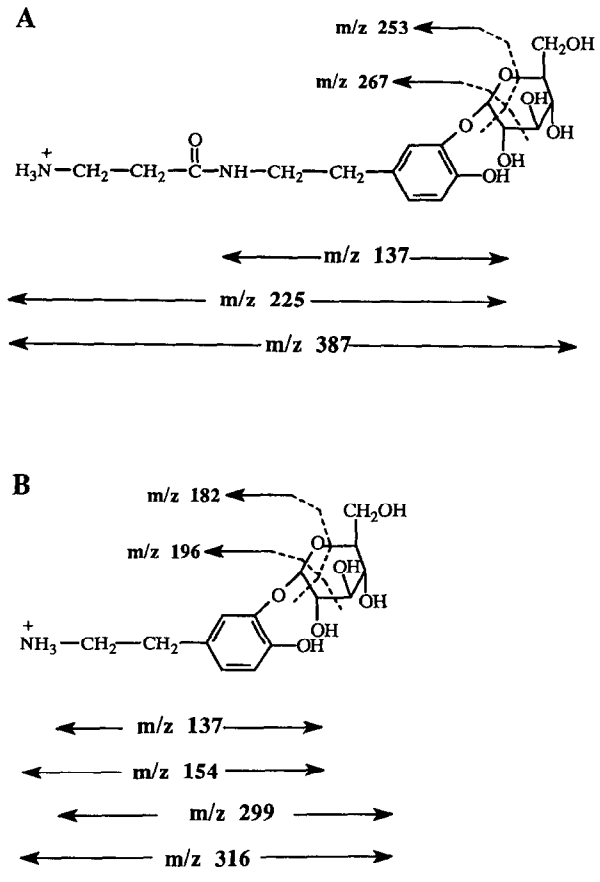


FIGURE 4. Interpretation of CAD spectra (Fig. 3) of (A) NBAD glucoside and (B) DA glucoside isolated from the hemolymph of newly ecdysed pupae of *M. sexta*.

catecholamine glucosides (Mueller *et al.*, 1993) strongly suggested that they represented NADA 4-*O*-glucoside. The remaining ~10% of the sample also appeared to be from a catecholamine, but it has not yet been identified.

DISCUSSION

Catecholamine precursors for cuticle sclerotization in *M. sexta* vary with developmental stage and the properties of the cuticle secreted in different regions of the exoskeleton (Hopkins *et al.* 1982, 1984; Hopkins and Kramer, 1991). N-Acetyldopamine, the major catecholamine in larval hemolymph, increases to peak concentrations as a conjugate in pharate and newly ecdysed larvae and then decreases to very low levels as the cuticle sclerotizes. A NADA conjugate(s) was the predominant catecholamine in adult hemolymph during cuticle stabilization, but lesser amounts of dopamine and NBAD conjugates were present in both larval and adult hemolymph. In larvae, NBAD was found to be associated with the hardening of such dark cuticular structures as the mandibles, whereas dopamine appeared to be a precursor for black melanic pigments (Hopkins *et al.*, 1984). However, hemolymph of pharate and newly ecdysed pupae accumulated very high concentrations (~4.0 mM) of an NBAD conjugate(s) and low levels of dopamine and NADA conjugates. These conjugates were identified tentatively as β-glucosides based on hydrolysis by a β-glucosidase and the labeling of the pupal NBAD conjugate with ^{14}C-glucose. The present study of the isolation and characterization of the catecholamine conjugates from hemolymph has now provided unequivocal evidence of their chemical structures. In all cases, glucose was the only sugar released by acid hydrolysis from pure samples of NADA, NBAD and dopamine conjugates. Glucosylation of the catechol hydroxyl groups was found to occur primarily on the 3-*O*-position in NADA, NBAD and dopamine in larval and pupal hemolymph. However, the major NADA glucoside in adult hemolymph had the 4-*O*-linkage. FAB-MS confirmed the correct molecular masses of the glucosides, as did the CAD spectra of the fragmentation ions. In a companion study, ^{1}H- and ^{13}C-NMR analyses provided additional evidence that the major NBAD conjugate and the dopamine conjugate isolated from pupal hemolymph are β-3-*O*-glucosides (Mueller *et al.*, 1993).

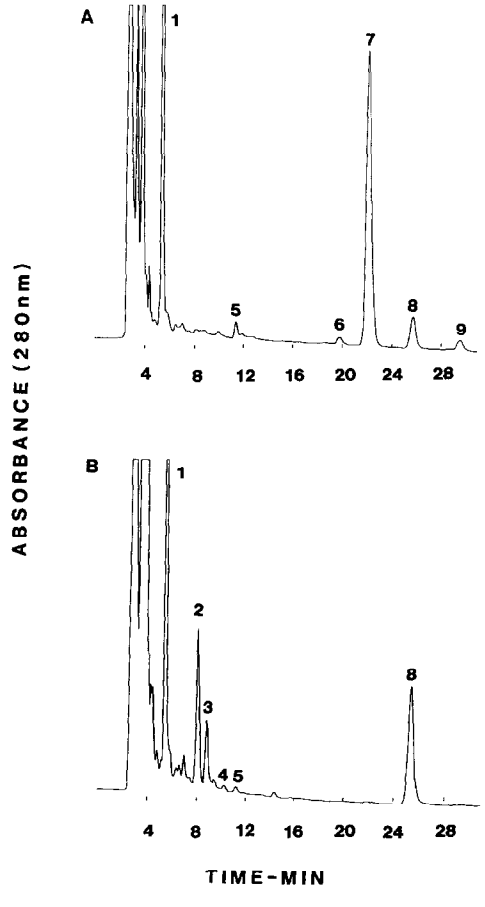


FIGURE 5. HPLC of *M. sexta* hemolymph extracts from (A) pharate pupa within 24 h of ecdysis; (B) pharate adult within 12 h of eclosion. Peak numbers are: (1) tyrosine; (2) NADA 4-*O*-glucoside; (3) NADA 3-*O*-glucoside; (4) NADA, (5) dopamine 3-*O*-glucoside; (6) NBAD 4-*O*-glucoside; (7) NBAD 3-*O*-glucoside; (8) tryptophan; and (9) NBAD. HPLC with 7.6% acetonitrile mobile phase and absorbance detection (280 nm) as described in Materials and Methods.

TABLE 1. Catecholamine glucosides (μM) in the hemolymph of larval, pupal and adult stages of *Manduca sexta**

Stage†	Catecholamine	Glucoside 3-*O*-Glc	Glucoside 4-*O*-Glc	Unconjugated	Total after‡ hydrolysis
Larva	DA	32 ± 4	N.D.	8 ± 1	122 ± 16
	NADA	454 ± 50	89 ± 8	19 ± 2	554 ± 112
	NBAD	50 ± 9	TR	23 ± 2	113 ± 16
Pupa	DA	100 ± 13	N.D.	2 ± 1	441 ± 78
	NADA	N.D.	N.D.	TR	36 ± 13
	NBAD	3313 ± 114	81 ± 5	104 ± 28	3904 ± 508
Adult	DA	~14	N.D.	~2	51 ± 12
	NADA	170 ± 54	341 ± 115	2 ± 1	422 ± 141
	NBAD	TR	N.D.	2 ± 1	9 ± 2

*Means of two animals ± $\frac{1}{2}$ range, N.D. = not determined, TR = trace (< 1 μM for free catecholamine; < 10 μM for conjugates).

†Hemolymph from newly ecdysed 5th instar larvae or pharate pupal and adult stages sampled approximately 12 h before ecdysis.

‡Approximately 10% or less of the amide bonds of NBAD and NADA are hydrolyzed in 1.2 M HCl at 100°C for 10 min, therefore accounting for the high free dopamine concentrations in the hydrolysates.

Comparison of ^{1}H-NMR parameters for the catecholamine metabolite isolated from pharate larvae of *M. sexta* with those of appropriate known glucoside conjugates indicated that the compound was NADA 3-*O*-glucoside, in agreement with HPLC, FAB-MS and chemical evidence. The aliphatic region of this metabolite, however, showed a multiplet for H-8 in contrast to a more normal triplet pattern for the same hydrogens in NADA and DA 3-*O*-glucoside and a significant downfield shift for the acetyl hydrogens compared with those of NADA. The appearance of a multiplet (probably an ABX_2 coupling pattern) for H-8 was similar to that seen for NBAD 3-*O*-glucoside, but not observed in either NBAD or in DA 4-*O*-glucuronoside (Mueller *et al.*, 1993). The appearance of nonequivalent H-8 hydrogens in NBAD 3-*O*-glucoside was attributed to an interaction, perhaps

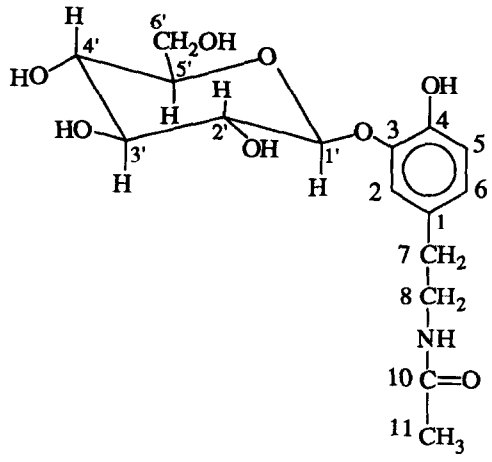
TABLE 2. Comparison of chemical shifts, (δ) and coupling constants (Hz) for NADA, NADA 3-*O*-glucoside and DA 3-O-glucoside

Hydrogen	DA glucoside* δ, ppm	NADA glucoside δ, ppm	NADA* δ, ppm
A. Aromatic ring hydrogens			
2-H	7.089	7.033	6.780
	(2.10)	(n.d.)†	(1.93)
5-H	6.96e	6.885	6.846
	(7.1e)	(n.d.)	(8.07)
6-H	6.93e‡	(*c.* 6.88)	6.687
	(7.5e)	(n.d.)	(8.08)
	(2.1e)	(n.d.)	(1.94)
B. Aliphatic C-H			
7-H	2.928	2.732	2.668
	(7.22)	(6.80)	(6.85)
8-H	3.249	*c.* 3.398	3.356
	(7.08)	(multiplet)	(6.84)
$C(=O)CH_3$		1.924	1.748
C. Glucoside C-H			
1-H	5.082	5.041	
	(7.10)	(7.54)	
6-Ha	3.970	3.942	
	(12.40)	(12.10)	
	(2.06)	(2.06)	
6-Hb	3.779	3.774	
	(12.31)	(12.43)	
	(6.20)	(5.63)	
2-,3- and	*c.* 3.64	*c.* 3.62	
5-H	(n.d.)	(n.d.)	
4-H	*c.* 3.52	*c.* 3.52	
	(n.d.)	(n.d.)	

*See Structure 1. From Mueller *et al.* (1993).

†N.D.: not determinable by inspection.

‡An "e" after a value indicates that it was estimated from peak positions that had some intensity distortion.



STRUCTURE I

H-bonding, between the glucosyl and amide groups which restricted rotation about the N-C_8 bond. Thus, it appears that a similar interaction occurs in NADA 3-*O*-glucoside. This conclusion is strengthened by the relatively large downfield shift of the acetyl group hydrogens compared to those of NADA (Table 2) as might be expected for hydrogen bonding to the carbonyl oxygen in the 3-*O*-conjugate. Apparently, N-acetylation of the terminal aliphatic amine of dopamine is sufficient to bring about an interaction with the 3-*O*-glucosyl group. A similar interaction is stronger and the motion is even more restricted in NBAD 3-*O*-glucoside.

β-Glucoside conjugates of catecholic precursors for quinone sclerotizing agents have been found in a number of insect species, including tanning agents of cockroach and mantid oothecal proteins (Brunet and Kent, 1955a, b; Kent and Brunet, 1959; Kawasaki and Yago, 1983; Yago and Kawasaki 1984; Yago *et al.* 1984; Tomino, 1965). As suggested by Brunet (1980), catechols can be sequestered by conjugation of a phenolic group with glucose. Thus, they are protected from premature oxidation to quinones by the ubiquitous phenoloxidases in insect hemolymph and tissues. Accumulation and storage in insect hemolymph of both tyrosine and catecholamines as metabolically inactive conjugates commonly occur prior to ecdysis and cuticle sclerotization (Brunet, 1980; Hopkins and Kramer, 1991, 1992). Conjugation may also be a mechanism that blocks the hormonal or neural effects of free catecholamines.

Synthesis of phenolic glucosides by glucosyltransferases and their release by *β*-glucosidases appear to be tightly regulated by the morphogenetic hormones in synchrony with cuticle secretion and ecdysis. Phenol *β*-glucosyltransferases occur in the tissues of larval *M. sexta*, with the highest activities in fat body and labial gland (Ahmad and Hopkins, 1992). We have observed in larval fat body UDP-glucosyltransferases that glucosylate tyrosine, NBAD and NADA (Ahmad *et al.*, unpublished data). The specificity for glucosylation of the ring hydroxyls may be related to the characteristics of the glucosyltransferases that are developmentally synthesized. Whereas glucosylation of NBAD in the pharate pupa occurred mainly on the 3-hydroxyl group, NADA glucosylation was less specific with 75–85% of NADA 3-*O*-glucoside and 15–25% of 4-*O*-glucoside in larval hemolymh. The reverse relationship occurred in the pharate adult in which NADA 4-*O*-glucoside was about twofold greater concentration than the 3-*O*-glucoside. The biological significance of the position of glucosylation is unknown, but may be related to the sequential storage and release of the glucosides of tyrosine and its catecholamine metabolites. Glucosyltransferases with different substrate specificities may therefore be involved in conjugating these sclerotization precursors. Likewise, the *β*-glucosidases responsible for releasing the aglucones may also have different specificities for the 3-*O*- and 4-*O*-glucosides necessary for regulating the availability of substrates for phenoloxidases. A more detailed understanding of these precursors and enzymes essential for sclerotization of the exoskeleton and their regulation during development could provide selective targets for biorational insect control agents.

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