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Catechols Involved in Sclerotization of Cuticle and Egg Pods of the Grasshopper, *Melanoplus sanguinipes,* and Their Interactions With Cuticular Proteins

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***N*-Acetyldopamine (NADA) is the major catechol in the hemolymph of nymphal and adult grasshoppers, *Melanoplus sanguinipes* (F.), and mainly occurs as an acid-labile conjugate indicated to be a sulfate ester. Its concentration increases in last instar nymphs and peaks during adult cuticle sclerotization. Dopamine (DA), the precursor of NADA and melanic pigments, is about 10 times lower in concentration than NADA, but shows a similar pattern of accumulation. NADA also predominates in cuticle, but its concentration is lowest during the active period of sclerotization, reflecting its role as a precursor for quinonoid tanning agents. Two other catechols, 3,4-dihydroxybenzoic acid (DOBA) and 3,4-dihydroxyphenylethanol (DOPET), also occur in hemolymph and cuticle, and their profiles suggest a role in cuticle stabilization. Solid-state NMR analysis of sclerotized grasshopper cuticle (fifth instar exuviae) estimated the relative abundances of organic components to be 59% protein, 33% chitin, 6% catechols, and 2% lipid. About 99% of the catechols are covalently bound in the cuticle, and therefore are involved in sclerotization of the protein-chitin matrix. To determine the types of catechol covalent interactions in the exocuticle, samples of powdered exuviae were heated in HCl under different hydrolytic conditions to release adducts and cross-linked products. 3,4-Dihydroxyphenylketoethanol (DOPKET) and 3,4-dihydroxyphenylketoethylamine (arterenone) are the major hydrolysis products in weak and strong acid, respectively, and primarily represent NADA oligomers that apparently serve as cross-links and filler material in sclerotized cuticle. Intermediate amounts of norepinephrine (NE) are released, which represent *N*-acetylnorepinephrine**

Abbreviations used: DA = 3,4-dihydroxyphenylethylamine (dopamine); DOBA = 3,4-dihydroxybenzoic acid; DOBAlc = 3,4-dihydroxybenzyl alcohol; DOPET = 3,4-dihydroxyphenylethanol; DOPKET = 3,4-dihydroxyphenylketoethanol; NADA = *N*-acetyldopamine; NANE = *N*-acetylnorepinehrine; NBAD = *N*-β-alanyldopamine; NE = norepinephrine.

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(NANE), a hydrolysis product of NADA bonded by the b-carbon to cuticular proteins and possibly chitin. Small quantities of histidyl-DA and histidyl-DOPET ring and side-chain C-N adducts are released by strong acid hydrolysis. Therefore, grasshopper cuticle appears to be sclerotized by both *o*-quinones and *p*-quinone methides of NADA and dehydro-NADA, which results in a variety of C-O and C-N covalent bonds linked primarily through the side-chain carbons of the catechol moiety to amino acid residues in cuticular proteins. The primary catechol extracted from both the female accessory glands/calyx and the proteinaceous frothy material of the egg pod is DOBA, which also commonly occurs in cockroach accessory glands and oothecae, presumably as a tanning agent precursor. 3,4-Dihydroxyphenylalanine (DOPA) was also detected in extracts of the accessory glands/calyx of grasshoppers, and may serve as a precursor for DOBA synthesis. Arch. Insect Biochem. Physiol. 40:119–128, 1999.

Key words: insect cuticle; sclerotization; catecholamines; catechols; *N*-acetyldopamine; grasshopper; *Melanoplus sanguinipes*; egg pod; ootheca

INTRODUCTION

The newly secreted cuticular exoskeleton of insects must be selectively stiffened and hardened by sclerotization processes to provide the necessary mechanical properties for locomotion and protection of each developmental stage. Sclerotization of cuticle partly involves the formation of covalent bonds between the quinone metabolites of two *N*-acyldopamines, NADA and *N*-β-alanyldopamine (NBAD), and functional groups of histidine and possibly other amino acid residues of cuticular proteins (Hopkins and Kramer, 1992; Andersen et al., 1996; Xu et al., 1997). The cuticle becomes increasingly dehydrated, dense, and hydrophobic as catechol metabolites replace water and modify the structural proteins by forming numerous non-covalent and covalent bonds. Although insects as a group produce a wide variety of sclerotized cuticles that differ in chemical and physical properties according to their functional adaptations, little is known about the molecular organization of any of these because of the complexity of their structures and their resistance to degradation for structural analysis.

The sclerotization of grasshopper cuticle has been studied mainly in the migratory locusts. When radiolabelled tyrosine was injected into *Schistocerca gregaria* a few hours before ecdysis, radioactivity accumulated in the sclerotized outer regions of the cuticle, indicating that tyrosine metabolites were important in sclerotization (Karlson and Schlossberger-Raecke, 1962; Schlossberger-Raecke and Karlson, 1964). *N*-Acetyldopamine (NADA), a metabolite of tyrosine, was first identified as a precursor for sclerotization of puparial cuticle of blowfly, *Calliphora erythrocephala*, and cuticle of the desert locust, *S. gregaria* (Karlson and Sekeris, 1962; Karlson and Herrlich, 1965). Andersen and coworkers have extensively studied sclerotization of the cuticle of two migratory locusts *S. gregaria* and *Locusta migratoria* (Andersen, 1990; Andersen et al., 1996). Catechol metabolites released from their cuticles by different conditions of acid hydrolysis were derived mainly from NADA, indicating its importance as a sclerotization precursor (Andersen, 1970, 1971; Andersen and Barrett, 1971). Dimers and oligomers of NADA or its metabolites linked primarily by carbon-oxygen bonded benzodioxan structures apparently function as cross-links and filler material in the protein matrix of the locust exocuticle (Andersen et al., 1992a). Other sclerotization mechanisms involving acid stable carbon-nitrogen adducts and cross-links have been studied mainly in model reactions in vitro using locust cuticular enzymes (Andersen et al., 1992 a,b), and very little is known concerning cuticle tanning in other species of grasshoppers.

This study reports for the first time a comprehensive analysis of the catechol precursors of cuticle tanning agents in an orthopteran insect represented by the grasshopper, *Melanoplus sanguinipes,* their concentrations in hemolymph and cuticle during the course of sclerotization, and an inventory of the types of covalent bonds be-

tween catechols and proteins that give new insights into the molecular organization of sclerotized cuticle. The catechols that may serve as precursors for egg pod tanning or other protective functions also were analyzed in the female accessory glands and their frothy proteinaceous secretions that form the protective covering of eggs buried in soil.

MATERIALS AND METHODS

Insects

The experimental insects were from a nondiapausing strain of *M. sanguinipes* obtained from the USDA/ARS Rangeland Insects Laboratory (Sidney, MT) and were reared by the methods of Henry (1985). They were fed wheat seedlings or romaine lettuce and ground guinea pig pellets and held at 28°C with a 16:8 LD photoperiod. Last instar nymphs and adults were selected from the cultures at different stages of development for hemolymph and cuticle sampling. Fifth instar exuviae were collected from the rearing cages shortly after adult ecdysis.

Hemolymph, Tissue, and Cuticle Preparations

Hemolymph was collected in calibrated capillary pipets from a cut tibia of the hind leg while slight pressure was applied to the abdomen. Known volumes (2–12 μl) were pipetted into cold 10% HCl (100 or 200 μl) and kept frozen at –20°C until analyzed. Abdominal cuticle was dissected and cleaned of tissue by scraping with a small spatula and washing with distilled water. The cleaned cuticles then were blotted dry, weighed, and homogenized in cold 10% HCl using ground glass tissue grinders. The hemolymph and cuticle homogenate samples in acid were centrifuged to remove protein and insoluble material, and the supernatants recovered for analysis. Accessory glands and calyx were dissected from females and egg pod froth was collected shortly after oviposition. These were homogenized in cold 10% HCl, and the supernatants processed for catechol analysis. To release catechols from their conjugates, 0.1-ml aliquots were heated at 100°C for 10 min. Unheated aliquots also were analyzed to determine amounts of free catechols. For enzyme studies of conjugates, hemolymph was extracted with cold methanol in a tissue grinder, centrifuged, and aliquots of the supernatant dried under nitrogen and incubated with sulfatase (Sigma, St. Louis, MO). Fifth instar exuviae representing fully sclerotized cuticle also were analyzed by grinding the cuticle into a fine powder in liquid nitrogen and then heating the samples in 10% HCl at 100°C for 30 min to release weakly bound catechols. For HPLC analysis of catechol-amino acid adducts, samples of powdered exuviae were hydrolyzed in 6 M HCl containing 5% phenol at 110°C for 24 h, and the catechol-containing products recovered on alumina (Xu et al., 1997).

HPLC Analysis of Catechols

Catechols were purified from the HCl extracts by adsorption on alumina as previously described (Hopkins et al., 1984). HPLC analysis of catechols was done according to the methods of Hopkins et al. (1984), Morgan et al. (1987), and Xu et al. (1997). Catechol standards of DA, DOBA, DOPA, NADA, and NE were from Sigma (St. Louis, MO); DOPET was a gift from Hoffman LaRoche, Nutley, NJ; DOPKET and arterenone were gifts from Dr. S.O. Andersen, University of Copenhagen, Denmark.

Solid-State NMR

Fifth instar exuivae were ground to a fine powder in liquid nitrogen, dried, and then analyzed for protein, chitin, catechol, and lipid composition by solid-state 50.3-MHz ^{13}C NMR according to Schaefer et al. (1987), Kramer et al. (1991), and Merritt et al. (1996).

RESULTS

Catechols in Hemolymph

NADA was the predominant catechol in grasshopper hemolymph in last instar nymphs and adults (Fig. 1). It increased in concentration during development of last instar nymphs and reached a peak in newly ecdysed adults at approximately four times the nymphal level. NADA levels remained high through 3-h postecdysis as sclerotization occurred and then decreased to near preecdysial levels by 24 h. Dopamine, the precursor for NADA and for melanin biosynthesis, was about 10-fold lower in concentration than NADA at the same time intervals but also showed a more than fivefold increase in concentration during cuticle sclerotization and pigmentation (Fig. 1). Other catechols also found in substantial concentrations in grasshopper hemolymph were DOPET and DOBA. Both increased during nymphal development and peaked in newly ecdysed adults (Fig. 2). However, unlike NADA,

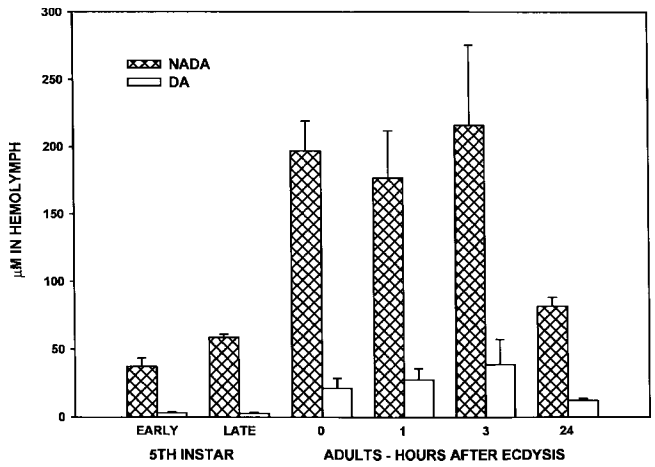

Fig. 1. Total concentrations of NADA and DA (free and conjugated) in extracts of the hemolymph of *Melanoplus sanguinipes* during fifth instar nymphal development and adult cuticle sclerotization. Means ± S.E., n = 3.

their levels decreased rapidly by 1 h after ecdysis and remained low during cuticle sclerotization. NBAD was not detected in the hemolymph or cuticle extracts.

All catechols found in hemolymph primarily occurred as acid labile conjugates. Very low concentrations of free catechols were observed in cold acid extracts, but heating the extracts at 100°C for 10 min released the bound catechols. Incubating the catechol conjugates with sulfatase released NADA and DOBA in quantities similar to the acid hydrolyzed samples.

Catechols in Cuticle

NADA was the predominent catechol extracted with cold 10% HCl from adult abdominal cuticle (Fig. 3). Its concentration remained relatively low for the first 24 h during the main period of cuticle sclerotization. However, after 72 h when the cuticle was fully sclerotized, extractable NADA had accumulated and increased eightfold over the level at 24 h. Dopamine also was detected in the cuticle extracts but at 8 to 10 times lower amounts than NADA (data not shown). DOBA and traces of DOPET were extracted with cold HCl from cuticle at all intervals after ecdysis. DOBA concentrations were similar to NADA levels during cuticle sclerotization and increased about twofold (0.03 nmol/mg) in fully sclerotized cuticle (data not shown). Concentrations of the catechols generally increased from 2 to 4 times after the cold acid extracts were heated for 10 min at 100°C, indicating that these compounds were partially deposited in cuticle as acid-labile conjugates.

Catechols in Fifth Instar Exuviae

When powdered exuviae were ground in cold 10% HCl and the supernatant was heated at 100°C for 10 min, NADA predominated in the extracts (Fig. 4). Intermediate amounts of DOPKET and lower concentrations of NANE also were released by heating, the latter calculated from the NE present in the extracts. NANE is readily hydrolyzed to NE under these conditions (Morgan et al., 1987). However, when exuviae were heated directly in 10%

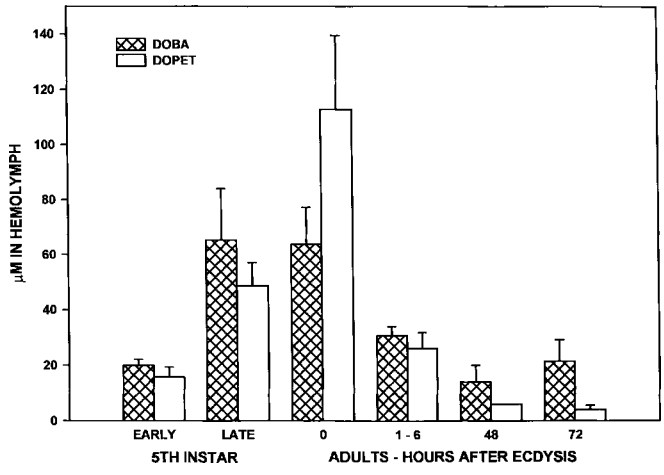

Fig. 2. Total concentrations of DOBA and DOPET (free and conjugated) in extracts of the hemolymph of *Melanoplus sanguinipes* during fifth instar nymphal development and adult sclerotization. Means ± S.E., n = 3.

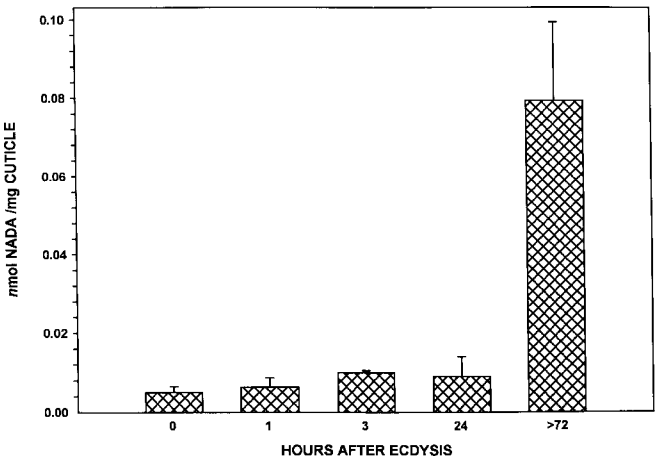

Fig. 3. Total concentrations of NADA (free and conjugated) in extracts of the cuticle of adult *Melanoplus sanguinipes* during sclerotization. Means ± S.E., n = 3.

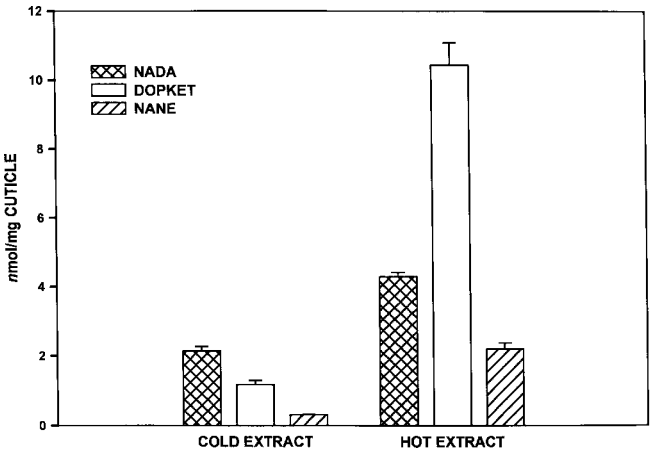

Fig. 4. Concentrations of NADA, DOPKET, and NANE released from *Melanoplus sanguinipes* fifth instar exuviae by cold and hot 10% HCl. See Materials and Methods for details. Means ± S.E., n = 3.

HCl for 30 min, DOPKET was the predominant catechol released, increasing about 10-fold in concentration, whereas NANE increased about sixfold and NADA twofold (Fig. 4).

When powdered fifth instar exuviae and wings from *M. sanguinipes* were heated in 6 M HCl at 110°C for 24 h, the major catechol released was arterenone and lesser amounts of an NE-phenol adduct, DA, and both histidyl-DA and histidyl-DOPET adducts were present (Fig. 5, Table 1). Data from the HPLC analyses of hydrolysates of larval head capsule and pupal cuticle exuviae of the tobacco hornworm, *Manduca sexta*, are shown in Table 1 for comparison, because the catechols and their adducts were identified first from the pupal cuticle hydrolysates of this species (Xu et al., 1997). The clear stiff cuticles from the grasshopper wings and larval exuviae and the head capsule exuviae of the hornworm larvae show close similarities in the relative amounts of the catechol-containing compounds. Arterenone was the primary catechol released by strong acid hydrolysis of these cuticles (56–73%), whereas the stiff dark pupal exuvial cuticle of *M. sexta* had six-fold less of this compound. In contrast, the pupal cuticle was high in DA, NE-phenol adduct, and the histidyl-DA adducts.

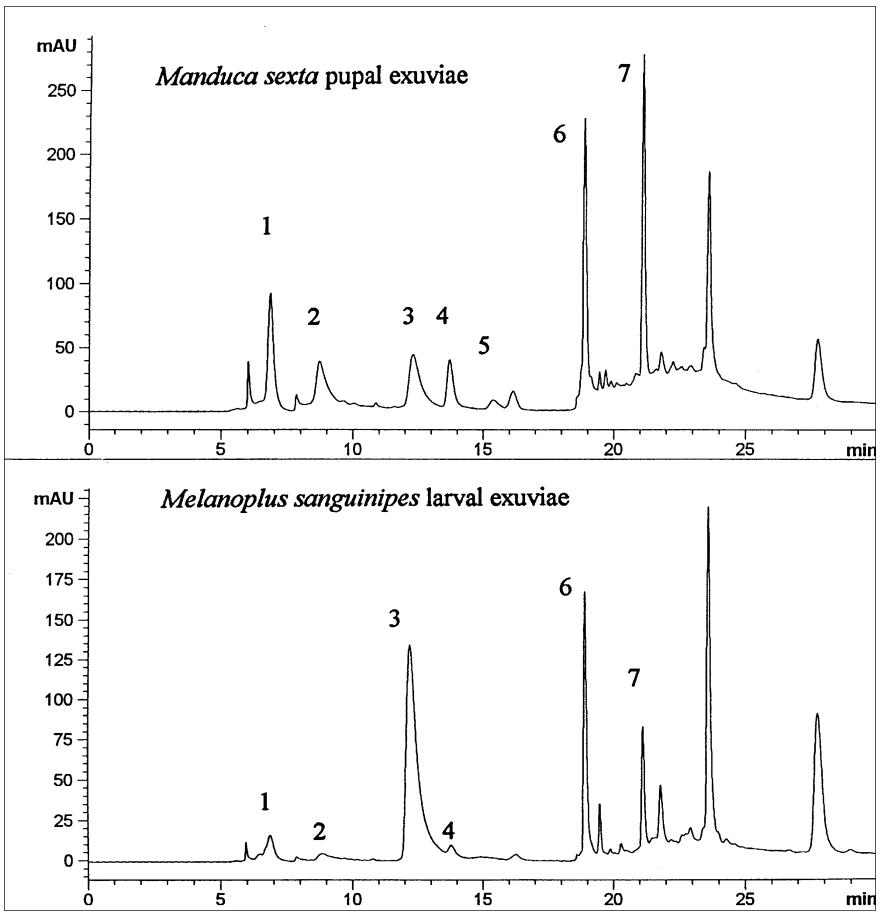

Fig. 5. HPLC analysis of catechol-containing compounds released by hydrolysis of *Manduca sexta* pupal exuviae and *Melanoplus sanguinipes* fifth instar larval exuviae in 6 M HCl at 110°C for 24 h. 1: 7-*N*τ-histidyl-DA; 2: 6-*N*τ-histidyl-DA; 3: arterenone; 4: 7-*N*τ-histidyl-DOPET; 5: 6-*N*τ-histidyl-DOPET; 6: DA; 7: NE-phenol adduct.

TABLE 1. Catechol-Containing Compounds in 6 M HCl Hydrolysates of Cuticles From the Grasshopper, *Melanoplus sanguinipes*, and the Tobacco Hornworm, *Manduca sexta

		Relative amounts (%)						
Species	Stage/cuticle-type	7-N-I	6-N-I	7-N-II	6-N-II	ART	DA	NE-P
Melanoplus sanguinipes	Larval exuviae	3	2	2	ND	58	23	11
Melanoplus sanguinipes	Forewings	2	ND	5	1	73	12	7
Melanoplus sanguinipes	Hindwings	3	ND	8	1	56	23	8
Manduca sexta	Larval head capsule exuviae	7	2	5	ND	60	15	12
Manduca sexta	Pupal exuviae	12	7	5	3	9	32	31

*7-N-I = 7-Nτ-histidyldopamine; 6-N-I = 6-Nτ-histidyldopamine; 7-N-II = 7-Nτ-histidyl DOPET; 6-N-II = 6-Nτ-histidyl-DOPET; ART = arterenone; DA = dopamine; NE-P = norepinephrine-phenol adduct; ND = none detected.

Organic Composition of Sclerotized Cuticle

Powdered exuviae from fifth instar *M. sanguinipes* were analyzed by solid-state ^{13}C NMR to obtain a natural abundance spectrum (Fig. 6) for estimation of organic composition (Table 2). Protein at nearly 60% was the major component of sclerotized cuticle. Chitin was next in abundance at 33%, whereas total catechols accounted for 6%, and lipid 2%. The compositions of the exuviae of several species of cockroaches (Kramer et al., 1991) compare closely with that of grasshopper exuviae (Table 2).

Catechols in Female Accessory Glands and Egg Pods

The major catechol extracted from female accessory glands and calyx of the lateral oviducts and the frothy material secreted on the egg pod of *M. sanguinipes* was DOBA. It occurred as an acid-labile conjugate along with lesser quantities of DOPA in extracts of the accessory glands/calyx extracts of females with fully developed eggs. DOBA content of the paired accessory glands/calyx of each female was estimated to range from 0.06 to 0.1 nmol. In young females with undeveloped eggs or those that had recently oviposited, no DOBA or only trace amounts of it were detected in the gland extracts. The dried egg plug froth contained highly variable amounts of DOBA ranging from 0.02 to 0.4 nmol/mg. For comparison, the female accessory glands of the cockroaches *Periplaneta australasiae* and *Blaberus discoidalis* were extracted and analyzed. The major catechol in *P. australasiae* was DOBA and low amounts of 3,4-dihydroxybenzyl alcohol (DOBAlc) were also detected. However, the reverse was true in *B. discoidalis,* with DOBAlc predominating and minor quantities of DOBA observed in the gland extracts.

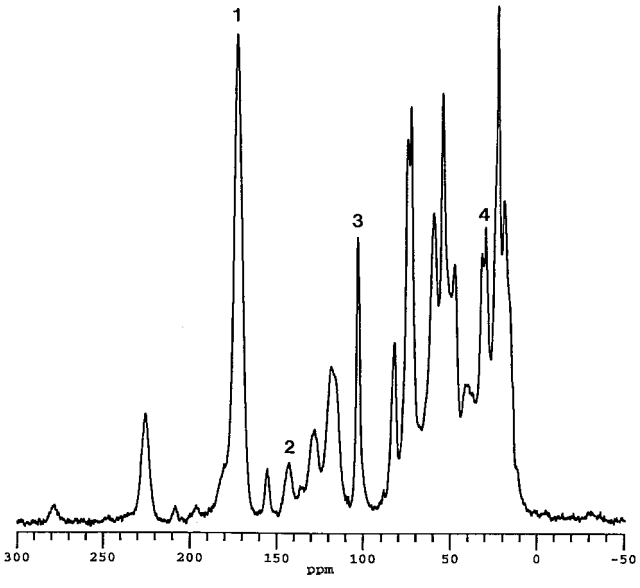

Fig. 6. Natural-abundance 50.3-MHz ^{13}C NMR spectrum of fifth instar exuviae of *Melanoplus sanguinipes* with magic-angle spinning at 5 kHz. 1: Proteins, 175 ppm (corrected for chitin and catechols). 2: Catechols, 144 ppm. 3: Chitin, 102 ppm. 4: Lipids, 33 ppm (corrected for chitin and proteins).

DISCUSSION

M. sanguinipes is similar to the migratory locusts *L. migratoria* and *S. gregaria* in that all three species use primarily NADA as a precursor for quinones that sclerotize the newly secreted exoskeleton (this study and Andersen et al., 1996). However, for the first time concentrations of NADA and other catechols were determined in hemolymph and cuticle in an orthopteran species during the course of metamorphosis and cuticle sclerotization. NADA is the predominant catechol in both the hemolymph and cuticle of *M. sanguinipes*, and its storage as a sulfate conjugate in high concentrations prior to and during stabilization of the newly secreted cuticle correlates with such a role. NADA concentrations then decline in the hemolymph after the exoskeleton becomes hardened and stiffened. Although no

TABLE 2. Organic Composition of Last Instar Larval Exuviae From a Grasshopper and Five Species of Cockroaches*

Species	Relative %[a]			
	Protein	Chitin	Catechol	Lipid
Melanoplus sanguinipes	59	33	6	2
Blaberus craniifer	52	42	5	1
Leucophaea maderae	61	35	4	1
Gromphadorhina portentosa	53	38	8	1
Blattella germanica	59	30	9	2
Periplaneta americana	49	38	11	2

*Cockroach data from Kramer et al. (1991).
[a]Estimated by ^{13}C solid-state natural abundance NMR.

other studies of catechols in grasshopper or locust hemolymph are available, several species of cockroaches also show similar patterns of accumulation and utilization of catechols for cuticle sclerotization (Czapla et al., 1988, 1989, 1990). *M. sanguinipes* most closely resembles the blaberoid cockroaches in which NADA conjugates predominate in hemolymph, rather than the blattid species in which mainly dopamine sulfate is sequestered (Czapla et al., 1990). Unlike cockroaches, no N-β-alanyldopamine was detected in either hemolymph or cuticle. NADA is also the predominant catechol extracted in cold dilute acid from adult cuticle of *M. sanguinipes*. However, its concentrations remain low until after sclerotization, indicating its rapid metabolism into quinone tanning agents. NADA then increases over eightfold in the fully sclerotized adult cuticle, perhaps serving as a precursor for quinones involved in wound healing reactions. The accumulation of NADA and NBAD in cuticle after the main period of sclerotization has been reported in several insect species including cockroaches (Czapla et al., 1990; Hopkins and Kramer, 1991). DOBA and DOPET also may play roles in cuticle stabilization of *M. sanguinipes*, because they increase in hemolymph to peak concentrations in newly ecdysed adults and decrease rapidly as sclerotization occurs the first 24 h. However, these catechols that can be extracted from fully sclerotized cuticle may have other functions including antimicrobial agents, antioxidants, and substrates for wound-healing reactions (Brunet, 1980; Hopkins and Kramer, 1991; Andersen et al., 1996).

The organic composition of the fifth instar exuviae of *M. sanguinipes* as determined by solid-state NMR analysis was very similar to those of several cockroach species (Kramer et al., 1991), particularily *Leucophaea maderae,* a species that primarily uses NADA rather than NBAD for cuticle sclerotization (Czapla et al., 1989, 1990). Protein was the main component in all of these species, ranging from 49 to 61%, and was nearly two thirds higher than the chitin content. Total catechols ranged from 4 to 11% in cockroaches compared to 6% in *M. sanguinipes.* The relatively small amount (~1%) extractable with cold dilute acid indicated that most of the total catechols in grasshopper exuviae are bound covalently to the protein-chitin matrix. The exuviae shed during ecdysis is composed largely of the sclerotized exocuticular layer but also other structures (ecdysial membrane, mesocuticle, etc.) that are resistant to the molting fluid enzymes or are only partially digested before molting occurs.

NADA oligomers with benzodioxan-type C-O linkages predominate in the exuviae of last instar nymphs and the wing cuticles of *M. sanguinipes*, as evidenced by the large amount of ketocatechols released by either weak acid hydrolysis (DOPKET) or strong acid hydrolysis (arterenone). Andersen and Roepstorff (1981) first identified benzodioxan dimers of NADA in extracts of locust cuticle, and later were able to synthesize them and also higher oligomers in vitro by incubating NADA with locust and other types of cuticle (Andersen et al., 1992a). They suggested that NADA oligomers can form longer cross-links between protein chains and also act as filler material in the cuticular matrix. Formation of C-N bonds between histidyl and lysyl amino acid residues of cuticular proteins and the terminal residues of NADA oligomers also can be catalyzed by locust cuticle enzymes as demonstrated by in vitro experiments (Andersen et al., 1992a, b). The head capsule exuviae of *M. sexta* larvae, a clear stiff cuticle sclerotized by NADA quinones (Hopkins et al., 1984), is similar to *M. sanguinipes* abdominal cuticle in having 60% arterenone in the hydrolysate, indicative of a high content of NADA oligomers involved in stabilization. In contrast, the pupal cuticle exuviae of *M. sexta*, a stiff dark

brown cuticle sclerotized by NBAD quinones, has only 9% arterenone and higher amounts of catechol β-carbon and ring C-O and C-N adducts. Evidence for another type of catechol-protein bond in grasshopper exuvial and wing cuticle is the presence of NE or an NE-phenol adduct in weak and strong acid hydrolysates, respectively. NADA oxidized to NADA quinone and then isomerized to NADA quinone methide could react with a number of amino acid nucleophiles to form both C-O and C-N adducts bonded to the β-carbon of NADA (reviewed by Sugumaran, 1988; Hopkins and Kramer, 1992; Andersen et al., 1996). Acid hydrolysis will cleave the weak C-O linkages, releasing NANE, which then is hydrolyzed rapidly to NE and acetate (Morgan et al., 1987). When hydrolysis occurs in 6 M HCl containing phenol as an antioxidant, NE forms an adduct with phenol (Okot-Kotber et al., 1994). In both weak and strong acid hydrolysates of grasshopper exuviae, about 84% of the C-O linkages were estimated to be from NADA oligomers, as evidenced by the ketocatechol content, and 16% were from β-carbon adducts of catechols linked to proteins, as estimated from the levels of NE and the NE-phenol adduct. Grasshopper wings have comparable ratios of these adducts.

The C-N ring and side chain adducts in strong acid hydrolysates of grasshopper exuviae and wings were compared to those adducts first identified in the pupal exuvial cuticle of *M. sexta* (Xu et al., 1997; Kerwin et al., 1999): 6-*N*τ-histidyl-DA, 7-*N*τ-_histidyl-DA, 6-*N*τ-histidyl-DOPET, and 7-*N*τ-histidyl-DOPET. The grasshopper exuvial adducts consist of nearly equal amounts of 6-*N*τ-histidyl-DA, 7-*N*τ-histidyl-DA, and 7-*N*τ-histidyl-DOPET or about 71% catechol β-carbon and 29% catechol ring C-N adducts. Hydrolysates of grasshopper wing cuticle contain mainly the 7-*N*τ-histidyl-DA and 7-*N*τ-histidyl-DOPET adducts, with the latter about twofold higher in amount. The C-N adducts account for about 9% of the total adducts in exuvial hydrolysates, with the remainder occuring as C-O bonds. However, this may be a low estimate for the C-N adducts and cross-links, because some of the catechol-containing products in the strong acid hydrolysates have not yet been identified (Xu et al., 1997). However, these unknown products are relatively low in quantity in the grasshopper cuticle hydrolysates. Therefore, grasshopper cuticle appears to be sclerotized by both *o*-quinones and *p*-quinone methides of NADA and dehydro-NADA, very similar to the cuticles of the migratory locusts (Andersen et al., 1996). This results in a variety of C-O and C-N covalent bonds linked primarily through the side-chain carbons of the catechol moiety. The histidyl-DA and histidyl-DOPET adducts provide the primary evidence so far obtained for catechol-protein C-N cross-links in grasshopper cuticle. Andersen et al. (1992a) reported that locust cuticle, incubated in vitro with NADA and *N*-acetylhistidine (NAH), catalyzes the formation of a NAH-NADA β-carbon adduct. Further, incubation of the NAH-NADA adduct with NADA and locust cuticle containing a 1,2-dehydro-NADA enzyme generating system will catalyze the formation of NADA oligomers coupled to NAH. These model adducts formed in vitro with locust cuticle enzymes are consistant with the 7-*N*τ-histidyl-DA and 7-*N*τ-histidyl-DOPET adducts obtained from grasshopper cuticle in this study.

Unlike the cuticle, the catechols that may be precursors for tanning the grasshopper egg pod frothy secretion were identified as DOBA and DOPA. These compounds accumulate in the glands from very low levels in young females or those that have recently oviposited to high amounts in females with fully developed eggs. The catechols occurred as acid labile conjugates in extracts of the female accessory glands plus the calyx of the lateral oviducts, and DOBA was extracted from the frothy egg plug. The β-glucoside of DOBA was identified previously in the accessory gland and calyx of the two lined grasshopper, *M. femoratus (M. bivittatus),* and DOBA was released by incubation with a β-glucosidase (Stay and Roth, 1962). For comparison, we extracted and analyzed the colleterial glands of the cockroaches, *Periplaneta australasiae* and *Blaberus discoidalis*, since DOBA and 3,4-dihydroxybenzyl alcohol (DOBAlc) have been reported as precursors for cockroach oothecal tanning in several species (Pryor et al., 1946; Brunet and Kent, 1955; Kent and Brunet, 1959; Stay and Roth, 1962; Pau and Acheson, 1968). Both catechols were found in the two species, with DOBA predominating in *P. australasiae*, and DOBAlc in *B. discoidalis.* DOPA has not been reported previously in accessory gland secretions, and its functions are unknown, although it does serve as a precursor for the biosynthesis of other catechols (Hopkins and Kramer, 1992). Catechol-protein adducts and cross-links so far have not been investigated in the sclerotization of insect oothecal proteins by quinones from DOBA or other related catechols.

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