Archives of Insect Biochemistry and Physiology 7:13–28 (1988)

Diphenols in Hemolymph and Cuticle During Development and Cuticle Tanning of *Periplaneta americana* (L.) and Other Cockroach Species

T.H. Czapla, T.L. Hopkins, K.J. Kramer, and T.D. Morgan

Departments of Entomology (T.H.C., T.L.H., T.D.M.) and Biochemistry (K.J.K.), Kansas State University and U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture (K.J.K), Manhattan

Diphenolic compounds in cockroach hemolymph and cuticle were extracted with 1.2 N HCl, partially purified by alumina adsorption, and analyzed by liquid chromatography. Dopamine (DA) is the major catecholamine in hemolymph of *Periplaneta americana, Blatta orientalis, Blattella germanica, Gromphadorhina portentosa,* and *Blaberus craniifer* at adult ecdysis, while N-acetyldopamine (NADA) predominates in hemolymph of *Leucophaea maderae.* In *P. americana,* NADA is the second most abundant catecholamine, while N-β-alanyldopamine (NBAD), norepinephrine (NE), 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylethanol, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxybenzoic acid occur in lesser quantities. Catecholamines occur mainly as acid labile conjugates in hemolymph. Dopamine, conjugated primarily as the 3-sulfate ester, increases in hemolymph from 0.1 to 0.8 mM during the last instar. Concentrations decrease by 75% in pharate adults, partially because of an increase in hemolymph volume. A second smaller peak of DA sulfate occurs after ecdysis followed by a rapid disappearance as the cuticle tans. A conjugate of catechol (*o*-dihydroxybenzene) is also present in relatively high concentrations at all ages examined. In cuticle, N-β-alanylnorepinephrine accumulates during the early period of adult tanning, while NBAD, NADA, N-acetylnorepinephrine, and DA increase more slowly. The N-β-alanyl and N-acetyl derivatives of DA

Acknowledgments: Contribution No. 87-348-J from the Kansas Agricultural Experiment Station, Manhattan, Kansas. Cooperative investigation between ARS, USDA, and the Kansas Agricultural Experiment Station. K.J.K. is a research chemist and adjunct professor at the U.S. Grain Marketing Research Laboratory and the Department of Biochemistry, Kansas State University, respectively. Supported in part by National Science Foundation grant DCB 86-09717. The authors thank Georgia Grodowitz and Cheryl Armendariz for their assistance and Dr. J. Stephen Kennedy, Neurosciences Research Branch, National Institute of Mental Health, for providing the dopamine sulfate standard compounds.

Received April 20, 1987; accepted September 17, 1987.

Address reprint requests to T.L. Hopkins, Department of Entomology, 123 Waters Hall, Kansas State University, Manhattan, KS 66506.

© 1988 Alan R. Liss, Inc.

and NE occur in relatively high concentrations in tanned cuticle of *P. americana* and probably play an important role in the stabilization process.

Key words: catecholamine, dopamine, sclerotization, dopamine sulfate, catechol

INTRODUCTION

Sclerotization, or tanning of insect cuticle, involves cross-linking and dehydration of the chitin-protein matrix by diphenols and quinones derived from tyrosine [1–3]. NADA* and NBAD are two major precursors for highly reactive quinonoid intermediates that form covalent bonds with other cuticular components during the sclerotization process [4–6]. They are derived from DA by N-acylation with acetate or β-alanine, respectively. The former compound has long been considered to be the main precursor for sclerotizing agents of insect cuticle [4], while NBAD has more recently been shown to be associated with tanning of pupal cuticle in *Manduca sexta* and certain other holometabolous insects forming stiff brown cuticles [5,7–9].

A number of diphenolic compounds, presumed to be tanning agent precursors , have been identified in cockroach tissues and oothecae. Dopamine-3-sulfate (3-hydrosulfato-4-hydroxyphenethylamine) was isolated from whole body extracts of newly ecdysed *Periplaneta americana* [10]. NADA or its conjugates were identified in tissues of *P. americana* [11–13], whereas NBAD was detected in hemolymph at ecdysis [5]. Several other diphenolic acids, aldehydes, and alcohols were identified in the exuvia and oothecae of *P. americana* and other cockroach species [14–16].

In this study, we have further investigated the diphenols that are important for the sclerotization and pigmentation of cuticle in *P. americana*. Hemolymph and cuticle concentration profiles were determined during development of the last instar and sclerotization of the adult cuticle. A comparison of their major diphenols in hemolymph from recently ecdysed adults of related cockroach species was also made.

MATERIALS AND METHODS

Insects

Cockroaches were reared in containers bedded with wood shavings at 28 ± 2°C with a photoperiod of 16L:8D. Water and Purina® Lab Chow were provided ad libitum. Insects were collected for analysis at different ages during the last nymphal period through several weeks after adult ecdysis.

*Abbreviations: DHBZ = 3,4-dihydroxybenzoic acid; DHBA = 3,4-dihydroxybenzylamine; DOPAC = 3,4-dihydroxyphenylacetic acid; DOPA = 3,4-dihydroxyphenylalanine; DOPET = 3,4-dihydroxyphenylethanol; DA = dopamine; DDC = DOPA decarboxylase; 3-DAS = 3-dopamine sulfate; 4-DAS = 4-dopamine sulfate; LCEC = liquid chromatography with electrochemical detection; AMD = α-methyl DOPA; NADA = N-acetyldopamine; NANE = N-acetylnorepinephrine; NBAD = N-β-alanyldopamine; NBANE = N-β-alanylnorepinephrine; NE = norepinephrine; SOS = sodium octyl sulfate; TLC = thin-layer chromatography; TH = tyrosine hydroxylase.

Last-stage nymphs were selected from the colonies, sexed, and placed in individual rearing cages made from square plastic dishes. The ages of individual insects were determined from the last nymphal ecdysis. Pharate stages were timed using eye color as an indicator. The eye changes from a shiny black to a dull grayish blue color at apolysis [17]. It assumes a brighter blue appearance as the cockroach nears adult ecdysis.

Hemolymph Extraction

Cockroaches were anesthetized with CO_2, quick-frozen in dry ice powder, and stored at −20°C until analyzed. After thawing in a desiccator (approximately 5 min), the insects were placed in a spring steel clip that applied light pressure to the abdomen. The insect was suspended head down for 1 min, the coxa of a front leg was severed, and hemolymph was collected with microcapillary pipettes (5–10 μl) and diluted 10-fold in 1.2 N HCl containing 5 mM ascorbic acid and 6 μg $ml^{-1}$of an internal standard, either DHBA or AMD. The extracts were centrifuged at 6,500*g* for 10 min and the supernatant removed for further sample preparation [7]. Freshly collected hemolymph had similar diphenol profiles.

Cuticle Extraction

The pronotum was the major area of cuticle analyzed, but abdominal and thoracic areas were also examined in some cases. Integument was dissected and placed in distilled water containing a few crystals of phenylthiourea. After adhering muscle and fat body were teased away from the cuticle, the inner surface was scraped and rinsed with distilled water to remove the epidermis. Pieces of cuticle were blotted dry on absorbent tissue, weighed (0.5 to 5.0 mg), and then homogenized in 0.3 ml of 1.2 N HCl and 5 mM ascorbate containing 0.18 μg of DHBA or 1.8 μg AMD in a ground glass tissue grinder. The homogenate was then centrifuged at 6,500*g* for 10 min and the supernatant saved for further sample preparation.

Hydrolysis and Recovery of Diphenols for LCEC Analysis

Aliquots of hemolymph acidic extracts (0.25 ml) were heated at 100°C under nitrogen for 10 min to release diphenols from their acid labile conjugates [5,7]. Both hydrolyzed and unhydrolyzed extracts (50 to 100 μl) were adsorbed on alumina and the diphenols recovered in 1 M acetic acid for LCEC analysis [18]. The primary mobile phase for hemolymph analysis (Fig. 1) consisted of 17.5% methanol, 0.34 mM SOS, 90 μM sodium EDTA, and 0.1 M H_3PO_4 adjusted to pH 3.1 with NaOH. The primary mobile phase for cuticle samples consisted of 15% methanol, 160 μM SOS, 90 μM sodium EDTA, and 0.1 M H_3PO_4 adjusted to pH 2.9 with NaOH[1]. A third mobile phase for identification consisted of 26% acetonitrile, 1.1 mM sodium lauryl sulfate, 50 μM sodium EDTA, and 0.1 M H_3PO_4 adjusted to pH 3.3 with

[1]LCEC chromatograms of cuticle extracts can be found in Morgan et al [19].

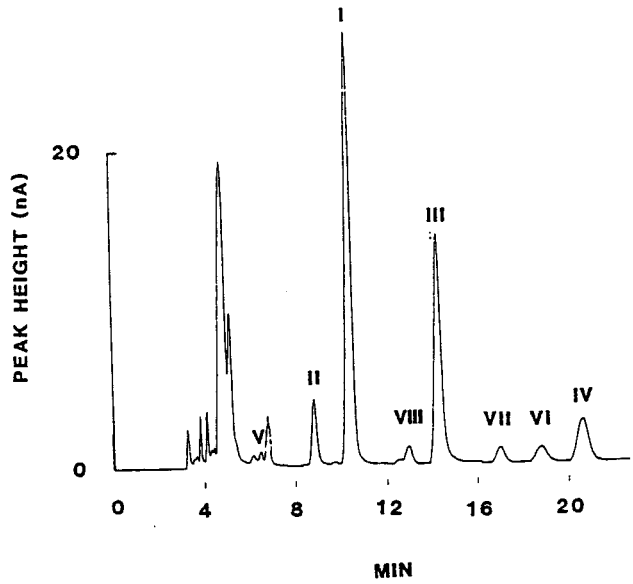

Fig. 1. LCEC chromatogram of 0.5-day hemolymph sample (hydrolyzed) of *P. americana*. **I:** AMD (internal standard); **II:** DA, **III:** Catechol, **IV:** NADA; **V:** DOPA; **VI:** NBAD; **VII:** DOPAC; **VIII:** DOPET. NE (4.77 min) is unresolvable in this mobile phase because of interferences.

NaOH[1]. Diphenols were detected with Bioanalytical Systems (W. Lafayette, IN) LC4A or LC4B electrochemical detector operated at +720 mV, while the dopamine sulfates were detected at +900 mV. The retention times of diphenol standards were compared with those of electroactive compounds in hemolymph and cuticle extracts using two mobile phases. Quantities of individual diphenols were determined by comparing peak heights with that of an internal standard in each extract and then correcting for recoveries established using standard compounds. The percentage of conjugation of each diphenolic compound was calculated from the difference in amounts present in unhydrolyzed and hydrolyzed samples.

Chemical Sources

Tyr, DA, NADA, NE, DHBA, AMD, DOPA, DOPAC, and DHBZ were from Aldrich Chemical Company (Milwaukee, WI) or Sigma Chemical Company (St. Louis, MO). DOPET was a gift from Hoffmann-La Roche Inc. (Nutley, NJ). Dopamine-3-sulfate and DA-4-sulfate were supplied by Dr. J. Stephen Kennedy of the Neurosciences Research Branch, NIMH (Rockville, MD). NBAD, NBANE, and NANE were synthesized as previously described [5,19,20]. The sulfate esters of Tyr, DA, and DOPA were synthesized by sulfation at low temperature in concentrated sulfuric acid [21,22]. [$U^{14}C$]Tyr was obtained from Schwarz/Mann (Cleveland, OH; specific activity 50 nCi μl^{-1}), and [^{35}S] sulfate was obtained from ICN (Irvine, CA; specific activity, 5 μCi μl^{-1}).

Two-Dimensional Mapping of Radiolabeled Tyrosine Metabolites

Last nymphal instars of *P. americana* were anesthetized with CO_2 and then injected with 2.5 μCi of [U^{14}C]Tyr or 5.0 μCi of [^{35}S]sulfate between the third and fourth dorsal abdominal terga lateral to the heart. Insects were collected at three different developmental stages, including feeding last instars (6 h after injection), pharate adults (approximately 72 h after injection), and adults (3–12 hr after ecdysis, injected at ecdysis), and quick frozen.

Hemolymph and whole body samples were extracted and analyzed before and after hydrolysis as previously described [32]. Two-dimensional separation was done by electrophoresis and TLC on cellulose sheets (Eastman, Rochester, NY; 20 × 20 cm). Electrophoresis was conducted for 2.7 to 5 h at pH 1.9 (8% acetic acid, 2% formic acid) and 200 V per plate. This procedure was followed by TLC with a butanol:pyridine:acetic acid:water mixture (15:10:3:12) [23]. The cellulose sheets were then sprayed with either ninhydrin [24] or diazotized sufanilic acid [25] to locate amino acids or phenols and diphenols, respectively. Radioactive compounds were located by autoradiography with X-ray film (Kodak XAR; Eastman) or by a chromatogram scanner. To confirm the identity of these metabolites, the radioactive zones from unsprayed plates were removed and extracted with 80% aqueous methanol containing 5 mM ascorbic acid solution. Aliquots were then analyzed by LCEC either directly or after acid hydrolysis to release conjugated diphenols. In other studies, extracts of hemolymph from [U^{14}C]Tyr-injected cockroaches were hydrolyzed and then analyzed by LCEC. Individual electroactive metabolites were collected as they eluted from the column, and their radioactivity was determined by liquid scintillation counting.

RESULTS

Diphenols in Hemolymph

Diphenols identified by LCEC analysis of *P. americana* hemolymph extracts after mild acid hydrolysis in order of decreasing concentration are DA, NADA, NE, NBAD, DOPET, DOPA, DOPAC, and DHBZ (Fig. 1). In addition, a major electroactive unknown compound was isolated after hydrolysis by the alumina adsorption procedure. We have tentatively identified it as catechol (*o*-dihydroxybenzene) by comparison of retention times and cochromatography with authentic catechol.

Dopamine is the major catecholamine in hemolymph, while lesser amounts of NADA, NE, and NBAD are also present (Figs. 2,3). Dopamine increases nearly 10-fold during the last nymphal stadium, reaching a peak in the early pharate adult stage (1 mM at 25–28 days). More than 80% of the DA is sequestered as an acid labile conjugate(s) in hemolymph throughout development (Fig. 2). A three-fold decrease of DA occurs as the insect approaches ecdysis (0.3 mM at 28–30 days), followed by an increase to 0.4 mM 1 h after ecdysis. A decline to approximately 10 μM 24 h later occurs as the new cuticle tans. Hemolymph DA remains at about that level through 5 months after ecdysis. Conjugation during this later time is only about 20–30% of the total DA.

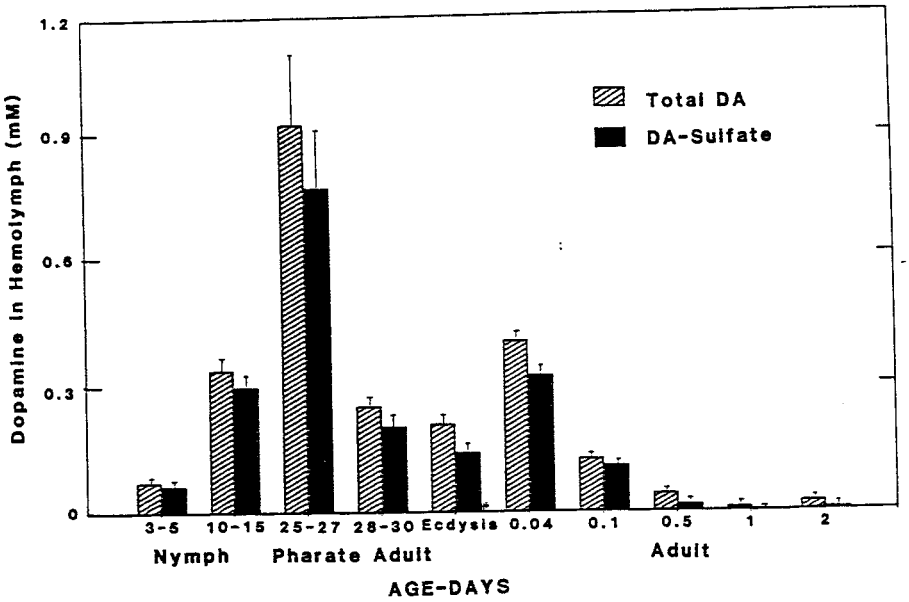

Fig. 2. Dopamine and dopamine sulfate concentrations in hemolymph of *P. americana* during last instar and adult ecdysis. Data are means of three to eight animals ± SE.

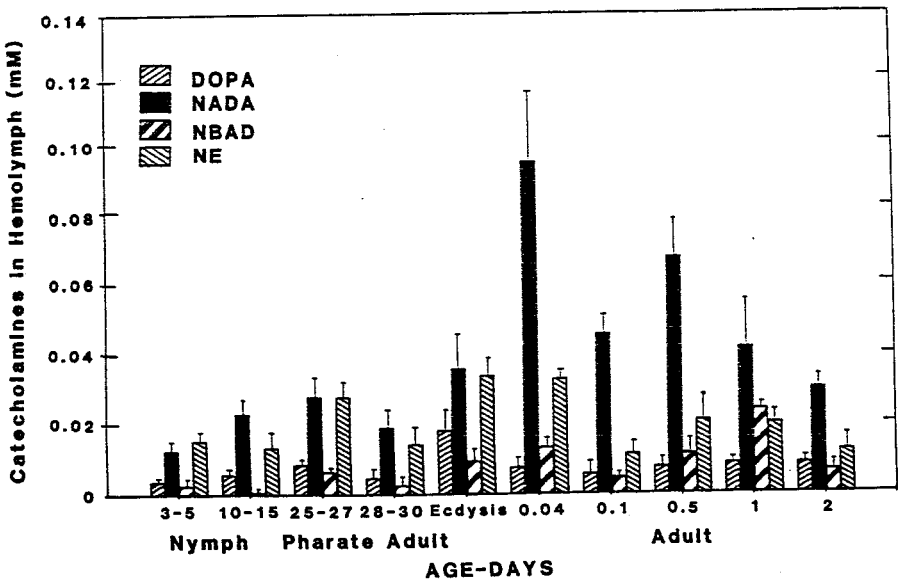

Fig. 3. Catecholamine concentrations in hemolymph (hydrolyzed) of *P. americana* during last instar and adult ecdysis. Data are means of three to eight animals ± SE.

At adult ecdysis, hemolymph DA (210 μM) is about five to 20 times greater than NADA, NE, and NBAD (Figs. 2,3). N-Acetyldopamine is the second most abundant catecholamine during the period of adult cuticle morphogenesis. Like DA, NADA increases during the last nymphal stadium and reaches a peak level 1 h after ecdysis (0.1 mM) followed by a gradual decline to 30 μM as the cuticle is tanned (Fig. 3). At ecdysis, approximately 80% of NADA is sequestered as acid labile conjugates. Other catecholamines in measurable quantities are NE, DOPA, and NBAD with highest titers near the time of ecdysis (Fig. 3). Approximately two-thirds of the NBAD is conjugated. Other diphenols detected in *P. americana* hemolymph are DOPAC and DHBZ in trace quantities (< 2 μM), while DOPET is found at a higher level at 24 h (15 μM).

The major electroactive compound (Fig. 1, peak III) that was released from a conjugate in hemolymph was found to become radiolabeled after injecting cockroaches with [^{14}C]Tyr. It had retention times identical to catechol when analyzed by LCEC with two separate mobile phases. The conjugate of catechol occurs in the hemolymph of last-nymphal instars and adults in relatively high concentrations (0.2–0.4 mM) and is the major diphenolic compound at all times except in pharate adults when DA increases to a greater level. Its concentration remains relatively constant in both nymphs and adults, with an apparent decrease in pharate adults.

Radiolabeled Metabolites of [U^{14}C]Tyr and [^{35}S]Sulfate

Radioisotopic studies of Tyr metabolites and their conjugates in *P. americana* were conducted after injecting [U^{14}C]Tyr and [^{35}S]sulfate into nymphal and adult stages, followed by two-dimensional separations of extracts by electrophoresis and TLC on cellulose. Unhydrolyzed extracts of either hemolymph or whole body from last instars (before apolysis) contained radioactivity from [U^{14}C]Tyr that migrated mainly into two zones as determined by autoradiography (Fig. 4B). The radiolabeled zones corresponded in mobility to the standard compounds Tyr and DA sulfate (Fig. 4A). The metabolite corresponding to DA sulfate is electrophoretically neutral at pH 1.9 and has slightly more mobility than Tyr in the TLC solvent. Extracts heated at 100°C for 10 min in 1.2 N HCl did not contain the radiolabeled metabolite corresponding to DA sulfate.

A similar two-dimensional analysis of hemolymph and whole body extracts of [^{35}S]sulfate-injected *P. americana* showed that the radioactivity was incorporated into a single radiolabeled zone with a mobility identical to those of both the DA sulfate standard and the unknown conjugate from the [U^{14}C]Tyr-injected insects (Fig. 4C). The plates used to resolve metabolites present in heated acidic extracts of [^{35}S]sulfate-labeled hemolymph or whole body did not exhibit any radiolabeled zones.

In either pharate adults or adults that were injected with [U^{14}C]Tyr or [^{35}S]sulfate, both hemolymph and whole body extracts also yielded two major zones of radioactivity with mobilities identical to those observed with the nymphal extracts. Acid hydrolysis destroyed the compound corresponding to DA sulfate. Evidence from the two-dimensional fingerprint analysis strongly suggested that DA is formed from Tyr and stored as a sulfate

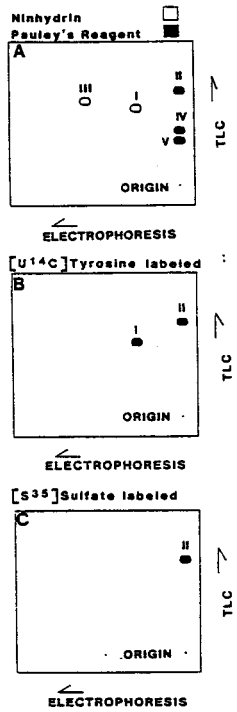

Fig. 4. Reconstructed two-dimensional TLC-electrophoresis fingerprint maps of **A:** standard compounds identified by ninhydrin or Pauley's reagent, including I, tyrosine; II, dopamine sulfate; III, dopamine; IV, DOPA sulfate; V, tyrosine sulfate; **B:** developed X-ray film of hemolymph extract (80% aqueous methanol) from [^{14}C]tyrosine-injected pharate adult *P. americana*; and **C:** developed X-ray film of hemolymph extract (80% aqueous methanol) from [^{35}S]sulfate-injected pharate adult *P. americana.*

conjugate. Removal of the radioactive DA zone from TLC plates followed by acid hydrolysis and LCEC analysis revealed that DA is the only detectable metabolite. Two-dimensional separation of nonradiolabeled extracts followed by LCEC analysis after acid hydrolysis also showed that DA is the only electroactive compound recovered from this zone. This evidence supports the identity of this conjugate as a DA sulfate. Analysis by LCEC of the cockroach conjugate recovered from the cellulose plates revealed two peaks with retentions times identical to those of the standard compounds DA-3-sulfate and DA-4-sulfate. The 4-sulfate is a minor metabolite and varies greatly in pharate adult cockroaches, with ratios ranging from 1:8 to 1:20 or less (N = 7) with respect to the 3-sulfate (Fig. 5A,B).[2]

Diphenols in Cuticle

Several catecholamines were extracted in 1.2 M HCl from the cuticle of nymphal and adult *P. americana* (Fig. 6). The N-β-alanyl and N-acetyl deriva-

[2]Hemolymph mobile phase was used for LCEC analysis. Ratio is based on area of peaks produced by the oxidation of the compounds.

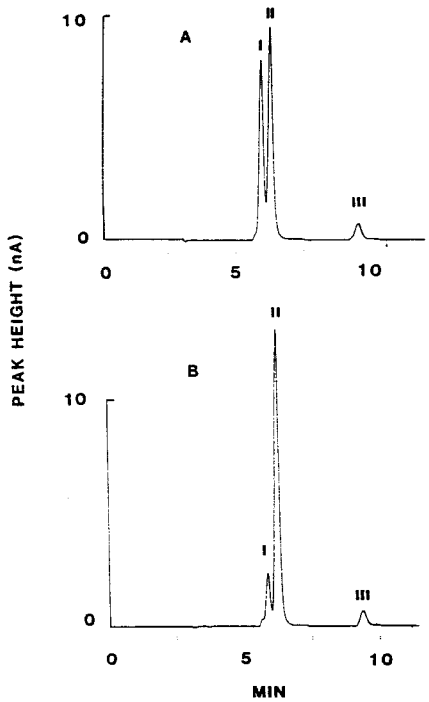

Fig. 5. LCEC chromatograms of **A:** standard compounds—I, 4-DA-sulfate; II, 3-DA-sulfate; III, DA; and **B:** hemolymph extracted from *P. americana* and recovered from two-dimensional TLC-electrophoresis separation.

tives of DA (NBAD and NADA) and NE (NBANE and NANE) predominate in tanning cuticle, while free DA is only a minor component. Only trace levels of catecholamines are detected in the newly secreted cuticle of pharate adults approximately 2 days before ecdysis (data not shown). However, by the time of ecdysis low levels of NBANE are present (0.1 μmol g^{-1}) together with traces of NBAD, NADA, and NANE (Fig. 6). N-β-Alanylnorepinephrine increases rapidly to relatively high concentrations (1.5 μmol g^{-1}) by 1 h postecdysis and is the major extractable catecholamine in cuticle during the first 12 h of tanning. Other catecholamines accumulate more slowly in tanning cuticle except for NBAD, which reaches concentrations equivalent to NBANE by 24 h (2.1 μmol g^{-1}). The two N-acetyl derivatives NADA and NANE accumulate to peak levels at 48 h, when they become comparable to the N-β-alanylcatecholamines in concentration. The accumulation of large amounts of freely extractable N-acylcatecholamines as tanning takes place is dramatic. Accumulations increase from barely detectable levels in newly ecdysed cuticle to a total of nearly 8 μmol g^{-1}or about 0.2% of cuticle weight by 48 h later. Dopamine, the precursor of the N-acylcatecholamines, also increases slightly during the stabilization of cuticle. Other diphenols detected at low levels in tanning cuticle are DOPET (560 nmol g^{-1} at 24 h), DOPAC (300 nmol g^{-1} at 24 h), and several minor unidentified electroactive compounds.

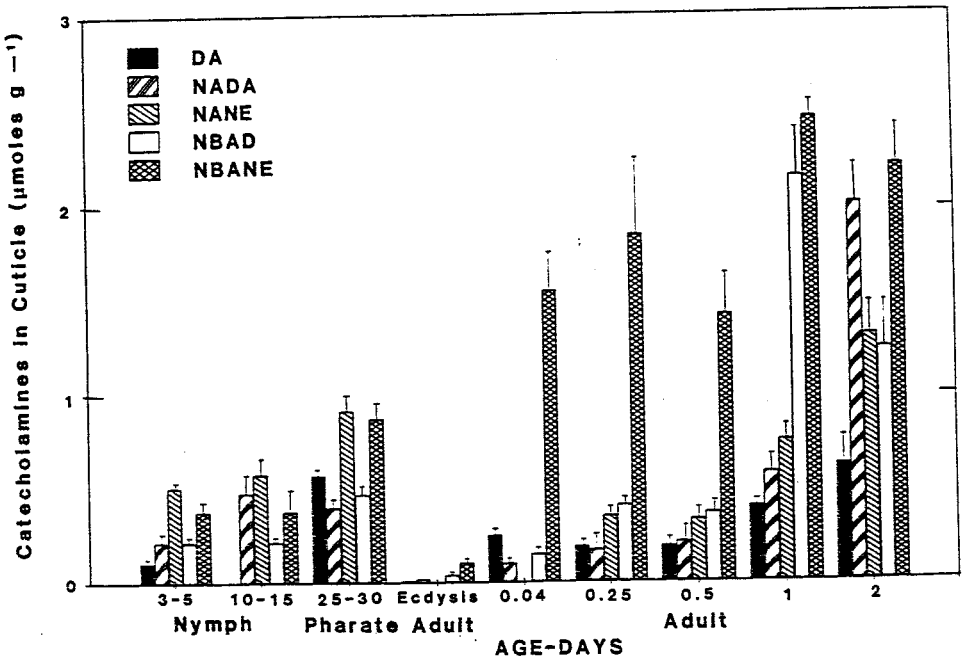

Fig. 6. Catecholamines extracted with cold 1.2 N HCl from cuticle of *P. americana* during last-instar and adult development. Data are means of three to five samples ± SE. Nymphal cuticle was analyzed at 25–30 days; pharate adult cuticle at 25–30 days did not contain measureable diphenol concentrations and is not shown.

TABLE 1. Catecholamines in Hemolymph (Hydrolyzed) of Six Cockroach Species at Adult Ecdysis*

Species	DA	NADA	NBAD
Periplaneta americana	210 ± 30	40 ± 3	10 ± 3
Blatta orientalis	250 ± 58	45 ± 6	25 ± 6
Blattella germanica	100 ± 30	7 ± 1	< 1
Gromphadorhina portentosa	50 ± 9	14 ± 6	< 1
Blaberus craniifer	160 ± 12	30 ± 12	7 ± 1
Leucophaea maderae	15 ± 1	150 ± 20	25 ± 6

*Units = µM. Mean value ± SE (N = 3–7).

The composition and relative amounts of extractable catecholamines in cockroach nymphal cuticle are similar to those in adult cuticle. However, the individual and total concentrations in the former are only about one-half those of adult cuticle (Fig. 6).

Catecholamines in Hemolymph of Other Cockroach Species

Dopamine is the major hemolymph catecholamine in four of five other cockroach species sampled shortly after ecdysis (Table 1). It is present mainly as an acid labile conjugate(s), but we have not yet determined if the conjugate is DA sulfate, as was found in *P. americana*. N-Acetyldopamine and NBAD are the next most abundant catecholamines, except for *L. maderae*, in which NADA is highest in concentration at ecdysis followed by DA and NBAD.

DISCUSSION

Dopamine is the major catecholamine in hemolymph of several species of cockroaches at ecdysis. One exception is *L. maderae*, which has relatively high concentrations of NADA in comparison to DA. Conversely, *B. craniifer* and *G. portentosa*, the blaberoid species most closely related to *L. maderae* [26], had DA as the major diphenol in hemolymph. In all of the other species examined, including *P. americana*, *B. orientalis*, and *B. germanica*, NADA is second in abundance, while NBAD is present at relatively low levels.

Dopamine accumulates to high levels in hemolymph of the last nymphal instar of *P. americana* and is largely conjugated with sulfate on a phenolic oxygen. Dopamine-3-sulfate has been previously identified as a precursor for cuticle tanning agents in newly ecdysed *P. americana* [10]. We have confirmed that the DA in hemolymph is primarily conjugated as the 3-sulfate ester. Although catecholamine sulfates in vertebrates are thought to be end products of metabolism for excretion [27], their role(s) in insects is quite different, where they serve as storage molecules for cuticle tanning substrates. Conjugation of DA with sulfate is suggested to be a mechanism for protecting the diphenol against premature oxidation to quinone and subsequent indolization to melanin in *P. americana* [10]. Dopamine upon oxidation to its quinone either chemically or enzymatically by phenoloxidases or tyrosinases rapidly undergoes cyclization to a leucoaminochrome in the melanin pathway [28]. Conjugation may also affect catecholamine transport, because the sulfate moiety does not appear to enter the cuticle with DA or its acyl derivatives [10]. N-Acetyldopamine 3-sulfate and NADA 3-phosphate have also been isolated from homogenates of newly ecdysed *P. americana* nymphs [13]. In our study approximately 80% of the NADA and 67% of the NBAD in hemolymph was conjugated, but we have not identified the nature of these conjugates.

Dopamine sulfate begins to accumulate in hemolymph of *P. americana* approximately 1–3 days after ecdysis of the last nymphal instar and builds to peak concentrations by nymphal-adult apolysis. Although both Tyr hydroxylating and DOPA decarboxylating activities are low during the feeding period of last nymphal *P. americana* [29,30], sufficient levels of these enzymes are present to convert Tyr to DA. Radiorespirometric studies on the metabolism of Tyr side chain carbons in live insects showed a low but constant level of synthesis of a two-carbon side chain metabolite of Tyr, presumably dopamine, throughout the last nymphal stadium and the pharate adult period. Only after ecdysis was there a rapid and more than 10-fold increase in synthesis of this metabolite [12,29,30]. Dopamine is then largely sequestered in hemolymph as two sulfate conjugates for later use in the sclerotization and pigmentation of adult cuticle.

The storage of catecholamine conjugates throughout the last immature stage of the cockroach differs from the tobacco hornworm *M. sexta*. In the latter case, catecholamine conjugates are not stored in the hemolymph in large amounts until after apolysis to pharate stages of larvae, pupae, and adults [5,7]. However, β-D-glucopyranosyl-O-L-tyrosine (Tyr glucoside) is sequestered in hemolymph during the final larval feeding period of *M. sexta*

for subsequent metabolism to catecholamines during pupal cuticle tanning [23,31]. No Tyr glucoside or Tyr sulfate was found in *P. americana* [23,32].

The large decrease of DA sulfate in hemolymph of pharate adults before tanning of the new cuticle was unexpected. This could be accounted for in part by the increase in hemolymph volume in preparation for ecdysis of the pharate adults. Hemolymph volume increases by approximately 50% during this time [33]. However, DA sulfate concentrations decrease about 75% during this period, suggesting that other factors also affect DA levels. Metabolism of DA to other diphenols may account for part of the decrease. N-Acylation of DA to NADA or NBAD takes place in pharate adults, but the levels of these products and catechol in hemolymph also decrease during this period. Catecholamines may be transported to other tissues such as epidermis and fat body for subsequent metabolism to tanning agents. During pharate adult development DDC (EC 4.1.1.28) activity and free Tyr concentrations increase, but TH (EC 1.14.18.1) activity remains low, thereby preventing a rapid synthesis of DA until after ecdysis [29,30,34]. The concentration of DA in hemolymph increases a second time by 1 h following ecdysis. This profile of DA concentrations correlates with greatly increased DDC and TH activities [29,30] and in vivo synthesis of two-carbon side chain metabolites of Tyr [12]. Other hemolymph diphenols do not increase significantly in concentration during this period, although their overall total amounts are greater because of a larger hemolymph volume. The transport of DA and its N-acyl derivatives NADA and NBAD into the integument could account for the steady state level of these metabolites in hemolymph during the early stages of cuticle tanning.

Hemolymph DA drops rapidly between 1 and 12 h after ecdysis, and at 24 h it is less than 2% of the maximum level. However, other hemolymph catecholamines, such as NADA and NBAD, remain relatively constant throughout this time period. The rapid disappearance of DA from hemolymph cannot be attributed to a cessation in synthesis because of a decrease in DDC or TH activity. On the contrary, apparent DDC and TH activities are at their highest levels 6 h after ecdysis and slowly decrease during the next few hours [29,30]. Continued synthesis of catecholamines is further indicated by a gradual decrease in hemolymph Tyr from peak concentrations at adult ecdysis to near preapolysis levels at 24 h [34]. Therefore, the decrease in hemolymph DA that occurs when precursor titers and enzyme activities are at their highest levels suggests that DA or its metabolites NADA and NBAD are being transported to the epidermis and cuticle for conversion to sclerotizing agents.

A conjugate of catechol was also found in hemolymph of both nymph and adult *P. americana* in relatively high concentrations. Catechol extracted from hemolymph was approximately 90% conjugated in adults, but only 65% conjugated in pharate adults [32]. Earlier studies on the metabolism of Tyr side chain carbons to CO_2 in live insects had shown that a low rate of synthesis of ring compounds lacking the aliphatic side chain occurred throughout development and suggested that catechol or a conjugate was the end metabolite [12,35]. The present data confirm this, but the role of such a conjugate is unclear. This compound is the major diphenol in hemolymph at

all ages tested except during the large buildup of DA sulfate in pharate adults. Catechol conjugate levels decrease somewhat during this time, apparently because of dilution by increasing hemolymph volume. We have not determined whether catechol is transported into epidermis or cuticle, but none was detected at nmol g^{-1} levels in the latter. Free catechol has previously been reported to occur in locust cuticle [36].

The N-acyl derivatives of DA (NBAD, NADA) and NE (NBANE, NANE) appear to be the major metabolites involved in the sclerotization of new cockroach cuticle. Previous studies have shown NADA associated with hard, colorless cuticle, NBAD with hard brown cuticle, and DA with black pigmentation [5,7,8]. Therefore, NBAD would be expected to be a major precursor for sclerotization of *P. americana* cuticle. While NBAD is a minor catecholamine in hemolymph of *P. americana*, it accumulates to relatively high levels in tanning cuticle. However NBANE, the β-hydroxylated derivative of NBAD, is the most abundant diphenol that can be extracted from the cuticle of *P. americana* during the period of sclerotization. Studies with *M. sexta* show that NBANE is synthesized from NBAD by a cuticular enzyme [19]. The former compound increases more than 10-fold in new cockroach cuticle during the first hour after ecdysis, while NBAD is barely detectable. However, by 24 h NBAD concentrations are similar to those of NBANE. The accumulation of the N-β-alanylated catecholamines in tanning cuticle correlates with the increase of β-alanine in *P. americana* that was observed in hydrolyzed cuticle [37,38]. Those studies reported that β-alanine was absent in newly ecdysed cuticle, but increased thereafter and reached a plateau value several hours later.

The early appearance of NBANE in untanned cuticle and its rapid buildup supports the hypothesis that it is a primary substrate for sclerotizing enzymes in hard brown cuticle. However, NBANE may also be a hydrolysis artifact from NBAD covalently linked via the β-carbon to the chitin-protein matrix [19]. Both NADA and its β-hydroxylated derivative NANE accumulate to high levels in cuticle by 48 h at similar rates. Our previous studies with *M. sexta* cuticular enzymes have demonstrated that NADA is a good substrate for the biosynthesis of NANE [19]. Free DA concentrations in cuticle remain relatively low, and, since *P. americana* lacks black pigmentation, DA may serve more as a substrate for N-acylation than as a substrate for melanin biosynthesis [39].

The accumulation of large amounts of unconjugated catecholamines in insect cuticle after the main period of sclerotization suggests additional functions for these metabolites [7]. In *Schistocera gregaria*, sclerotization has been demonstrated to continue for several weeks after adult ecdysis [40,41]. These compounds may participate in wound healing and additional stabilizing reactions in the exoskeleton. Catecholamines may stiffen the cuticle by a dehydrating mechanism, in which water molecules are displaced by diphenols [42,43], or may act as antioxidants to protect labile compounds such as lipids in the epicuticle [14].

The proposed pathway for biosynthesis of catecholamines in *P. americana* is shown in Figure 7. Tyr is metabolized via hydroxylation, decarboxylation, and sulfation to DA sulfate, which is stored in the hemolymph until ecdysis.

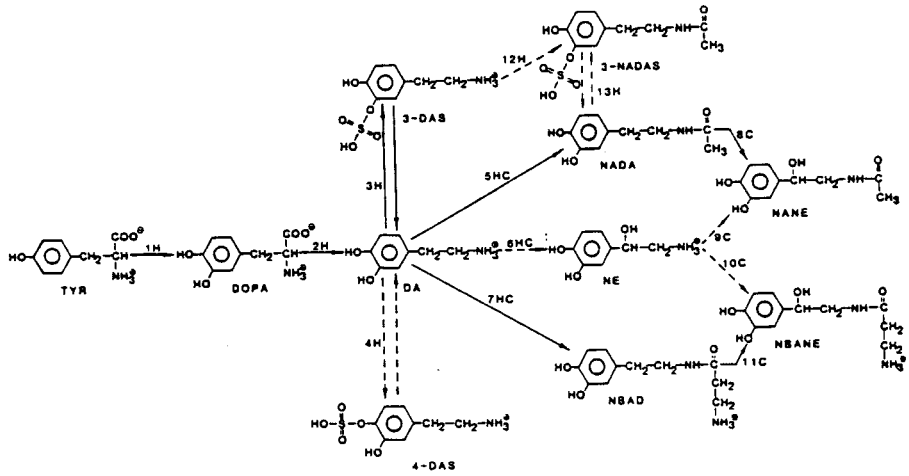

Fig. 7. Proposed pathway of catecholamine metabolism for cuticle tanning in *P. americana*, including Tyr, DOPA, DA, 3-DAS (dopamine-3-sulfate), 4-DAS (dopamine-4-sulfate), NE, NADA, NBAD, NBANE, and NANE. Solid arrows indicate major reaction pathways. Dashed arrows indicate minor reaction pathways. 1, tyrosinase or tyrosine hydroxylase; 2, DOPA decarboxylase; 3 and 4, DA sulfotransferase (reverse reaction catalyzed by sulfatase); 5, DA N-acetyltransferase; 6, DA β-hydroxylase or tyrosinase; 7, DA N-β-alanyltransferase; 8, tyrosinase or NADA β-hydroxylase; 9, NE N-acetyltransferase; 10, NE N-β-alanyltransferase; 11, tyrosinase or NBAD β-hydroxylase; 12, 3-DAS N-acetyltransferase; 13, NADA sulfotransferase (reverse reaction catalyzed by sulfatase). Reactions designated "C" and "H" probably occur in the integument and in the hemolymph or fat body, respectively.

Release of free DA from the conjugate may occur in the epidermis after which N-acylation and β-hydroxylation produce the precursors for sclerotizing agents. β-Hydroxylation of catecholamines in vivo has been shown to be catalyzed by enzyme preparations from cuticle. [19]. This enzyme after partial purification has been shown to β-hydroxylate both NADA and NBAD (unpublished work by Yonekura, Morgan,and Czapla). Other cockroach diphenolic compounds derived from Tyr include catechol, DOPET, DOPAC, and DHBZ.

Cuticle sclerotization and pigmentation appear to depend on a complex pattern of catecholamine biosynthesis from Tyr, and utilization depends on the function of different regions of the exoskeleton [5,7–9]. The present study with *P. americana* supports the role of DA and the derived N-β-alanyldiphenols as precursors for dark, heavily sclerotized cuticle. However, *P. americana* cuticle differs from the hard brown cuticle of *M. sexta*, *T. castaneum*, and *M. domestica* [7–9] in that the N-acetyl catecholamines NADA and NANE also appear to play significant roles in the stabilization process.

LITERATURE CITED

1. Andersen SO: Biochemistry of insect cuticle. Annu Rev Entomol 24, 29 (1979).
2. Andersen SO: Sclerotization and tanning in cuticle. In: *Compar Insect Physiol Biochem Pharmacol*. Kerkut GA, Gilbert LI, eds. Pergamon, Oxford, Vol 3, pp 59–74 (1985).
3. Brunet PCJ: The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. Insect Biochem *10*, 467 (1980).

4. Karlson P, Sekeris CE: N-Acetyldopamine as sclerotizing agent of the insect cuticle. Nature (Lond) *195*, 183 (1962).
5. Hopkins TL, Morgan TD, Aso Y, Kramer KJ: N-β-Alanyldopamine: Major role in insect cuticle tanning. Science *217*, 364 (1982).
6. Schaefer J, Kramer KJ, Garbow JR, Jacob GS, Stejskal EO, Hopkins TL, Speirs RD: Aromatic cross-links in insect cuticle: Detection by solid-state ^{13}C and ^{15}N NMR. Science *235*, 1200 (1987).
7. Hopkins TL, Morgan TD, Kramer KJ: Catecholamines in haemolymph and cuticle during larval, pupal and adult development of *Manduca sexta*. Insect Biochem *14*, 553 (1984).
8. Kramer KJ, Morgan TD, Hopkins TL, Roseland CR, Aso Y, Beeman RW, Lookhart GL: Catecholamines and β-alanine in the red flour beetle *Tribolium castaneum*. Insect Biochem *14*, 293 (1984).
9. Roseland CR, Grodowitz MJ, Kramer KJ, Hopkins TL, Broce AB: Stabilization of mineralized and sclerotized puparial cuticle of muscid flies. Insect Biochem *15*, 521 (1985).
10. Bodnaryk RP, Brunet PCJ: 3-O-Hydrosulphato-4-hydroxyphenethylamine (dopamine 3-O-sulfate), a metabolite involved in the sclerotization of insect cuticle. Biochem J *138*, 463 (1974).
11. Mills RR, Lake CR, Alworth WA: Biosynthesis of N-acetyldopamine by the American cockroach. J Insect Physiol *13*, 1539 (1967).
12. Murdock LL, Hopkins TL, Wirtz RA: Tyrosine metabolism in vivo in teneral and mature cockroaches, *Periplaneta americana*. J Insect Physiol *16*, 555 (1970).
13. Bodnaryk RP, Brunet PCJ, Koeppe J: On the metabolism of N-acetyldopamine in *Periplaneta americana*. J Insect Physiol *20*, 911 (1974).
14. Atkinson PW, Brown WV, Gilby RA: Phenolic compounds from insect cuticle: Identification of some lipid antioxidants. Insect Biochem *3*, 309 (1973).
15. Brunet PCJ, Kent PW: Observations on the mechanism of tanning reaction in *Periplaneta* and *Blatta*. Proc R Soc Lond [Biol] *144*, 259 (1955).
16. Pryor MGM, Russell PB, Todd AR: Protocatechuic acid, the substance responsible for the hardening of the cockroach ootheca. Biochem J *40*, 627 (1946).
17. Flint RA, Patton RL: Relation of eye color to molting in *Periplaneta americana* (L.). Bull Brooklyn Entomol Soc *54*, 140 (1959).
18. Murdock LL, Omar D: N-Acetyldopamine in insect nervous tissue. Insect Biochem *11*, 161 (1981).
19. Morgan TD, Hopkins TL, Kramer KJ, Roseland CR, Czapla TH, Tomer KB, Crow FW: N-β-Alanylnorepinephrine: Biosynthesis in cuticle and possible role in sclerotization. Insect Biochem *17*, 255 (1987).
20. Anderson SO: Cuticular sclerotization. In: *Cuticle Techniques in Arthropods*. Miller TA, ed. Springer, Berlin pp 185–212 (1980).
21. Tallan HH, Bella ST, Stein WH, Moore S: Tyrosine-O-sulfate as a constituent of normal human urine. J Biol Chem *217*, 703 (1955).
22. Jenner WN, Rose FA: Studies on the sulfation of 3,4-dihydroxyphenylethylamine (dopamine) and related compounds by rat tissues. Biochem J *135*, 109 (1973).
23. Kramer KJ, Hopkins TL, Ahmed RF, Mueller D, Lookhart G: Tyrosine metabolism for cuticle tanning in the tobacco hornworm, *Manduca sexta* (L.) and other Lepidoptera: Identification of β-D-glucopyranosyl-O-L-tyrosine and other metabolites. Arch Biochem Biophys *205*, 146 (1980).
24. Heilmann J, Barollier J, Watzke E: Beitrag zur aminosaurebestimmung auf papierchromatogrammen. Hoppe-Seyler Z Physiol Chem *309*, 219 (1957).
25. Block RJ, Durrum EL, Zweig G: A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press, New York, p 253 (1955).
26. McKittrick FA: Evolutionary studies of cockroaches. Cornell Univ Agric Exp Sta Mem *389*, 197 (1964).
27. Demassieux S, Bordleau L, Gravel D, Carriere S: Catecholamine sulfates: End products of metabolic intermediates. Life Sci *40*, 183 (1986).
28. Kramer KJ, Nuntnarumit C, Aso Y, Hawely MD, Hopkins TL: Electrochemical and enzymatic oxidation of catecholamines involved in sclerotization and melanization of insect cuticle. Insect Biochem *13*, 475 (1983).

29. Hopkins TL, Wirtz RA: Dopa and tyrosine decarboxylase activity in tissues of *Periplaneta americana* in relation to cuticle formation and ecdysis. J Insect Physiol 22, 1167 (1976).
30. Hopkins TL: Tyrosine hydroxylase activity and tyrosine titers during cockroach metamorphosis. Experientia *38*, 235 (1982).
31. Ahmed RF, Hopkins TL, Kramer KJ: Tyrosine and tyrosine glucoside titres in whole animals and tissues during development of the tobacco hornworm *Manduca sexta* (L.). Insect Biochem *13*, 369 (1983).
32. Czapla TH: Catecholamines in the Hemolymph and Cuticle of the American Cockroach, *Periplaneta americana* (L.) and the Madeira Cockroach, *Leucophaea maderae* (F.) Master's Thesis, Kansas State University (1985).
33. Wheeler R: Studies on total haemocyte count and haemolymph volume in *Periplaneta americana* (L.) with special reference to the last moulting cycle. J Insect Physiol *9*, 223 (1963).
34. Wirtz RA, Hopkins TL: Tyrosine and phenylalanine concentrations in hemolymph and tissues of the American cockroach, *Periplaneta americana*, during metamorphosis. J Insect Physiol *20*, 1143 (1974).
35. Hopkins TL, Murdock LL, Wirtz RA: Tyrosine side-chain metabolism in larval and adult cockroaches, *Periplaneta americana*: Post-ecdysial patterns. Insect Biochem *1*, 97 (1971).
36. Malek SRA: Polyphenols and their quinone derivatives in the cuticle of the desert locust, *Schistocerca gregaria* (Forskal). Comp Biochem Physiol *2*, 35 (1961).
37. Kennaugh JH: Amino acid metabolism, polyphenol production and the process of tanning in the cockroach *Periplaneta americana*. J Insect Physiol *2*, 97 (1958).
38. Hackman RH, Goldberg M: Studies on the hardening and darkening of insect cuticle. J Insect Physiol *17*, 335 (1971).
39. Mills RR, Fox FR: The sclerotization process by the American cockroach: Contribution of melanin. Insect Biochem *2*, 23 (1972).
40. Andersen SO, Barrett FM: The isolation of ketocatechols from insect cuticle and their possible role is sclerotization. J Insect Physiol *17*, 69 (1971).
41. Andersen SO: Cuticular sclerotization in larval and adult locusts, *Schistocerca gregaria*. J Insect Physiol *20*, 1537 (1974).
42. Vincent JFV, Hillerton JE: The tanning of insect cuticle—A critical review and a revised mechanism. J Insect Physiol *25*, 653 (1979).
43. Hillerton JE, Vincent JFV: The stabilization of insect cuticles. J Insect Physiol *25*, 957 (1979).