

Catecholamines in the Cuticles of Four Strains of the German Cockroach *Blattella germanica* (L.) During Sclerotization and Melanization

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Catecholamines were extracted from the cuticles of four strains of the cockroach *Blattella germanica* at different times 48 h after adult ecdysis and analyzed by reverse phase HPLC with electrochemical detection. The wild (*VPI*), black (*Bl*), orange (*or*), and yellow (*y*) phenotypes differ in cuticular pigmentation, particularly in the extent of melanization. *N*- β -Alanyldopamine (NBAD) and *N*- β -alanyl norepinephrine (NBANE) were major *o*-diphenolic compounds in extracts from cuticle of all strains during the main period of sclerotization. *N*-Acetyldopamine (NADA) and *N*-acetyl norepinephrine (NANE) were minor the first day after ecdysis, but accumulated to higher levels thereafter. Dopamine (DA) concentrations were higher in the darker pigmented cuticles of strains *Bl* and *or* than in the lighter-colored cuticles of strains *VPI* and *y*. Extractable DA rapidly increased in *VPI*, *Bl*, and *or* cuticles shortly after ecdysis, reached peak levels 6-24 h later, and then decreased after melanization. Only small amounts of DA were detected in strain *y* cuticle, whereas NBANE concentrations were very high. Therefore, high DA levels in cuticle are correlated with melanization that occurs during the first few hours after adult ecdysis, whereas sclerotization is correlated with high levels of the *N*- β -alanyl catecholamines. Sclerotization appears to be delayed in strain *Bl*, since only low concentrations of the *N*-acylated catecholamines accumulate until after melanization is completed.

Key words: dopamine, *N*-acetyldopamine, *N*- β -alanyldopamine, *N*- β -alanyl norepinephrine, *N*-acetyl norepinephrine, mutants, cockroaches

INTRODUCTION

The role of catecholamines as precursors for quinonoid compounds that sclerotize and melanize insect cuticle has been investigated extensively [1-5].

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N-acetyldopamine and *N*- β -alanyldopamine have been identified as major precursors for quinone sclerotizing agents in most insect cuticles [6–8], whereas dopamine appears to be an important precursor for insect melanins [9–12]. Dopamine may be *N*-acylated with acetate or β -alanine to form NADA* and NBAD, respectively, in the sclerotization pathway, or it may be oxidized directly to dopamine *o*-quinone, eventually leading to melanin biosynthesis [13].

The temporal patterns of catecholamine metabolism for sclerotization and melanization have been investigated previously in brown (wild-type) and black cuticles of mutant phenotypes of the red flour beetle, *Tribolium castaneum* [10,12]. Melanization of the black phenotype occurs during the first day after ecdysis and is correlated with high cuticular concentrations of DA and an absence of both β -alanine and NBAD. The delay in sclerotization caused by the shunt of DA into the melanin biosynthetic pathway results in a temporarily weaker cuticle. However, after melanization is completed, *N*-acylation of DA with β -alanine increases and cuticular strength becomes equal to the wild strain within a few days. Therefore, the partitioning of DA between two competing pathways for either melanization or sclerotization of insect cuticle appears to be regulated by the availability of *N*-acylating substrates and by the *N*-acyltransferases that catalyze the conjugation of DA.

To further investigate the temporal sequence of catecholamine metabolism underlying insect cuticle melanization and sclerotization, we have analyzed the profiles and concentrations of catecholamines and related *o*-diphenols in tanning adult cuticle of four strains of the German cockroach, *Blattella germanica*, wild (*VPI*), black (*Bl*), orange (*or*), and yellow (*y*). A correlation between certain catecholamines and phenotypic pigmentation of the strains was observed.

MATERIALS AND METHODS

Insects

Strains of *B. germanica* were obtained from Dr. Mary Ross, Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA [14], and reared in plastic containers bedded with wood shavings at $28 \pm 2^\circ\text{C}$ with a light:dark photoperiod of 16:8 h. Water and Purina® "Lab Chow" were provided ad lib. Adults were collected at ecdysis for analysis at different times during the period of cuticle tanning, quickly frozen with dry ice, and then stored at -20°C until analyzed.

Extraction of Catecholamines From Cuticle

Abdominal integument was dissected and cleaned of adhering muscle and fat body in distilled water, and the inner surface was scraped to remove epidermis. Pieces of cuticle were rinsed and blotted dry on absorbent tissue and weighed on a microbalance, with the final weight being recorded after water

*Abbreviations used: AMD = α -methyl dopa; ANOVA = analysis of variance; DA = dopamine; DDC = dopa decarboxylase; DOPAC = 3,4-dihydroxyphenylacetic acid; DOPET = 3,4-dihydroxyphenylethanol; DOPKET = 3,4-dihydroxyphenylketoethanol; LSD = least significant difference; NADA = *N*-acetyldopamine; NANE = *N*-acetylnorepinephrine; NBAD = *N*- β -alanyldopamine; NBANE = *N*- β -alanylnorepinephrine; NE = norepinephrine; SAS = Statistical Analysis System.

loss from evaporation was less than $1 \mu\text{g s}^{-1}$. Cuticle (1–2 mg) was homogenized in a ground glass tissue grinder containing 0.15 ml of 1.2 M HCl, 5 mM ascorbic acid, and 0.120 mg of the internal standard, AMD. The homogenate was centrifuged at 6,500g for 10 min, and the supernatant was removed for analysis by HPLC with electrochemical detection.

Analysis of Catecholamines

Extracts (0.1 ml) were adsorbed on alumina and recovered in 1 M acetic acid [15]. *o*-Diphenols were resolved by a reversed phase, C18 5 μm column at a flow rate of 1 ml min^{-1} and detected with a Bioanalytical Systems LC4B electrochemical detector at +720 mV. The primary mobile phase consisted of 15% methanol, 160 μM sodium octyl sulfate, 90 μM sodium EDTA, and 0.1 M H_3PO_4 adjusted to pH 2.9 with NaOH. A second mobile phase consisted of 26% acetonitrile, 1.1 mM sodium dodecyl sulfate, 50 μM sodium EDTA, and 0.1 M H_3PO_4 adjusted to pH 3.3 with NaOH. The retention times of catecholamine standard compounds were compared to those of electroactive compounds in cuticular extracts using both mobile phases. Chemical standards were obtained from commercial sources or were synthesized [15]. Quantities of individual catecholamines were determined by comparing peak areas with those of an internal standard recovered in each extract and then correcting for recoveries established using standard compounds.

Statistical Analysis

Data were analyzed by SAS [16], using ANOVA with the LSD for each variable (catecholamines vs. strains) as a function of time.

RESULTS

Catecholamine Profiles in Cuticle During Melanization and Sclerotization

Dopamine and the *N*-acyl catecholamines were the major *o*-diphenols found in tanning cuticle of the four strains of *B. germanica*. In general, DA concentrations were correlated positively with extent of melanization, whereas the *N*- β -alanyl catecholamines, for the most part, were inversely correlated with DA during the early stages of cuticle tanning (Figs. 1–4). Minor amounts ($\leq 50 \text{ nmol g}^{-1}$) of DOPAC, DOPET, and DOPKET also were detected in cold acid cuticular extracts, but their concentration profiles did not correlate with tanning or pigmentation.

NBANE was the major *o*-diphenol extracted from cuticle of the wild-type strain VPI at ecdysis (60 nmol g^{-1}). Maximal amounts were extractable by 3 h after ecdysis (320 nmol g^{-1}), and a slightly lower level was maintained during the remaining period of sclerotization (Fig. 1). NBAD concentrations were initially low ($< 20 \text{ nmol g}^{-1}$), but reached levels comparable to those of NBANE 12 h after ecdysis (200 nmol g^{-1}). Extractable DA was also low at ecdysis ($< 20 \text{ nmol g}^{-1}$), but rapidly increased to 180 nmol g^{-1} at 3 h, followed by a gradual decline. The *N*-acetyl catecholamines, NADA and NANE, were present only at relatively low levels during the first 24 h ($< 0.1 \mu\text{mol g}^{-1}$). However, NANE gradually increased to levels comparable to those of *N*- β -alanyl catecholamines at 48 h (180 nmol g^{-1}).

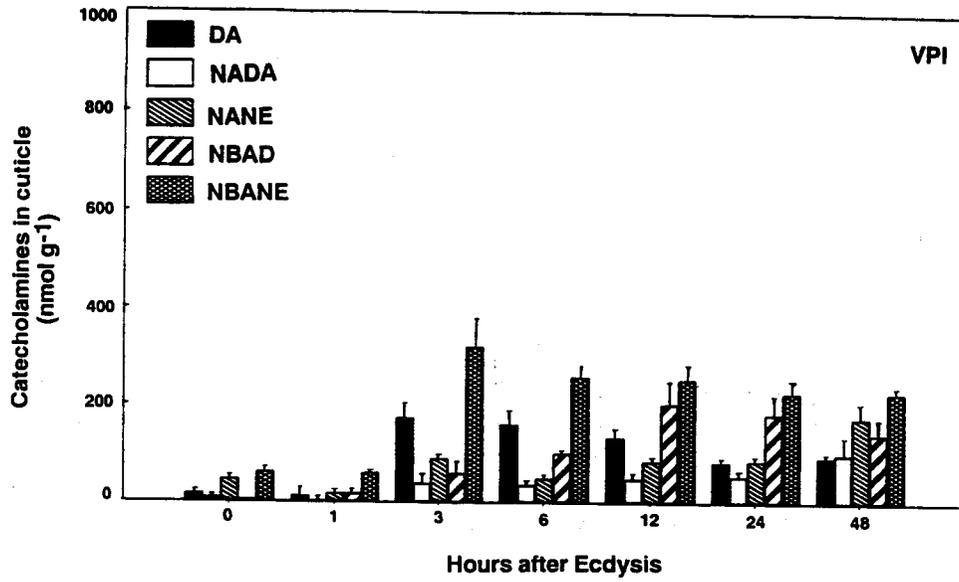


Fig. 1. Concentrations of catecholamines extracted with cold 1.2 M HCl from cuticle of *Blattella germanica* wild-type VPI strain after adult ecdysis. Data are means of three to seven samples \pm SE.

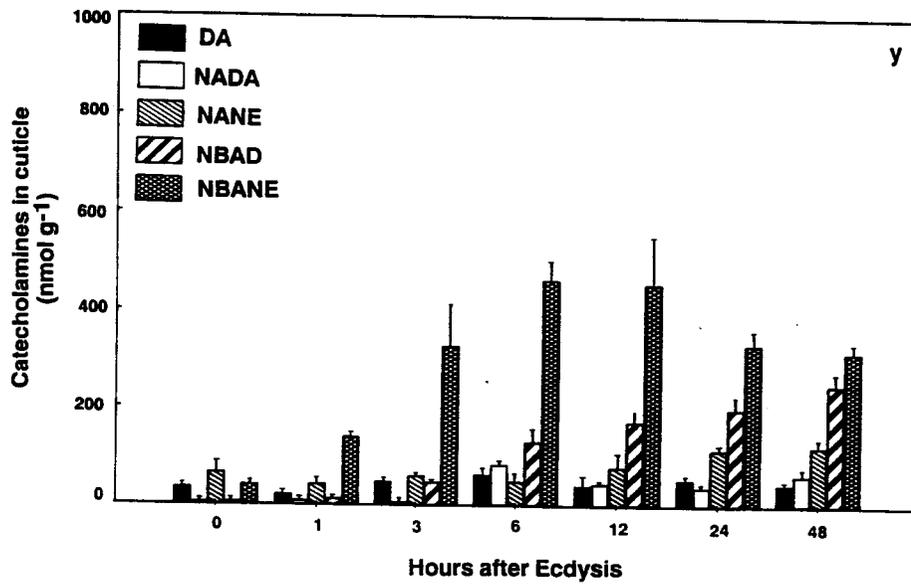


Fig. 2. Concentrations of catecholamines extracted with cold 1.2 M HCl from cuticle of *Blattella germanica* strain y after adult ecdysis. Data are means of three to seven samples \pm SE.

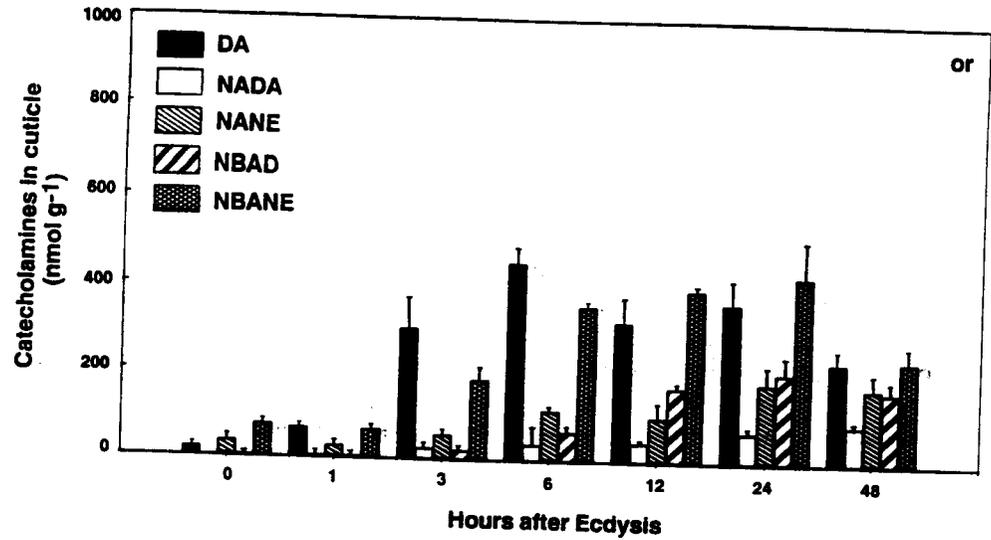


Fig. 3. Concentrations of catecholamines extracted with cold 1.2 M HCl from cuticle of *Blattella germanica* strain *or* after adult ecdysis. Data are means of three to seven samples \pm SE.

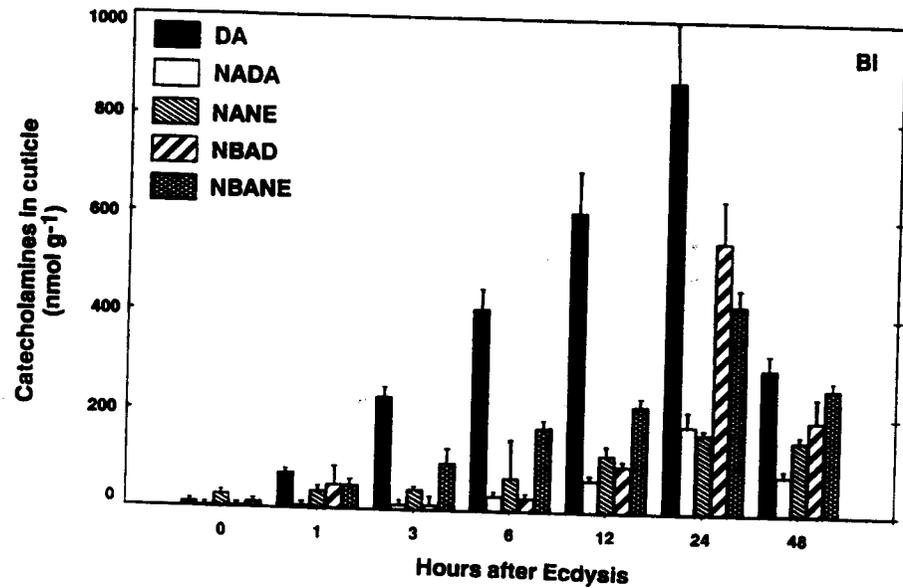


Fig. 4. Concentrations of catecholamines extracted with cold 1.2 M HCl from cuticle of *Blattella germanica* strain *BI* after adult ecdysis. Data are means of three to seven samples \pm SE.

NBANE was also the major extractable diphenol in strain γ cuticle and increased about tenfold between 3 and 6 h after ecdysis (460 nmol g^{-1}) (Fig. 2). Less was extractable between 12 and 24 h as sclerotization proceeded. NBAD gradually accumulated in cuticle from less than 50 nmol g^{-1} to equal the concentration of NBANE at 48 h. DA, NADA, and NANE concentrations were very low in strain γ ($< 80 \text{ nmol g}^{-1}$) during sclerotization, and NANE increased only slightly to 120 nmol g^{-1} at 48 h.

DA and NBANE were the major catecholamines present in extracts of strain *or* cuticle. DA reached a peak (450 nmol g^{-1}) between 3 and 6 h after ecdysis, remained high through 24 h, but then declined about twofold at 48 h (Fig. 3). NBANE increased at a slower rate to reach a peak (450 nmol g^{-1}) between 6 to 24 h. About twofold less was extractable by 48 h. NBAD and NANE were slower to increase, but both catecholamines reached a maximum (170 and 180 nmol g^{-1} , respectively) at 24 h. NADA was detected only at very low concentrations throughout the sclerotization period ($< 90 \text{ nmol g}^{-1}$).

In the cuticle of strain *Bl*, DA was the major extractable catecholamine, reaching a peak (880 nmol g^{-1}) at 24 h after ecdysis, corresponding to an 80-fold increase. At 48 h, threefold less DA was recovered (Fig. 4). The β -alanyl catecholamines, NBANE and NBAD, remained at low concentrations until 6 and 12 h after ecdysis, respectively. NBAD then increased by 200-fold to 550 nmol g^{-1} , whereas NBANE increased about 35-fold to 430 nmol g^{-1} at 24 h after ecdysis. *N*-Acetyl catecholamines increased from low levels to about 150 nmol g^{-1} at 24 h.

Comparison of Cuticular Catecholamines Between Strains

Extractable DA concentrations were significantly greater in cuticles of strains *Bl* and *or* than in those of strains *VPI* and *y* from 1 through 48 h after ecdysis (Table 1). *Bl* cuticle also contained more extractable DA than *or* cuticle at 12 and 24 h. Although mean DA levels in strain *y* cuticle were less than those in wild-type strain *VPI*, the differences were not significant, except at 6 h.

Extractable NBANE concentrations were substantially less in *Bl* cuticle than in cuticles from other strains, except very late in the sclerotization period (24 and 48 h, Table 2). Conversely, NBANE concentrations were significantly higher in the cuticle of *y* than in *VPI* from 6 through 48 h after adult ecdysis.

Differences between *B. germanica* strains in total *N*- β -alanyl catecholamine concentrations (NBAD + NBANE) in cuticle were very similar to those of NBANE alone. *Bl* cuticle had substantially less total *N*- β -alanyl catecholamines than the other strains, whereas at several times, *y* cuticle displayed the highest levels of these sclerotizing precursors. *N*-Acetyl catecholamines (NADA and NANE), however, did not differ significantly between strains.

Few differences were observed in total extractable amounts of catecholamines present in cuticles from all strains, with a major exception occurring at 24 h after ecdysis (Fig. 5). At this time, strain *Bl* cuticle had over twice the extract-

TABLE 1. Dopamine (nmol g^{-1}) in Cuticular Extracts From Different Strains of *Blattella germanica* After Adult Ecdysis*

Hours after ecdysis	Strains			
	<i>VPI</i>	<i>y</i>	<i>or</i>	<i>Bl</i>
0	20ab	40a	20ab	10b
1	10b	20b	70a	70a
3	170ab	50b	300a	230a
6	160b	60c	450a	410a
12	130c	40c	320b	610a
24	80c	50c	370b	880a
48	90b	40b	230a	280a

*Means with same letter and in the same row are not significantly different at $P \leq .05$ level (ANOVA by time, $n = 17-23$).

TABLE 2. *N*- β -Alanyl norepinephrine (nmol g⁻¹) in Cuticular Extracts From Different Strains of *Blattella germanica* After Adult Ecdysis*

Hours after ecdysis	Strains			
	<i>VPI</i>	<i>y</i>	<i>or</i>	<i>Bl</i>
0	60a	40ab	80a	10b
1	60b	140a	70b	50b
3	320a	330a	180ab	90b
6	260c	460a	360b	170c
12	250b	460a	400ab	220b
24	220b	330ab	420a	430a
48	230b	320a	290ab	260ab

*Means with same letter and in the same row are not significantly different at $P \leq .05$ level (ANOVA by time, $n = 17-23$).

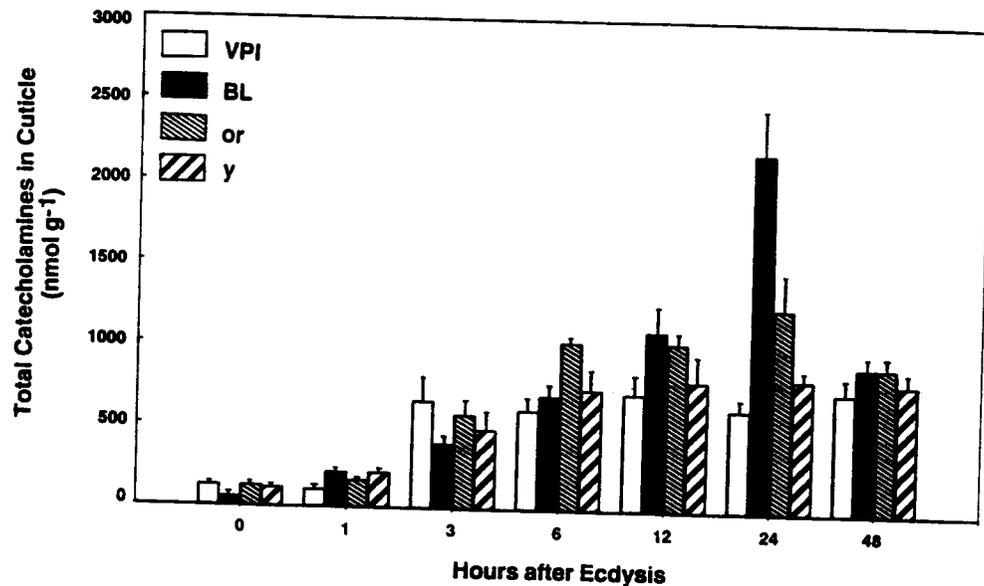


Fig. 5. Total cold-acid extractable catecholamines from cuticles of *Blattella germanica* strains after adult ecdysis. Data are means of three to seven samples \pm SE.

able catecholamines than did the cuticles of other strains. Most of this difference was accounted for by the high concentrations of DA in strain *Bl*. However, the extractable concentrations of the *N*- β -alanyl catecholamines were also higher in strain *Bl* than in the other *B. germanica* strains at 24 h.

DISCUSSION

Four strains of German cockroaches display substantial differences in concentrations of extractable catecholamines in adult cuticle during melanization and sclerotization. Cuticular DA is very abundant in *Bl* and *or* from 1 through 24 h, but is significantly less in strains *VPI* and *y*. The *N*- β -alanyl catecholamines, NBAD and NBANE, are abundant in the cuticles of all *B. germanica* strains, but they accumulate at the slowest rate in the black phenotype. The

N-acetyl catecholamines, NADA and NANE, do not accumulate to high levels in cuticle except when NANE reaches concentrations similar to the *N*- β -alanyl catecholamines during the 24–48 h period after ecdysis.

Genetic linkage analysis has shown that mutations occurring in the *B. germanica* strains are single alleles localized on different linkage groups. Strain *or* is on linkage group 4, chromosome 7; strain *Bl* linkage is on group 6, chromosome 6; and strain *y* is on linkage group 10, chromosome 4 or 8 [14]. Mutation *or* is also epistatic to mutation *y*. Apparently, each strain has a unique biochemical lesion that produces different cuticle colorations. A bright yellowish cuticle and an absence of dark bands on the pronotum are characteristic features of strain *y* adults [17]. NBANE is the major catecholamine in *y* cuticle with levels substantially greater than those in the wild-type *VPI* cuticle. Other catecholamines occur at similar concentrations in both *VPI* and *y* cuticles, although DA tends to be lower in *y* cuticle that lacks black pigmentation. DA accounts for only 6% of the total extractable catecholamines in *y* cuticle, but 25% in *VPI* cuticle at 6 h after adult ecdysis. This difference in catecholamine composition, coupled with high levels of NBANE in strain *y*, may explain the lighter coloration of strain *y* and the lack of black pronotal stripes. Total *o*-diphenol concentrations, however, are very similar in cuticles from both strains.

Adults of the *or* strain are easily distinguished from wild-type *VPI* adults by their orange-brown color and brown, rather than black, pronotal bands [18]. The catecholamine profile of *or* cuticle differs from that of *VPI* cuticle, with DA and NBANE concentrations being the major catecholamines occurring at about twofold higher levels during tanning. Because of relatively high DA levels in strain *or* (DA accounts for 44 and 29% of all extractable catecholamines in *or* cuticle at 6 and 24 h, respectively), one might expect to observe a darker coloration in *or* cuticle and pronotal stripes than in *VPI* cuticle. However, the stripes are a brownish orange and cuticle coloration is similar to that of *VPI* cuticle. DA may be unavailable or protected from oxidation by phenoloxidases and subsequent polymerization to melanins in *or* cuticle or the phenoloxidase may be deficient. The formation of a phaeomelanin (yellow-brown), in which quinones of DOPA, DA, or other catecholamines bind with cysteine rather than eumelanin (black) also may account for coloration of strain *or* cuticle [13,19].

Bl adults are characterized by extremely dark body pigmentation with black legs, antennae, and cerci compared to the light-brown coloration of wild-type strain *VPI* [17]. DA is significantly greater in *Bl* cuticle than in *VPI* cuticle throughout the sclerotization period and constitutes almost 60% of the extractable catecholamines at 6 h after ecdysis. In contrast to the other strains, buildup of the *N*-acyl catecholamines that presumably act as tanning precursors is delayed during the first 12 h after ecdysis when melanization occurs. DA reaches peak concentrations at 24 h, but at that time, melanization appears to be nearly completed. A large increase of extractable *N*- β -alanyl catecholamines occurs at that time, suggesting a high rate of sclerotization after an initial delay, when DA is being shunted into melanin biosynthesis.

Although *or* and *Bl* cuticles have similar concentrations of DA through 6 h, the cuticular coloration of each strain is quite different by that time. NBANE concentrations in *or* cuticle are about twice those of *Bl* and may be correlated with the lack of black pigmentation, in spite of high DA levels in the former.

The four strains of *B. germanica* produced similar total quantities of extractable catecholamines, but in different proportions. Total extractable catecholamines generally peaked at 24 h, and the following trend by age was observed for all strains $0 < 1 < 3 < 6, 12, 48 < 24$ h. An inverse relationship was observed between the relative amounts of DA and *N*- β -alanyl catecholamines in the four strains. Overall, relative percentages of DA in total catecholamines extracted by cold acid were $y < VPI < or < Bl$ (7, 26, 44, and 57%, respectively) at 6 and 24 h after ecdysis, whereas the relative percentages of *N*- β -alanyl catecholamines were in the reverse order (75, 60, 41, and 28%, respectively). The relative percentages of *N*-acetyl catecholamines were about the same for all strains at all times (14–22%).

Tyrosine is the major precursor of the catecholamines used for both sclerotization and melanization of insect cuticle, with DA being a common metabolite for both pathways [5,20]. *N*-Acylation of DA with either acetate or β -alanine produces the sclerotization precursors NADA or NBAD, respectively. Oxidation of these *N*-acyl catecholamines to *o*-quinone and *p*-quinone methides apparently leads to sclerotization of cuticle through formation of adducts and crosslinks with cuticular proteins and/or chitin [2,4,5]. However, oxidation of free DA to DA *o*-quinone leads to indolization and polymerization of these compounds to form melanins. The early appearance of relatively high concentrations of NBANE in cuticles of strains *or*, *y*, and *VPI* appears to correlate with the suppression of general melanin production, as observed in the black phenotype. In strain *Bl*, *N*-acylation of DA is delayed until after melanization. High concentrations of DA indicate a possible metabolic role for that catecholamine in the melanization pathway of insect cuticle [10–12]. Late occurrence of *N*-acylation of DA to form NBAD and subsequently NBANE indicates that the rate of sclerotization is possibly slowed in strain *Bl* and that hardening of the cuticle is completed after melanization. Presence of β -hydroxylated catecholamines suggests substantial conversion of *o*-quinones to *p*-quinone methides and the formation of side-chain adducts and crosslinks [4,5,20].

Several biochemical differences could account for phenotypes that exhibit different cuticular coloration patterns. Free β -alanine levels in each strain could either limit or increase *N*-acylation of DA. Abundant amounts would lower the concentration of free DA available for melanization and give a light-colored cuticle, as seen in strain *y*. Deficient quantities of β -alanine would have the opposite effect of increasing the availability of DA for melanin biosynthesis and darker coloration as found in strain *Bl*. Similar effects could occur if a mutation of an enzyme such as *N*- β -alanyltransferase or tyrosinase resulted in a deficiency or excess of free DA. A correlation between body coloration and levels of free DA and *N*- β -alanyl catecholamines in cuticle exists in all strains of *B. germanica*.

The increase in the *N*- β -alanyl catecholamines during sclerotization of *B. germanica* strain *Bl* cuticle is different from that displayed by the cuticle of *Tribolium castaneum* black mutant [12]. The flour beetle mutant exhibits extremely low levels of NBAD throughout the sclerotization process. Injection of β -alanine just after ecdysis rescues the wild-type pigmentation of the flour beetle mutant [12]. Similar injections into *Blattella* strain *Bl* also changed its coloration to that of the wild-type strain (K.J. Kramer, unpublished data). The lack of

β -alanine in dark-body mutants of *Drosophila melanogaster*, *D. virilis*, *Musca domestica*, and *Bombyx mori* has been reported [21–23]. Microinjections of β -alanine into the *D. melanogaster* black mutant rescue normal pigmentation, but not in the case of the *ebony* mutant [23,24]. β -Alanine supplemented in the diet produces the normal pigmentation in pupal cuticle for the *M. domestica* *bp* mutant [22]. When a mutant strain has a darker body pigmentation than the wild-type strain, a likely cause is an abnormal processing of β -alanine for DA conjugation. Results of β -alanine analogue treatment studies also suggest a major role for β -alanine as a determinant of cuticle coloration. Hydrazino and aminoxy derivatives of β -alanine injected in *Manduca sexta* interfere with β -alanine utilization, reducing NBAD and increasing DA levels, such that blackening (melanization) of the pupal cuticle occurs [25]. Coinjection of the analogues with excess β -alanine, however, results in normal brown color.

The presence or absence of JH may also have an effect on melanization of insect cuticle. In *M. sexta* last instar larvae, melanization of larval cuticle occurs if JH is absent during an 8 h period following head capsule apolysis [11,26]. In a color polymorphic species, *Spodoptera litura*, a decrease in JH titer at head capsule slippage is necessary for activation of the melanization-and-reddish-coloration hormone [27]. Suppression of melanization by JH also occurs in other color polymorphic species, like *Locusta migratoria* [28] and *Cephododes hylas* [29]. However, in *B. germanica*, JH has the opposite effect of enhancing melanization in the cuticle of nymphs treated 12 h before ecdysis [30]. It is possible that the mutation in strain *Bl* affects the regulation of JH titers rather than β -alanine or DA metabolism directly.

The catecholamine profile in cuticle of *B. germanica* wild-type VPI is similar to that of *Periplaneta americana* [15], but concentrations are approximately tenfold lower in the former. Both cockroach species have high extractable NBANE levels shortly after ecdysis, with NBAD accumulating later as sclerotization progresses. The *N*-acetyl catecholamines in both species are low during the first 24 h, but increase in the latter stages of sclerotization, whereas DA remains low. The cuticle of *B. germanica* differs from that of *P. americana* in that black pigments, presumably melanin, occur primarily in the abdomen and pronotum of the former and the overall cuticle coloration is a yellow-brown, rather than red-brown. Although DA concentrations in cuticle are similar in both species, DA constitutes a larger percentage of the extractable catecholamines in *B. germanica* (VPI) cuticle (26 and 13% at 6 and 24 h respectively) than in *P. americana* cuticle (6% at both times) [15]. The percentage of extractable catecholamines in *y* cuticle closely resembles that determined in *P. americana* cuticle at both 6 and 24 h. However, the coloration of *y* cuticle is similar to that of VPI cuticle but lacks the black pigmentation. The catecholamine profile of *Leucophaea maderae* cuticle differs from that of VPI or *P. americana* cuticle, because of high levels of the *N*-acetyl catecholamines NADA and NANE [31]. These may play a more important role in sclerotization of this species.

The wild-type and mutant strains of *B. germanica* are useful for relating chemical composition to cuticle type. Additional work is required to determine the genetic basis for regulation of cockroach catecholamine metabolism underlying melanization and sclerotization, as well as the resulting effects on the physical properties of the exoskeleton.

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