

Tyrosine Metabolism for Insect Cuticle Tanning

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Insects have become one of the most successful animal groups in diversity and numbers through the development of a multifunctional exoskeleton and skin, which must be shed periodically in order for them to grow and develop into adults. The evolutionary choice of certain structural materials for the assembly and stabilization of a cuticle with remarkable mechanical and chemical properties has allowed insects to invade terrestrial environments and to evolve flight mechanics for dispersion relatively early in geological history. Diphenolic compounds derived from tyrosine play a central role in sclerotization or tanning of the new cuticle. The phenolic amino acid is stored during larval feeding, and it is mobilized for the production of both structural proteins and diphenolic tanning precursors that are transported into the cuticle. The latter compounds permeate the cuticle and serve as precursors for quinonoid derivatives that both sclerotize and pigment the exoskeleton. This report focuses on how tyrosine and derived diphenolic structures are stored as inactive molecules in preecdysial stages, and how they are released and metabolized to tanning chemicals that stabilize the new cuticle.

Key words: sclerotization, catechol, catecholamines, chitin, protein, ecdysteroid, quinonoid, conjugate, solid state NMR, diphenols, quinone methide

INTRODUCTION

Tyrosine is a highly versatile amino acid for insects because of the phenolic, amino, and carboxylic acid functional groups. It serves as a precursor for

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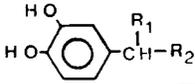
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diphenolic compounds that stabilize and pigment the cuticle (exoskeleton and skin) and other proteinaceous structures such as cocoon silks and egg cases, and biogenic amines that function as neurotransmitters, neurohormones, and neuromodulators. Other roles include precursors for defensive or communicating chemicals in some species. Tyrosine metabolism is therefore a topic worthy of continued research because of its vital importance in the physiology of the skeletal, nervous, hormonal, and behavioral systems of insects [1-3].

Tyrosine or its precursor phenylalanine is mainly supplied to the insect by digestion of dietary protein. The latter amino acid is rapidly hydroxylated by phenylalanine hydroxylase, a rather ubiquitous enzyme in insect tissues, after absorption from the gut to yield tyrosine. Tyrosine may then be stored or sequestered as conjugates for later conversion to diphenols, which are the main class of compounds serving as precursors of cuticle-sclerotizing agents. For example, dopamine (see Table 1 for structure) or N-acetyldopamine may participate in sclerotization, pigmentation, or neural function depending upon tissue localization and stage of development. Because of the blood-brain barrier and conjugating systems that block metabolism, the coexistence of similar if not identical tyrosine metabolites and associated enzymes in both skeletal and neural tissues is possible, and there are interactions between the two different kinds of tissues for regulation of tyrosine metabolism. In this paper, we will address the metabolic reactions that occur in insect tissues to stabilize and pigment the cuticle or other tanned structures such as oothecae and cocoon silks.

TABLE 1. Structures of Principal Catechols Extracted From Insect Cuticle

			
		R ₁	R ₂
Dopamine	DA	H	CH ₂ -NH ₂
Dihydroxyphenylalanine	DOPA	H	COOH CH-NH ₂
N-Acetyldopamine	NADA	H	O CH ₂ -NH-C-CH ₃
N-β-Alanyldopamine	NBAD	H	O CH ₂ -NH-C-CH ₂ -CH ₂ -NH ₂
Norepinephrine	NE	OH	CH ₂ -NH ₂
N-Acetylnorepinephrine	NANE	OH	O CH ₂ -NH-C-CH ₃
N-β-Alanylnorepinephrine	NBANE	OH	O CH ₂ -NH-C-CH ₂ -CH ₂ -NH ₂
Dihydroxyphenylacetic acid	DOPAC	H	COOH
Dihydroxyphenylethanol	DOPET	H	CH ₂ OH

Diphenolic Compounds in Cuticle and Other Tanned Structures

Diphenols often occur in abundance in insect cuticle. For example, 3,4-dihydroxyphenylacetic acid constitutes nearly 2% of the dry weight of beetle cuticle [4,5]. Solid state NMR* studies on chemical composition indicate that catecholic metabolites increase 3.5-fold during sclerotization of *Manduca sexta* pupal cuticle and, in the pupal exuvia, are greatly enriched, accounting for >10% of the total carbon mass [6]. Experiments using radiolabeled tyrosine or its diphenolic metabolites in vivo show incorporation into cuticle that becomes sclerotized [7]. Both epicuticle and procuticle become labeled either diffusely or in discrete bands [8,9]. The diffuse labeling is believed to represent primarily tyrosine or a metabolite(s) being transported, while the discrete banding probably corresponds to incorporation of metabolites (diphenols) used for cuticle sclerotization.

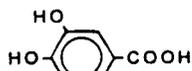
The search for insect tanning agents derived from tyrosine first occurred using a noncuticular structure, the cockroach egg capsule (ootheca), and the accessory gland, which contained β -glucosides of DHBZ and 3,4-dihydroxybenzyl alcohol (Fig. 1) [10-13]. A number of diphenolic acids, aldehydes, and alcohols were subsequently reported to occur in cuticle of insects from several different orders [14]. Although derived from tyrosine by way of DOPA, the compounds lacked an amino group in the side chain, presumably to prevent the formation of indole and subsequently of catecholamine melanin during sclerotization. The discovery of NADA in 1962 as the phenolic precursor of sclerotizing intermediates in *Calliphora* cuticle pointed to a critical role played by N-acylated catecholamine derivatives in the tanning of cuticular structures [7]. In 1982, NBAD was identified to be the major precursor for sclerotizing agents in stiff brown cuticle [15]. Since then, the N-acetyl and N- β -alanyl derivatives of norepinephrine have been discovered to be the major metabolites of NADA and NBAD, respectively, in cuticle, and as such, appear to play a significant role in the sclerotization process [16-18]. Other noncuticular tanning agents include NMAD and N-(N-acetyl- β -alanyl)dopamine in mantid oothecae [19-21] as well as DOPA, N-(3,4-dihydroxyphenyllactyl)-DOPA, and 3-hydroxyanthranilic acid in silkworm cocoons [22,23].

Tyrosine metabolites found in cuticle may have functions other than cuticle sclerotization. Catecholic compounds also serve as precursors for brown and black pigmentation, prevent entry of microbial or fungal pathogens, or facilitate proper orientation of epicuticular lipids [24]. N- β -Alanyl catecholamines are often associated with brown sclerotins, while high dopamine levels often result in melanization [1,5]. The yellow wing pigment in certain papilionid butterflies is also an NBAD derivative [25]. Certain catecholic acids

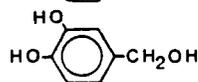
*Abbreviations: cAMP = adenosine 3':5'-monophosphate; DA = dopamine; DDC = DOPA decarboxylase; DHBZ = 3,4 dihydroxybenzoic acid; DOPA = 3,4-dihydroxyphenylalanine acid; DOPAC = 3,4-dihydroxyphenylacetic acid; DOPKET = 3,4-dihydroxyphenylketoethanol; EDTA = ethylenediaminetetraacetate; NADA = N-acetyl-dopamine; NANE = N-acetylnorepinephrine; NBAD = N- β -alanyldopamine; NBANE = N- β -alanylnorepinephrine; NMAD = N-malonyldopamine; PTF = puparial tanning factor; TH = tyrosine hydroxylase; NMR = nuclear magnetic resonance.

A. COCKROACH EGG CAPSULE

3,4-Dihydroxybenzoic acid

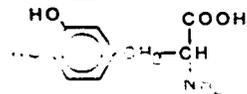
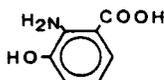


3,4-Dihydroxybenzylalcohol

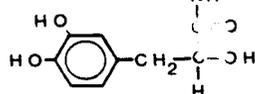
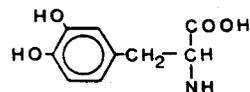


B. SILK MOTH COCOON

3-Hydroxyxanthranilic acid

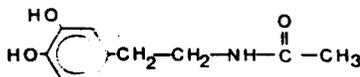


N-(3,4-Dihydroxyphenyllactyl) DOPA

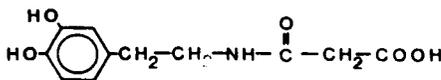


C. MANTID EGG CASE

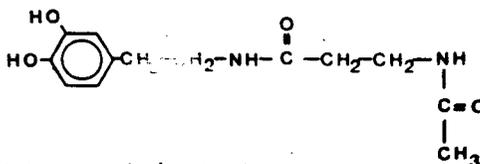
N-Acetyldopamine



N-Malonyldopamine



N-(N-Acetyl-β-alanyl)dopamine



1. Tanning diphenols in noncuticular structures.

and alcohols in cockroach cuticle have been shown to act as antioxidants in protecting the lipid waterproofing layer of the epicuticle [26,27].

Synthesis and Release of Tyrosine and Diphenolic Conjugates

The storage of tyrosine during larval feeding periods is a prerequisite for the large requirement of diphenolic compounds needed to sclerotize and pigment a newly elaborated cuticle during each molting cycle [1,28]. Tyrosine is such a sparingly soluble amino acid that insects have had to resort to chemical modifications or conjugations in order to sequester enough to meet the need for cuticle morphogenesis. To increase solubility, it is coupled with polar substituents such as phosphate, glucose, or β-alanine (Table 2). The solubility of β-D-glucopyranosyl-O-L-tyrosine (tyrosine glucoside) found in

TABLE 2. Tyrosine Conjugates in Insecta

Conjugate	Lepidoptera	Diptera
Tyrosine-O-glucoside ^a	<i>Anavitrinella pampinaria</i>	<i>Drosophila busckii</i>
	<i>Bombyx mori</i>	<i>Ceratitis capitata</i>
	<i>Cephanonodes hyalis</i>	
	<i>Ceratomia catalpae</i>	
	<i>Diatraea grandiosella</i>	
	<i>Ephestia cautella</i>	
	<i>Lampides boeticus</i>	
	<i>Leucania separata</i>	
	<i>Manduca sexta</i>	
	<i>Maruca testulalis</i>	
	<i>Ostrinia nubilalis</i>	
	<i>Papilio bianor</i>	
	<i>Papilio helenus</i>	
	<i>Papilio proteno</i>	
	<i>Papilio xuthus</i>	
	<i>Peridroma saucia</i>	
	<i>Phalera flavescens</i>	
	<i>Pieris melete</i>	
	<i>Pieris rapae</i>	
	<i>Plodia interpunctella</i>	
<i>Spodoptera littoralis</i>		
<i>Theretra japonica</i>		
<i>Thyridopteryx ephemeraeformis</i>		
<i>Trichoplusia ni</i>		
Tyrosine-O-phosphate ^b		<i>Drosophila americana</i>
		<i>Drosophila ananassae</i>
		<i>Drosophila funebris</i>
		<i>Drosophila hydei</i>
		<i>Drosophila macropina</i>
		<i>Drosophila melanogaster</i>
		<i>Drosophila mercatorium</i>
		<i>Drosophila nigromelanica</i>
		<i>Drosophila povani</i>
		<i>Drosophila simulans</i>
		<i>Drosophila subfunebris</i>
		<i>Drosophila subobscura</i>
		<i>Drosophila virilis</i>
N- β -Alanyltyrosine ^c		<i>Agria housei</i>
		<i>Sarcophaga bullata</i>
		<i>Sarcophaga barbata</i>
		<i>Sarcophaga crassipalpus</i>
		<i>Sarcophaga peregrina</i>
	<i>Phryxe caudata</i>	

^a[From 29,33-39].^b[From 72,76].^c[From 42,52,142].

many Lepidoptera is nearly tenfold greater than that of tyrosine itself [29]. Tyrosine may also be conjugated with phosphate as in most *Drosophila* species [30]. The developmental profile and isotopic labeling experiments with [¹⁴C]-tyrosine of this phosphate ester indicate that it is hydrolyzed prior to its incorporation into the puparium [31]. Tyrosine phosphate serves as a tyrosine reservoir for sclerotization in ten *Drosophila* species belonging to three different subgenera [32]. In one species, *D. busckii*, however, tyrosine is not conjugated with phosphate but instead is coupled with glucose as tyrosine glucoside, which also serves as a tyrosine reservoir for sclerotization of the puparium. The occurrence of this conjugate has been verified in one other dipteran species, the Mediterranean fruit fly, *Ceratitis capitata* [33], and in over 20 species of Lepidoptera [29,34-37]. In general, the glucoside is synthesized at a high rate in the last larval instar and hydrolyzed abruptly at the larval-pupal transformation, when free tyrosine is utilized for pupal or puparial cuticle tanning [34,37-39]. A second increase in tyrosine glucoside has been observed in pharate adults, with a decrease after adult eclosion, suggesting a similar role in adult cuticle tanning [34,35,39]. Tyrosine glucoside appears to be bifunctionally important in molting metabolism because it is also a source of glucose that may be used for chitin synthesis during cuticle morphogenesis.

In *Calpodes ethlius* and perhaps other species including *Leptinotarsa decemlineata*, *Phormia regina*, *M. sexta*, *Hyalophora cecropia*, and *Chironomus tentans* [40], free tyrosine appears to be available for larval cuticle stabilization at levels forty-fold greater than the solubility limit in watery vacuoles present in fat body that begin to disappear at molting. In *Drosophila* hemocytes, tyrosine is stored in situ as a crystalline inclusion [41]. Whether tyrosine in the vacuoles or inclusions is actually free or in the form of a highly labile conjugate that is rapidly converted to tyrosine when the tissue is extracted during analytical procedures is uncertain.

Dipeptide and protein forms of tyrosine are also well documented in the Insecta. The flesh fly, *Sarcophaga bullata*, accumulates in the hemolymph N- β -alanyl-L-tyrosine, which is rapidly hydrolyzed into the two amino acids for incorporation into the puparial cuticle [42,43]. Large molecular weight hemolymph proteins or arylphorins that contain high levels of tyrosine (about 10 mole percent) are believed to be storage forms for amino acids sequestered during the latter stages of larval life to be used for construction of the pupal and adult animals [44-46]. However, recent immunological evidence obtained using *Manduca* and *Calliphora* indicates that the fate of some of the arylphorin is to leave the hemolymph, traverse the epidermal cells, and enter the cuticle in toto in an undegraded state [47-49]. Thus, arylphorin may serve as a structural protein in the cuticle or perhaps as a primer for chitin synthesis, i.e., chitinogenin, much like the glycoprotein glycogenin serves as a primer for glycogen synthesis in muscle [50]. Glycogenin is linked to glycogen carbohydrate residues via a novel linkage involving the hydroxyl group of tyrosine, a residue in relatively high abundance in the arylphorin class of proteins. Thus, it appears that both low and high molecular weight forms of tyrosine supply the amino acid for cuticular tanning. The low molecular weight type of conjugate probably plays the dominant role. For example, in

the fifth larval instar of *M. sexta*, the ratio of low molecular weight tyrosine equivalents, i.e. tyrosine or tyrosine glucoside, to high molecular weight equivalents, i.e. arylphorin, is approximately 5:1 [40,45].

The release of tyrosine from storage in the larval fat body of *Calliphora* nearing pupariation was suggested to be controlled by ecdysone [51]. Experimental evidence has shown this to be the case in another dipteran, the flesh fly *Sarcophaga bullata* [52]. The storage molecule, N- β -alanyl-L-tyrosine, is hydrolyzed by a specific dipeptidase whose synthesis is directed by 20-hydroxyecdysone. The released tyrosine is then incorporated into the sclerotized puparium [42]. N- β -alanyl-L-tyrosine synthetase and hydrolase enzymes were found in *Sarcophaga* fat body, with the hydrolytic activity rising sharply during pupariation in order to release tyrosine [43]. We have found that 20-hydroxyecdysone injected into isolated abdomens of late fifth larval instars of *M. sexta* results in the hydrolysis of stored tyrosine glucoside, suggesting the induction or activation of the tyrosine glucoside hydrolase system by the hormone [53]. DA, NADA, and NBAD are mainly found as β -glucoside conjugates in the hemolymph of *M. sexta* prior to and during cuticle tanning [15,16; Kramer and Hopkins, unpublished data]. Substrate availability by activation of conjugate hydrolyzing systems would then be an important means of regulating cuticle tanning.

In *M. sexta*, the synthesis and hydrolysis of tyrosine glucoside for utilization in cuticle is under hormonal control to synchronize metabolism of the storage molecule with demand for free tyrosine and glucose (Fig. 2). 20-Hydroxyecdysone regulates tyrosine glucoside hydrolase activity in the fat body, the major tissue source of the enzyme [53]. When molting hormone is injected into ligated larval abdomens, the titers of tyrosine glucoside and tyrosine in the hemolymph decrease and increase, respectively. Low levels of hydrolase activity occur in vivo subsequent to the first small pulse of 20-hydroxyecdysone that initiates gut purging and wandering behavior of fully grown larvae. A later peak of hydrolase activity at ecdysis follows the second large pulse of 20-hydroxyecdysone. Juvenile hormone is a negative effector for tyrosine glucoside synthetase [54]. If juvenile hormone or a mimic is injected into the fifth larval instar at 24-h intervals after ecdysis, tyrosine glucoside synthesis is almost completely suppressed. The eventual disap-

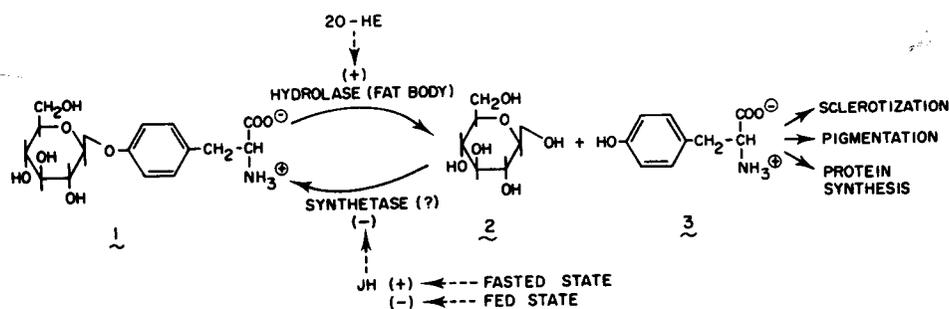


Fig. 2. Scheme for the regulation of tyrosine glucoside synthesis and hydrolysis in *M. sexta* [54]. 1, tyrosine glucoside; 2, glucose; 3, tyrosine. 20-HE, 20-hydroxyecdysone; JH, juvenile hormone.

pearance of juvenile hormone after ecdysis of the last larval instar is a necessary prerequisite for the synthesis or activation of tyrosine glucoside synthetase along with the initiation of other metamorphic events.

In all probability, 20-hydroxyecdysone also induces tyrosine release in other insects. A small release of molting hormone occurs to initiate wandering in fifth-instar *Manduca* larvae followed by a large release associated with the start of new cuticle formation [55,56]. Morgan and Poole [57] found a similar pattern of 20-hydroxyecdysone titers in the locust, *Schistocerca gregaria*. Wirtz and Hopkins [58,59] have shown that free tyrosine titers in *Periplaneta americana* start to increase a few hours before ecdysis and peak at ecdysis. The two-stage buildup of substrate in the cockroach for the eventual tanning of the new exoskeleton suggests a close temporal relationship with ecdysone release, and this phenomenon should be investigated further to determine if a direct causal relationship exists. Accumulation of free tyrosine in hemolymph and other tissues is correlated with very low TH and aromatic amino acid decarboxylase activities in *Periplaneta* pharate stages [60-62]. Thus, conservation of substrate pools by regulation of their enzymes until the critical time for utilization may be an important mechanism in controlling tanning.

Tyrosine Metabolism to Diphenols

There are many reactions that tyrosine undergoes in insect metabolism, including hydroxylation of the ring and side-chain carbons, transamination, decarboxylation, side-chain desaturation or lysis, and oxidation [1]. One of the most critical steps for tanning involves the formation of a diverse number of diphenols, which have been proposed to act as dehydrating agents, protein denaturants, and as precursors of pigments and cross-linking agents for the unsclerotized protein-chitin cuticular matrix [1,2].

The first reaction in the biochemical pathway of tanning is the hydroxylation of tyrosine to DOPA, but the identity and location of the enzyme responsible has not been firmly established. This reaction in insects appears to be catalyzed by a tyrosinase or phenoloxidase, which also oxidizes *o*-diphenols to *o*-quinones [63-68]. These enzymes, which require copper ions for activity, are widely distributed in plants and animals. Phenol-*o*-monooxygenase has been proposed as a name for the enzyme system that hydroxylates monophenols to *o*-diphenols and further catalyzes their oxidation to *o*-quinones [69].

Most studies on insect phenoloxidases have been concerned with the oxidation of diphenols to quinones [1,14,70-73]. Phenoloxidases are ubiquitous in insect hemolymph and integument, and in addition to their apparent role in tanning substrate production, they are involved in wound healing, parasite encapsulation, immune responses, and melanization [74]. Cuticular tyrosinases (EC 1.14.181), laccases (EC 1.10.3.2), and peroxidases (EC 1.11.1.7) may generate the reactive quinonoid or free-radical intermediates that are involved in sclerotization [1]. Tyrosinases are commonly present in cuticle, and we have isolated and characterized a tyrosinase from the pupal integument of *M. sexta* that hydroxylates monophenols and dehydrogenates diphenols and dihydroxyindoles, giving rise to catechols, *o*-quinones,

p-quinoneimines, and indole quinones [67]. The properties of the hemolymph protyrosinase after activation by a cuticular enzyme are the very similar to the cuticular tyrosinase, suggesting that the hemolymph proenzyme may be the precursor of the cuticular tyrosinase [68]. Tyrosinases oxidize catecholamines and other *o*-diphenols to reactive *o*-quinones that cross-link proteins or polymerize to melanin [75], and they can also hydroxylate monophenols. Laccases appear to be present in the cuticle of at least four species of insects [76], and this type of enzyme can oxidize *o*-diphenols, *p*-diphenols, aminophenols, and diamines [77]. The insoluble laccase in the cuticle of *Schistocerca gregaria* might also be responsible for oxidation of NADA to an intermediate with a reactive β -carbon [78]. Purification of the enzyme(s) responsible for quinone- and β -sclerotization and identification of the reactive intermediates remains a major challenge.

A specific tyrosine-3-monooxygenase (EC 1.14.16.1) that requires ferrous ions and a hydropterin cofactor has so far not been found in insects [79]. We have examined insect tissues for this kind of enzymatic activity using liquid chromatography with electrochemical detection, and have preliminary evidence that the enzyme is present only in nervous tissues of *M. sexta* and therefore only involved in neurotransmitter or neurohormone synthesis [Aso, Kramer, and Hopkins, unpublished data]. This enzyme does not appear to function in hydroxylating tyrosine for cuticle tanning in this species. The cuticular (integumentary?) tyrosinase present in *M. sexta* could be the enzyme that is needed for the synthesis of DOPA [67], but it has never been proven that DOPA synthesis is a function of tyrosinase in any organism except when associated with melanization [80]. After hydroxylation of tyrosine, tyrosinase may oxidize DOPA to DOPA quinone, which eventually forms melanin in many species. However, insect melanins appear to be derived from DA [81]. A reducing agent such as ascorbic acid could suppress quinone or melanin formation by reducing the quinones back to diphenols before indolization and polymerization. The diphenols could be rapidly inactivated by conjugation (for example, synthesis of DA glucoside) or rapidly transported into a compartment that lacks tyrosinase to avoid wasteful consumption of sclerotizing precursors. If a tyrosinase is present in insect cells and is involved in DOPA synthesis, tight regulation is needed to avoid cellular death due to the cytotoxic *o*-quinones produced by subsequent dehydrogenation of diphenols by the same enzyme [1].

The discovery by Karlson and Sekeris [7] that NADA was the principal tanning substrate in *Calliphora* and in several other insect species [82,83] suggested the importance of the DDC system. The *Calliphora* DDC had a high specificity for DOPA [84] and was located in the epidermis, where its synthesis was directed by 20-hydroxyecdysone during pupariation [7,85]. Karlson and coworkers [86] consider DDC to be the rate-determining enzyme system controlling tanning substrate production. DDC increases again before eclosion of adult flies, but since no corresponding peak of 20-hydroxyecdysone was found, some other factor may be responsible for inducing enzyme synthesis [87]. We have found that DDC activity begins to increase in both hemolymph and integument of pharate adult cockroaches a few hours before ecdysis, reaching maximal activity approximately 6 h after ecdysis [61,62].

Like in the adult fly, control of DDC activity in the cockroach is unknown. In cockroaches, *in vivo* decarboxylation rates are not elevated until after ecdysis [60,62]. This indicates that DOPA, the substrate of DDC, is not synthesized until after ecdysis. We later found this to be the case in that TH was not activated until ecdysis in *P. americana* [88]. Therefore, TH appears to be the rate-limiting enzyme for tanning substrate production in the cockroach and Diptera [89] and not DDC as first proposed [86].

The production of tyrosine ring hydroxylation, DOPA, does not appear to occur at substantial levels in insect tissues. Instead, DA or its N-acetylated derivatives build up to high levels in both integument and hemolymph of *M. sexta*, *Tribolium castaneum*, *Musca domestica*, *M. autumnalis*, and *P. americana*. Although there is a general correspondence of catecholamine profiles in cuticle and hemolymph, the specific compounds that occur differ from species to species and from one type of cuticle to another. Even localized areas of cuticle exhibiting varying degrees of stiffness and pigmentation differ considerably in catecholamine composition. For example, the concentration of NADA in the hard, colorless head capsule of *M. sexta* larvae is 100-times higher than NBAD or DA, but in the dark, hard larval mandibles, NBAD is more than double the level of NADA [16]. In brown pupal abdominal tergite cuticle, NBAD or its β -hydroxylated derivative, NBANE, is at least tenfold higher than either NADA or DA [18]. Rust-red elytra of *T. castaneum* have more than fourfold higher NBAD than DA or NADA [5]. However, the predominant diphenol in 7-day-old *Tribolium* elytra is not a catecholamine, but instead is a catechol acid, DOPAC, which accumulates to levels >100 times higher than NBAD. Apparently, a monoamine oxidase is responsible for DOPAC production in *T. castaneum*. *M. domestica* brown puparial cuticle is similar in catecholamine content to that of *M. sexta* pupal cuticle [90]. However, the white, highly mineralized puparial cuticle of *M. autumnalis* contains DA as its major catecholamine, which disappears during hardening. The yellow-brown pronotum of *P. americana* contains numerous catecholamines including NBANE, DA, NBAD, NANE, and NADA (Fig. 3) [91-93]. These compounds function as precursors for crosslinking agents and polymeric pigments in the cockroach, but their high concentrations in fully sclerotized cuticle suggests additional roles in cuticle stabilization, such as dehydration.

Although free diphenols mainly occur in the cuticle, they are, for the most part, conjugated in the hemolymph. In *M. sexta* hemolymph NBAD, NADA and DA are conjugated almost totally as glucosides during the period before and shortly after pupal ecdysis [15,16, Kramer, Morgan, and Hopkins, unpublished data]. As tanning proceeds, the percentage of conjugated catecholamines decreases as total hemolymph titers decrease, suggesting that hydrolysis occurs prior to transport into the cuticle. In *P. americana*, DA and NADA are conjugated with sulfate (Fig. 3) [92-94]. Conjugates of catecholamines commonly occur in insects and usually are assigned a protective and possibly a transport role in the regulation of substrate availability for tanning [1]. Glucoside, sulfate, or phosphate derivatives of a diphenol moiety decrease the susceptibility of the diphenols to oxidation, while acyl derivatives of the primary amino group decrease its nucleophilicity and stabilize quinonoid intermediates that are formed during tanning by preventing indolization and melanization.

ruvic acid in early larvae to hydroxylation and decarboxylation, forming catecholamines with NADA, the main tanning substrate [7]. Protoxyrosinase that accumulates in hemolymph of developing larvae is activated near pupation by a cuticular enzyme, probably a protease [63]. Ecdysone has no effect on induction of the proenzyme or its activation, but it was found to direct the synthesis of the activator enzyme [96] and to shift metabolism from transamination to ring hydroxylation. Karlson [86] concluded that the main effect of ecdysone was to induce de novo synthesis of DDC, the key enzyme in the switch to tanning-substrate production. Sekeris and Fragoulis [87] state that the induced synthesis of DDC is directed by ecdysone-mediated DDC mRNA synthesis. However, the decline of ecdysteroid titers at the end of a molt also appears to be necessary for DDC synthesis [97]. Thus, not only exposure to ecdysone but also decline of its titers is necessary for proper regulation of DDC. Chen and Hodgetts [98] demonstrated the action of 20-hydroxyecdysone in vitro on wing discs of *S. bullata*. DDC was induced by the hormone, and a fine cuticle deposited that became lightly sclerotized. Those results suggest that ecdysone acts directly on the integument to induce the entire sequence of tanning reactions. Mitsui and Riddiford [99,100] have likewise shown that 20-hydroxyecdysone induces tanning in vitro of pupal cuticle of *M. sexta*. Molting hormone was shown to act directly on the integument, which appeared capable of producing all reactants necessary for tanning.

Fraenkel et al. [101] and Seligman et al. [89], working on puparial tanning in *S. bullata*, found that ecdysone mediates the release of a neurosecretory hormone, PTF, which is involved in two actions: synthesis of a protein in the TH complex and activation via cyclic AMP of components of the TH system. However, they excluded activation of the hemolymph prophenoloxidase from the PTF scheme, since this occurs even before release of PTF. The nature of the cyclic-AMP activation of TH is as yet unexplained. Therefore, Fraenkel, Seligman, and co-workers proposed that ecdysone controls secondarily the metabolic event initiating tanned substrate production from tyrosine. Major changes in ecdysone titers may also regulate epidermal synthesis of cuticular proteins, which further affects cuticular structure [102].

The second hormone controlling tanning of insect cuticle is the neurosecretory protein, bursicon [reviewed in 103,104]. Cottrell [105] and Fraenkel and Hsiao [106,107] discovered a blood factor released from the brain or thoracic ganglion of adult flies shortly after ecdysis that initiated hardening and darkening of the cuticle. Ecdysone was completely inactive in this system. The hormone, which is a protein [108-110], has been found in several orders of hemi- and holometabolous insects and is generally associated with adult cuticle tanning [107,111-114]. Pagham [115,116] has shown that both tanning and melanization of first-instar locusts, *S. gregaria*, are regulated by bursicon.

Two hypotheses have emerged concerning the mode of action of bursicon. Seligman et al. [117], on evidence that bursicon increased DOPA synthesis, suggested that the hormone regulated the activation of tyrosine hydroxylation. Cyclic AMP was found to mimic the action of bursicon by causing tanning to occur in adult flies [118,119] and in cockroaches [120]. In vivo

stimulation of DOPA synthesis was one effect of injected cyclic AMP [118], while enhanced incorporation of tyrosine metabolites into cuticle was observed by Vandenberg and Mills [120]. The role of cyclic AMP as a secondary messenger in the activation of TH has also been demonstrated in puparial tanning [89]. In this case, another neurosecretory hormone, PTF, which differs in properties from bursicon, appears to control the system.

The second proposed mechanism of action suggests that bursicon increases membrane permeability of the hemocytes to tyrosine [121], with cyclic AMP acting as a second messenger in the system [120]. The tyrosine hydroxylating system within the hemocytes then initiates production of diphenols. Post [122] has substantiated this hypothesis in that hemocytes of *Pieris brassicae* were impermeable to tyrosine when stabilized with EDTA and a tyrosinase inhibitor. However, when a partially purified bursicon preparation was added, tyrosine uptake occurred, and penetration was further stimulated by Ca^{++} and Mg^{++} . Bursicon may also mediate the translocation of protein-bound catecholamines from the hemolymph to the cuticle by inducing permeability changes in the epidermis [123,124].

Truman [125] discusses the interactions between ecdysone, eclosion hormone, and bursicon titers in *M. sexta* that bring about cuticle digestion, molting fluid resorption, cuticle formation, ecdysis, expansion, and hardening of new cuticle. The decline of ecdysone titers apparently triggers release of eclosion hormone, which ultimately induces ecdysis and bursicon release. There may be differential responses to 20-hydroxyecdysone, with low titers inducing primarily apolysis and cuticle degradation, and higher titers causing cuticle deposition.

Interaction of Diphenols With Other Cuticular Components

The interactions of diphenols or quinones with cuticular proteins has been extensively reviewed by Andersen [78,126] and Brunet [1]. Two primary pathways have been proposed for generating cross-linking agents in insect cuticle via oxidation of catecholamines. One oxidizes the *o*-diphenols to *o*-quinones (quinone sclerotization), [127], while the other activates the beta carbon of aliphatic side chain (β -sclerotization) [126,128]. Covalent bonds between nucleophilic groups of protein and the aromatic or side-chain carbons of diphenols are hypothesized to result in different types of sclerotized cuticle. Sugumaran and Lipke and co-workers have studied mechanisms of sclerotization, primarily in puparial tanning of Diptera [95,129-134]. Their approach is to break down and fractionate cuticle into various constituents such as peptide adducts and then attempt to model the "pieces" back together. Their model incorporates compounds such as *p*-quinone methides, *o*-quinones, aromatic polymers, bityrosine, 1,4-quinonoid cross-links, and benzenoid adducts. The quinonoid mechanism is an especially attractive intermediate because it can lead to either aromatic-ring (*o*-quinone sclerotization) or aliphatic side-chain (β or quinone methide sclerotization) cross-linking reactions. However, no definitive proof for the existence of any of these structures except bityrosine and *o*-quinone mediated ring adducts has been presented to date. None of these sclerotization models have thus far

incorporated the role of the β -alanylcatecholamines that are closely associated with the stabilization of certain types of hard dark cuticle.

Although there is no direct evidence about the identity of the reactive intermediates that are generated from catecholamines during sclerotization, it is clear that there is more than one type. During the hardening of the colorless cuticle of insects such as *S. gregaria*, there is little release of tritium from the aromatic carbons of ring- ^3H -NADA and abundant release of tritium from β - ^3H -NADA [128]. The *o*-quinone of 1,2-dehydro-NADA might be the reactive intermediate involved in this type of sclerotization [135]. DOPKET is a major hydrolysis product of the covalently bound catecholamines that are produced by this pathway [78]. During the early stages of hardening of dark-brown cuticle, there is release of tritium from ring- ^3H -NADA and β - ^3H -NADA [128]. Other studies have shown that substantial amounts of labeled precursors such as DA or NBAD are covalently bound in dark-brown cuticle by acid-resistant bonds [47,75,134]. These results suggest the *o*-quinone of NBAD or NADA is an important sclerotization agent in brown cuticle. Although there may also be differences in the enzymatic activity of colorless and brown cuticles, we have found that NADA is the predominant catecholamine in hard colorless cuticle, while NBAD is the major catecholamine in the brown cuticles examined [5,15,16,90]. Both of these catecholamines are good substrates for side-chain modification by the insoluble cuticular enzyme(s) that is present in the pupal cuticle of *M. sexta*. However, NADA is the best substrate for synthesis of compounds that yield DOPKET during hydrolysis, while NBAD is the best substrate for β -hydroxylation [18]. An interesting hypothesis is that β -hydroxylation is an important pathway in brown cuticle and that β -hydroxylated catecholamines are good substrates for *o*-quinone tanning. There is little evidence concerning the fate of β -hydroxylated catecholamines in cuticle [136,137], but Roseland et al. [138] found that NBANE stimulates tanning of *M. sexta* cuticle in vitro more effectively than NBAD does.

Synthesis of NANE occurs when NADA is incubated with cuticular homogenates of *M. sexta* pupae [17] or with puparial cuticle of *S. bullata* [134]. Instead of direct hydroxylation, the formation of a *p*-quinone methide intermediate was proposed, which then reacts spontaneously with water to form the β -hydroxylated derivative. *p*-Quinone methides have also been proposed as intermediates involved in sclerotization [2]. The fact that β -methoxy-NBAD is synthesized during oxidation of NBAD by the cuticular enzymes of *M. sexta* when 20% methanol is present indicates that an intermediate with an electropilic β -carbon is present [Morgan, Hopkins, and Kramer, unpublished data]. Although several products of NADA metabolism that are covalently linked through both the α - and β -carbons have been isolated [74], the only identified catecholamine metabolite that is covalently linked through the β -carbon but not the α -carbon is a yellow wing pigment of a butterfly [139]. This pigment consists of NBAD linked by its β -carbon to the aromatic amine of kynurenine, and it is converted by mild acid hydrolysis to free NBANE and kynurenine. We have found that large quantities of the NBANE present in acidic extracts of the brown cuticle of *M. sexta*, *P. americana*, and *M. domestica* are not extracted by nonacidic solvents [18], and we are investi-

gating the possibility that some of the NBANE in the acidic extracts is actually the hydrolysis product of a covalently bound catecholamine.

Peter et al. [139] have used cross-polarization magic-angle spinning NMR to examine the structure of moth, locust, and cockroach cuticles. Their spectra show the relative abundance of a rather large number of phenolic and diphenolic carbons, as well as chitin and protein carbons. They suggested that the importance of covalent interactions between cuticular polymers has been overemphasized by others, and that sclerotization of cuticle may occur by the denaturation of structural protein by noncovalent interactions with phenolic polymers. However, the abundance of ^{13}C in their samples was too low to demonstrate covalent protein-catechol linkages. Grun and Peter [75] have developed an excellent *in vitro* model system for the cross-linking of cuticle proteins. Mushroom tyrosinase was used to catalyze oxidation of the sclerotizing agents NBAD and NADA in the presence of cuticle or serum proteins. High molecular weight protein multimers were found as the result of cross-linking, presumably by quinonoid derivatives of the catecholamines. The possibility that the same reaction occurs *in vivo* is quite high, since similar components are present in cuticle during tanning.

Because sclerotized cuticle is an intractable material and the putative cross-linking agents highly reactive and transient in nature, conventional analytical techniques used in the past to study the chemistry of sclerotization have not yielded convincing evidence for the cross-link structure. Nevertheless, since the proteins in sclerotized cuticle are difficult to extract, it has long been suggested that they are stabilized by cross-links involving covalent bonds to other cuticular components [140]. Using solid state NMR spectroscopy, we have investigated the possibility of nitrogen-containing amino acid side chains in cuticular proteins acting as potential nucleophiles for the formation of carbon-nitrogen cross-links to components such as quinonoid intermediates in cuticle. Spectral data on *M. sexta* pupal cuticle protein labeled with 1,3- $^{15}\text{N}_2$ -histidine or ϵ - ^{15}N -lysine demonstrate that a side-chain histidyl or lysyl nitrogen becomes attached to an aliphatic or aromatic carbon atom during the process of sclerotization [6]. NMR analysis of pupal cuticle that has been double labeled with both 1,3- $^{15}\text{N}_2$ -histidine and ring- $^{13}\text{C}_6$ -DA reveals that an aromatic carbon of the catecholamine is covalently bonded to a ring nitrogen of histidine (Fig. 4). Thus, a carbon-nitrogen cross-link between proteins and diphenols arises in cuticle that is consistent with a protein side-chain aromatic nitrogen attacking an *o*-quinone cross-linking agent. No nitrogen from histidine was observed to bond to the β -carbon of DA. Instead, an oxygenated β -carbon adduct was detected, which is due to either endogenous NBANE or perhaps a β -carbon-O-alkylated adduct formed between NBAD and a seryl, threonyl, tyrosyl, aspartyl, glutamyl, or 2-acetamido-2-deoxy-D-glucopyranosyl residue. Because of the relative concentration of the adducts, the aromatic carbon-nitrogen cross-link that is due to an *o*-quinone tanning mechanism is more important in this relatively hard type of cuticle than any aliphatic side-chain linkages proposed for a β -sclerotization mechanism.

Chitin may also be involved in the cross-linking reactions of insect cuticle. Solid state NMR analysis of pupal chitin prepared by alkali extraction from

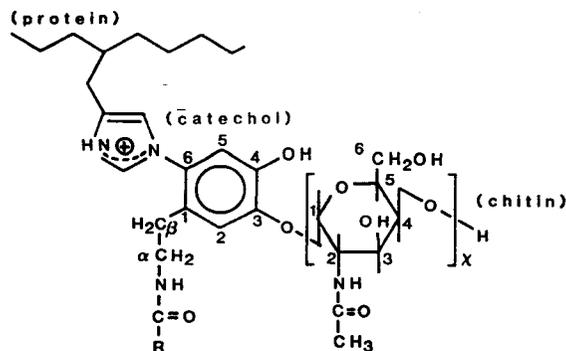


Fig. 4. Proposed structure for catecholamine-mediated cross-link between protein and chitin in *M. sexta* pupal cuticle [6].

exuviae labeled with 1,3- $^{15}\text{N}_2$ -histidine reveals a chemical shift expected for the substituted imidazole nitrogen cross-link structure shown in Figure 4 [6]. Virtually all of the substituted histidyl rings appear to be coupled to chitin in *M. sexta* pupal cuticle. Natural abundance ^{13}C -NMR spectra of the pupal cuticle chitin preparation confirms the presence of aromatic compounds such as diphenols. Apparently, the chitin is not coupled directly to protein but instead to a diphenol, the latter serving as a bridging cross-link for protein and chitin macromolecules. A catecholamine-chitin carbon-oxygen linkage between the phenoxy carbon 3 and the GlcNAc carbon 1 has been proposed as the chitin-catecholamine cross-link, but phenoxy carbon 4 as well as other GlcNAc carbons may also participate (Fig. 4). Further evidence for a chitin-catecholamine interaction is the finding that chitinous lamellae of *Drosophila* pupal sheaths are compacted in vitro by exposure to nascent quinones of NADA or NBAD [141]. In order to directly measure a carbon-oxygen cross-link between diphenol and chitin molecules, solid state NMR analysis of cuticle enriched with ^{13}C and ^{17}O will need to be undertaken.

CONCLUSIONS

The identification of many tyrosine metabolites, primarily catecholamines as well as their N-acylated and O-conjugated derivatives, in association with proteins and chitin in tanning cuticle points to a complex interplay of structural materials such as protein, chitin, minerals, diphenols, lipids, pigments, cross-linking agents, enzymes, hormones, and even water for the stabilization of cuticles with a wide diversity of mechanical and chemical properties. Cuticle assembly and stabilization can be generalized to occur as follows. First, protein, chitin, and lipid are secreted into a laminated procuticle that is later impregnated with diphenols derived from tyrosine. Both tyrosine and catecholamines are sequestered in hemolymph as phenolic conjugates to block premature oxidation and to increase solubility and transport properties. The storage and release of these compounds and the enzyme pathways utilizing tyrosinases, phenoloxidases, or laccases, and aromatic amino acid decarboxylases appear to be precisely regulated by the ecdysteroid hormones

that also initiate new cuticle synthesis and by certain neuropeptide hormones that initiate tanning. Much more work is needed to define the precise roles of those hormones in regulating tyrosine metabolism. Second, cross-links (covalent couplings) form between proteins and/or chitin via reactive intermediates such as *o*-quinones or *p*-quinone methides. Carbon-nitrogen and carbon-oxygen bonds form, the nature of which depends on the reactive cross-linking agent and the nitrogen- or oxygen-donating cuticular components in proximity. Third, free diphenols or mineral salts accumulate in the cuticle over a longer period of time, some of which may displace water, thereby dehydrating and stiffening the cuticle.

Two outstanding problems regarding the chemistry of sclerotization and the wide range of mechanical properties associated with the insect exoskeleton are the chemical structures of the highly reactive quinonoid compounds that interact with the protein and chitin components found in the procuticle, and the nature of the covalent bonds established between the aromatic reactants and the biopolymers of the cuticle. Utilization of the noninvasive procedure of solid state ^{13}C , ^{15}N , and ^{17}O NMR together with selective isotopic enrichment of the protein, chitin, and aromatic constituents is providing direct structural information about the aromatic cross-links in insect cuticle. Additional advances in NMR, electrochemical techniques, fast atom bombardment mass spectrometry, and high-performance liquid chromatography have greatly advanced our understanding of the structures of aromatic metabolites derived from tyrosine and their functional relationships to cuticle stabilization.

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