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INSECT CUTICLE SCLEROTIZATION[1]

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PERSPECTIVES AND OVERVIEW

Sclerotization or tanning is a complex process that stabilizes proteinaceous animal structures through the reaction of quinones with the functional groups of the proteins. It has been postulated that aromatic cross-links are inserted between adjacent polypeptide chains, causing progressive hardening, dehydration, and close packing of the polymers. Proteins covalently bonded together by the process of quinone tanning were first named sclerotins by Pryor in 1940 (91, 92). Sclerotins are generally very tough and resistant to chemical or physical degradation.

Sclerotized structures are found throughout the animal kingdom from protozoa to the lower chordates (93, 149). Insects, however, have utilized sclerotization more extensively than any other group of animals for the stiffening and hardening of their exoskeletons, egg shells, oothecae, cocoons, and various silk structures. The process of sclerotization, therefore, is prob-

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ably an ancient one in the evolution of structural materials with high strength and durability. The early success of insects in evolving flight mechanisms and colonizing a wide array of terrestrial habitats resulted in large part from the development of a light-weight organically hardened exoskeleton more than 300 million years ago. The organic structure was much better adapted to terrestrial life than the mineralized exoskeleton of the insects' marine ancestors or that of some present-day arthropod relatives. Rare examples of mineralized insect exoskeletons are found in certain aquatic larvae and puparia of Diptera, although these may also be partially sclerotized as are some crustacean exoskeletons (93, 106).

The initial step in the sclerotization process involves formation of highly reactive tanning agents by the oxidation of catechols or *o*-diphenols to their corresponding electrophilic *o*-benzoquinones. This reaction is catalyzed by copper oxidases in the phenoloxidase group and requires molecular oxygen as a hydrogen acceptor. The *o*-quinones then can react directly with protein amino or imidazole groups to form covalent bonds between aromatic ring carbons and protein nitrogens. Other amino acid side-chain nucleophiles such as thiol, hydroxyl, and carboxyl groups may participate in cross-linking as well. That proteins may also form cross-links through the β-carbon of the catecholamine side chain was an important discovery, which Andersen named *β-sclerotization* (2, 4). Recent research shows that *o*-benzoquinones with N-acyl side chains can undergo isomerizations to other quinonoid tanning agents capable of forming both α and β aliphatic carbon cross-links between polypeptide chains (68, 110, 114, 128). Additional impregnation with diphenols or their polymeric oxidation products further dehydrates and stabilizes the protein matrix by formation of numerous noncovalent bonds (40, 93). In cuticular structures containing chitin, cross-links between the N-acetylglucosamine or glucosamine residues and catecholic ring or side-chain carbons are also possible (115).

Sclerotized cuticles vary considerably in stiffness and hardness depending upon their evolved functional role, but all are characterized by their high mechanical strength and their resistance to chemical or enzymatic degradation. Sclerotization provides not only the requisite mechanical properties for muscle attachment and locomotion, but also stability against hydrolytic enzymes widely distributed in organisms. The outer cross-linked exocuticle is highly resistant to digestion, although some pathogenic fungi can penetrate less sclerotized areas.

The advantages of an exoskeleton consisting primarily of organically stabilized polymers of protein and chitin are its strength, flexibility, and lightness of weight. The main disadvantage, particularly those exoskeletons armored with extensive sclerites, is the limit of expandability for growth, thereby requiring insects to molt periodically to complete development to the adult

stage. During each molting cycle, a new cuticle must be secreted by the epidermis and the old one partially digested to make escape possible. The amino acids and sugars from the digested endocuticle moreover may be reabsorbed and reused as building blocks for protein and chitin in the new cuticle. Prior to ecdysis or shedding of the exuviae, sclerotization of specific regions of the newly secreted exoskeleton, i.e. mandibles, tarsal claws, or other structures required during the teneral period, may occur. The new cuticle then briefly becomes extensible for expansion to accommodate growth, followed by sclerotization and pigmentation to complete the functional exoskeleton. The patterns of cuticle sclerotization are genetically fixed for each stage of development and are regulated by the underlying epidermis and other tissues through little-understood mechanisms.

The diversity of sclerotized cuticles with variable physical and chemical properties, ranging from the extremely hard cutting edges on mandibles of chewing insects to the thin stiffened cuticle of membranous wings, strikingly demonstrates the potential complexities of sclerotization mechanisms. Among the types of cuticle so far studied, the pupal cuticle of *Manduca sexta* has served as a useful model for investigating sclerotization and the cross-link structure of hard brown cuticle. Puparial cuticles of Diptera and the stiff colorless cuticle of migratory locusts also have been extensively studied as models for other types of sclerotization. Newly secreted undifferentiated cuticle primarily consists of multiple layers of a soluble protein matrix containing embedded chitin microfibrils as illustrated in the pupal wing of *M. sexta* (Figure 1*A*). These lamellae become dense and less distinct as sclerotization progresses in the outer exocuticular region (Figure 1*B*). Additional layers of endocuticle continue to be secreted for several days (Figure 1*C*), all of which will be digested by molting fluid enzymes prior to adult ecdysis. The wing exuviae consist primarily of a hard brittle exocuticle (Figure 1*D*). The pupal abdominal cuticle of *M. sexta* is partially sclerotized at ecdysis, and the outer layers are densely packed (Figure 1*E*). Pore canals that presumably transport sclerotizing substrates and other materials are clearly visible. The abdominal exuviae contain not only the very dense exocuticle but also several partially stabilized inner lamellae that have resisted digestion by molting fluid enzymes (Figure 1*F*). Understanding this entire process remains a major challenge 50 years after Pryor (91) proposed the first model for sclerotization.

Numerous reviews in recent years have examined aspects of insect cuticle sclerotization and other proteinaceous structures (4, 21, 39, 65, 66, 68, 73, 86, 128, 156). However, new advances are rapidly being made in deciphering the structure of sclerotized cuticle through the use of solid-state nuclear magnetic resonance (NMR) analysis, and a much more complex picture of the enzymes that catalyze the biosynthesis of quinonoid tanning agents is emerg-

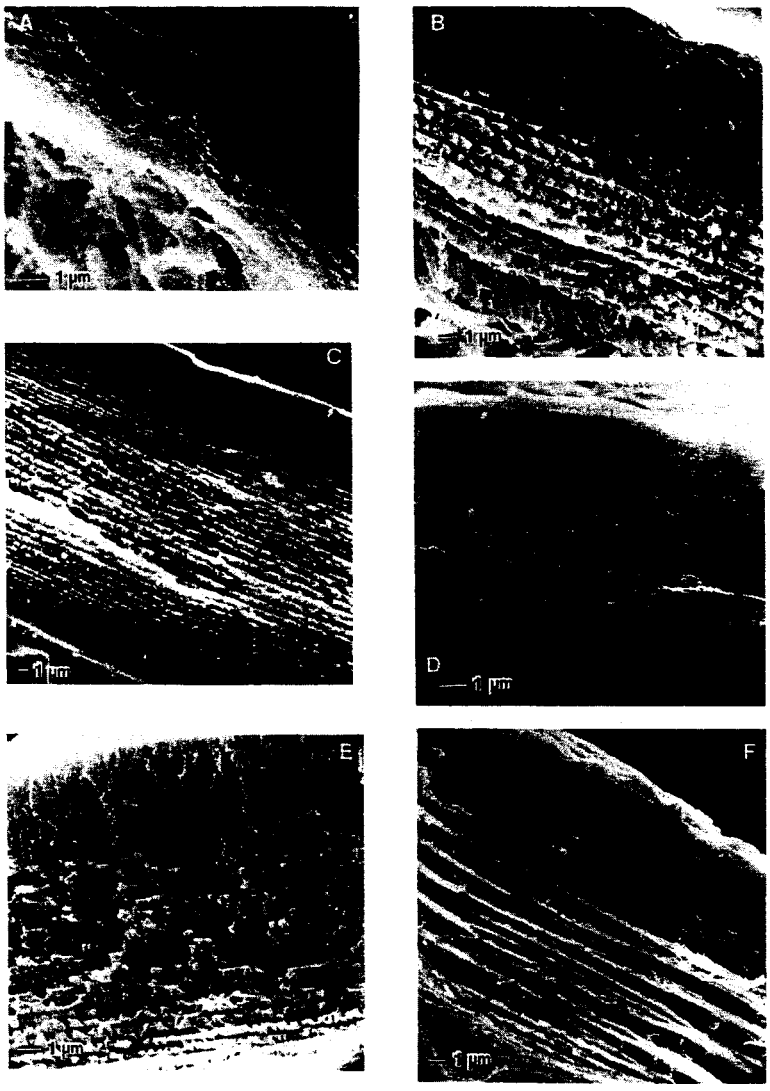


Figure 1 Scanning electron micrographs of newly ecdysed pupal wing and abdominal cuticles of *Manduca sexta* undergoing sclerotization. (*A*) Unsclerotized wing cuticle at ecdysis. Procuticle contains many thin layers or lamellae destined to be sclerotized. (*B*) Wing cuticle 24 h after ecdysis. Procuticle from *A* now fused into hardened exocuticle. Below the exocuticle, thick differentiated layers of endocuticle have been secreted. (*C*) Wing cuticle 72 h after ecdysis. Exocuticle remains at the same thickness as at 24 h, but many more layers of endocuticle have been secreted. (*D*) Wing exuviae. Only sclerotized exocuticle remains after digestion by molting fluid. (*E*) Abdominal cuticle at ecdysis showing preecdysial sclerotization and vertical pore canals. (*F*) Abdominal exuviae with outer layer of highly sclerotized exocuticle and inner stabilized thin layers and connecting fibers not digested by molting fluid.

Kramer, unpublished data). The synthesis is only initiated when the juvenile hormone titer decreases after ecdysis of the fifth instar (1). Release of 20-hydroxyecdysone (20-HE) into the hemolymph initiates the onset of new cuticle secretion and also appears to be the primary stimulus for the release of the aromatic amino acids or catecholamines from their conjugates (65). Hormonal events would therefore appear to regulate β-glucosidases, sulfatases, or dipeptidases that hydrolyze the conjugates at the critical time for biosynthesis of tanning agents.

Tyrosine has also been found sequestered in fat-body vacuoles of the larvae of certain Lepidoptera and Diptera (78). The dipeptide N-β-alanyltyrosine appears to be the primary storage form of tyrosine for puparial tanning in *Sarcophaga* and a few tachinid species (17, 65). This compound in the hemolymph of last-instar larvae reaches peak concentrations at the time of pupariation. It is then rapidly hydrolyzed, and the two amino acids or their metabolites are incorporated into the puparial cuticle. In *Ceratitis capitata*, two hemocyte proteins were recently shown to specifically bind tyrosine, presumably for transport into cuticle (144). One of the proteins is incorporated into cuticle, but whether tyrosine and/or its derivatives are unloaded in the epidermal cells or in the cuticle is unknown.

Low-molecular-weight storage forms of tyrosine or phenylalanine have not been identified in other insect orders, although similar conjugating mechanisms for the sequestration of aromatic amino acids are probably common in insects given the periodic requirement for sclerotization precursors.

Catecholamines

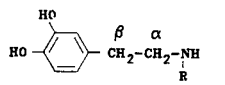
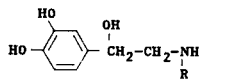
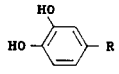
The immediate precursors for quinonoid sclerotizing agents in insect cuticle appear to be mainly the N-acyl derivatives of 3,4-dihydroxyphenethylamine [dopamine (DA), Table 1]. The discovery of N-acetyldopamine (NADA) in several insect species (59) focused attention on this compound and its biosynthetic pathways in the integument. Previously, catechols with acidic side chains were primary candidates for tanning-agent precursors because of their reported involvement in the tanning of cockroach oothecal proteins (21). Much of the research in recent years on mechanisms of cuticle sclerotization has been based on the assumption that NADA, rather than catechols with acidic, aldehyde, or alcohol side chains, is the common precursor for such reactions. The basic catecholamine N-β-alanyldopamine (NBAD) was later discovered to be a major precursor for tanning of stiff brown cuticles in several species (50, 65). The N-acyl derivatives of norepinephrine are cuticular metabolites of NADA and NBAD or may be hydrolysis products of their β-carbon adducts (51, 81, 85, 136). They may also serve as precursors for sclerotization because they can be oxidized to their corresponding *o*-quinones, though at a much slower rate in vitro than the parent compounds (82, 143). N-acetylnorepinephrine (NANE) was first reported as a product of NADA

incubation with insect and mushroom phenoloxidases (85). N-β-alanylnorepinephrine (NBANE) is a major catecholamine extracted from the brown pupal cuticle of *M. sexta* (51, 81) and the dark cuticles of several species of cockroaches undergoing sclerotization (31–34). It is also a major product of NBAD oxidation catalyzed by insect and fungal phenoloxidases in vitro (7, 81, 82, 137, 143). *M. sexta* accumulates its catecholamine reserves for larval, pupal, and adult cuticle tanning only after apolysis to the pharate stage and shortly before ecdysis (51). Like tyrosine, NBAD, NADA, and DA occur as glucoside conjugates in the hemolymph of *M. sexta,* and their concentrations decrease to low levels during the course of sclerotization (51).

Cockroaches sequester conjugates of catecholamines in hemolymph during the nymphal feeding periods for later use as tanning-agent precursors (65). Dopamine-3-O-sulfate is the major storage molecule in *Periplaneta americana* and certain other cockroach species (18, 31–33, 124) and accumulates to high levels shortly before adult ecdysis. Conjugates of NADA have also been identified in the hemolymph of newly ecdysed *P. americana* and other cockroach species (19, 32, 33).

A second possible mechanism for sequestration of catecholamines was discovered in fifth stadial larvae of *M. sexta* (69). Unconjugated NBAD, the major precursor for pupal cuticle tanning agents, progressively increases to high concentrations in larval integument with a peak level at the wandering stage. After apolysis and as digestion of larval cuticle by molting fluid

Table 1 Catecholamine precursors for sclerotization and related catechols extracted from insect cuticle

COMPOUND	ABBREV.	FORMULA	R
DOPAMINE	DA		H
N-β-ALANYLDOPAMINE	NBAD		$-C(=O)-CH_2-CH_2-NH_2$
N-ACETYLDOPAMINE	NADA		$-C(=O)-CH_3$
α,β-DEHYDRO-N-ACETYLDOPAMINE	DEHYDRO NADA	···· CH=CH ····	
NOREPINEPHRINE	NE		H
N-β-ALANYLNOREPINEPHRINE	NBANE		$-C(=O)-CH_2-CH_2-NH_2$
N-ACETYLNOREPINEPHRINE	NANE		$-C(=O)-CH_3$
3,4-DIHYDROXYPHENYLETHANOL	DOPET		CH_2-CH_2OH
3,4-DIHYDROXYPHENYLKETOETHANOL	DOPKET		$-C(=O)-CH_2OH$
3,4-DIHYDROXYPHENYLACETIC ACID	DOPAC		CH_2COOH
3,4-DIHYDROXYBENZOIC ACID	DOBA		COOH

enzymes occurs, NBAD levels decrease in the integument and simultaneously rise in the hemolymph. This temporal pattern suggests that the NBAD released from larval cuticle is transported into the hemolymph to be utilized for tanning of pupal cuticle.

Biosynthesis of Catecholamines

The biosynthetic pathway for catecholamine tanning precursors primarily involves three enzyme-catalyzed steps: (*a*) hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), (*b*) decarboxylation of DOPA to dopamine, and (*c*) acylation of the dopamine amino group with either acetate or β-alanine. Other secondary enzyme–regulated steps include the conjugation of catecholamines with either glucose or sulfate for sequestration and the subsequent release of free diphenols by β-glucosidases or sulfatases as previously discussed.

TYROSINE HYDROXYLATION The initial reaction and perhaps most critical regulatory step in catecholamine biosynthesis is the hydroxylation of tyrosine to DOPA. Tyrosinase (EC 1.14.18.1) may be important because a specific tyrosine hydroxylase (EC 1.14.16.1), which catalyzes the rate-limiting reaction for vertebrate catecholamine biosynthesis (83), has not been demonstrated in insects. Insect tyrosinases in hemolymph and integument catalyze hydroxylation of tyrosine (9, 21, 82), but since they also have catechol oxidase activity, the latter would have to be suppressed. For example, the presence of a reducing agent such as ascorbic acid would inhibit the oxidation of catechols to quinones and favor the tyrosine hydroxylase activity. Because of the multifunctional properties of tyrosinases, the difficulty of isolating and purifying these particulate enzymes and proenzymes, and the problems of differentiating between naturally activated enzymes and those activated during the isolation procedures, researchers have so far been unable to determine the role of tyrosinases in DOPA synthesis during each molting cycle. Purified tyrosinases isolated from the tanning integument of pharate pupal *M. sexta* have low tyrosine hydroxylase activity (82), and in vitro cultures of *M. sexta* integument after exposure to 20-HE can be induced to lightly tan newly secreted cuticle, indicating that the enzymes necessary to convert tyrosine to tanning precursors are present (79, 80). However, supplementation of the culture medium with NBAD and NBANE greatly stimulates tanning, indicating only a low endogenous level of precursor synthesis (105). DA supplementation of cultured *M. sexta* integument is also necessary for high rates of NBAD synthesis (69), perhaps because of low levels of tyrosine hydroxylase and DOPA decarboxylase (DDC) activity.

The hemolymph or other tissues may also be a source of DOPA production for sclerotization (65). Protyrosinases that, after activation, hydroxylate tyro-

sine have been obtained from the hemolymph of several species (9, 21). However, the hemolymph phenoloxidases are generally believed to have primary functions in immune reactions and wound healing.

In vivo studies on tyrosine hydroxylation in cockroaches and *M. sexta* show a large increase in DOPA production near the start of cuticle tanning that persists for a few hours during the main period of sclerotization (48; T. L. Hopkins & K. J. Kramer, unpublished data). However, only low DOPA concentrations are found in the hemolymph or integument during this period (45, 50, 51), unless DDC inhibitors are administered (52). High levels of DDC are already present before the increase of DOPA production, so that DOPA normally never accumulates but is instead rapidly decarboxylated to dopamine, which may then be N-acylated or converted to melanins. The rate-limiting enzyme for catecholamine biosynthesis, therefore, is most likely a tyrosine hydroxylase or tyrosinase system under ecdysteroid and neurohormonal control (120) that makes DOPA available to previously synthesized DDC.

The neurohormone bursicon regulates the onset of cuticle tanning in synchrony with other ecdysial events (65, 97). The tyrosine hydroxylating enzyme may be activated, or substrate accessibility to the enzyme increased, by this protein hormone after its release from the ganglia of the ventral nerve cord. Cyclic AMP also plays a role in the process, but little progress has been made in recent years in understanding the mode of action of this important regulatory hormone that initiates postecdysial tanning. The puparial tanning factor, a much larger protein than bursicon in the ganglia of dipteran larvae, plays a similar role in initiating tyrosine hydroxylation through cAMP (120). Preecdysial tanning and melanization are apparently not regulated by bursicon, but rather are correlated more closely with ecdysteroid releases (103).

DECARBOXYLATION OF DOPA The discovery of NADA as a major cuticle-tanning precursor directed attention to its biosynthetic pathway and, particularly, the importance of DDC (EC 4.1.1.28) as a regulatory step mediated by 20-HE (118). Both in vivo and in vitro studies with several insect species have shown a close correlation between increases in DDC activity and cuticle tanning, suggesting hormonal control of catecholamine biosynthesis (65). DDCs from epidermis and integument have been purified and studied extensively (25, 35, 63, 117, 157). This enzyme is highly specific for DOPA, with little or no activity towards tyrosine or other aromatic amino acids, unlike the mammalian aromatic amino acid decarboxylases (83). The hemolymph of some species also contains high levels of enzyme activity at ecdysis (49, 154).

DDC synthesis is induced by ecdysteroid at the transcriptional level for puparial tanning in Diptera (118). However, DDC activity increases again

before eclosion of adult insects, but only in the absence of hemolymph ecdysteroids (49, 70, 154). Hiruma & Riddiford (42–44) provided an understanding of the differential regulation of DDC synthesis by ecdysteroids in that de novo synthesis of DDC in *M. sexta* larvae and adults only occurs in response to declining ecdysteroid titers. A differential response of *Drosophila* tissues to ecdysteroid titer also has been reported. In this insect, the DDC gene in mature larval epidermis exposed to 20-HE rapidly produces DDC transcripts, whereas the imaginal discs require a decrease in hormone titer for DDC transcripts to accumulate (26). Therefore, the gene coding for DDC synthesis is apparently regulated differently for puparial tanning than for cuticular tanning and melanization in other developmental stages.

N-ACYLATION OF DOPAMINE Acylation of the amino group of DA with either acetate or β-alanine represents the final step in the biosynthesis of the main sclerotization precursors, NADA and NBAD. N-acetyl dopamine transferases (NAT, EC 2.3.1.5), which catalyze the acetylation of DA and other biogenic amines with acetyl CoA, have been most extensively studied in Diptera (58, 76, 77, 123, 156). Peaks of NAT activity tend to correlate with puparial sclerotization in *Drosophila*.

N-acylation of DA with β-alanine is catalyzed by N-β-alanyl dopamine transferase (NBAT), which requires ATP and Mg^{2+} for maximum activity (69, 156). NBAT activity is highest in the integument of *M. sexta* fifth-instar larvae shortly before wandering and again in pharate pupae starting to tan (69). Therefore, peaks of NBAT activity correlate with sequestration of NBAD in larval cuticle and again in pupal hemolymph. NBAT activity in fat body was approximately 100-fold less than in the integument. When pharate pupal integument is cultured in vitro, supplementation with DA is necessary for maximal NBAD synthesis. 20-Hydroxyecdysone does not affect NBAT activity in vitro, although in vivo injection of the hormone into isolated larval abdomens induces synthesis of NBAD (69).

The N-acyl transferases may catalyze important regulatory steps that determine the amounts of DA shunted into the melanization versus the sclerotization pathways. Acylation of the free amino group of DA is necessary to block the rapid cyclization of dopamine quinone and eventual formation of melanins. In black phenotypes of *Blattella germanica, Drosophila melanogaster*, and *Tribolium castaneum*, free DA accumulates to very high concentrations during the melanization phase, whereas NBAD synthesis is delayed possibly because of low levels of NBAT activity during this period (34, 107, 156). However, β-alanine may not be synthesized by these mutants, or transport of this amino acid into the integument may be deficient (46). Hydrazino and aminooxy derivatives of β-alanine, when injected into pharate pupae of *M. sexta* four or more hours before ecdysis, cause melanization of

the normal brown cuticle (147). These β-alanine derivatives are thought to interfere either with β-alanine synthesis or N-acylation of DA with β-alanine.

The N-acyl dopamine transferases undoubtedly play an important role in regulating both sclerotization and melanization of insect cuticle in genetically determined patterns, but more research is needed to advance our understanding of these important developmental enzymes.

Proteins

Little information about cuticular proteins involved with sclerotization of the exoskeleton is available (152). Generally accepted is the idea that proteins extracted from different cuticular regions of a single animal reflect the regional rigidity, flexibility, and plasticity of insect cuticles (3, 20, 22, 27–30). Besides modifications by quinones during sclerotization, some cuticular proteins undergo posttranslational glycosylations, but the functional significance remains unknown (30). Recent studies on the sequence analysis of cuticle proteins, either direct or deduced from the cloned genomic or cDNA sequences, have further expanded our knowledge on the structural characteristics of insect cuticle proteins and their genes (47, 53, 84, 95, 125–127, 153). Novel repetitive sequences have been identified in both hydrophobic and hydrophilic domains of individual proteins, and some similarities in amino acid sequences between proteins from different species have been found. The conserved Ala-Ala-Pro-(Ala/Val) sequence found in the cuticle proteins of pharate adult *Locusta migratoria* (47) occurs many times in the hydrophilic domains of *Bombyx mori* pupal cuticle protein (84). Researchers have also noted similarities between a larval cuticle protein gene from *D. melanogaster* and genes from *Manduca, Sarcophaga,* and *Hyalophora* spp. (95).

Cuticular proteins are derived from the underlying epidermal cells or are transported from the hemolymph (22, 29, 30, 104). Some proteins are synthesized by epidermal cells in a temporally regulated program (60) and secreted into the cuticle where they may contribute to flexibility, hardness, and/or pigmentation, either as structural proteins or as specific enzymes (3, 21). In *D. melanogaster*, each developmental stage has cuticle proteins encoded by discrete genes, except for the second instar, which is largely identical to the first (22). Changes within stages are seen in proteins from third-instar and adult cuticles. The pattern of cuticular protein synthesis by the epidermis of *M. sexta* larvae changes during the final day of feeding, leading to an alteration in cuticular structure and a stiffening of the cuticle (53). Ecdysteroid effects the dramatic switch in cuticular protein synthesis that occurs in final-instar *M. sexta* larvae and causes epidermal cells to become pupally committed (155), permanently shutting off the larval-specific genes for larval cuticular proteins (100, 101). Juvenile hormone prevents both ecdysteroid release and the associated epidermal changes (99, 122, 155).

Several years ago, biochemical and immunological studies revealed similarities between hemolymph and cuticular proteins (61, 90). The best known examples of specific protein incorporation from hemolymph are the arylphorins, which are synthesized mainly by the fat body and are secreted into the hemolymph during larval development (24, 71, 119, 142). Experiments using immunoblotting, immunofluorescence, and autoradiography have demonstrated that arylphorins from *Ceratitis capitata* (57) and *Calliphora vicina* (62, 116) are internalized by the epidermal cells and then secreted into the cuticle. During the larval molt of *M. sexta*, injected radiolabeled arylphorin is incorporated into the newly forming fifth-instar cuticle (151). Grun & Peter (37, 38) showed that arylphorin is not metabolized in the hemolymph of *M. sexta* and is cross-linked in vitro with the sclerotizing agents, NADA quinone and NBAD quinone, generated by tyrosinase-catalyzed oxidation of their precursors. Another cellular source of cuticle proteins is the hemocyte. Comparisons between synthetic and accumulation profiles of hemocyte proteins in *C. capitata* revealed that some of those proteins are internalized into the integument from the hemolymph (144). Immunofluorescence experiments suggested that hemocyte proteins are first internalized into the epidermal cells and subsequently into the cuticle.

SCLEROTIZING AGENTS AND ENZYMES

The quinone tanning hypothesis advanced by Pryor (91, 92) and later refined in more detail proposes the establishment of extremely stable covalent bonds between the ring carbons of the quinonoid nucleus and the amino nitrogens of proteins (Figure 2). The resultant reduction of the quinone and subsequent oxidation of the catechol-protein adduct enables the establishment of a cross-link between two protein chains by a second addition reaction. Because of the stability of these bonds, little progress was made in obtaining direct evidence for the quinone tanning mechanism until the development of solid-state NMR techniques (115). Terminal amino groups of proteins and lysyl ϵ-amino functions were considered primary reactive sites for C-N linkages (39, 40, 135). Other nucleophilic groups on protein side chains, particularly the imidazole nitrogens of histidine, are also involved in quinone tanning to varying extents (115, 135). Because of the potential distances between cross-linking sites in the protein matrix, larger spans may be bridged by polymeric forms of quinonoid tanning agents that can also serve as space fillers (40, 93).

The participation of the catecholamine side-chain carbons in forming adducts and cross-links with proteins was the second major advance in understanding sclerotization mechanisms (2, 4). The first term applied by Andersen to describe covalent modification of the β-carbon in the catecholamine side chain was β-sclerotization (2, 3). However, subsequent research

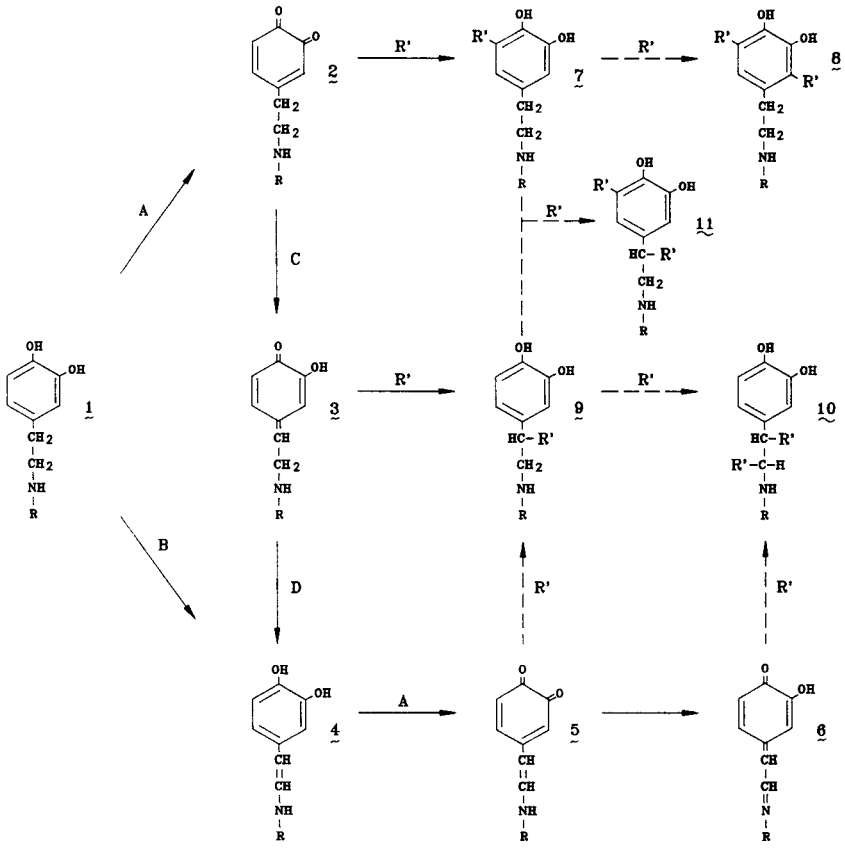


Figure 2 Metabolic pathways proposed for synthesis of quinonoid sclerotizing agents in insect cuticle and interactions with structural proteins. 1. N-acyldopamine precursor. 2. *o*-Benzoquinone derivatives. 3. *p*-Quinone methide derivatives. 4. N-acyl α,β-dehydrodopamine. 5. *o*-Quinone derivative of No. 4. 6. *p*-Quinone methide derivative of No. 4. 7. N-acyldopamine ring adduct; R' could be on ring carbons 2, 5, or 6. 8. N-acyldopamine ring cross-link. 9. N-acylcatecholamine β-carbon adduct. 10. N-acylcatecholamine α,β-carbon cross-link. 11. N-acylcatecholamine ring, β-carbon cross link. A represents phenoloxidase (laccase, tyrosinase); B, N-acyl α,β-dopamine desaturase; C, *o*-quinone:*p*-quinone methide isomerase; D, *p*-quinone methide:N-acyl α,β-dehydrodopamine tautomerase; R, acetyl or β-alanyl group; R', protein.

has indicated that the α-carbon may be a cross-linking site as well (4, 114) (Figure 2). To account for the reactivity of the β-carbon in forming adducts with various nucleophiles, Lipke, Sugumaran, and coworkers (73, 128) have proposed that quinone methides of N-acyl catecholamines are the main sclerotizing agents for side-chain cross-linking mechanisms (Figure 2). The term *quinone methide sclerotization* has been proposed to replace β-

sclerotization, and is based on the tanning agent rather than the cross-linking site, conforming to the earlier term of quinone tanning (114, 128). However, the use of tanning-agent names to designate precise cross-linking mechanisms is ambiguous since more than one ring or side-chain carbon may be involved in a cross-link structure, and the identity of most quinonoid tanning agents and products has not been directly established.

Apparently, cuticular phenoloxidases initially catalyze metabolic reactions for generating quinonoid sclerotizing agents, while quinone isomerases or tautomerases catalyze subsequent reactions (Figure 2). However, the unequivocal identification of transient quinonoid or radical intermediates and the purification and characterization of the enzyme catalysts and their regulating mechanisms pose major research challenges. Attempts to extrapolate results from laboratory experiments conducted on one species to general models for cuticle tanning in vivo may be unrealistic given the diversity of sclerotized structures. More effort will be needed to understand the complexities of sclerotization in cuticles that have evolved with differing mechanical properties, taking into account the characteristics of the sclerotizing agents, structural proteins, and chitin; their chemical interactions; and the enzymes that direct the assembly and stabilization of the exoskeleton outside the boundary of the epidermis.

Cuticular Oxidases

The phenoloxidases are copper-containing enzymes that catalyze the oxidative dehydrogenation of diphenols to quinones in animals and plants (96, 102). Two distinct types with substrate and inhibitor specificities that resemble typical tyrosinases and laccases are generally found in insect cuticle (4, 11–13, 21, 82, 143). Evidence suggests that some phenoloxidases may be initially secreted into cuticle as inactive proenzymes and that regulation of sclerotization may partially reside in their activation by cuticular proteases (21, 68).

TYROSINASES Tyrosinase is a multifunctional enzyme that catalyzes both hydroxylation of monophenols (monophenol monooxygenase, EC 1.14.18.1) and the oxidation of catechols to *o*-benzoquinones [*o*-diphenol: O_2 oxidoreductase, catechol oxidase, (EC 1.10.3.1)] (102). It is widely distributed in the cuticle, hemolymph, and other tissues of insects (21). Catechol oxidase activity is typically found in flexible larval cuticles where its function may be associated more with wound healing than with sclerotization (4, 12, 13). However, tyrosinases have also been isolated from pharate pupal integument of *M. sexta* undergoing cuticle sclerotization, so their role in oxidation of N-acylcatecholamines for this purpose has not been ruled out (10, 82). N-acyldopamines may be preferred substrates for tyrosinases since NADA

and NBAD are oxidized at a much higher rate than other substrates tested (11–13, 82). Tyrosinase is probably secreted into cuticle as a proenzyme that is activated by proteases upon wounding. In *Lucilia cuprina* larval cuticle, active tyrosinase was detected in epicuticular filaments, and a protyrosinase that was activated by wounding was present in procuticle (15). On the basis of their activity during dipteran larval development, tyrosinases have been assigned a role mainly in wound healing rather than puparial tanning (4, 11–13).

LACCASES Laccase-type phenoloxidases [diphenol: O_2 oxidoreductase (EC 1.10.3.2)] (96) have been detected in the cuticles of several species of insects (4, 11, 13, 143), and their developmental activity profile correlates with the main period of sclerotization. Laccase activity has also been observed in situ in the inner epicuticle of fly larvae close to pupariation [but not in younger larvae (15)] and in tanning pupal cuticle of *M. sexta* (143). N-acylcatecholamines also are preferred substrates for the cuticular laccases as well as for the tyrosinases (11–13, 143). Therefore, cuticular laccases have been assigned the major role of catalyzing the synthesis of quinonoid sclerotizing agents.

PEROXIDASES These heme-containing oxidases [phenol: hydrogen peroxide oxidoreductase (EC 1.11.1.7)] reportedly are present in the integument or cuticle during sclerotization (74, 133) and can catalyze the oxidation of catechols to quinones through one-electron steps. Quinones may then react spontaneously with nucleophilic groups for adduct and cross-link formation. More specifically, peroxidases have been associated with catalyzing formation of carbon-to-carbon cross-links between tyrosyl rings for synthesis of the rubber-like protein, resilin, in wing hinges (3, 4). However, before a significant role in cuticular tanning can be assigned to peroxidases, more data must be gathered concerning their properties and activity during the molting cycle.

Enzymes Involved in Catechol Side-Chain Reactions

Whereas the main role of phenoloxidases in cuticle sclerotization is to generate *o*-quinones from N-acyl dopamine substrates, other enzymes have recently been discovered that catalyze the conversion of these initial quinones to highly transient intermediates that appear to be *p*-quinone methides (8, 110, 111, 138). The proposal that the quinone methides of NADA and NBAD are tanning agents is mainly based on the nonenzymatic addition of water or other nucleophiles to the side-chain β-carbon after oxidation of the catechols to quinones (73, 128). However, because of their extreme reactivity, quinone methides of NADA and NBAD have not yet been directly observed either in vitro or in vivo, in contrast to the more stable *o*-quinones. Whenever NADA

or NBAD is oxidized by cuticular preparations or phenoloxidases from various sources, β-hydroxylation occurs (5, 81, 82, 85, 111, 136–138). The β-hydroxylation product is usually one of the major catecholamines extracted from early tanning cuticle (51, 81) and is racemic, suggesting spontaneous addition of water to a quinone methide or free radical derivative (85, 89). The electrophilic intermediate will also react with other nucleophiles including methanol and kynurenine to form β-carbon adducts (5, 65, 110, 137, 139). The mechanisms for generating reactive sclerotizing agents from diphenols and quinones may also include free radical formation, during either oxidation or subsequent spontaneous reactions. The existence of catecholic free radicals as sclerotizing agents is difficult to establish because of their tendency to undergo rapid nonenzymatic reactions in aqueous systems (86).

Quinone isomerases (*o*-quinone:*p*-quinone methide isomerases) have been extracted and partially purified from cuticles of several insect species (8, 68, 112). Unlike the phenoloxidases, these enzymes are soluble and have no activity towards catechols, but rather catalyze the conversion of *o*-quinones into reactive intermediates, most likely *p*-quinone methides that then react nonenzymatically with water or other nucleophiles. Some of the products of these reactions are catecholic β-carbon adducts. Although quinone methides were originally reported to be direct oxidation products of alkyl-substituted catechols (73, 128, 136), subsequent studies have shown that an *o*-quinone is the initial product that then undergoes isomerization (111, 138, 141). A significant amount of nonenzymatic β-hydroxylation occurs after oxidation of N-acyldopamines (68, 82, 140), but quinone isomerases appear to be specialized cuticular enzymes that have evolved for accelerating the production of side-chain activated cross-linking agents. A laccase partially purified from pharate pupal cuticle of *M. sexta* also catalyzes β-hydroxylation of N-acyldopamines at rates higher than nonenzymatic or tyrosinase-catalyzed reactions (143).

Another type of isomerization recently reported involves enzymatic conversion of NADA quinone methide to α,β-dehydro NADA (112–114). Dehydro NADA appears to be an important precursor of quinonoid tanning agents implicated in certain types of cuticle as first discovered by Andersen and colleagues (2–4). They observed that this precursor does not accumulate in tanning cuticle preparations of *L. migratoria* unless its rapid consumption is blocked by nucleophilic reagents or inhibitors (4, 5). Sugumaran et al (132) also demonstrated rapid consumption of dehydro NADA in vitro by cuticular phenoloxidase presumably through a quinone methide intermediate. However, trapping experiments that used radiolabeled NADA as a precursor indicated that this compound was not a precursor for sclerotization of puparial cuticle in *Sarcophaga bullata* (128, 129). Recently, however, an isomerase or tautomerase from larval cuticle of *S. bullata* was reported that apparently converts the transient NADA quinone methide to dehydro NADA (113, 114).

This result is consistent with a pathway in which NADA is oxidized to NADA quinone by cuticular phenoloxidase followed by an initial isomerization to NADA quinone methide, and tautomerization to α,β-dehydro NADA. NADA quinone and NADA quinone methide are putative agents for ring and β-carbon side-chain cross-linking reactions, respectively (Figure 2). Dehydro NADA, formed by tautomerization of NADA quinone methide, then may be reoxidized by a phenoloxidase to a quinone and isomerized to a quinone methide that could participate in covalent bonding to nucleophiles through either the α or β-carbons (114, 130, 134). Recently, the *o*-quinones of DOPA derivatives, blocked from internal cyclization, were shown to tautomerize nonenzymatically to their α,β-dehydro derivatives, apparently through a quinone methide intermediate (108, 109). However, NADA quinone did not tautomerize nonenzymatically to dehydro NADA. This observation perhaps explains the role of an enzyme-catalyzed pathway in insect cuticle. The extremely short life of the proposed quinone methide intermediates and their reactivity towards water and nucleophilic groups on proteins in the cuticular environment make it difficult to envision them as typical enzyme substrates. The properties of quinone isomerases or tautomerases and their catalytic mechanisms require further elucidation to understand how they catalyze the biosynthesis of these labile tanning agents.

Direct formation of α,β-dehydro NADA by an insoluble desaturase in locust cuticle was the first reported pathway for synthesis of this sclerotizing-agent precursor (4) (Figure 2). So far, the desaturase has not been isolated or characterized because of its insolubility and/or instability. However, the differential formation of dehydro NADA and dimeric products from NADA versus NANE under conditions that favor either desaturase or laccase activity suggests a unique desaturase enzyme in locust cuticle (5, 6).

NBAD as a precursor for quinonoid tanning agents differs considerably from NADA in that α,β-dehydrogenation of the side chain does not appear to occur in appreciable amounts with the former compound (7, 137). Instead, NBAD appears to be preferentially involved in ring and β-carbon cross-links in stiff brown cuticles, whereas NADA may more often be a precursor for cross-links to both α- and β-carbons in stiff colorless cuticles (4, 7, 50, 51, 81).

Catecholamines and Cuticular Strength

Quantities of different N-acylcatecholamine tanning agent precursors are correlated to some extent to the hardening and mechanical strengthening of cuticle during the course of sclerotization. Extractable catecholamines increase from nearly undetectable amounts in newly ecdysed cuticle to peak concentrations usually within 24 h (31–33, 51, 81). Hard cuticles such as mandibles, head capsules, and stiff brown pupal or puparial cuticles often accumulate the highest concentrations of N-acylcatecholamines in compari-

son to less sclerotized cuticles. For example, the cutting edge of larval mandibles of *M. sexta* has about 10 times more total extractable catecholamines as does head capsule cuticle and 50 times more than the dorsal abdominal cuticle 24 h after ecdysis (51, 81). Most of the N-acylcatecholamines are found in the outer exocuticular layer of the cuticle; only minor amounts occur in endocuticle of pupal *M. sexta* sampled one and three days after ecdysis (66; T. D. Morgan, K. J. Kramer & T. L. Hopkins, unpublished data).

The type of tanning agent precursor may also determine to some extent the physical properties of sclerotized cuticle. The clear hard cuticle of the larval head capsule contains mostly the N-acetyl derivatives NADA and NANE, whereas the hard dark cuticle of the larval mandible and pupal cuticle have primarily the N-β-alanyl derivatives NBAD and NBANE (51, 81). The hard brown puparial cuticles of Diptera (106) and elytral cuticle of *Tribolium castaneum* (107) also accumulate primarily NBAD and NBANE during the course of sclerotization. NBAD is probably metabolized to *o*-quinone and *p*-quinone methide tanning agents that form covalent bonds with nucleophilic groups via the ring and side-chain β-carbons (7, 81, 115, 137). NBANE, which is the predominant catecholamine extracted with weak acid from stiff brown cuticle during sclerotization, is thought to be a hydrolytic product of a NBAD-cuticle adduct weakly bonded via the β-carbon through oxygen (81). Much less NBANE can be extracted with methanol or aqueous solvents. Although NADA may also be a precursor for ring and β-carbon cross-links, it may be more important for bonding through both α and β side-chain carbons via its dehydro metabolite (4–6, 82, 114, 134). The two N-acyldopamines and their cuticular metabolites may confer special physical properties to different types of cuticle in which they predominate, but few data on this relationship are available. The total quantities of diphenols, both covalently bonded and weakly bound, increase progressively during sclerotization as determined by natural-abundance ^{13}C-NMR spectra (66–68, 87, 115). However, most of the diphenols occur as insoluble covalently bound adducts or cross-links (67).

Mechanical strength of tanning cuticle of *B. germanica* (34) and *T. castaneum* (107), as measured by resistance to puncture, increases as concentrations of extractable N-β-alanylcatecholamines rise. The black mutants of both species divert more dopamine into the melanization pathway, and their cuticles are therefore weaker because of deficiencies of cross-linking agents.

CROSS-LINKING IN CUTICLE

Covalent cross-linking of biopolymers via aromatic or side-chain carbons of N-acyl catecholamines stabilizes the three-dimensional structure of insect cuticle and appears to be the primary mechanism of sclerotization. The types

of bonding of proteins and chitin to the reactive intermediates include C-C links leading to modification of tyrosyl residues (38, 133) and C-N bonds involving histidyl, lysyl, and β-alanyl residues (23, 40, 68, 115, 135). Because cysteinyl residues are virtually absent from insect cuticular proteins, C-S couplings are unlikely (40). More likely are C-O bonds, but only indirect evidence for these cross-links in cuticle has been presented (72, 115). Recovery of bityrosine and tertyrosine from hydrolysates of insoluble residues supports the occurrence of C-C cross-links in cuticular polypeptide chains present in certain species (135). This kind of bridging unit could result from free-radical substitution reactions initiated by a peroxidase-type enzyme. However, C-C cross-links are apparently not ubiquitous in cuticles. Insoluble chitin-catecholamine-protein complexes have been extracted from fly and moth cuticles, but the structure of the chitin linkage has not been delineated (115, 133).

Solid-state NMR spectroscopy has been used to identify bonds from catecholamine aromatic and aliphatic carbons to protein nitrogens in insect cuticle. Rotational-echo, double resonance (REDOR) ^{13}C and ^{15}N NMR (23, 68) and ^{1}H-^{13}C-^{15}N double-cross polarization magic angle spinning (DCPMAS) (115) analyses revealed linkages between an aromatic carbon or β-carbon of dopamine and a histidyl ring nitrogen in *M. sexta* pupal cuticle (Figure 3). Approximately two-thirds of the bonds to histidine nitrogens are made through catecholamine ring carbons and about one-third through the β-carbon (68). We have also detected modifications of the α-carbon of dopamine and the amino group of β-alanine using solid-state NMR, but the substituents on those atoms have not yet been identified (A. M. Christensen, J. Schaefer, K. J. Kramer, T. D. Morgan & T. L. Hopkins, unpublished data). Thus, N-β-alanyldopamine apparently contributes several covalent bonding sites to *M. sexta* pupal cuticle sclerotization via aromatic ring carbons 2, 5, or 6: α- and β-carbons of the aliphatic side chain; and the terminal amino group of β-alanine.

Cross-linking experiments in vitro using arylphorin or other cuticular proteins of *M. sexta* and quinones derived from the oxidation of NBAD and NADA have documented the formation of multimeric protein forms (37, 38, 88). Whereas NADA quinone does not polymerize bovine serum albumin and ferritin (37), it does polymerize some noninsect test proteins, i.e. lysozyme, ribonuclease, and cytochrome *c*, indicating that protein requirements for this system are relatively nonspecific (131). Several types of phenoloxidases including mushroom and insect tyrosinase and insect laccase catalyze the protein polymerizations. The choice of N-acyldopamine influences the extent of reaction, however. NBAD was substantially more effective as a cross-linking agent precursor than NADA (37).

Another type of covalent bonding in insect cuticle occurs when pigments are formed. When oxidized, dopamine undergoes indolization and

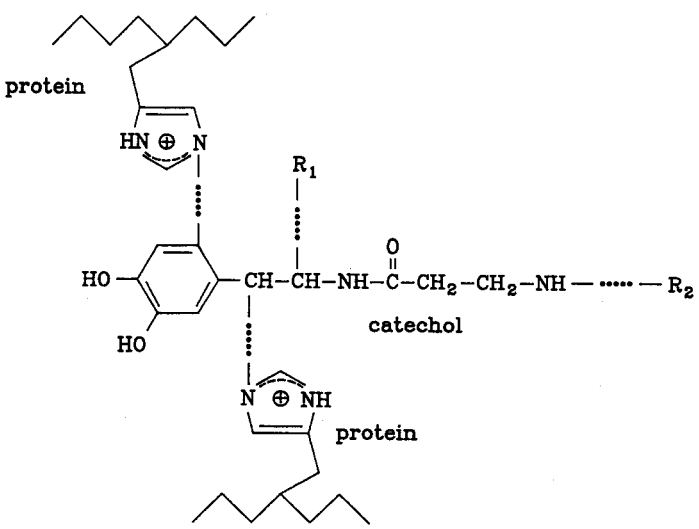


Figure 3 Proposed cross-link sites in *Manduca sexta* sclerotized pupal cuticle involving the tanning agent precursor N-β-alanyldopamine. The structures of R_1 and R_2 are not identified.

polymerization to eumelanin. Solid-state NMR analysis of cuticle from wild-type and black strains of *T. castaneum* revealed that the latter have substantially more melanin than the former (67). Melanins have been proposed to interact covalently with other cuticular macromolecules (38), but little structural information is available about possible linkages, and the degree to which they contribute to cuticle stabilization is unknown.

INHIBITORS OF SCLEROTIZATION

Inhibitors of sclerotization may disrupt the metabolism and/or deposition of phenolic compounds, protein, or other components that participate in cuticular stabilization mechanisms. Normal tanning requires the biosynthesis of N-acylated dopamines from tyrosine and metabolism to quinonoid derivatives. Thus, tyrosine hydroxylase or tyrosinase, DDC, N-acyl dopamine transferases, phenoloxidases, and quinone isomerases constitute potential targets of insecticidal action.

DDC is a key enzyme in the pathway for tanning-agent synthesis, and DDC inhibitors are toxic to blowfly larvae, apparently by specific interference with sclerotization (145, 146). Larvae exposed to DDC inhibitors such as α-methyl DOPA appear normal until molting, at which time they desiccate and die without tanning the newly secreted exoskeleton. The epicuticle is abnormal in its ultrastructure and defective as a water-permeability barrier. Coadministra-

CATECHOLAMINE METABOLISM AND CUTICLE SCLEROTIZATION MECHANISMS IN THE TOBACCO HORNWORM MANDUCA SEXTA

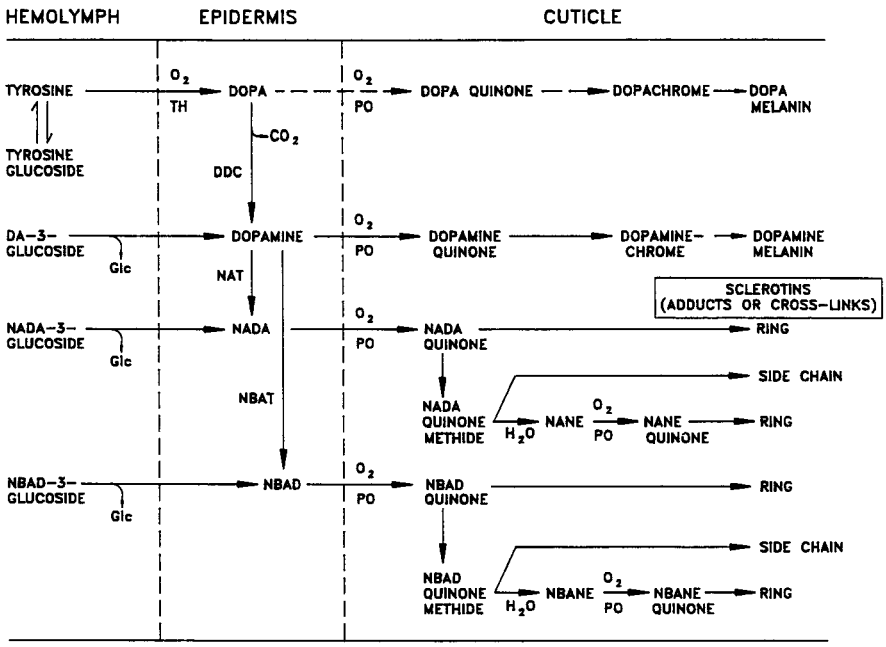


Figure 4 Roles of hemolymph, epidermis, and cuticle in the transport, biosynthesis, and metabolism of catecholamines for sclerotization and melanization of the insect exoskeleton as represented by *Manduca sexta*. Abbreviations are: DDC, dopa decarboxylase; Glc, glucose; NAT, N-acetyldopamine transferase; NBAT, N-β-alanyldopamine transferase; PO, phenoloxidase (tyrosinase, laccase); TH, tyrosine hydroxylase (tyrosinase). See Table 1 for other abbreviations.

hornworm, *M. sexta,* has served as a useful model insect in this respect (Figure 4).

Progress in deciphering the molecular organization and superstructure of sclerotized cuticle and the mechanisms of its assembly has been exceedingly slow because of the intractable nature of the finished product and the irreversibility of the tanning process. However, the recent development of noninvasive solid-state NMR procedures and selective labeling with ^{13}C and ^{15}N of putative cross-linking agent precursors has for the first time provided direct evidence for covalent bonds between the catecholamine-ring and β-carbons and the imidazole nitrogens of histidyl residues in protein of sclerotized cuticle. Solid-state NMR offers excellent potential for further elucidation of the molecular structures of different types of stabilized cuticles that have widely varying physical properties.

The characterization of the cuticular enzymes that catalyze the synthesis of

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