Sclerotization of insect cuticle is an extremely important biochemical process for the successful survival of most insects. N-Acetyldopamine and N-β-alanyldopamine are two widely used sclerotizing precursors. N-Acetyldopamine is converted by phenoloxidase, quinone isomerase, and quinone methide isomerase to generate the reactive intermediates quinone, quinone methide, and quinone methide imine amide for use in quinone tanning, quinone methide sclerotization, and α,β-sclerotization, respectively. N-β-Alanyldopamine has been claimed to be used only by phenoloxidase and quinone isomerase for quinone tanning and quinone methide sclerotization thereby attributing biochemical diversity to the coloration and strength of different cuticles. However, we demonstrate here that cuticular enzymes isolated from the larvae of Calliphora possess the capacity to generate 1,2-dehydro-N-β-alanyldopamine from N-β-alanyldopamine. Chemical synthesis of 1,2-dehydro-N-β-alanyldopamine and its further oxidation are reported for the first time. Comparative biochemical studies confirm that both the sclerotizing precursors, N-acetyldopamine and N-β-alanyldopamine, are used by all three different mechanisms of the unified theory of sclerotization.

In most insects the conversion of soft and pale cuticle into hard and tanned exoskeleton is achieved by sclerotization reactions (1). Sclerotization is a vital process for the survival of most insects. Arrest or even delay of sclerotization can have devastating effects on several insects. During the last few decades massive enhancements into the understanding of the biochemistry of cuticular sclerotization have been made (2-9). Sclerotization involves unconjugated soluble structural proteins and chitin fibers cross-linking and being rendered insoluble by chemical reactions with enzymatically generated sclerotizing agents derived from the cathecholamine sclerotizing precursors. Two of the widely used sclerotizing precursors are N-acetyldopamine (NADA) and N-β-alanyldopamine (NBAD). The bulk of the studies carried out with NADA have led to the development of three different modes of sclerotization mechanisms. They are quinone tanning involving quinones as sclerotizing agents, quinone methide sclerotization using quinone methides as the sclerotizing agents and α,β-sclerotization also commonly known as β-sclerotization which uses 1,2-dehydro-NADA and its derivatives (2-9). Discovery of two new enzymes, viz. quinone isomerase which generates NADA quinone methide from NADA quinone and quinone methide isomerase that produces 1,2-dehydro-NADA from NADA quinone methide (10-17) in recent years, led to the unification of the above three mechanisms (Fig. 1). Although NBAD is also widely used as a tanning precursor in numerous insects (6-9, 18, 19), its usage is believed to be limited to quinone tanning and quinone methide sclerotization to account for the variation in coloration and strength of different cuticles (7, 19). In this study, however, we present evidence for the first time to the formation of dehydro-NBAD by soluble enzymes isolated from the cuticle of Calliphora larvae and report on its chemical and enzymatic oxidation pathways. Our studies confirm that both the universal sclerotizing precursors NADA (Fig. 1; R = CH₃) and NBAD (Fig. 1; R = CH₂CH₃NH₂) are participating in the unified mechanism of sclerotization.

**MATERIALS AND METHODS**

**Preparation of Cuticular Enzymes—**Calliphora larvae were obtained from commercial supplier (Grub Co., Hamilton, OH) and used at wandering stages. All operations were carried out at 0-5 °C unless stated otherwise. The larvae were suspended in 1% sodium borate, pH 8.5, and homogenized in a Waring Blendor at 30-s pulse until their internal contents were extracted in buffer. The transparent cuticle obtained was suspended in 1% sodium borate, pH 8.5, for approximately 18 h in the cold room. The extracted proteins were subjected to ammonium sulfate fractionation. The proteins precipitated between 0 and 30% ammonium sulfate saturation were collected by centrifugation at 15,000 g for 15 min. The pellet obtained was either used immediately or stored at -80 °C until use (within 3-4 weeks).

The ammonium sulfate pellet was dissolved in a minimum amount of 10 mm sodium phosphate buffer, pH 6.0, and desalted on Sephadex G-25 column (2.5 x 20 cm) using the same buffer. The protein fraction was used as the enzyme source.

**Chemicals—**Dehydro-NADA was synthesized by the published procedures (29). NBAD was synthesized by coupling dopamine and N-α-t-butyloxy-carbonyl-β-alanine-N-hydroxysuccinimide ester in 10% potassium tetraborate, pH 9.0, and deblocking the conjugate with trifluoroacetic acid. After removal of acid, NBAD was purified by repeated column chromatography on fresh Sephadex LH-20 columns using 0.2 μ acetic acid as the eluant. NBANE was similarly prepared by hydrolyzing the adduct obtained by condensation of norepinephrine with N-α-t-butyloxy-carbonyl-β-alanine-N-hydroxysuccinimide ester in borate. Dehydro-NBAD was synthesized in the same way as NBANE except treatment with trifluoroacetic acid was over a longer time period (18 h) and column chromatography was carried out using Bio-Gel P-2 with 0.2 μ acetic acid as the eluant. NADA quinone N-acetylcysteine adduct was prepared by the published procedure (21). β-Methoxy-NBAD was synthesized as previously reported (14). Papiliochrome Ia and Ib isolated from the yellow wing scales of the butterfly Papilio demoleus by the procedure outlined by Umebachi and Yoshida (22) were generously supplied by Dr. Motoko Yago of Iwate Medical College, Morioka, Japan.

**HPLC Analysis—**HPLC analyses of reaction mixtures were carried out using a Beckman (Berkeley, CA) model 332 liquid chromatography system equipped with two model 110 B pumps, a model 420 controller, a model 180 absorbance detector, and a model 427 integrator. Separation...
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**FIG. 1. Unified mechanism for sclerotization of insect cuticle.** Sclerotizing precursors such as NADA (R = CH₃) and are oxidized by phenoloxidases (1), both laccases and o-diphenoloxidases, to the corresponding quinones that participate in quinone tanning. Quinone isomerase (3) converts quinones to quinone methides and provide them for quinone methide sclerotization. NADA quinone methide is specifically converted to dehydro-NADA by quinone methide isomerase (4). Dehydro-NADA is further oxidized by phenoloxidases to dehydro-NADA quinone which is rapidly tautomomerized to form the quinone methide imine amide that serves as the sclerotizing agent for α,β-sclerotization. 2, nonenzymatic reactions. The same scheme is also applicable to N-β-alanyldopamine (R = CH₂CH₂NH₂), but the dehydro-NBAD formation and its subsequent oxidation has not been reported previously to this publication.

**RESULTS AND DISCUSSION**

Partially purified soluble cuticular enzymes isolated from the last instar larvae of Calliphora possessed all three enzymes involved in the unified sclerotization, viz., phenoloxidase, quinone isomerase, and quinone methide isomerase. Accordingly, when NADA was incubated with the enzyme preparation, dehydro-NADA formation could be witnessed. Fig. 2 for instance, shows the UV spectral changes associated with the oxidation of NADA. The formation of a shoulder at about 315 nm to the main peak is consistent with the generation of dehydro-NADA reported earlier for the sarcophagid system (11). HPLC analysis of the reaction mixture revealed the formation of both NANE and 1,2-dehydro-NADA (Fig. 2, left inset, peaks A and C, respectively). These identifications are based on the retention time on HPLC, UV spectra, and co-chromatography with synthetic material. The formation of these products can be accounted for by the action of (a) phenoloxidase which is responsible for NADA quinone production, (b) quinone isomerase which causes the isomerization of NADA quinone to quinone methide (the latter undergoes a nonenzymatic reaction with water to form NANE), and (c) quinone methide isomerase that generates 1,2-dehydro-NADA from NADA quinone methide.

The dehydro-NADA formed does not accumulate in the reaction but undergoes further oxidation by phenoloxidase to form dimeric products as reported for the sarcophagid system (23).

The presence of 10 μM phenylthioureia, a well known inhibitor of phenoloxidase, completely inhibited the formation of these products indicating that phenoloxidase action is essential for the production of both NANE and dehydro-NADA. As was established in the sarcophagid system (12, 23), NADA quinone served as an essential intermediate for the biosynthesis of dehydro-NADA. When NADA quinone, synthesized by silver oxide oxidation of NADA, was provided as the substrate for the Calliphora enzymes, they decomposed this intermediate rapidly (Fig. 2, right inset). The decay accompanied the formation and subsequent disappearance of the red-colored NADA quinone methide imine amide intermediate (24) that can be monitored at 475 nm (Fig. 2, right inset). Both the NADA quinone and the red colored intermediate exhibited an approximate half-life of 20 s in the presence of cuticular enzymes. The decomposition of these quinonoid compounds was accompanied by the appearance of NADA dimers. The results of Calliphora cuticular enzyme reactions agree well with those reported for Sarcophaga bullata reactions, enhancing the contention that Calliphora cuticular enzymes also use the unified mechanism of sclerotization for metabolizing NADA.

We then focused our attention on the study of enzymatic oxidation of NBAD. The same enzyme preparation used above for NADA studies also oxidized NBAD to NBANE (Fig. 3B). The NBANE formation could occur via two methods, viz., either by a direct β-hydroxylation of NBAD or by nonenzymatic hydration of NBAD quinone methide produced by the tautomeration of phenoloxidase generated NBAD quinone (14). The presence of NBAD quinone in the HPLC tracing (Fig. 3B) indicated the latter possibility which was confirmed by the following studies. When the NBAD-cuticular enzyme reaction was carried out in the presence of 10 μM phenylthioureia, a specific inhibitor of phenoloxidase, complete inhibition of NBANE formation was noticed suggesting the requirement of phenoloxi-
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Fig. 3. HPLC analysis of NBAD oxidation. A reaction mixture containing 9.1 mM NBAD and 0.5 mg of enzyme protein in 1.0 ml of 100 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature, and 15-μl aliquots of the reaction mixture were subjected to HPLC analysis. Solvent system II was used to separate NBANE (3.38-min peak), NBAD (6.55-min peak), and NBAD quinone (4.51-min peak). Chromatogram A, zero time reaction; chromatogram B, 10-min reaction; chromatogram C, HPLC analysis of a 10-min NBAD N-acetylcysteine reaction. Conditions as above except for the inclusion of 2 mM methanol, was witnessed (Fig. 4B). This new adduct was not matched that of dehydro-NADA very well. In addition NMR spectral studies (Fig. 4A) showed that the UV spectrum of this compound was identical to authentic NBAD quinone methide (Fig. 1; inset), which afforded two new adducts identified as papiliochromes IIa and IIb based on the HPLC and UV spectral similarities with those of authentic samples (Fig. 4D and inset). These studies confirmed the enzymatic generation of NBAD quinone methide and its subsequent nonstereoselective addition with available nucleophiles in the NBAD cuticular enzyme reaction mixture.

The possibility of dehydro-NBAD formation was examined. To pursue the formation of dehydro-NBAD, we first chemically synthesized this compound using the established chemical procedures. The UV spectrum of this compound matched that of dehydro-NADA very well. In addition, NMR spectrum confirmed the structure of the product. 1H NMR in (dimethyl sulfoxide-d_{6}) had the following signals at δ = 1.95 (m, 2H, C-11); 2.2 (m, 2H, C-12); 6.0 (dd, 1H, J = 17.5 and 1.5 Hz, C-7); 6.75 (m, 1H, Ar); 6.85 (m, 1H, C-8); 7.00 (m, 1H, Ar); 7.20 (m, 1H, Ar); and 10.05 (m, 1H, amide).

From the NADA-cuticular enzyme reaction mixture dehydro-NBAD production can be clearly seen by HPLC studies (15). However, in the case of NBAD-enzymatic reaction, although the UV spectral studies accompanying the enzymatic oxidation clearly resembled the NADA reaction, unequivocal identification of dehydro-NBAD could not be made with HPLC studies. Under most of the HPLC conditions tried, both NBAD and synthetic dehydro-NBAD exhibited the same retention time without significant resolution, making it difficult to monitor the biochemical conversion of NBAD to dehydro-NBAD. However, chromatography of the entire enzymatic reaction mixture on Sephadex LH-20 with 0.2 M acetic acid as eluant resolved these two compounds readily. Fig. 5 shows a typical chromatographic pattern of a NBAD-cuticular reaction mixture on Sephadex LH-20. The major peak observed in the chromatogram contained both unreacted NBAD and NBANE, while the small minor peak contained a single compound. The UV spectrum of this compound (Fig. 5, inset) showed a typical dehydrocatechol type peak and compared very favorably with authentic dehydro-NBAD. Based on rechromatography on HPLC and LH-20 columns and UV spectral studies, this compound was identified as dehydro-NBAD, which was confirmed by comparison of the FT-IR spectrum of isolated product with that of the synthetic material. The FT-IR spectrum of the isolated product exhibited
To half its original absorbance is consistent with dimerization.

A continual drop in absorbance well below the half initial absorbance was reported from this laboratory analyzed oxidation of dehydro-NADA of a compound which resembled the dehydro-NAD dimer in its NH-CO-R), found to be sensitive to alkaline pH. At pH values higher than of starting material but dimers strongly bound to the column.

HPLC analysis of the reaction showed complete disappearance of starting material but dimers strongly bound to the column and resisted elution. Column chromatography on Sephadex LH-20 (Fig. 7, inset) resulted in the isolation of trace amounts of a compound which resembled the dehydro-NADA dimer in its UV spectrum.

Similar to dehydro-NADA (27), dehydro-NBAD was also found to be sensitive to alkaline pH. At pH values higher than 8.0, rapid nonenzymatic oxidation of dehydro-NBAD occurred (Fig. 7, bottom). As indicated by the absorbance spectrum, continual drop in absorbance well below the half initial absorbance (seen for enzymatic dimerization) accompanied the nonenzymatic oxidation indicating eventual polymerization of the compound. The enzymatic oxidation of dehydro-NBAD accompanied the transient accumulation of a red colored intermediate (Fig. 5). The visible spectrum of this intermediate (Fig. 8, inset) resembled the visible spectrum of the reactive quinone methide imine amide generated from dehydro-NADA by tyrosinase-catalyzed oxidation of dehydro-NADA (24). Therefore, analogous to dehydro-NADA, dehydro-NBAD also seems to generate the same type of reactive intermediate upon enzymatic oxidation.

Our results can be summarized as follows. NBAD is oxidized by phenoloxidase to its quinone. NBAD quinone can be trapped as its N-acetylcysteine adduct. Alternatively, NBAD quinone in the presence of quinone isomerase is converted to quinone methide. NBAD quinone methide undergoes nonenzymatic and hence nonstereoselective addition with (a) water to form NBANE, (b) methanol to form β-methoxy-NBAD, and (c) L-kyurenine to form papiliochromes Ila and Iib. Quinone methide also serves as a substrate for quinone methide isomerase to form dehydro-NBAD. Finally, dehydro-NBAD upon oxidation generates reactive quinone methide imine amide intermediate that dimerizes and polymerizes. These findings strongly support the contention that NBAD is used by the unified mechanism of sclerotization (Fig. 1; R = CH₂CH₂NH₂) just as NADA in Calliphora cuticle.

Since the discovery in 1962 of the involvement of NADA as an important sclerotization precursor (28), several authors have examined the fate of this compound in numerous cuticles.
generate the three known reactive intermediates of tanning, viz. quinones, quinone methides, and dehydrodopamine derivatives. However, both NADA and NBAD have a major structural difference. Since NBAD has an amino group in its side chain, one or more of the reactive intermediates generated from this compound might exhibit intramolecular cyclization reactions as observed in the case of dopaquinone (4) or carboxyethyl-o-benzooquinone (31). Solid state NMR studies seem to point out to such a possibility (32). As NADA does not have any reactive groups on it, it will not exhibit such internal reactivity. Therefore, these two sclerotizing precursors can still show marked differences in their mode of incorporation into insect cuticle after generating the three common reactive intermediates (quinone, quinone methide, and quinone methide imide amide) accounting for the differences in coloration and strength of different insect cuticle.

Fig. 8. Generation of a reactive intermediate during tyrosinase catalyzed oxidation of dehydro-NBAD. A reaction mixture containing 1 mg dehydro-NBAD, 50 μg of mushroom tyrosinase in 0.5 ml of 100 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature and the generation of the reactive intermediate was continuously monitored at 475 nm. Inset, visible spectrum of the transient reactive intermediate formed in the above reaction at 3 s.

Andersen and his associates (6, 7) who pioneered the studies on cuticular enzyme catalyzed oxidation of NADA, first reported the formation of dehydro-NADA as a key sclerotizing precursor (29). A few years ago, our laboratory reported the successful solubilization of the cuticular enzymes and identified quinone isomerase and quinone methide isomerase as new enzymes involved in the sclerotization of insect cuticle, apart from the well known phenoloxidase (10-17). Our studies also led to the unification of the three different mechanisms of tanning, viz. quinone tanning, quinone methide sclerotization, and β-sclerotization (Fig. 1). A different but related sclerotization precursor was discovered by Hopkins and his associates (30). Hopkins and co-workers identified NEAD as the major sclerotizing precursor of Manduca sexta and several other organisms (8, 9, 30). After examining the oxidation chemistry of this compound (7, 14, 18, 19), a general consensus which claims that NADA is utilized by the unified mechanism, while NBAD is used only by quinone tanning and quinone methide sclerotization mechanisms, was reached. These conclusions are also based on the empirical observation that NADA is utilized for the production of soft and colorless cuticle, while NBAD is used for making hard and colored cuticle (7, 19). In addition, cuticles utilizing NADA seem to produce ketocatechols as a degradation product, while cuticles treated with NBAD generate NBANE as the major identifiable degradation product (18). These differences led to the belief that variation in temporal hardening and coloration could be accounted for by the use of different sclerotizing precursors by different mechanisms. On the contrary, studies presented in this report demonstrate that the sclerotizing enzymes do not discriminate between NADA and NBAD to generate the three known reactive intermediates of tanning.