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CUTICLE-CATALYZED COUPLING BETWEEN *N*-ACETYLHISTIDINE AND *N*-ACETYLDOPAMINE

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Abstract—Several types of insect cuticle contain enzymes catalyzing the formation of adducts between *N*-acetyldopamine (NADA) and *N*-acetylhistidine (NAH). Two such adducts, NAH-NADA-I and NAH NADA-II, have been isolated and their stuctures determined.

In one of the adducts the link connecting the two residues occurs between the 1-position (β-position) in the NADA side chain and the 1-N atom (τ-N) in the imidazole ring of histidine. Diphenoloxidase activity alone is not sufficient for formation of this adduct, whereas extracts containing both diphenoloxidase and *o*-quinone-*p*-quinone methide isomerase activities catalyze the coupling reaction. The adduct consists of a mixture of two diastereomers and they are presumably formed by spontaneous reaction between enzymatically produced NADA-*p*-quinone methide and *N*-acetylhistidine.

The other adduct has been identified as a ring addition product of *N*-acetylhistidine and NADA. In contrast to the former adduct it can be formed by incubation of the two substrates with mushroom tyrosinase alone.

An adduct between *N*-acetylhistidine and the benzodioxan-type NADA-dimer is produced *in vitro*, when the *N*-acetylhistidine-NADA adduct is incubated with NADA and locust cuticle containing a 1,2-dehydro-NADA generating enzyme system.

Trimeric NADA-polymerization products of the substituted benzodioxan-type have been obtained from *in vivo* sclerotized locust cuticle, confirming the ability of cuticle to produce NADA-oligomers.

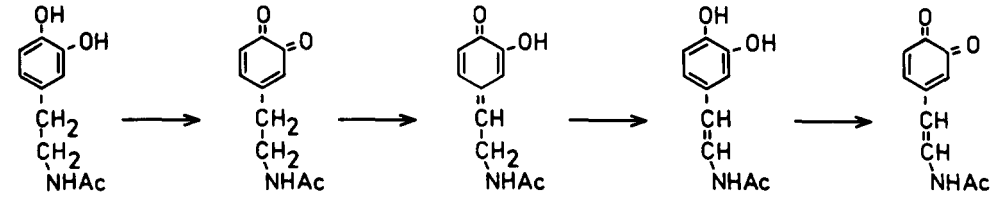
The results indicate that some insect cuticles contain enzymes promoting linkage of oxidized NADA to histidine residues. It is suggested that histidine residues in the cuticular proteins can serve as acceptors for oxidized NADA and that further addition of NADA-residues to the phenolic groups of bound NADA can occur, resulting in formation of protein-linked NADA-oligomers. The coupling reactions identified may be an important step in natural cuticular sclerotization.

Key Word Index: sclerotization; quinone; quinone methide; *o*-quinone isomerase; *Hyalophora cecropia*; *Locusta migratoria*

INTRODUCTION

During sclerotization of insect cuticle *o*-diphenolic compounds, such as *N*-acetyldopamine (NADA) and *N*-β-alanyldopamine (NBAD), are oxidatively incorporated into the cuticle. For a recent review of cuticular sclerotization see Andersen (1990). The activation of these *N*-acyldopamines is due to the combined action of several enzymes: in addition to various types of phenoloxidases (tyrosinases and laccases) oxidizing the diphenols to *o*-quinones, an enzyme isomerizing *o*-quinones to *p*-quinone methides occurs in cuticle (Saul and Sugumaran, 1988; Saul *et al.*, 1991; Andersen, 1989a), and an enzyme isomerizing NADA-*p*-quinone methide to 1,2-dehydro-NADA (Saul and Sugumaran, 1989a, b, 1991a) have been described.

A likely scheme (Scheme 1) for the metabolism of NADA during cuticular sclerotization will accordingly be:



NADA | NADA-*o*-quinone | NADA-*p*-quinone methide | 1,2-dehydro-NADA | 1,2-dehydro-NADA-*o*-quinone

According to the scheme NADA (or NBAD) is enzymatically oxidized to the corresponding *o*-quinone,

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which can be isomerized to the corresponding *p*-quinone methide (Peter, 1980) in an enzyme catalyzed reaction (Saul and Sugumaran, 1988; Saul *et al.*, 1991; Andersen, 1989a). In some types of cuticle, such as locust hard cuticle, NADA-*p*-quinone methide can be isomerized to dehydro-NADA, which is oxidized enzymatically to dehydro-NADA-*o*-quinone; apparently NBAD-*p*-quinone methide is not isomerized to dehydro-NBAD (Andersen, 1989b; Sugumaran *et al.*, 1991).

The various quinones shown in the scheme may react with amino acid residues in the cuticular proteins (Peter, 1989), thereby contributing to the stabilization of the cuticle, but it is still uncertain which residues will react with the various quinones. The *o*-quinones of both NADA and NBAD react readily with SH-groups to give ring-substituted catechols (Sugumaran *et al.*, 1989), but this reaction may be of minor importance since insect cuticles appear to contain little, if any, cysteine (Andersen, 1979). Dehydro-NADA-*o*-quinone reacts readily with catechols to give substituted benzodioxan-derivatives (Andersen and Roepstorff, 1982), but reactions with amino acid residues have not yet been reported. The *p*-quinone methides react readily with water and methanol to give β-hydroxy- and β-methoxy-acyldopamines, respectively (Peter and Vaupel, 1985; Sugumaran *et al.*, 1989), and papiliochrome-II has been obtained by spontaneous reaction between L-kynurenine and enzymatically generated NBAD-*p*-quinone methide (Yago, 1989; Saul and Sugumaran, 1991b). Recently, adduct formation between *N*-acetylhistidine (NAH) and NADA has been obtained by co-incubation with larval cuticle from the American silkmoth, *Hyalophora cecropia* (Andersen *et al.*, 1991). The adduct contains a bond between the 1-nitrogen atom in the histidine imidazole ring and the beta-position in NADA and it is probably formed by reaction between the imidazole ring and NADA-*p*-quinone methide.

Solid state NMR studies have revealed a complex polyphenolic component in the cuticle (Peter *et al.*, 1984), containing covalent links between the aromatic ring of *N*-acyldopamines and histidine and lysine residues (Schaefer *et al.*, 1987). By means of REDOR NMR spectroscopy the presence of bonds between imidazole nitrogen in histidine residues and the beta carbon atom in the side chain of dopamine derivatives has recently been demonstrated (Christensen *et al.*, 1991).

From degradation studies and indirect evidence, it has also been suggested that histidine may be involved in adduct formation (Sugumaran and Lipke, 1982). Other amino acid side chains are probably also involved in sclerotization, but the evidence is incomplete, as well-defined derivatives containing acyldopamines linked to amino acids have not yet been isolated and characterized from sclerotized cuticle. In this paper we describe the cuticle-catalyzed *in vitro* formation of *N*-acetylhistidine-NADA adducts and report that such adducts may take part in further NADA-polymerization.

METHODS AND MATERIALS

Cuticle incubations

Cleaned cuticle (*ca* 500 mg wet wt) from late fifth instar larvae of *H. cecropia* was incubated in 50 ml 0.2 M Na-phosphate, pH 7.0, containing 10 mM NADA and 10 mM *N*-acetylhistidine (NAH), at 40°C for at least 8 h. The reaction was monitored by analyzing 100 μl aliquots at regular intervals by reversed phase high performance liquid chromatography (RP-HPLC). The incubation conditions for other types of cuticle were similar, except that either 100 mg wet wt of femur cuticle from 1 day old adult locusts (*Locusta migratoria*) or 50 mg of puparial cuticle from *Drosophila melanogaster* were used per 50 ml medium.

To purify the *N*-acetylhistidine-NADA adducts (NAH-NADA-I and NAH-NADA-II), the pooled media from several incubations with *H. cecropia* cuticle were acidified, concentrated and fractionated on a column (1.6 × 15 cm) of BioBeads SM-16 (BioRad Laboratories) in equilibrium with 0.2 M acetic acid and eluted with a linear gradient of ethanol from 0 to 100%. The adducts eluted between *N*-acetylnorepinephrine (NANE) and NADA.

Products from incubation of NADA plus NAH-NADA-I adduct

The conditions for reaction between NADA and the NAH-NADA-I adduct were: 10 ml 0.2 M Na-acetate, pH 5.6, containing 10 mM NADA and 7 mM NAH-NADA-I plus 30 mg wet wt of locust femur cuticle, were incubated at 40°C. The progress of reaction was monitored by HPLC-analysis. The femurs were removed after 18 h incubation, and the supernatant was acidified, concentrated in vacuum to *ca* 2 ml, and subjected to preparative HPLC, using the same conditions as for analytical HPLC. The peaks of interest were collected and either analyzed by mass spectrometry or hydrolyzed.

Reversed phase HPLC separations were performed on a Spherisorb ODS-2 C_{18} column (4 × 250 mm) in equilibrium with 0.1% trifluoroacetic acid and eluted with a linear gradient from 0 to 70% methanol. The u.v. absorption of the eluate was monitored from 220 to 370 nm by means of a LKB diode array detector.

Formic acid extraction of sclerotized cuticle

Cleaned and powdered cuticle (20 g) from about 6 weeks old adult *L. migratoria* were extracted overnight in 150 ml anhydrous formic acid at 4°C. The filtered extract was concentrated to about 30 ml in vacuum, diluted with water and reconcentrated to remove most of the formic acid. The concentrate was centrifuged, divided into three equal portions, which were consecutively fractionated on a column (2.6 × 30 cm) of BioGel P-2 (BioRad Laboratories) eluted with 0.2 M acetic acid. The peaks of interest were pooled and refractionated on a column of BioBeads SM-16, in equilibrium with 0.2 M acetic acid and eluted with a linear gradient from 0 to 100% methanol. The fractions of interest were pooled and concentrated, subjected to preparative HPLC, and fractions were collected for mass spectrometry and hydrolysis.

Hydrolysis

Samples purified by HPLC were taken to dryness in a vacuum centrifuge, redissolved in 1 ml 1 M HCl, and hydolyzed at 100°C for 4 h. The hydrolyzed samples were redried, dissolved in 200 μl water, and analyzed by HPLC using the same elution conditions as above. The relative amounts of catechols and ketocatechols were estimated from the peak areas, using molar extinction coefficients of 2000 and 8200 for catechols and ketocatechols, respectively (Andersen, 1980).

Plasma desorption mass spectrometry (PDMS)

The samples, approx. 5 μg, dissolved in 0.1% trifluoroacetic acid were applied to a nitrocellulose covered target by the spin technique (Nielsen *et al.*, 1988). The mass spectra were recorded on a BioIon 20 plasma desorption mass spectrometer for 10^6 start events.

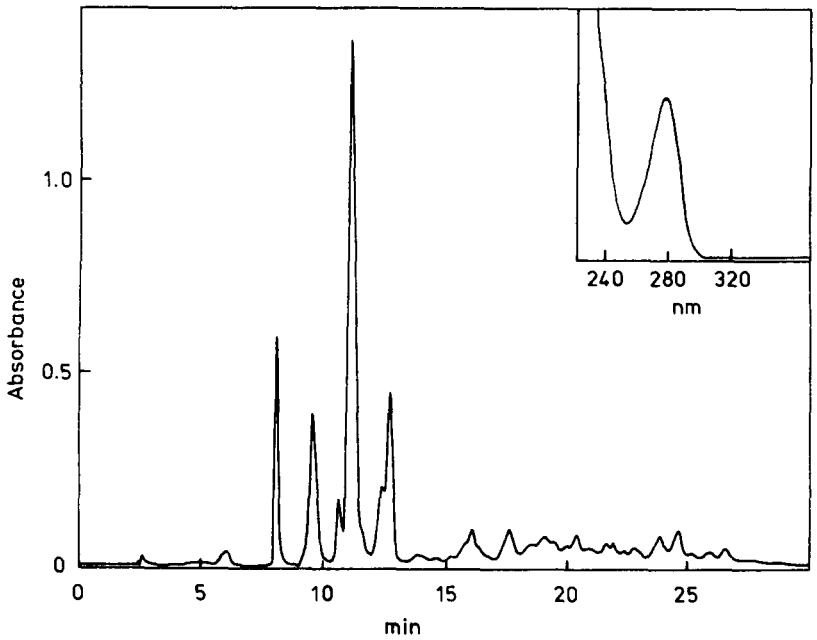


Fig. 1. Reversed phase chromatogram of sample from medium after 7 h incubation of 500 mg wet *H. cecropia* larval cuticle in 50 ml 10 mM *N*-acetylhistidine, 10 mM NADA in 0.2 M Na-phosphate, pH 7.0, at 40°C. Absorbance at 280 nm is recorded. Peak at 8.1 min: NANE; 9.6 min: NAH-NADA-I; 10.5 min: NAH-NADA-II; 11.2 min: NADA; 12.7 min: X_1. The insert shows the u.v. spectrum of the NAH-NADA-I peak at 9.6 min.

NMR-spectroscopy

^{1}H- and ^{13}C-NMR spectra were recorded using [D_6]-DMSO as solvent at ambient temperature with a Bruker WM 200 (200 resp. 50.3 MHz) NMR spectrometer. The internal standard was tetramethylsilane for ^{1}H-NMR ($\delta = 0$ ppm) and solvent signals for ^{13}C-NMR.

RESULTS

N-acetylhistidine-NADA adduct formation

Incubation of pieces of soft cuticle from fifth instar larvae of the American silkmoth *H. cecropia* together with NADA and *N*-acetylhistidine (NAH) results in the formation of several products (Fig. 1), some of which have been identified. During RP-HPLC, NANE elutes at 8.1 min and NADA at 11.2 min. The product eluting at 12.7 min, formerly called X_1 (Andersen, 1989c), has recently been identified as an oxidized adduct (1), composed of NADA and *N*-acetyl-trihydroxyphenylethylamine (Andersen *et al.*, 1992). The product eluting at 9.5 min in Fig. 1 has by combined use of mass spectrometry and ^{1}H- and ^{13}C-NMR studies been identified as an adduct of NAH

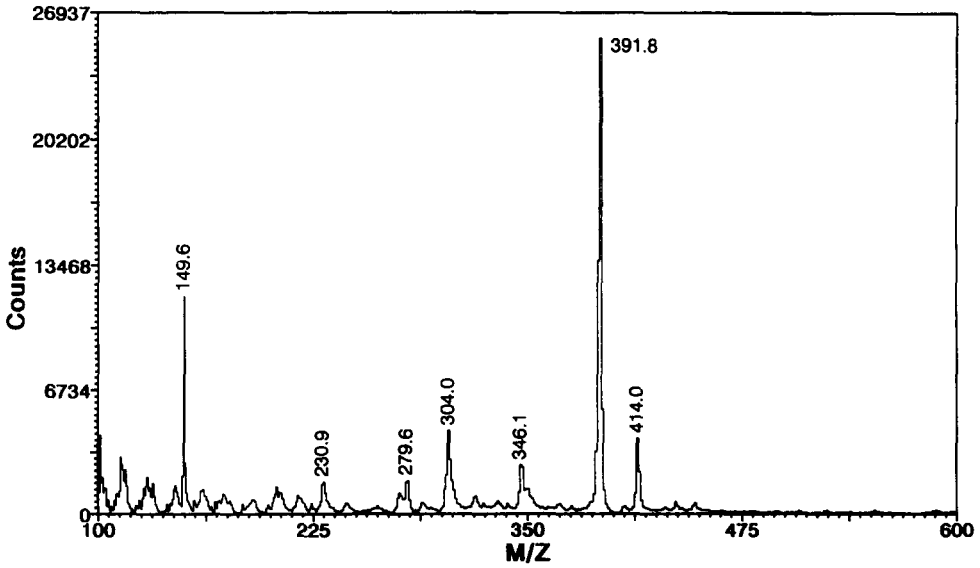


Fig. 2. Plasma desorption mass spectrum of the compound eluted at 10.5 min in Fig. 1.

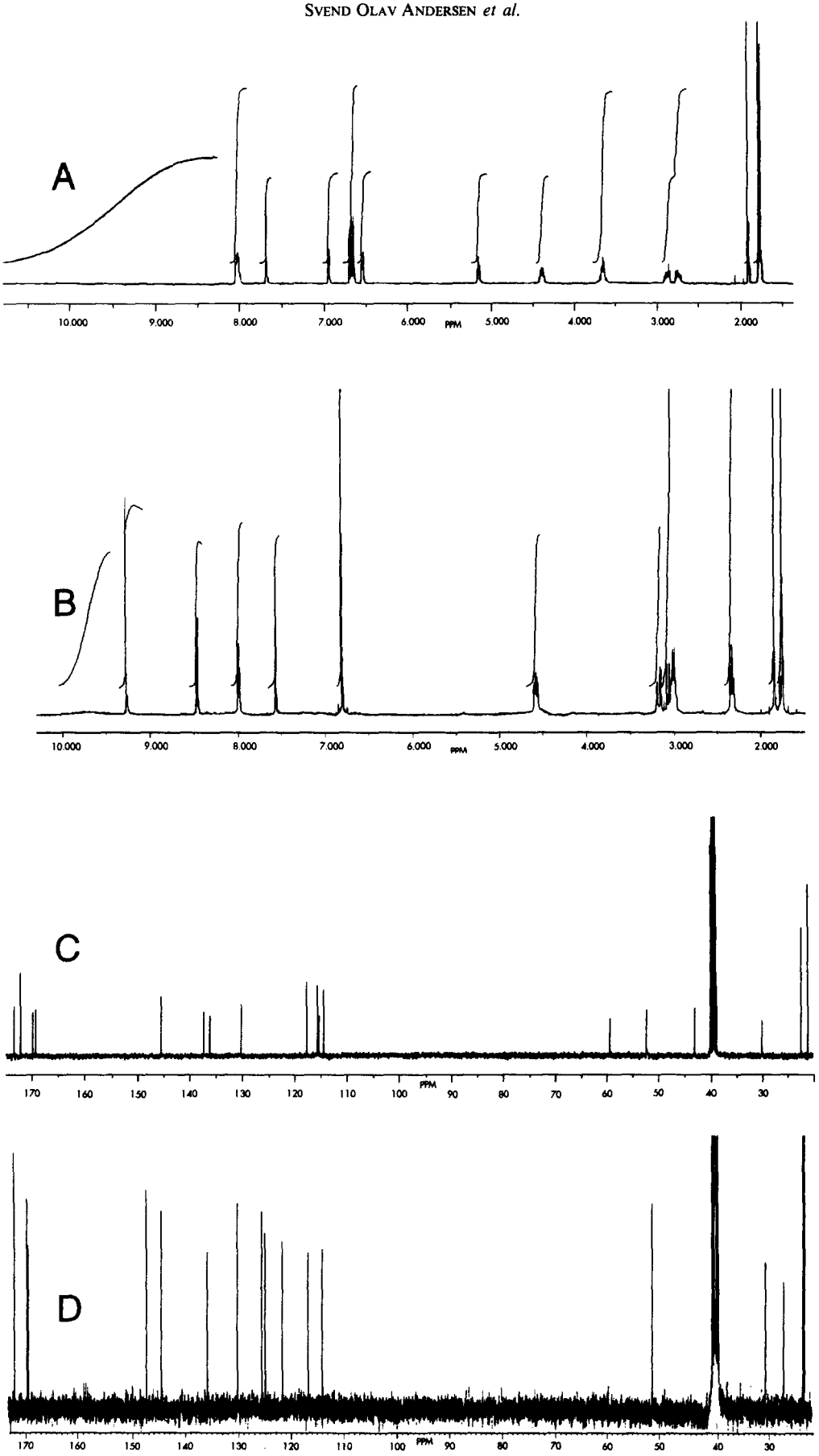
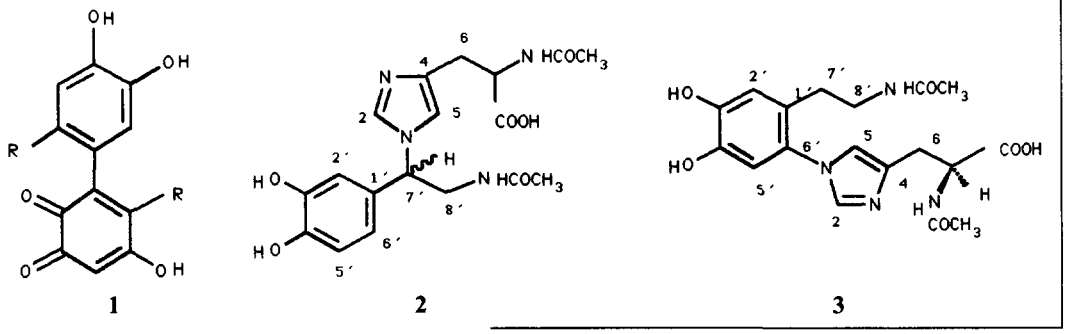


Fig. 3. *Caption on facing page.*

and NADA (**2**, NAH-NADA-I) (Andersen *et al.*, 1991).

A second product, NAH-NADA-II, was isolated from the incubation mixture. It elutes at 10.5 min from the C_{18}-column. The u.v. spectrum shows λ_{max} at 224 and 281 nm. Plasma desorption mass spectrometry (Fig. 2) reveals the protonated molecular ion at *m/z* 391.8, indicating that NAH-NADA-II could be an isomer of NAH-NADA-I. The structure of NAH-NADA-II was finally resolved by NMR spectroscopy as depicted in structure **3**. Comparison of the NMR spectra of NAH-NADA-I [Fig. 3(A) and (C); for full assignment of the data, see Andersen *et al.* (1991)] and NAH-NADA-II [Fig. 3(B) and (D)] shows that the latter contains the intact side chains of both NAH and NADA. The aromatic region reveals, besides the two NH protons, two coupled (J = 1.6 Hz) doublets at 9.27 and 7.56 ppm of an N^{τ}-substituted imidazole and two closely resonating protons at *ca* 6.8 ppm, indicative for a 1,2,4,5-tetrasubstituted benzenoid ring. The ^{13}C-NMR spectrum of NAH-NADA-II is in complete agreement with structure **3**. Selective C-H-decoupling proves that the imidazole proton at 9.27 ppm is directly linked to the carbon atom resonating at 135.78 ppm, whereas the other imidazole proton at 7.56 ppm is connected to the carbon atom at 121.78 ppm. Likewise, the two protons at 6.8 ppm are linked to the carbons appearing at 116.78 and 113.93 ppm.



1 **2** **3**

NAH-NADA-I is also obtained, although in lower yields, when NADA and NAH are incubated with either puparial cuticle from *D. melanogaster* or with pharate adult locust cuticle (Fig. 4). The peaks at 21.0 and 22.5 min are the main products during the early incubation period [Fig. 4(A)], they are isomeric forms of the benzodioxan-type NADA-dimer, and they are also produced when NADA is the sole substrate (Andersen, 1989c,d). After more prolonged incubation both NANE, X_1 and NAH-NADA-I are formed in significant amounts [Fig. 4(B)]. Formation of NAH-NADA-II by means of locust cuticle has not been established with certainty.

The NAH-NADA-I and NAH-NADA-II adducts can also be produced by incubation of NAH and NADA with a centrifuged extract of fifth stage larval cuticle of *H. cecropia*, containing both diphenol-oxidase and *o*-quinone isomerase activities. Incubation of NAH plus NADA with mushroom tyrosinase did not give measurable amounts of the NAH-NADA-I adduct, whereas NAH-NADA-II was produced (data not shown).

The NAH-NADA-I adduct was obtained in mg amounts by preparative chromatography of the *H. cecropia* incubation media on BioBeads SM-16 (Fig. 5). The NAH-NADA-I peak is incompletely separated from the NANE peak, but the two compounds could be well separated by cation-chromatography at pH 3, as shown in Fig. 6.

Extraction of sclerotized cuticle

In a previous publication (Andersen and Roepstorff, 1981) we reported on the presence of a series of benzodioxan-type compounds in sclerotized cuticle, indicating that some cuticles contain an enzyme system catalyzing the formation of covalent links between the two carbon atoms in the NADA side chain and the phenolic groups in other catechols. To search further for compounds with a benzodioxan-type structure, a formic acid extract of sclerotized locust cuticle was fractionated on a BioGel P-2 column, and pooled fractions were refractionated on BioBeads SM-16 and reversed phase HPLC. Peaks of interest were collected and subjected to mass spectrometry as well as to acid hydrolysis followed by analysis of the hydrolysis products. Figure 7 shows a HPLC-run of one of the BioGel P-2 pools, and the insert shows the u.v spectrum of the peak at 27.1 min. The other peaks eluting in the group between 26 and 29 min all have similar u.v. spectra.

The early eluting peaks have been identified as NANE at 8.0 min, dihydroxyphenylketoethanol (DOPKET) at 9.8 min, NADA at 11.3 min, and

(*See facing page.*) Fig. 3. NMR spectra ([D_6]-DMSO) of NAH-NADA-I and NAH-NADA-II. A: ^{1}H-NMR (200 MHz) of NAH-NADA-I (for full assignment of signals see Andersen *et al.*, 1991). B: ^{1}H-NMR (200 MHz) of NAH-NADA-II. Assignments: δ = 9.27 ppm [*d*, J = 1.6 Hz, H-C(2)]; 8.46 [*d*, J = 8.0 Hz, NH-C(7)], 7.99 [*t*, J = 6.0 Hz, NH-C(8′)], 7.56 [*d*, J = 1.6 Hz, H-C(5)], 6.804 [*s*, H-C(5′), 6.801 (narrow *t*, J ~ 1.2 Hz, H-C(2′)], 4.57 [*ddd*, $J_{7,6A}$ = 9.2, $J_{7,NH}$ = 8.0, $J_{7,6B}$ = 4.8 Hz, H-C(7)], 3.18 [*dd*, $J_{6A,6B}$ = 15.2, $J_{6B,7}$ = 4.8 Hz, HA-C(6)], 3.05 *dd*, $J_{6B,6A}$ = 15.2, $J_{6A,7}$ = 9.2 Hz, HB-C(6)], 3.00 [*dt*, $J_{8',7'} \sim J_{8',NH} \sim$ 6 Hz, H-C(8′)], 2.33 [*dt*, $J_{7',8'}$ = 6.5, $J_{7',2'} \sim$ 1.2 Hz, H-C(7′), 1.84 (*s*, $COCH_3$), 1.74 (*s*, $COCH_3$). C: ^{13}C-NMR (50.3 MHz) of NAH-NADA-I (for full assignment of signals see Andersen *et al.*, 1991). D: ^{13}C-NMR (50.3 MHz) of NAH-NADA-II. Assignments: δ = 172.02 ppm (COOH), 169.56 (HNCO), 169.40 (HNCO), 147.38 [C(3′)], 144.39 [C(4′)], 135.78 [C(2)], 130.15, 125.66, 125.01 [C(4,6′, 1′)], 121.78 [C(5)], 116.78, 113.93 [C(2′,5′)], 51.21 [C(7)], 39.52 [C(8′)], 29.87 [C(6)], 26.38 [C(7′)], 22.58 (CH3), 22.36 (CH_3).

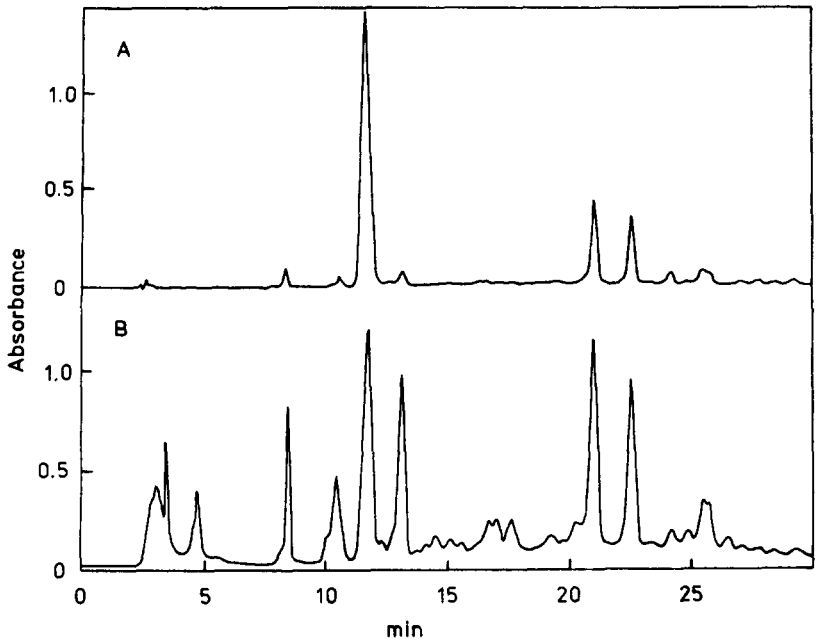


Fig. 4. Reversed phase chromatogram of sample from medium after incubation of 100 mg wet *L. migratoria* femur cuticle in 50 ml 10 mM *N*-acetylhistidine, 10 mM NADA in 0.2 M Na-phosphate, pH 7.0, at 40 °C. Absorbance at 280 nm is recorded. A: after 6 h; B: after 24 h. Peak at 8.4 min: NANE; 10.4 min: NAH-NADA-I; 11.6 min: NADA; 13.2 min: X_1; 21.0 and 22.5 min: benzodioxan-type NADA-dimers. The other peaks are yet unidentified.

N-acetylarterenone at 11.8 min, respectively, and the two peaks at 20.2 and 21.8 min are the benzodioxan-type dimers of NADA.

Each of the peaks at 26.4, 27.1, 28.0, and 28.8 min were collected and subjected to plasma-desorption mass-spectrometry and acid hydrolysis. By mass spectrometry all four compounds gave identical spectra. The spectra (Fig. 8) are dominated by a peak at *m/z* 601 (range 600.9–601.2 in the different spectra), corresponding within the mass accuracy of the method (0.1%) to MNa^+ of a compound containing three interconnected NADA-residues. A minor peak is present at *m/z* 579 (range 578.4–579.2), corresponding to MH^+. As the suggested structure does not contain a natural protonization site, dominance of MNa^+ should be expected. The spectra further contain a number of fragment ions, nominal *m/z* 193, 342, and 386, supporting the interpretation.

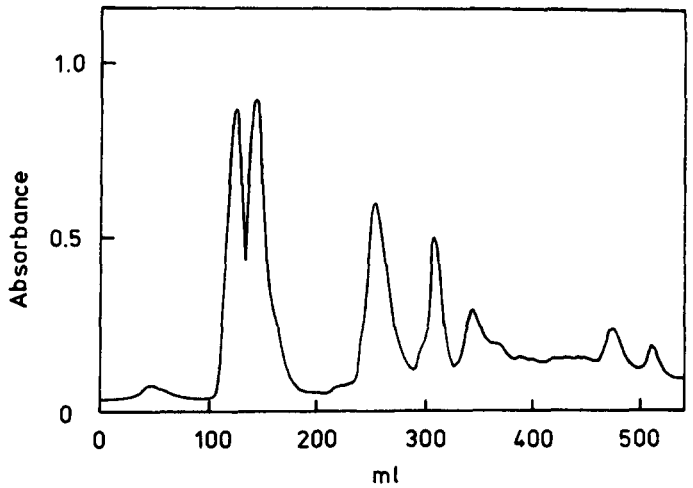


Fig. 5. Fractionation of products obtained by incubation of *H. cecropia* larval cuticle with NADA plus *N*-acetylhistidine. Incubation conditions as in Fig. 1. Separation was achieved on BioBeads SM-16 (1.6 × 15 cm), and elution was by a linear gradient from 0 to 100% methanol in 0.2 M acetic acid. Absorbance at 280 nm was recorded. NANE is eluted at 110–130 ml, NAH-NADA-I is eluted at 130–150 ml, NADA at 240–265 ml, and X_1 at 300–320 ml.

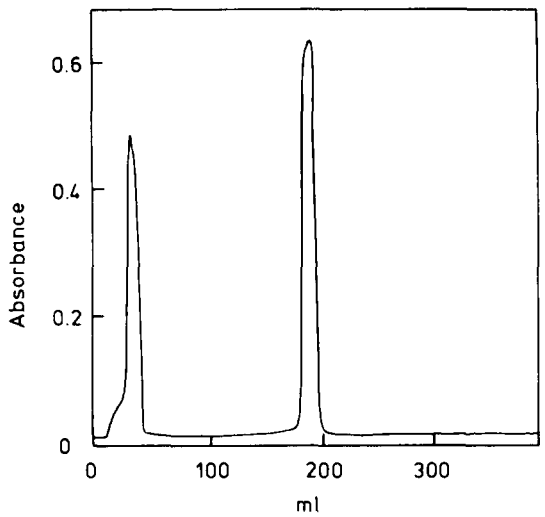


Fig. 6. Purification of NAH-NADA-I from the peak at 130–150 ml in Fig. 5. Fractionation was on a column (1.6 × 15 cm) of SP–Sepharose in equilibrium with 0.2 M acetic acid; elution with a linear gradient from 0 to 0.2 M NaCl in 0.2 M acetic acid. Absorbance at 280 nm was recorded. Peak at 30 ml = NANE; peak at 190 ml = NAH-NADA-I.

Acid hydrolysis gives dopamine and DOPKET with nearly twice as much DOPKET as dopamine (1.93 mol DOPKET per mol dopamine). The observations are in agreement with the trimeric structure (**4**), which has a molecular weight of 577.6 Da and where two NADA-residues, having their side chain involved in a benzodioxan-structure, will give DOPKET on hydrolysis, and the third NADA-residue will give dopamine.

Coupling of NADA and NAH-NADA-I adduct

The presence of small amounts of trimeric NADA derivatives in naturally sclerotized locust cuticle demonstrates that the cuticle is able to add NADA-residues by their side chain to available *o*-diphenolic groups. Accordingly, cuticle should also be able to add NADA-residues to already formed NAH-NADA adducts. To test this hypothesis, locust cuticle was incubated with a solution of NADA and NAH-NADA-I, and the resulting products were analyzed by HPLC (Fig. 9). Besides the usual products two new peaks appeared at 16.0 and 18.2 min, respectively, being more hydrophobic than NAH-NADA-I at 9.7 min and less hydrophobic than the benzodioxan-type NADA-dimers at 21.0 and 22.6 min. The mass spectra (Fig. 10) of the purified compounds were identical within the mass accuracy, indicating two isomeric forms. A peak at m/z 582.8 is in agreement with MH^+ for a compound having structure **5** (MW = 581.3). In this case MH^+ is dominating because the histidyl residue has a natural protonization site.

Samples of the two compounds were hydrolyzed in 1 M HCl, and the products analyzed by HPLC. Two products were obtained from both peaks: an early eluting non-identified catechol derivative and DOPKET. The two products were in nearly equimolar amounts, based on the assumption that the catechol has an u.v. absorption coefficient corresponding to that of dopamine.

Coupling of NADA to the NAH-NADA-II adduct has not been attempted, as the latter compound is obtained in much lower yields than NAH-NADA-I

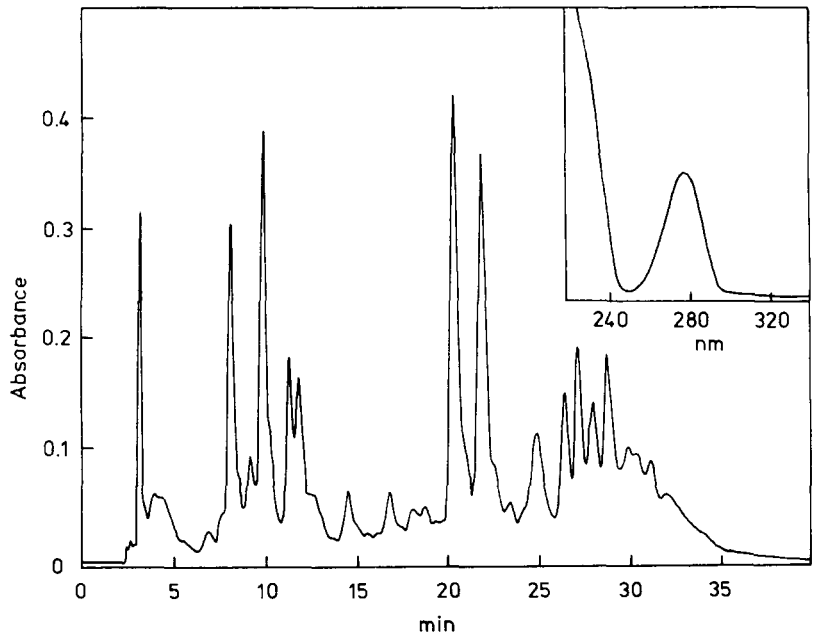
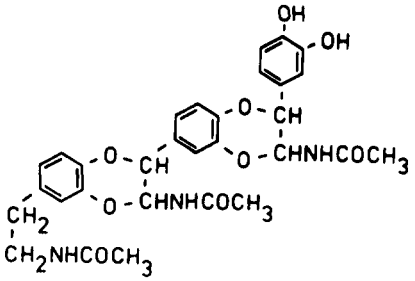


Fig. 7. Sclerotized locust cuticle extracted in anhydrous formic acid, and the extract fractionated on a column of BioGel P-2. Aliquot from peak containing benzodioxan-type NADA-dimers analyzed on reversed phase HPLC. Absorbance at 280 nm is recorded. Peak at 8.1 min: NANE; 11.3 min: NADA; 9.9 and 11.8, min: ketocatechols; 20.2 and 21.8 min: benzodioxan-type NADA-dimers; 26.4, 27.1, 28.0, and 28.8 min: benzodioxan-type NADA-trimers. The insert shows the u.v. spectrum of the peak at 27.1 min.

under the incubation conditions used, and it is therefore difficult to obtain it in sufficient quantities.



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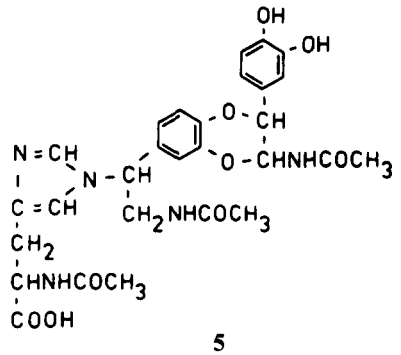

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DISCUSSION

Sclerotization of insect cuticle is a complex process, involving a series of reactive intermediates formed by oxidation of the catechol derivatives NADA and NBAD as outlined in Scheme 1.

The three quinoid structures occurring in the sequence may react with several amino acid residues in the cuticular proteins, and the resulting product pattern will depend upon the rate of generation of the individual quinones as well as on the reactivity and number of the various residues present.

Two coupling products between *N*-acetylhistidine and NADA (NAH-NADA-I and NAH-NADA-II), formed when *H. cecropia* larval cuticle is incubated together with the two compounds have now been identified. The structures of the coupling products (**2** and **3**) support the hypothesis that cuticular enzymes can catalyze formation of both side chain and ring addition products of histidine to NADA via enzymatically generated NADA- *p*-quinone methide and NADA-*o*-quinone, respectively, as reactive intermediates. This result agrees with the observation that the coupling reactions are catalyzed by pieces of intact larval cuticle as well as by cuticular extracts containing phenoloxidase and *o*-quinone-*p*-quinone methide isomerase, whereas mushroom tyrosinase will not catalyze the formation of NAH-NADA-I in measurable amounts, although it catalyzes formation of NAH-NADA-II.

It is interesting that *H. cecropia* larval cuticle gives a higher yield of NAH-NADA-I than of NAH-NADA-II, but both adducts and NANE are formed in significant amounts. In contrast to *H. cecropia* larval cuticle, locust cuticle contains relatively large amounts of an enzyme activity producing 1,2-dehydro-NADA (Andersen, 1989c), presumably by isomerisation of NADA-*p*-quinone methide. Locust cuticle initially gives much lower yields of both NANE and NAH-NADA-I than does *H. cecropia* cuticle, and the main products are benzodioxan-type NADA-dimers formed by reaction between dehydro-NADA-*o*-quinone and NADA. The initial low yields of both NANE and NAH-NADA-I indicate that the major part of the NADA-*p*-quinone methide is enzymatically transformed to dehydro-NADA before a reaction with either water or *N*-acetylhistidine can happen, and that the dehydro-NADA generating enzyme is gradually inactivated during the incubation period, whereby the chances for formation of NANE and NAH-NADA-I will be increased. Formation of NAH-NADA-II in measurable amounts has not yet been observed with locust cuticle.

Dehydro-NADA-*o*-quinone is a highly reactive compound: it reacts spontaneously with *o*-diphenols to give benzodioxan-derivatives (Andersen and Roepstorff, 1982), and it may also react with other

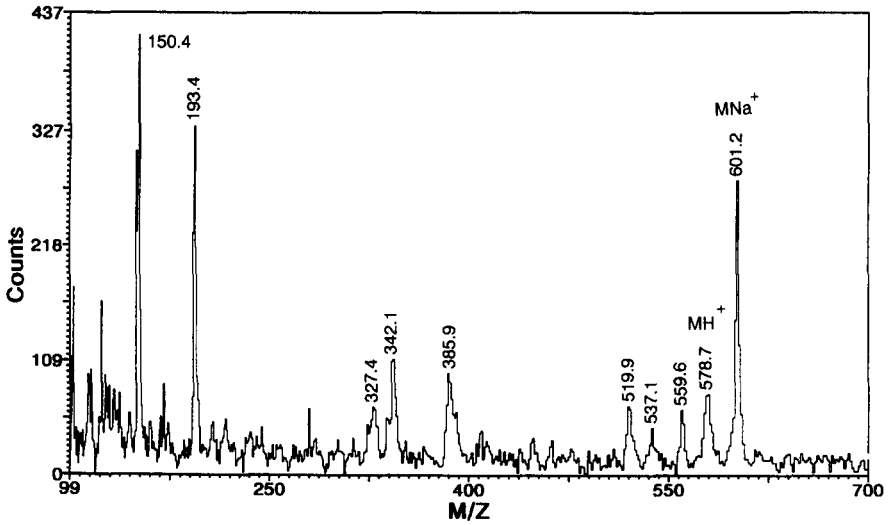

Fig. 8. Plasma desorption mass spectrum of the compound eluted at 27.1 min in Fig. 7.

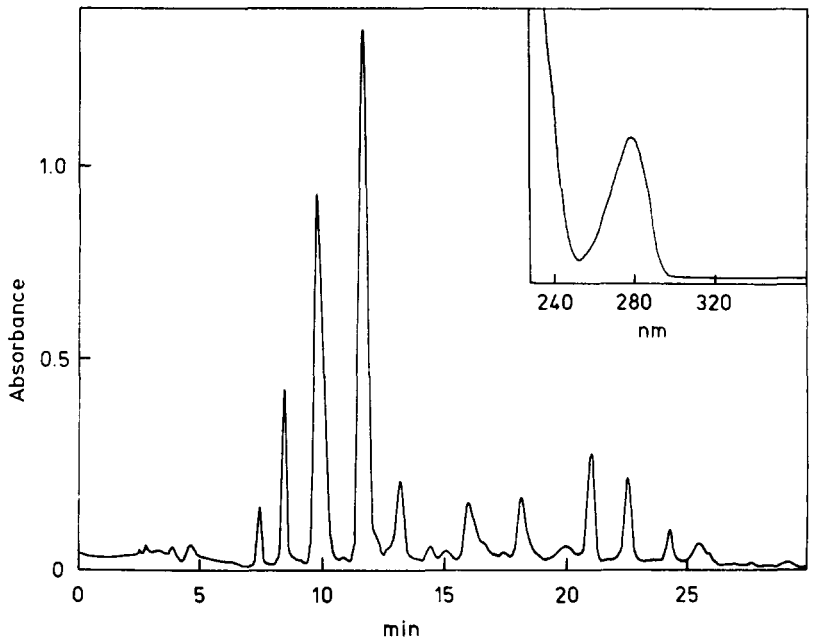

Fig. 9. Reversed phase chromatogram of sample from medium after incubation of 32 mg wet locust femur cuticle in 20 ml 7 mM NAH-NADA-I, 10 mM NADA in 0.2 M Na-acetate, pH 5.6, at 40°C for 18 h. Absorbance at 280 nm is recorded. Peak at 8.3 min: NANE; 9.7 min: NAH-NADA-I; 11.6 min: NADA; 13.2 min: X_1, 16.0 and 18.2 min: adducts between *N*-acetylhistidine and NADA-dimers; 21.0 and 22.6 min: benzodioxan-type NADA-dimers. The insert shows the u.v. spectrum of the peak at 18.2 min.

groups, although this has not yet been established. The results presented here together with earlier results indicate that dehydro-NADA-*o*-quinone will react spontaneously with any available *o*-diphenol to form dimeric, trimeric, and maybe also higher oligomeric NADA-derivatives. Since NADA attached to histidine residues via either its side chain or its aromatic ring still contains an *o*-diphenolic structure, it is to be expected that *in vivo* both types of bound NADA will be starting points for polymerization reactions involving addition of oxidized dehydro-NADA to the *o*-diphenolic groups.

Covalent links between imidazole nitrogen and ring carbon in dopamine derivatives (Schaefer *et al.*, 1987) as well as between imidazole nitrogen and the β-carbon atom (Christensen *et al.*, 1991) have been demonstrated in sclerotized *Manduca sexta* pupal cuticle by means of solid state NMR spectroscopy. The adducts produced *in vitro* between NAH and NADA appear thus to represent structures actually present in sclerotized cuticle.

Both attachment of NADA to histidine residues and NADA-polymerization can be assumed to be part of the natural sclerotization process in insect cuticle,

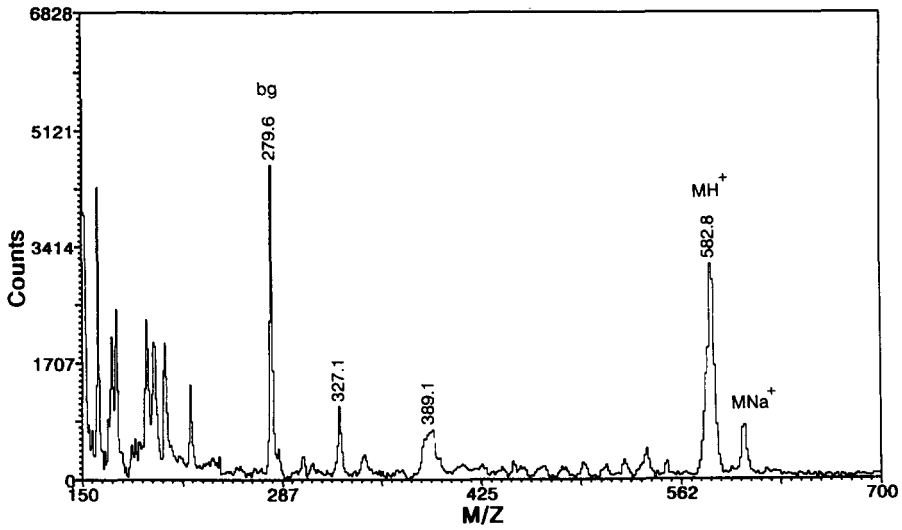

Fig. 10. Plasma desorption mass spectrum of the compound, eluted at 16.0 min in Fig. 8. bg: background peak.

but the process may well be more complicated than outlined. Oxidized derivatives of NADA could also react with free amino groups in lysine and with phenolic groups in tyrosine (Grün and Peter, 1984), giving more points for growth of NADA-oligomers. In this way the cuticular proteins can be highly modified by addition of aromatic groups, increasing the tendency for the peptide chains to adhere to each other. When two protein-bound NADA-oligomers are sufficiently close to each other, covalent cross-links may be formed by oxidative coupling between the oligomers. We have recently reported on cuticle-catalyzed formation of phenolic dimers (Miessner *et al.*, 1991; Andersen *et al.*, 1992), where two substituted diphenols are linked via their aromatic rings, leaving the side chains free to be involved in linkage to other sites, for example to the imidazole ring of histidine. In fact, phenolic polymerization as an important aspect of sclerotization has been emphasized earlier (Pryor, 1940; Andersen, 1977; Peter, 1980). We believe that with respect to the chemistry of sclerotization, much can be learned from the chemistry of other polyphenolic biopolymers (Peter, 1989).

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