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Model Insect Cuticle Sclerotization: Reactions of Catecholamine Quinones with the Nitrogen-Centered Nucleophiles Imidazole and *N*-Acetylhistidine

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The catecholamines *N*-acetyldopamine (NADA) and *N*-β-alanyldopamine (NBAD) are two precursors for quinonoios used as sclerotizing agents in insect cuticle. This study focused on the reaction pathways of the quinones of NADA and NBAD by using two nitrogen-centered nucleophiles, imidazole and *N*-acetylhistidine, to model cuticular proteins containing histidyl residues. The quinones were prepared by electrochemical oxidation, using either a coulometric microcell or a flow-through cell. The reactions of the quinones with the nucleophiles were investigated at physiological pH using electrochemical, chromatographic, and spectroscopic methods. The major products were purified by semipreparative liquid chromatography and identified by mass spectrometry and nuclear magnetic resonance spectroscopy to be nucleophilic addition products of the quinones with the nucleophiles bonded to two carbons in the aromatic ring. The predominant products for both nucleophiles were C6 adducts of NADA and NBAD. C2 adducts of *N*-acetylhistidine were minor products. © 1997 Academic Press

INTRODUCTION

Insect cuticle sclerotization or hardening is a vital process required for the survival of insects (*1–4*). During sclerotization, the chemical and mechanical properties and the appearance of the cuticle are changed: the water content decreases, the matrix proteins become much more resistant to extraction and degradation, and the cuticle becomes stiff and hard. This process has been under extensive study for several decades and especially so in recent years. In a recent model for the insect sclerotization process, catechols are first oxidized to their corresponding electrophilic *o*-quinones by cuticular phenoloxidases (*1*, *5*). The *o*-quinones are subsequently isomerized, first to *p*-quinone methides by quinone isomerases and then to α,β-dehydrocatechols by quinone methide isomerases. The α,β-dehydrocatechols are then oxidized to form the corresponding dehydrocatechol *o*-quinones, which are subsequently isomerized into *p*-quinone methides. All these quinonoid intermedi-

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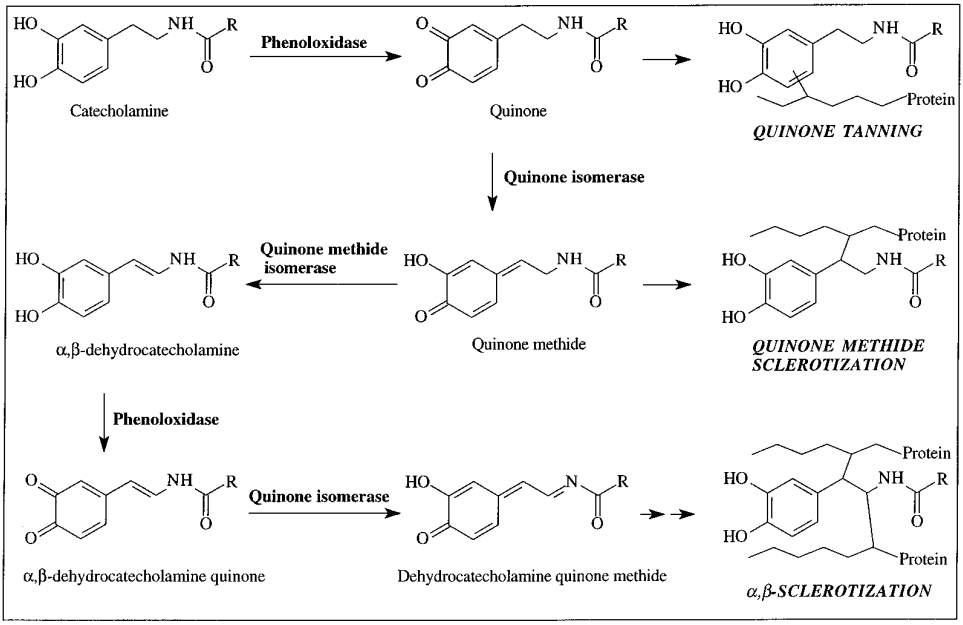
ates can act as sclerotizing agents and react directly with nucleophilic groups of cuticular proteins to form covalent bonds. The three different pathways involving reactions of nucleophiles with quinones, quinone methides, and α,β-dehydrocatechols are denoted as quinone tanning, quinone methide sclerotization, and α,β-sclerotization, respectively (*1*, *6*, *7*). The protein-bonded catechols, then, might be oxidized again and form another round of sclerotizing agents, which can react with nucleophilic groups in other protein molecules. This second addition results in covalent cross-linking between polypeptide chains via aromatic or side-chain carbons of catechols. In addition to these covalent cross-links, replacement of water in cuticle with catechols or their polymeric oxidation products and formation of numerous noncovalent interactions might also further stabilize the protein–chitin matrix of the cuticle (*1*, *8*).

Catechols that act as immediate precursors for quinonoid-sclerotizing agents in insect cuticle are primarily *N*-acyl derivatives of dopamine, such as *N*-acetyldopamine (NADA)[2] and *N*-β-alanyldopamine (NBAD) (*1*). Although other catechols, for example, 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylacetic acid, *N*-acetylnorepinephrine, and *N*-β-alanylnorepinephrine, have also been detected in extracts of insect cuticles, their roles in sclerotization are less certain (*2*). Until now, convincing evidence for precursor roles was available only for NADA and NBAD. NADA was found to be present in several insect species in 1962 and has been demonstrated to be incorporated into cuticle during the sclerotization process (*9*, *10*). In 1982, NBAD was discovered to be a major precursor for tanning agents in stiff brown cuticles (*11*, *12*). Although the involvement of NADA and NBAD in sclerotization has been established, the mechanisms (Scheme 1) by which these compounds serve as precursors for quinonoios used as sclerotizing agents have not been fully elucidated and some aspects remain controversial.

The nucleophilic groups of cuticular proteins that react with the quinonoid agents have been suggested to be α-amino groups, ε-amino groups of lysyl residues, and imidazole nitrogens of histidyl residues (*1*, *13*). Other nucleophilic groups of cuticular proteins, such as thiol, hydroxyl, and carboxyl groups, might participate in cross-linking as well, but their involvement is probably minor (*1*). Direct evidence for the existence of covalent bonds between catecholamines and nucleophilic groups in cuticular proteins had not been obtained until solid-state nuclear magnetic resonance (NMR) was used to examine the C–N covalent linkages in the pupal cuticle of the tobacco hornworm (*14–17*). It was determined by double cross-polarization magic-angle-spinning ^{13}C NMR that the histidyl nitrogens of cuticular protein were attached to aromatic ring carbons of a catecholamine in pupal exuviae prepared from *Manduca sexta* larvae injected with both [1,3-$^{15}N_2$]histidine and [ring-$^{13}C_6$]dopamine

[2] Abbreviations used: NADA, *N*-acetyldopamine; NBAD, *N*-β-alanyldopamine; NMR, nuclear magnetic resonance spectroscopy; NAcH, *N*-acetylhistidine; DA, dopamine; DMF, *N*,*N*-dimethylformamide; MS, mass spectrometry; LC, liquid chromatography; MALDI-MS, matrix-assisted laser desorption mass spectrometry; EC, electrochemical detection; 6-Imid-NADA, 6-(*N*-imidazole)-*N*-acetyldopamine; 6-Imid-NBAD, 6-(*N*-imidazole)-*N*-β-alanyldopamine; HMQC, heteronuclear multiple quantum coherence; TOCSY, total correlation spectroscopy; 6-NAcH-NADA, 6-(*N*-acetylhistidyl)-*N*-acetyldopamine; 2-NAcH-NADA, 2-(*N*-acetylhistidyl)-*N*-acetyldopamine; 6-NAcH-NBAD, 6-(*N*-acetylhistidyl)-*N*-β-alanyldopamine; 2-NAcH-NBAD; 2-(*N*-acetylhistidyl)-*N*-β-alanyldopamine.



SCHEME 1

(*14*). In addition, a linkage between a histidyl nitrogen and the β-carbon in the side-chain of catechols in the pupal exuviae from larvae that had been injected with both [1,3-$^{15}N_2$]histidine and [β-$^{13}C_6$]dopamine was found by rotational echo double resonance ^{13}C NMR (*15*). The results of these solid-state NMR studies are consistent with a sclerotization mechanism in which quinones and quinone methides derived from *N*-acylcatecholamines form covalent bonds with functional groups of cuticular proteins. However, the exact structures of the histidyl-catechol ring or side-chain carbon addition adducts could not be determined by the solid-state NMR studies.

In vitro evidence for cuticle-catalyzed formation of adducts between NADA and amino acid derivatives was obtained from the analysis of products derived from their incubation with cuticle or cuticular enzymes (*18–21*). The structures of a few of these model adducts have also been determined. Recently, four histidyldopamine adducts were isolated and characterized from an acid hydrolysate of *M. sexta* pupal cuticle (*22*). These adducts apparently are formed from Michael 1,4- and 1,6-addition reactions with quinonoios used as sclerotizing agents. Thus, the imidazole side chain of histidine residues in cuticular proteins are nucleophiles critical for sclerotization.

Although knowledge about the chemistry of cuticular sclerotization has increased substantially in recent years, many aspects are still unclear. For example, the identities of most quinonoios used as sclerotizing agents and products have not been directly established and the kinetics of the reactions between the sclerotizing agents and functional groups of proteins have not been determined. The objective of this

study was to elucidate the reaction pathways of the quinones of the catecholamines NADA and NBAD, which are immediate precursors for sclerotizing agents in insect cuticle, by using amino acid derivatives to model the functional groups in cuticular protein. Two nitrogen-centered nucleophiles, imidazole and *N*-acetylhistidine (NAcH), were employed.

EXPERIMENTAL

Syntheses of Catecholamines

Although some NADA was obtained commercially (Sigma Chemical Co., St. Louis, MO),[3] most was synthesized from dopamine (DA) using a procedure modified from that of Andersen (*23*). First, 0.80 g DA hydrochloride (Sigma Chemical Co.) was dissolved in 40 ml of 10% potassium tetraborate that had been purged with nitrogen. Then, 0.44 ml of 99% acetic anhydride (Fisher Scientific Co., Pittsburgh, PA) was slowly added over a period of 30 min to the DA hydrochloride solution under a nitrogen atmosphere. To verify the completeness of the acetylation reaction, 1-μl aliquots of the product mixture were subjected to thin-layer chromatography on silica gel sheets (Eastman Kodak Co., Rochester, NY) using methanol as the developing solvent. The retention factors of DA and NADA were 0.40 and 0.83, respectively. After all DA had reacted, 2 ml of concentrated formic acid (Fisher Scientific Co., Pittsburgh, PA) were added to the product mixture to precipitate the boric acid. After filtration through Whatman No. 1 paper, the supernatant was subjected to gel filtration chromatography on a column packed with Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, CA) using 0.02% formic acid as the mobile phase. The effluent was monitored spectroscopically at a wavelength of 280 nm. Fractions were collected and analyzed by analytical liquid chromatography (LC) for the presence of NADA. Those fractions showing only NADA were pooled and lyophilized. NADA was obtained as a white powder in 80% overall yield. Mass spectrometry (MS), NMR, and LC analyses confirmed that the desired product had been prepared.

NBAD was synthesized from DA using a method modified from Kramer *et al.* (*24*) and Yamasaki *et al.* (*25*). First, 0.96 g DA hydrochloride was dissolved in 17.5 ml *N*,*N*-dimethylformamide (DMF) (Fisher Scientific Co., Pittsburgh, PA) that had been deaerated with nitrogen and predried with molecular sieves (size: 5 Å). The molecular sieves had been activated by heating at 600°C for a period of 24 h in the stream of nitrogen. *N*-Methylmorpholine (0.55 ml) (Sigma Chemical Co.) and *N*-*t*-butyloxycarbo-β-alanine-*N*-hydroxysuccinimide ester (17.2 g) (Chemalog, Chemical Dynamics Co., NJ) were dissolved in a second 17.5-ml volume of DMF. The two DMF solutions were combined and stirred for 48 h under a nitrogen atmosphere in an ice water bath. 1-(2-Aminoethyl)piperazine (0.26 ml) (Aldrich Chemical Co., St. Louis, MO) was then added to the product mixture in an ice water bath, after which the solution was allowed to warm to room temperature.

[3] Mention of a proprietary product does not constitute a recommendation by the USDA.

Another 0.52 ml of 1-(2-aminoethyl)piperazine was added to the mixture, which was then stirred for another hour. The product mixture was acidified with 100 ml of 0.25 M H_2SO_4 and extracted with 100 ml of ethyl acetate. Extraction of the aqueous layer with 30 ml of ethyl acetate was repeated three times. The extracts were subsequently pooled, washed with saturated NaCl solution three times, and dried over sodium sulfate under nitrogen overnight. The ethyl acetate extract was then filtered through Whatman paper and reduced to a volume of about 10 ml using a rotary evaporator, at which time a white solid formed. The suspension was filtered and the solid was collected and allowed to dry under vacuum for about 4 h. The dried solid was dissolved in 4 ml of a HCl : 1,4-dioxane (1 : 2) solution and left at room temperature for 3 h. The solution was reduced to a small volume using a rotary evaporator. Diethyl ether was added to the concentrated solution in sufficient volume to ensure precipitation of NBAD · HCl, which was collected by filtration, washed with diethyl ether, and dried under vacuum overnight. The yield was 68% (0.90 g). MS and NMR analyses confirmed that the desired product had been prepared.

Preparation of Catecholamine Quinones

Electrochemical oxidation was used to prepare the quinones. If chemical oxidation were to be used to prepare quinones, excess oxidizing agents would be generally employed to ensure complete oxidation of the catecholamines. However, if quinones thus obtained were to react with nucleophiles directly and form adducts, then the presence of the excess oxidizing agent might cause complications by oxidizing the adducts. In contrast, the electrochemical oxidation method results in the preparation of quinones that are devoid of excess oxidizing agent.

Since the quinones are relatively stable at low pH, the electrosynthesis of the quinones was conducted at pH 2.0 in the absence of nucleophiles. The supporting electrolyte was 0.01 N HCl and 0.09 N KCl. The potential for oxidation of NADA or NBAD was controlled at 0.7 V vs Ag/AgCl (saturated KCl). A custom-made, three-electrode coulometer (*26*) was used to apply a constant potential for electrolysis and to give instantaneous digital coulomb and analog current readouts. A Hewlett–Packard (Palo Alto, CA) 3390A integrator was used to monitor the electrolysis current. Either a coulometric microcell or a coulometric flow-through cell was used for the oxidation.

Coulometric microcell. A coulometric microcell was designed to prepare small quantities of quinones (Fig. 1) (*27*). Volumes of catecholamine solutions used for each batch preparation ranged from 0.4 to 1.5 ml. The large surface area to volume ratio of the working electrode and efficient mixing of the solutions permit rapid and quantitative coulometric conversion. Separation of the auxiliary and working electrodes by the membrane filter prevents mixing of the anode and cathode products, thereby permitting complete electrolysis. The close proximity of the reference electrode to the working electrode minimizes uncompensated iR drop and allows accurate control of the potential under high-current conditions. In addition, the small volume of the sample compartment and short electrolysis time make the cell suitable for use when starting materials are available in

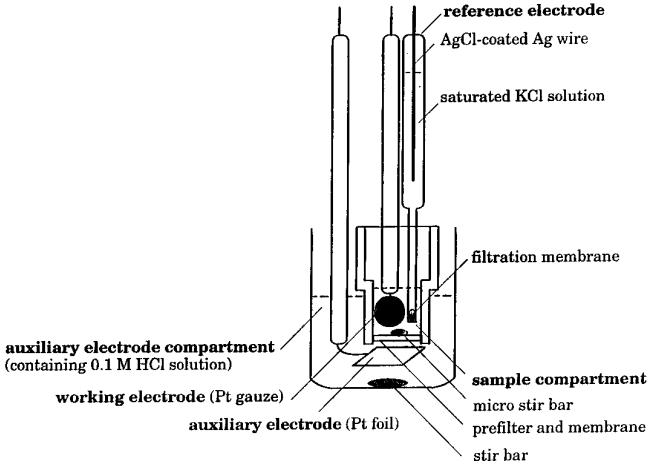


FIG. 1. Diagram of the microelectrochemical cell. The sample compartment is a polypropylene tube containing a prefilter and a 0.2-μm-pore size nylon 66 membrane at the tip. The working electrode is a piece of platinum gauze (20 $\times$ 50 mm) that was folded and shaped into a small sphere (diameter, ~5 mm). The reference electrode is a AgCl-coated silver wire placed inside a glass tube containing a saturated KCl solution; a 0.2-μm-pore size filtration membrane was used as the divider and sealed inside the tube tip. The auxiliary electrode is a platinum foil (5 $\times$ 10 mm). The stir bars in the sample compartment and the auxiliary electrode compartment are 5 and 20 mm, respectively.

limited quantity and/or when relatively reactive compounds are to be synthesized. This microcell was used for the electrosyntheses of reactive quinones that were immediately used for kinetics studies and subsequent reactions with nucleophiles.

Flow-through coulometric cell. To synthesize sufficient quantities of catecholamine–nucleophile adducts for structural analysis and characterization, relatively large-scale electrosyntheses of catecholamine quinones were required. Since catecholamine quinones are reactive, batch preparation is not desirable when a long electrolysis time is required. Hence, a flow-through coulometric cell was constructed that permits electrolysis of a relatively large amount of catecholamine and allows the quinone to react with a nucleophile shortly after formation of the quinone (Fig. 2) (*27*). Catecholamine solutions were introduced from a sample reservoir by gravity flow into the sample compartment and were electrolyzed to form quinones at a controlled potential (0.8 V). The quinone effluent was added to a stirred solution of the nucleophile to form adducts at pH 7.0. In this flow-through cell, rapid and efficient coulometric conversion was achieved with a large ratio of working electrode surface area to solution volume, a concentric arrangement of the working and the auxiliary electrodes, and a nylon membrane that separated the working and the auxiliary electrode compartments. Both a low uncompensated iR drop and accurate potential control were the result of the close proximity of the tip of the reference electrode to the working electrode. The cell design allowed the catecholamine quinones to react with nucleophiles

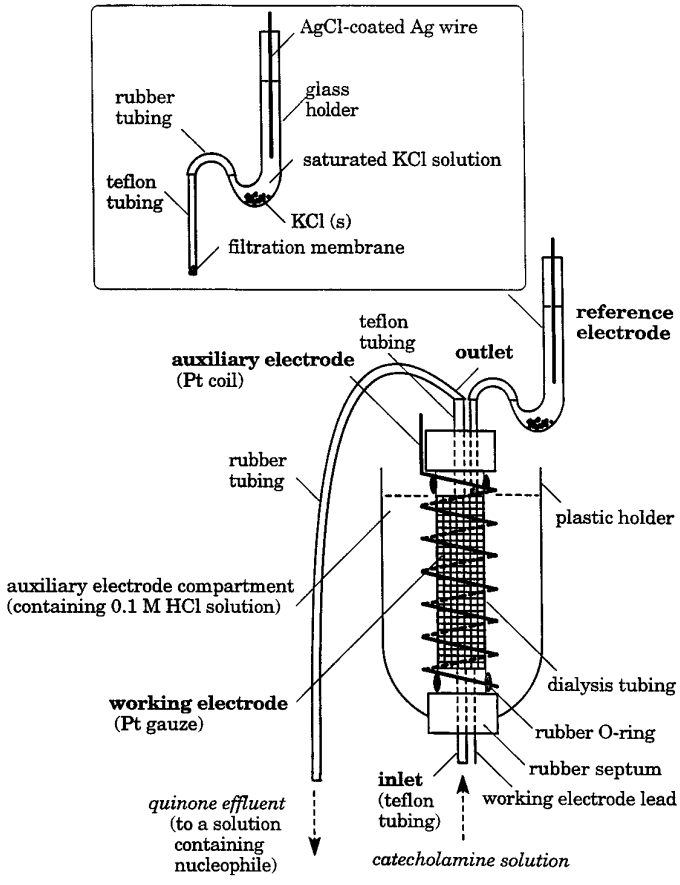


FIG. 2. Diagram of the flow-through electrochemical cell. The working electrode is a platinum gauze that was tightly folded and placed in the cellulose dialysis tubing. Rubber septa were inserted into both ends of the dialysis tubing. A tight seal between the dialysis tubing and the rubber septa was attained by addition of rubber o-rings that encircled the ends of the dialysis tubing. The Teflon tubing tip of the reference electrode was inserted into the dialysis tubing and positioned close to the upper portion of working electrode. The auxiliary electrode encircled the dialysis tubing. The auxiliary electrode compartment contained about 4.5 ml of 0.1 M HCl solution. Solutions were introduced by gravity flow into the dialysis tubing. The direction of fluid flow was from the bottom to the top of the cell. The dead volume of the sample compartment in the dialysis tubing was about 0.3 ml.

outside of the cell almost immediately after their formation. This minimizes quinone decay, which might occur during long-term electrolysis of a large quantity of catecholamines in a batch cell. It also precludes electrochemical oxidation of adducts that would have occurred if the catecholamine were electrolyzed in the presence of nucleophiles. This cell is efficient for relatively large-scale electrochemical syntheses and is especially useful when the electrogenerated intermediates are highly reactive and must be mixed with other substrates shortly after their formation to produce the desired products.

Syntheses of Catecholamine Adducts

Catecholamine adducts were synthesized at pH 7 by adding the effluent from the flow-through coulometric cell, which contained NADA or NBAD quinone at pH 2, into a solution of a nucleophile (*vide supra*). Purification of adducts formed in the reaction mixtures was carried out using either gel filtration or semipreparative LC.

Gel filtration of the product mixtures from the reactions of quinones and imidazole was performed using a column packed with Bio-Gel P-2 (Bio-Rad Laboratories). The mobile phase used was 0.05% formic acid. The effluent containing an adduct in dilute formic acid was lyophilized to yield the adduct as a white solid.

Purification using semipreparative LC was performed as described previously (*28*). A binary mobile phase system was used that consisted of solvents A and B. Solvent A was 150 mM formic acid and 30 mM ammonium formate (pH 3.0), whereas solvent B was 50% methanol (uv cutoff: 204 nm), 180 mM formic acid, and 8 mM ammonium formate (pH 3.0). For adducts of NBAD quinone with NAcH, the gradient employed was 0–15 min, 100% solvent A; and 15–30 min, linear gradient from 0 to 50% solvent B.

Analytical Liquid Chromatography

Analytical LC was conducted to analyze the compositions of product mixtures. The liquid chromatography system and the uv/vis and electrochemical detectors were the same as those used previously (*28*). Separation was achieved on a Microsorb-MV C18 stainless steel column (5 μm, 4.6 $\times$ 250 mm) (Rainin Instrument Co., Inc., Woburn, MA) using a flow rate of 1 ml/min. As was reported previously, during LC analysis of quinones, stainless steel in the columns, including the inlet and outlet frits, causes on-column reduction of quinones, which might lead to misidentification of chromatographic peaks (*29*). In the current LC studies of product mixtures from reactions of quinones with nucleophiles, samples were injected into columns after the quinones had been completely consumed by nucleophiles. Thus, on-column reduction of quinones was not a concern.

The binary mobile phase system, which consisted of solvents A and B, was used (see the preceding section). For the product mixtures produced from NADA quinone reacting with imidazole, the gradient was 0–15 min, 80% solvent A and 20% solvent B; and 15–30 min, linear gradient from 20 to 80% solvent B. For the product mixtures from the reaction of NBAD quinone with imidazole, the gradient was 0 min, 90% solvent A and 10% solvent B; 0–15 min, linear gradient from 10 to 14% solvent B; 15–20 min, linear gradient from 14 to 25% solvent B; and 20–25 min, linear gradient from 25 to 40% solvent B. For the product mixtures from NBAD quinone reacting with NAcH, the gradient was 0 min, 100% solvent A; 0–20 min, linear gradient from 0 to 20% solvent B; and 20–30 min, linear gradient from 20 to 30% solvent B.

UV/Vis Spectroscopy

In addition to on-line uv/vis detection conducted during analytical LC, uv/vis spectra of adducts in 0.01 M HCl solution and uv/vis spectral changes associated with reactions of NADA quinone or NBAD quinone with various nucleophiles

and/or in various buffers were recorded in a 1.0-cm quartz cuvette using a Hewlett–Packard (Palo Alto, CA) HP 8452A diode array spectrophotometer. For studies of reactions of quinones with nucleophiles, once electrolysis of varying concentrations of NADA or NBAD in 0.01 M HCl and 0.09 M KCl was completed, 0.195 ml of electrogenerated NADA quinone or NBAD quinone was added to 0.455 ml of either a nucleophile solution or a buffer in the quartz cuvette to give 0.650 ml of a 3 : 10 diluted quinone solution at the desired nucleophile or buffer concentration and pH. The uv/vis measurements within the wavelength range of 200 to 800 nm were initiated 15 s after mixing and conducted for 10 min at 15-s intervals. The cell temperature was controlled at 25°C by using a temperature-regulated circulating water bath.

The spectral data were used to estimate the initial rate constants for the reaction of NADA quinone or NBAD quinone with imidazole or *N*-acetylhistidine. To do this, the absorbance changes at 396 nm with time were extracted from each set of spectra. To compensate for instrument drift from one spectrum to the next, the absorbance at 396 nm was corrected by subtracting the absorbance at 700 nm, where there is no absorption from reactants or products. Regression analyses of log(corrected absorbance at 396 nm) vs time were performed to obtain the pseudo-first-order rate constants (Table 1). For the reaction between a quinone and nucleophile, the reaction rate law is expected to be

$$\text{reaction rate} = k[\text{quinone}][\text{nucleophile}] = k'[\text{quinone}]. \qquad [1]$$

Cyclic Voltammetry, Mass Spectrometry, and Nuclear Magnetic Resonance Spectroscopy

The cyclic voltammetry and NMR systems and conditions used were the same as described previously (*28*). Electrospray MS of adducts was carried out either at the National Institute of Environmental Health Sciences (Research Triangle Park, NC) or at the University of Kansas (Lawrence, KS). A few adducts were characterized by matrix-assisted laser desorption mass spectrometry (MALDI-MS) at Kansas State University.

RESULTS

Spectroscopic Studies of Reactions of N-Acetyldopamine and N-β-Alanyldopamine Quinones with Imidazole

The uv/vis spectroscopic studies of the kinetics of the reaction of NADA and NBAD quinones with imidazole were performed at various pH values and molar ratios. In all cases studied, the analytical concentration of imidazole was at least 10 times greater than that of the quinone, such that the kinetics behavior of the quinones was investigated under pseudo-first-order conditions.

The uv/vis spectra that were recorded for 0.3 mM NADA quinone in 200 mM

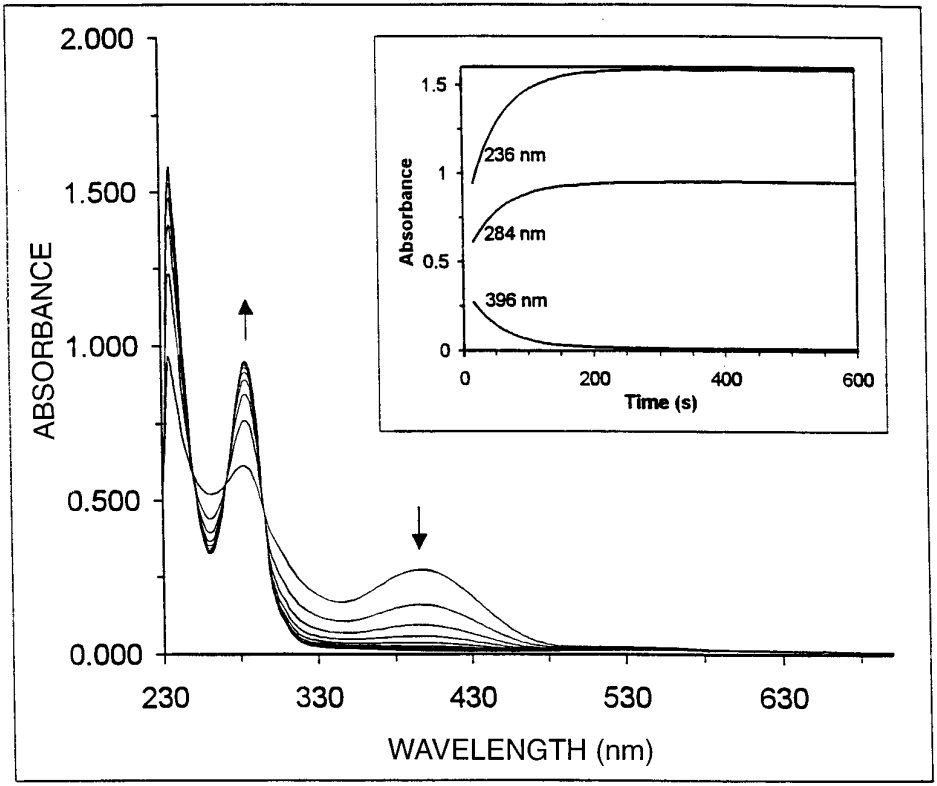


FIG. 3. Spectral changes associated with the reaction of 0.3 mM NADA quinone with 200 mM imidazole at pH 7.0. Although spectral measurements were conducted at 15-s intervals from 15 to 600 s after mixing of NADA quinone and imidazole solutions, for clarity only every other spectrum is shown here. (Inset) Absorbances at 236, 284, and 396 nm vs time.

imidazole at pH 7.0 are shown in Fig. 3, with three absorbance–time curves appearing as an inset. The absorbance at the λ_{max} of the quinone, 396 nm, decreases with time, indicating consumption of the quinone, whereas the absorbances at 236 and 284 nm increase, indicating the formation of a new product(s). The major product was determined to be an adduct of the corresponding catecholamine quinone with imidazole (*vide infra*). Similar changes in absorbances were also observed for quinones of various concentrations in the presence of various concentrations of imidazole and at different pH values. Comparable results were obtained for the kinetics behavior of NBAD quinone in the presence of imidazole (data not shown).

A broad absorption from ca. 300 to 600 nm is observed in the uv/vis spectra of some NBAD quinone reaction systems, such as in the case of 0.3 mM NBAD quinone in the presence of 50 mM imidazole at pH 7 (Fig. 4). This result indicates that another product(s) is forming in addition to the adduct of NBAD and imidazole. When the ratio of imidazole to NBAD quinone decreases or when the pH value

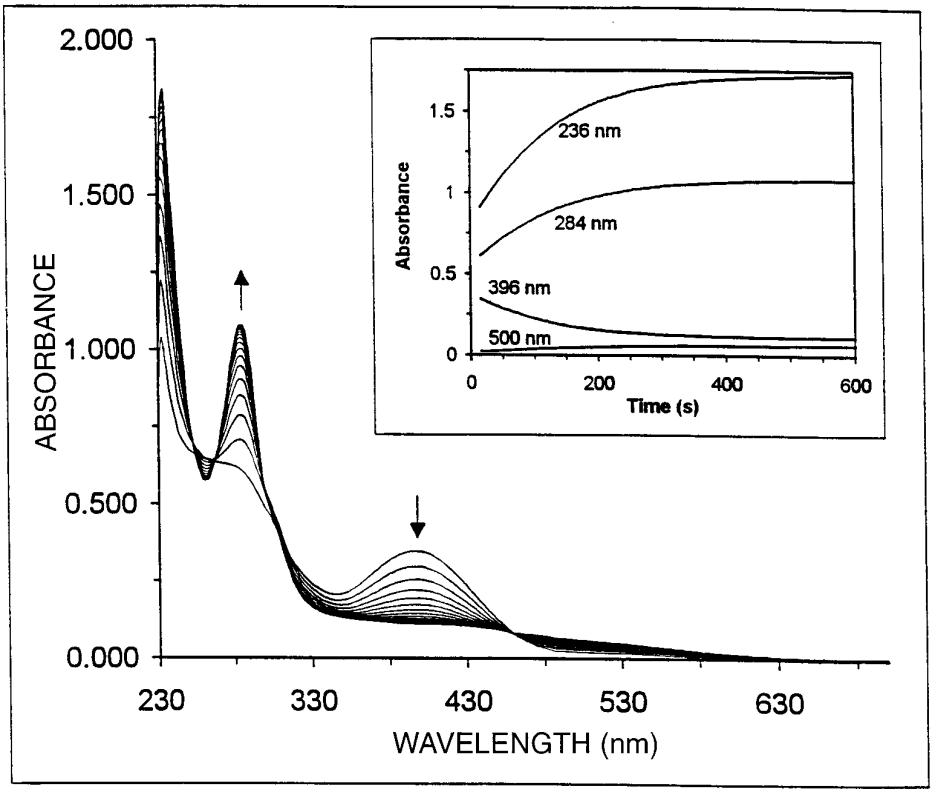


FIG. 4. Spectral changes associated with appearance of an unidentified species during the decay of 0.3 mM NBAD quinone in the presence of 50 mM imidazole at pH 7.0. Although spectral measurements were conducted at 15-s intervals from 15 to 600 s after mixing of NBAD quinone and imidazole solutions, for clarity only every other spectrum is shown here. The unidentified species has a broad absorption from ca. 300 to 600 nm. (Inset) Absorbances at 236, 284, 396, and 500 nm vs time.

increases, formation of the minor product becomes more evident (data not shown). A similar phenomenon also occurs for both NADA and NBAD quinones in buffer solutions in the absence of imidazole (unpublished data). Apparently, this phenomenon is due to a side reaction(s) of the quinones, which competes with the addition reaction of imidazole.

The effects of quinone concentrations, imidazole concentration, and pH on rate constants for the quinone reactions were examined and the expected rate law (Eq. [1]) was confirmed. First, the pseudo-first-order rate constants for reactions of 0.15, 0.3, 0.6, and 0.9 mM NADA quinone (Table 1) or NBAD quinone with 50 mM imidazole at pH 7.0 were found to be independent of the quinone concentration. Second, the pseudo-first-order rate constants for reactions of 0.3 mM NADA quinone (Table 1) or NBAD quinone with 20, 50, 100, and 200 mM imidazole at pH 7.0 showed the expected linear dependence on the concentration of imidazole.

TABLE 1
Rate Constants for NADA Quinone/Imidazole System

No.	[NADA quinone] (mM)	C_{Imid} (mM)[a]	pH	$[Imid]_{eq}$ (mM)[b]	Pseudo-first-order rate const.[c] (s^{-1}) × 10^3	Second-order rate const.[d] (M^{-1} s^{-1}) × 10^2
1	0.15	50	7.0	25	2.1	8.4
2	0.30	50	7.0	25	2.3	9.2
3	0.60	50	7.0	25	2.2	8.8
4	0.90	50	7.0	25	2.8	11
5	0.30	20	7.0	10	1.1	11
6	0.30	100	7.0	50	4.1	8.2
7	0.30	200	7.0	100	7.0	7.0
8	0.30	50	6.1	5.6	0.5	9.3
9	0.30	50	8.0	45.5	3.7	8.1

[a] C_{imid} is the analytical concentration of imidazole, i.e., the sum of the equilibrium concentrations of imidazole and its conjugate acid, imidazolium ion.

[b] $[Imid]_{eq}$, the equilibrium concentration of imidazole, is calculated using the corresponding analytical concentration and the pK_a of imidazolium ion, which is 7.0.

[c] The pseudo-first-order rate constants were obtained from slopes of plots for regression analyses of log(absorbance at 396 nm) vs time.

[d] The second-order rate constants were obtained by dividing the corresponding pseudo-first-order rate constants by the equilibrium concentrations of imidazole at the specified pH.

Third, the results obtained for reactions of 0.3 mM NADA quinone (Table 1) or NBAD quinone with 50 mM imidazole at pH 6.1, 7.0, and 8.0 show that pseudo-first-order rate constants increase with pH in this pH range. This is expected, since the fraction of imidazole that is not protonated increases with increasing pH. The pK_a of imidazolium ion is 7.0. At each pH, the equilibrium concentration of imidazole, i.e., the effective concentration of imidazole as a nucleophile was calculated from the corresponding analytical concentration of imidazole and the pK_a of imidazolium ion (Table 1). From the equilibrium concentration of imidazole and the pseudo-first-order rate constant (k'), the second-order rate constant for reaction of the quinone with the imidazole (k) was obtained by using $k = k'/[\text{Imidazole}]$ (Table 1). The average second-order rate constants are 9.0×10^{-2} M^{-1} s^{-1} for the reaction of NADA quinone with imidazole and 7.4×10^{-2} M^{-1} s^{-1} for the reaction of NBAD quinone with imidazole.

Liquid Chromatographic Studies of Product Mixtures from Reactions of N-Acetyldopamine and N-β-Alanyldopamine Quinones with Imidazole

The compositions of the product mixtures obtained from mixing 90 μl of 1 mM NADA quinone or NBAD quinone with 225 μl of 40 or 400 mM imidazole (1 : 100 or 1 : 1000 molar ratio) at pH 7.0 were analyzed by LC with uv/vis and electrochemical detection (EC) under different gradient conditions. LC-EC (reduction) chromatograms for product mixtures showed no discernible peaks (data not shown), which indicates that the quinone had been consumed completely. In the corresponding