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Electrochemical Oxidation of *N*-Acyldopamines and Regioselective Reactions of Their Quinones with *N*-Acetylcysteine and Thiourea[1]

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The metabolism of catechols often involves their oxidation to quinones and subsequent nucleophilic addition reactions with sulfur-containing compounds. Adducts formed during these reactions may play important roles in many biological systems. We have studied the electrochemical oxidation of *N*-acetyldopamine (NADA) and *N*-β-alanyldopamine (NBAD) in the presence of two sulfur-centered nucleophiles, *N*-acetylcysteine (NACySH) and thiourea (TU), and have characterized the adducts and reaction pathways. NADA and NBAD react similarly, but their adducts with NACySH and TU were formed regioselectively. NACySH yields mainly 5-adducts and TU only 6-adducts. The NACySH adducts are oxidized more easily than the parent *N*-acyldopamine, and their oxidations are chemically reversible. However, the TU adducts are more difficult to oxidize, and their oxidation products undergo further chemical reactions. An intramolecular base catalysis mechanism for adduct formation with NACySH is proposed, which facilitates removal of the proton from the sulfhydryl group of NACySH and directs formation of the 5-adduct via a 1,6-Michael addition reaction. The absence of a proton on the thioureylene sulfur atom leads to formation of the 6-thioureylene adduct via a 1,4-Michael addition reaction of TU. This mechanism is consistent with the formation of other sulfur-centered adducts of catechols previously reported in the literature.

Key Words: **catecholamine; *N*-acetyldopamine; *N*-β-alanyldopamine; *o*-quinone; adduct; thiol conjugate; *N*-acetylcysteine; thiourea; oxidation; nucleophilic addition; regioselectivity; cyclic voltammetry; electrochemistry; melanin; tumor; Parkinson's disease; insect cuticle.**

Catechols and their quinone derivatives are abundant in nature and play important roles in many biological systems. Interest in the reactions of sulfur-containing nucleophilic compounds with catechol-derived quinones has increased since several cysteinyl derivatives of catecholamines were detected in neural tissues. The 5-*S*-cysteinyl adduct of dopamine (DA),[3] 5-*S*-cysteinylDA, was first identified in dopaminergic regions of mammalian brain in 1985 (1). Because of its possible significance as an index of oxidative stress in the aging process and in neurodegenerative disorders, such as Parkinson's disease, this DA metabolite has attracted substantial attention (1–5). Recently, 5-*S*-cysteinylDA has been suggested to be a precursor of neuromelanin (6–8). Cysteine adducts of 3,4-dihydroxyphenylalanine (DOPA) also have been detected in human and other mammalian brain tissues (1–3) and in the urine of patients with malignant melanomas (9). Two thio-adducts of DOPA, 5-*S*-cysteinylDOPA and 5-*S*-glutathionylDOPA, are present in melanoma tissues (10–12).

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[3] Abbreviations used: DA, dopamine; NADA, *N*-acetyldopamine; NBAD, *N*-β-alanyldopamine; DOPA, 3,4-dihydroxyphenylalanine; NACySH, *N*-acetylcysteine; TU, thiourea; 5-NACyS-NADA, 5-*S*-(*N*-acetylcysteinyl)-*N*-acetyldopamine; 2,5-di-NACyS-NADA, 2,5-*S*,*S*′-(*N*-acetylcysteinyl)-*N*-acetyldopamine; 6-TU-NADA, 6-thiourea-*N*-acetyldopamine; 6-TU-NBAD, 6-thiourea-*N*-β-alanyldopamine; LC, liquid chromatography; EC, electrochemical; ESI-MS, electrospray ionization mass spectrometry; COSY-LR, long-range correlated spectroscopy; HMQC, heteronuclear multiple quantum coherence; CV, cyclic voltammetry.

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Possible cellular sources of thiol-containing nucleophiles are L-cysteine, glutathione, and proteins containing cysteinyl residues. An *in vitro* study demonstrated that DOPA quinone formed by incubation of DOPA with tyrosinase bound to proteins through sulfhydryl groups of cysteine (13). CysteinylDA conjugates resulting from the reactions between DA quinone and protein cysteinyl residues were produced following incubation of rat neostriata slices with [^{3}H]DA (14). Some compounds containing SH groups can inhibit melanin formation after DA oxidation in the nervous system (15). Melanin formation is associated with Parkinson's disease, where DA-containing cells are susceptible to degeneration (16, 17). The inhibitory role of thiols on melanin formation appears to depend mainly on the addition of SH-nucleophilic compounds to DA quinone, resulting in the production of thio-adducts of DA (15, 18–21).

In addition to compounds containing the SH group, nucleophiles containing the thioureylene moiety [—HN—C(═S)—NH—] affect melanogenesis and other biochemical processes in pigment-producing systems *in vivo* (22). Several structurally related nucleophilic thioureylenes effectively suppress dopachrome and subsequent pigment formation by reacting with DOPA quinone (23).

Two *N*-acyl derivatives of DA, *N*-acetyldopamine (NADA) and *N*-β-alanyldopamine (NBAD), were of interest in this study. They are physiologically important as precursors for quinone metabolites that harden the exoskeletons of insects and related arthropods through adduct and cross-link formation with protein nucleophilic groups (24). They also have been detected in insect nervous systems (25, 26) and occur at high concentrations in the hemolymph of some insect species as glucosyl conjugates, which serve as storage forms prior to sclerotization of new cuticle (27). NADA also has been found in the human kidney and urine, primarily as a sulfate-conjugated form (28). It has antitumor activity toward neuroblastoma cells *in vitro*, and its cytotoxicity may arise from the reaction of NADA quinone with nucleophilic species within the cell (29, 30).

Adduct formation of catechols in various biological systems involves enzyme-catalyzed oxidation of the catechols and subsequent addition reactions of the corresponding *o*-quinones with sulfur-centered nucleophiles. However, the mechanisms of formation of many of these adducts, which vary in structure according to the nature of the reactants and reaction conditions, remain unclear. Model reactions between DA and *N*-acetylcysteine (NACySH) have been carried out to elucidate the initial steps of the oxidation pathway when electrochemically prepared DA quinone reacted with excess NACySH (31). Studies of exhaustive electrolysis of DA in the presence of excess NACySH also explored some of the reactions in this oxidation pathway (32). Little work has been done so far on the reaction of *N*-acyldopamine quinones with sulfur-centered nucleophiles.

The objectives of this study were to elucidate the oxidative pathways of NADA and NBAD; to characterize the major reaction products of their quinones with two sulfur-centered nucleophiles, NACySH and thiourea (TU); and to determine the mechanisms and regioselectivity of adduct formation.

EXPERIMENTAL

Chemicals. The following chemicals were obtained from commercial sources: NADA, NACySH (Sigma Chemical Co., St. Louis, MO)[4]; formic acid, ammonium formate, disodium ethylenediaminetetraacetate (EDTA) (Fisher Scientific Co., Pittsburgh, PA); TU (Allied Chemical, Morristown, NJ); and methanol (HPLC grade, UV cutoff 204 nm) (Baxter Healthcare Corporation, Burdick & Jackson Division, Muskegon, MI). NBAD was synthesized as described previously (33). Quinones were prepared by electrochemical oxidation using either a coulometric microcell for analytical scale synthesis or a coulometric flow-through cell for semipreparative scale synthesis (33).

Electrochemistry. A Bioanalytical Systems (West Lafayette, IN) BAS-100W electrochemical system was used. The working, reference, and auxiliary electrodes were a glassy carbon electrode with an area of 7.1 mm^2, an Ag/AgCl (saturated KCl) electrode, and a Pt wire electrode, respectively. Cyclic voltammetry (CV) was performed on *N*-acyldopamines in the absence and presence of varying concentrations of nucleophiles and on their adducts in 0.1 M phosphate buffer (pH 7.0). The scan rate was 200 mV/s unless otherwise noted. Chronoamperometry was performed on 0.3 mM *N*-acyldopamines in the absence and presence of 3 mM nucleophiles in 0.1 M phosphate buffer (pH 7.0), using 0 and 700 mV as initial and high potentials, respectively, and 1000 ms as pulse width. Data were collected in a time range of 10 ms to 1 s.

Liquid chromatography. Analytical and semipreparative LC were conducted to analyze reaction products that resulted from mixing either NADA quinone or NBAD quinone with nucleophiles and also to purify the adducts that formed. The LC system with UV/vis and electrochemical detection has been described previously (33). Unless otherwise noted, separation of the reaction products was achieved using a Microsorb-MV C18 column (5 μm, 4.6 $\times$ 250 mm) (Rainin Instrument Co., Inc., Woburn, MA) for analytical LC and a Phenomenex (Torrance, CA) C18 semipreparative column (10 μm, 10 $\times$ 250 mm) for semipreparative LC. The flow rates were 1ml/min for the former and 4 ml/min for the latter. A binary mobile-phase system, which consisted of solvent A [150 mM formic acid, 30 mM ammonium formate, and 0.1 mM EDTA (pH 3.0)] and solvent B [50% methanol, 180 mM formic acid, 8 mM ammonium formate, and 0.1 mM EDTA (pH 3.0)], was used for both analytical and semipreparative LC. Four mobile-phase gradients, denoted gradients I to IV, were used for different sample solutions. Gradient I was 0–15 min, 80% solvent A and 20% solvent B; and 15–30 min, linear gradient from 20 to 80% solvent B. This gradient was used for analytical LC of the product mixtures produced from NADA quinone reacting with TU. Gradient II 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. This gradient was used for analytical LC of the product mixtures from the reaction of NBAD quinone with TU. Gradient III was 0 min, 80% solvent A and 20% solvent B; 0–

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

15 min, linear gradient from 20 to 40% solvent B; and 15–30min, linear gradient from 40 to 50% solvent B. This gradient was used for analytical LC of the product mixture from the reaction of NADA quinone with *N*-acetylcysteine. Gradient IV was 0–1 min, 80% solvent A and 20% solvent B; 1–16 min, linear gradient from 20 to 40% solvent B; and 16–31 min, linear gradient from 40 to 45% solvent B. This gradient was used for semipreparative LC to purify adducts formed in the reactions of NADA quinone with NACySH.

For analytical LC of the products from the reaction of NBAD quinone with TU, separation was achieved on an Ultracarb 5 ODS 30 column (C18, 5 μm, 3.2 × 150 mm) (Phenomenex) at a flow rate of 0.5 ml/min. The mobile phase was 5% methanol, 150 mM formic acid, 40 mM ammonium formate, and 0.1 mM EDTA (pH 3.0).

Spectroscopy. UV/vis spectra of adducts in the mobile-phase or 0.01 M HCl solution were recorded in a 1.0-cm quartz cuvette using a Hewlett–Packard (Palo Alto, CA) HP 8452A diode array spectrophotometer. Characterization of adducts by electrospray ionization mass spectrometry (ESI-MS) was carried out using either an Autospec-Q (VG Analytical Ltd., Manchester, UK) equipped with a Mark III ESI source at the University of Kansas, Lawrence, Kansas, or a comparable instrument at the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. Samples were dissolved in MeOH:0.1% HOAc (70:30) for infusion into the mass spectrometers. Identifications of adducts by 1-D proton, 2-D long-range $^1H-^1H$ correlated spectroscopy (COSY-LR) and heteronuclear multiple quantum coherence correlation (HMQC) NMR experiments were performed with a Varian (Palo Alto, CA) UNITY*plus* 400-MHz spectrometer at 27°C or with a Varian UNITY*plus* 500-MHz spectrometer at 30°C. Adducts were dissolved in 0.7ml of either D_2O or H_2O:D_2O (90:10).

Syntheses of N-acyldopamine adducts of NACySH and TU. Analytical scale syntheses were achieved by mixing the electrochemically synthesized NADA quinone or NBAD quinone solution in 0.01 M HCl and 0.09 M KCl (pH 2) with a solution of nucleophile, NACySH or TU, in either 0.01 M HCl (pH 2) or 0.1 M phosphate buffer (pH 7.4). Semipreparative scale syntheses were achieved by adding the effluent from the flow-through coulometric cell, which contained NADA or NBAD quinone at pH 2, into a solution of NACySH or TU in 0.1 M phosphate buffer (pH 7.4). NACySH adducts were purified using the semipreparative LC system and conditions described above. Each product effluent was collected and desalted using the semipreparative LC column. After a product solution was injected onto the column, the salts were eluted using distilled water as the mobile phase; then the product was eluted using 25% methanol and lyophilized to dryness. TU adducts were purified using a gel filtration column packed with Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, CA). The mobile phase used was 0.05% formic acid.

RESULTS

Reactions of N-Acyldopamine Quinones with N-Acetylcysteine

Cyclic voltammetric studies. The electrochemical behavior of 0.3 mM NADA in the absence and presence of NACySH (0 to 3 mM) in 0.1 M phosphate buffer at pH 7.0 was studied using cyclic voltammetry at a scan rate of 200 mV/s (Fig. 1A). In the absence of NACySH, the cyclic voltammogram consists of an anodic peak at 0.22 V, which is due to the two-electron oxidation of NADA to NADA quinone, and a cathodic peak at 0.10 V, which is due to the reduction of the NADA quinone back to NADA (Fig. 1A). No other discernible peak occurred, indicating that NADA quinone did not decompose to give other electroactive products under these reaction conditions.

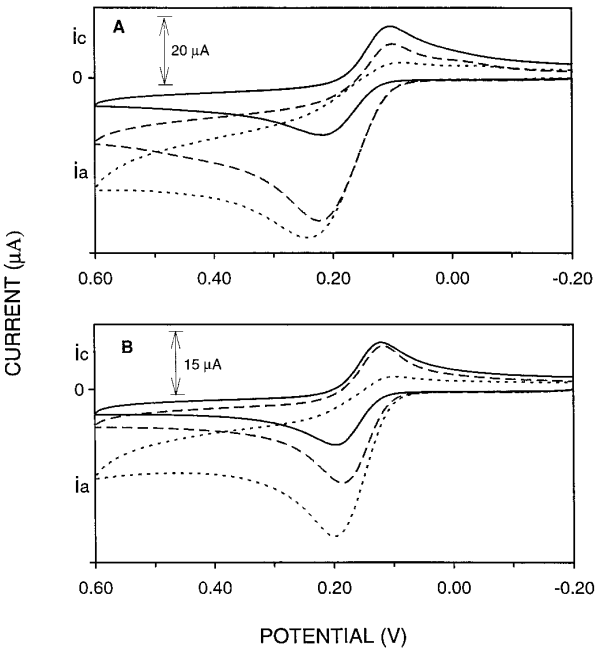


FIG. 1. Cyclic voltammograms of *N*-acyldopamines in the absence and presence of NACySH in 0.1M phosphate buffer at pH 7.0. The scan was initiated in the positive-going direction from −0.2 V at a rate of 200 mV/s. (A) NADA:NADA concentration was 0.3 mM. NACySH concentrations were 0 (—), 0.6 (---), and 1.5 mM (· · ·). (B) NBAD:NBAD concentration was 0.3 mM. NACySH concentrations were 0 (—), 0.6 (---), and 3 mM (· · ·).

When NACySH was present, the height of the anodic peak for the oxidation of NADA increased with an increasing molar ratio of NACySH to NADA; concomitantly, the height of the cathodic peak for the reduction of NADA quinone decreased. For instance, when the molar ratio of NACySH to NADA was 2:1, the anodic peak current for the oxidation of NADA increased by a factor of 2.6, whereas the cathodic peak current decreased approximately 50% (Fig. 1A). When the ratio of NACySH to NADA was 5:1, the anodic peak current increased by a factor of 3, whereas the cathodic peak nearly disappeared (Fig. 1A). In a separate control experiment (data not shown), cyclic voltammetric study of NACySH showed that it is not electroactive in this potential range (−0.2 to 0.6 V).

The observation that the cathodic peak largely disappeared when NACySH was present in excess suggests that both the original and any subsequently electrogenerated quinones were consumed by follow-up chemical and homogeneous electron transfer reactions under these conditions. The observation that the height of the anodic peak increased when NACySH was present indicates that NADA quinone reacted rapidly with NACySH to form a product(s) that also was oxidized at

the potential at which NADA is oxidized. Furthermore, because the initial anodic peak corresponds to a two-electron oxidation of NADA to NADA quinone, the increase of anodic current by a factor of 3 when excess NACySH is present indicated that six electrons are involved in the overall electron transfer process. This result suggests that NADA quinone undergoes two successive addition reactions, each of which generates a more highly substituted catecholamine derivative that was oxidized at the applied potential in a two electron process to its corresponding quinone.

In separate experiments, we attempted to determine the kinetics of these reactions at pH 7.0 by chronoamperometry (data not shown). The potential was stepped from a potential at which no oxidation occurs to a potential that is sufficiently positive such that the oxidations of NADA and NADA adducts occurred rapidly. Analysis of the current-time curves demonstrated that two molecules of NACySH were added to NADA quinone rapidly. The reaction was complete within 8 ms, which is the shortest time for which our instrumentation would allow quantitative measurements. At times longer than 8 ms, nucleophilic attack on the diadduct quinone to give additional electroactive species was indicated.

Cyclic voltammograms of NBAD in the presence of NACySH at pH 7.0 were similar to those of the NADA/NACySH system, with minor differences (Fig. 1B). While the cathodic peak in the system of NADA/NACySH disappeared when the molar ratio of NACySH to NADA was 5:1, the cathodic peak in the system of NBAD/NACySH was still discernible under these conditions. The latter peak did disappear when the ratio of NACySH to NBAD is increased to 10:1, which indicates that NBAD quinone reacted with NACySH at a rate slower than NADA quinone. As in the NADA/NACySH system, the anodic peak for the oxidation of NBAD increased by a factor of 3 when the ratio of NACySH to NBAD was 10. Like the oxidation of NADA to NADA quinone, the oxidation of NBAD to NBAD quinone involves two electrons. Therefore, the observation that the anodic current increased by a factor of 3 when NACySH was present in relatively large excess suggests that NBAD quinone also undergoes two successive addition reactions to form more highly substituted catecholamines that are also electroactive at the applied potential.

Characterization of reaction products. The composition of the product mixture from incubating 0.4 ml 1 mM NADA quinone with 0.2 ml 10 mM NACySH (1:5 molar ratio) at pH 7.4 was analyzed by LC-EC (oxidation), and the chromatogram showed several well-resolved peaks (Fig. 2). By comparison with standard compounds, the peak at 13.6 min corresponds to NADA and the peak at 5.2 min is due to NACySH. The predominant peak at 23.9 min and the peak at 26.4 min were identified after isolation as the adducts 5-*S*-(*N*-acetylcysteinyl)-*N*-acetyldopamine (5-NACyS-NADA) and 2,5-*S*,*S*′-di-(*N*-acetylcysteinyl)-*N*-acetyldopamine (2,5-di-NACyS-NADA). The LC-EC (oxidation) chromatogram for the product mixture at pH 2.0 has a similar pattern (data not shown), with the peak at 23.9 min (5-NACyS-NADA) also being predominant. However, the peaks at 13.6 min (NADA) and 26.4 min (2,5-di-NACyS-NADA) are much smaller than those obtained at pH 7.4.

5-NACyS-NADA exhibited UV absorption maxima at λ_{max} = 256 and 294 nm in mobile phase and CV peaks at $E_{p,a}$ = 0.21 V and $E_{p,c}$ = 0.15 V in 0.1 M phosphate buffer at pH 7.0. ESI-MS gave m/z = 357, which is consistent with the theoretical M+1 value for a monoaddition adduct of NACySH with NADA quinone. ^{1}H NMR (400 MHz, in D_2O) gave δ 6.92 (d, J = 2.1 Hz, 1H, H2), 6.81 (d, J = 1.8 Hz, 1H, H6), 4.43 (dd, J = 8.2, 4.3 Hz, 1H, H3′), 3.44 (dd, J = 14.4, 4.3 Hz, 1H, H2′a), 3.40 (t, J = 6.7 Hz, 2H, H8), 3.21 (dd, J = 14.4, 8.2 Hz, 1H, H2′b), 2.71 (t, J = 6.9 Hz, 2H, H7), 1.95 and 1.92 (2s, 6H, H6′, and H11).

2,5-di-NACyS-NADA exhibited UV absorption maxima at λ_{max} = 274 and 302 (sh) nm in mobile phase and CV peaks at $E_{p,a}$ = 0.19 V and $E_{p,c}$ = 0.15 V in 0.1 M phosphate buffer at pH 7.0. ESI-MS gave m/z = 518, which is consistent with the theoretical M + 1 value for a diaddition adduct of NACySH with NADA quinone. ^{1}H NMR (400 MHz, in D_2O) gave δ 7.00 (s, 1H, H6), 4.35 (dd, J = 7.9, 4.0 Hz, 1H, H3′), 4.23 (dd, J = 7.9, 4.0 Hz, 1H, H3″), 3.44 (m, 1H, H2′a), 3.40 (m, 2H, H8), 3.34 (dd, J = 14.0, 4.0 Hz, 1H, H2″a), 3.19 (dd, J = 13.7, 7.6 Hz, 1H, H2′b), 3.06 (dd, J = 13.4, 7.3 Hz, 2H, H7), 2.97 (dd, J = 13.9, 7.0 Hz, 1H, H2″b), 1.94, 1.88, and 1.87 (3s, 9H, H6′, H6″, and H11). Because the spectral pattern of this compound is comparable to that of 2,5-*S*,*S*′-di-(*N*-acetylcysteinyl)DA, which was produced from the reaction of DA quinone and NACySH (31), and also because the ring C6 site of the catecholamine quinones is the least reactive toward thiol nucleophiles (31), this adduct is identified as the 2,5-disubstituted adduct.

Relative molar ratios of the N-acetylcysteine adducts. The relative molar ratios of 5-NACyS-NADA and 2,5-di-NACyS-NADA that were produced from the reactions of NADA quinone (0.3 mM) with NACySH (1.5 mM) at pH 7.4 and 2 were estimated from spectroscopic data obtained during LC. Two chromatograms at 294 and 274 nm, which are the λ_{max} values of 5-NACyS-NADA and 2,5-di-NACyS-NADA, respectively, were obtained by extracting absorbances from the on-line UV/vis spectra recorded during LC (data not shown). The ratio of the area of the chromatographic peak for 5-NACyS-NADA at its λ_{max} of 294 nm to the area of the

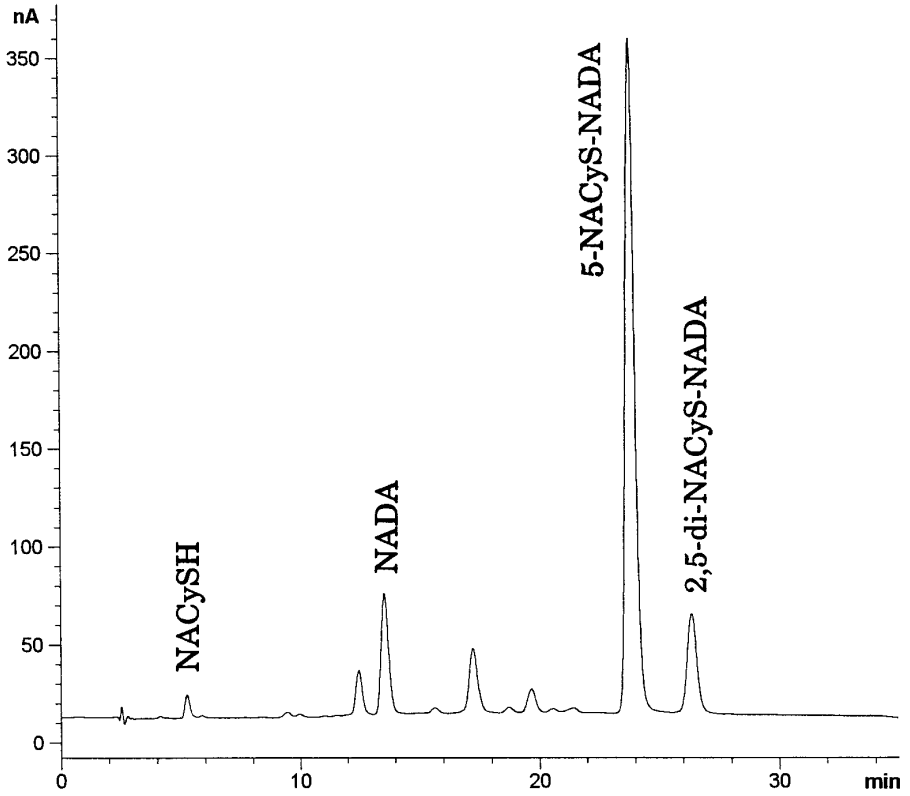


FIG. 2. LC-EC (oxidation) chromatogram of the product mixture from the reaction of NADA quinone with NACySH (1:5 molar ratio) at pH 7.4. Peaks were identified as follows: NACySH, 5.2 min; NADA, 13.6 min; 5-NACyS-NADA, 23.9 min; and 2,5-di-NACyS-NADA, 26.4 min. Unknown product (possibly 2-NACyS-NADA), 17.2 min.

chromatographic peak for 2,5-di-NACyS-NADA at its λ_{max} of 274 nm was calculated. The molar absorption coefficients reported in the literature (31) for the analogous C5 monoaddition and C2,5 diaddition *N*-acetylcysteine adducts of DA were used for the adducts of NADA, because the DA adducts and the NADA adducts have the same λ_{max} values at 294 and 274 nm for monoaddition (C5) and diaddition (C2,5), respectively. If the ratio of the molar absorption coefficient of 5-NACyS-NADA to that of 2,5-di-NACyS-NADA is assumed to be the same as the ratio of the analogous C5 monoaddition to C2,5 diaddition adducts of DA, which is 1:4, then the relative molar ratio of the adducts 5-NACyS-NADA and 2,5-di-NACyS-NADA would be approximately 10:1 at pH 7.4 and 32:1 at pH 2.0.

Reactions of N-Acyldopamine Quinones with Thiourea

Cyclic voltammetric studies. The electrochemical behavior of 0.3 mM NADA or NBAD in the presence of TU in 0.1 M phosphate buffer at pH 7.0 was studied using cyclic voltammetry. The concentration of TU ranged from 0.3 to 6 mM ($1 \leq$ [TU]/[NADA or NBAD] ≤ 20). Although there is no discernible evidence for a follow-up chemical reaction in the cyclic voltammogram of either catecholamine in the presence of TU on the first cycle, changes in cyclic voltammetric behavior of the catecholamines are observed during subsequent cycles (Figs. 3A and 3B). In both systems, a second anodic peak appears at a more positive potential, whereas the cathodic peak shifts to a less positive potential with an increasing number of cycles. Additionally, both anodic and cathodic peaks for NADA/NADA quinone or NBAD/NBAD quinone gradually decrease in height. These data indicate that products formed are less chemically and/or electrochemically reversible than the starting material. In addition, the new anodic peak that is observed at a more positive potential indicates the occurrence of a reaction between the quinone and TU to give an electroactive product(s) that is oxidized at a more positive potential. Furthermore, the relative ratio of the new anodic peak current to the initial anodic peak current is greater in the NADA/TU system (Fig. 3A) than in the NBAD/TU system (Fig. 3B), indicating that the reaction of NADA quinone with TU is faster than that of NBAD quinone with TU.

A significant increase in the background current was observed in the potential range from 0.4 to 0.6 V (Figs. 3A and 3B), which is due to the irreversible oxidation

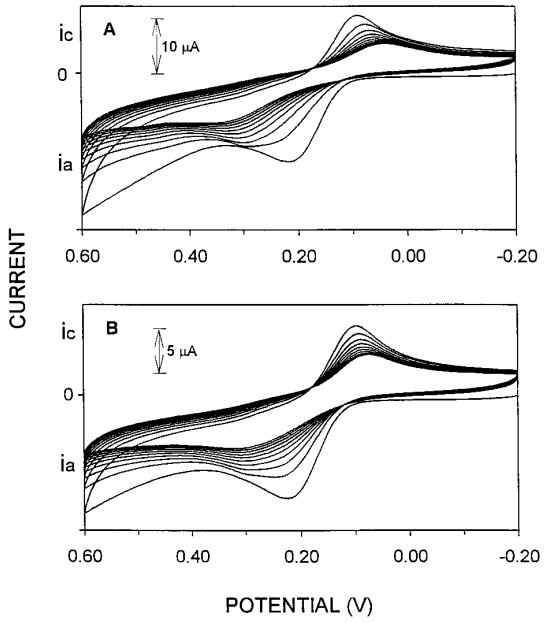


FIG. 3. Cyclic voltammogram of (A) NADA and (B) NBAD in the presence of TU in 0.1 M phosphate buffer at pH 7.0. The scan was initiated in the positive-going direction from −0.2 V at a rate of 200 mV/s. Concentrations of NADA and NBAD were 0.3 mM and that of TU was 1.5 mM.

of TU. In the cyclic voltammogram of TU (data not shown), the oxidation on the first positive-going sweep begins around 0.22 V and increases to give an ill-formed peak near 0.55 V. Successive scans indicate that the subsequent oxidations occur with more and more difficulty, suggesting that the oxidation of TU gives rise to some type of product that oxidizes less readily than the starting material.

Characterization of reaction products. The composition of the product mixture that results from incubating 0.4 ml 1 mM NADA quinone with 0.2 ml 10 mM TU (1:5 molar ratio) at either pH 7.4 or pH 2.0 was analyzed by LC. The LC-EC (oxidation) chromatogram of products at pH 7.4 is shown in Fig. 4. The chromatograms for either reaction condition are quite similar, except that the small unidentified minor peak at 12.2 min in the chromatogram of the reaction mixture at pH 7.4 is not observed in the reaction mixture at pH 2.0 (data not shown). Peaks at 3.4 and 17.5 min are due to TU and NADA, respectively. The major peak at 10.1 min is due to a reaction product, which was identified after isolation as the adduct 6-*S*-thiourea-*N*-acetyldopamine (6-TU-NADA).

6-TU-NADA exhibited UV absorption maxima at λ_{max} = 246 and 288 nm in 0.01 M HCl, and a CV peak at $E_{p,a}$ = 0.32 V in 0.1 M phosphate buffer at pH 7.0. ESI-MS gave m/z = 270, which is consistent with the theoretical M + 1 value for a monoaddition adduct of TU with NADA quinone. ^{1}H NMR (500 MHz, in H_2O:D_2O = 90:10) gave δ 7.82 (s, 1H, NH9), 7.07 (s, 1H, H5), 6.87 (s, 1H, H2), 3.29 (dt, J = 5.3, 5.3 Hz, 2H, H8), 2.76 (t, J = 5.3 Hz, 2H, H7), and 1.81 (s, 3H, H11). These assignments were confirmed by 2-D HMQC and COSY-LR. In COSY-LR, H2 shows connectivities with both H5 and H7, whereas H5 shows connectivity only with H2.

The composition of the product mixture that results from the reaction of NBAD quinone with TU also was studied using a different mobile-phase system (see Experimental for details). NBAD eluted as a minor peak at 9.7 min. Only one major product was detected at 5.6 min in the LC-EC (oxidation) chromatogram, which was identified after isolation as the adduct 6-*S*-thiourea-*N*-β-alanyldopamine (6-TU-NBAD).

6-TU-NBAD exhibited UV absorption maxima at λ_{max} = 246 and 288 nm in 0.01 M HCl and a CV peak at $E_{p,a}$ = 0.31 V in 0.1 M phosphate buffer at pH 7.0 (no cathodic peak). ESI-MS gave m/z = 299, which is consistent with the theoretical M + 1 value for a monoaddition adduct of TU with NBAD quinone. ^{1}H NMR (500 MHz, in H_2O:D_2O = 90:10) gave δ 7.98 (s, 1H, NH9), 7.09 (s, 1H, H5), 6.86 (s, 1H, H2), 3.33 (dt, J = 5.3, 5.3 Hz, 2H, H8), 3.08 (t, J = 5.3 Hz, 2H, H12), 2.77 (t, J = 5.3 Hz, 2H, H7), and 2.49 (t, J = 5.3 Hz, 2H, H11). These assignments were confirmed by 2-D HMQC and COSY-LR. In COSY-LR, H2 shows connectivities with both H5 and H7, whereas H5 shows connectivity only with H2.

Electrochemical behavior of thiourea adducts. The cyclic voltammetric behavior of 6-TU-NADA and 6-TU-NBAD in 0.1 M phosphate buffer at pH 7.0 was examined at a scan rate of 200 mV/s (Fig. 5). The anodic peak that corresponds to the oxidation of 6-TU-NADA occurs at 0.32 V (Fig. 5A), whereas the anodic peak that corresponds to the oxidation of 6-TU-NBAD occurs at 0.31 V (Fig. 5B). No corresponding cathodic peak for reduction of the quinone back to the adduct occurred in either voltammogram at this scan rate, demonstrating that both adducts are oxidized irreversibly. The anodic peak current in the second cycle is also significantly smaller than one would expect for a reversible process. This result, along with the absence of a cathodic peak for reduction of the adduct quinone, suggests that the adduct quinone is being consumed to give an unknown product(s). The small anodic peak at approximately 0.52 V might be due to the oxidation of the unknown product(s).

DISCUSSION

A mechanism for reactions that occur during cyclic voltammetry of NADA in the presence of a large excess of NACySH is proposed in Scheme 1. First, NADA is oxidized to form NADA quinone at the electrode surface

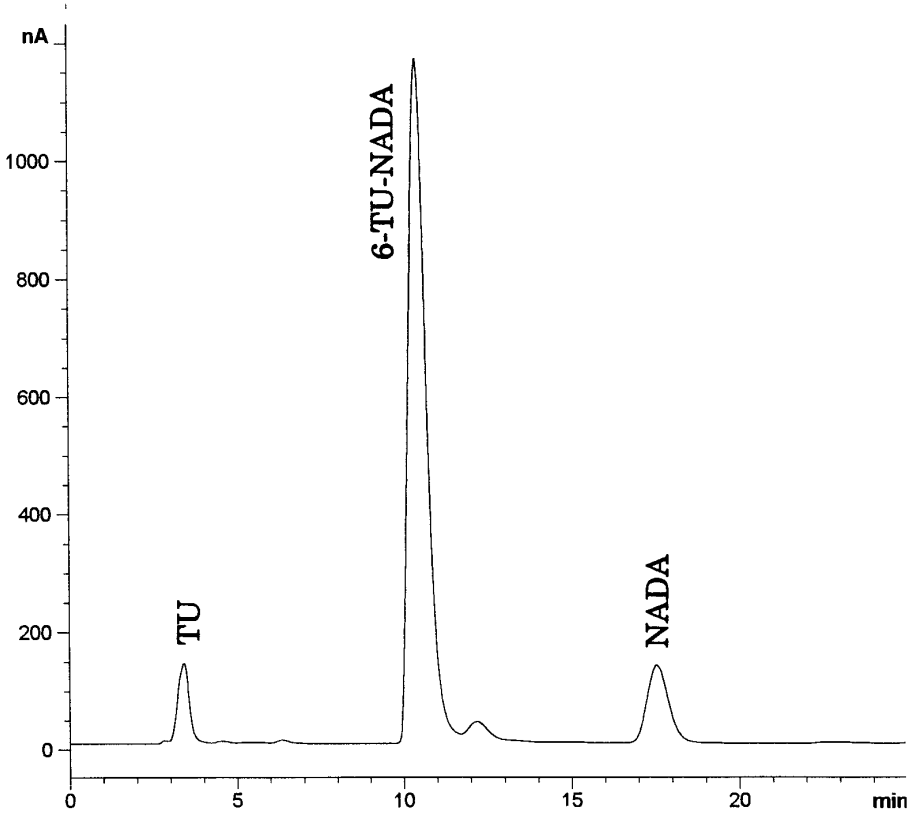


FIG. 4. LC-EC (oxidation) chromatogram of the product mixture from the reaction of NADA quinone with TU (1:5 molar ratio) at pH 7.4. Peaks were identified as follows: TU, 3.4 min; NADA, 17.5 min; and 6-TU-NADA, 10.1 min.

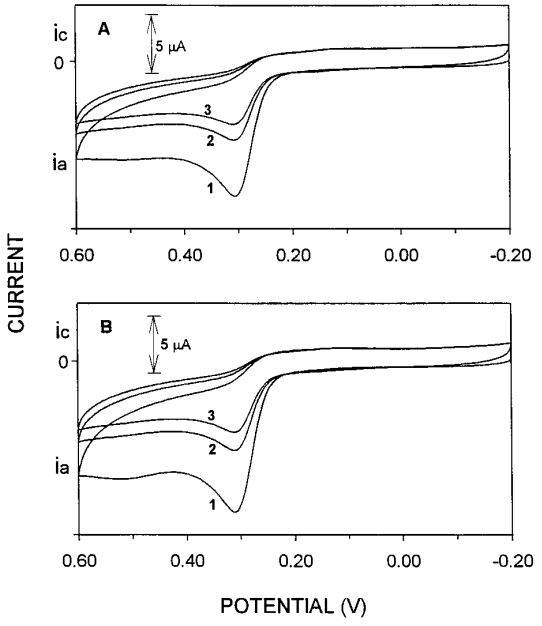
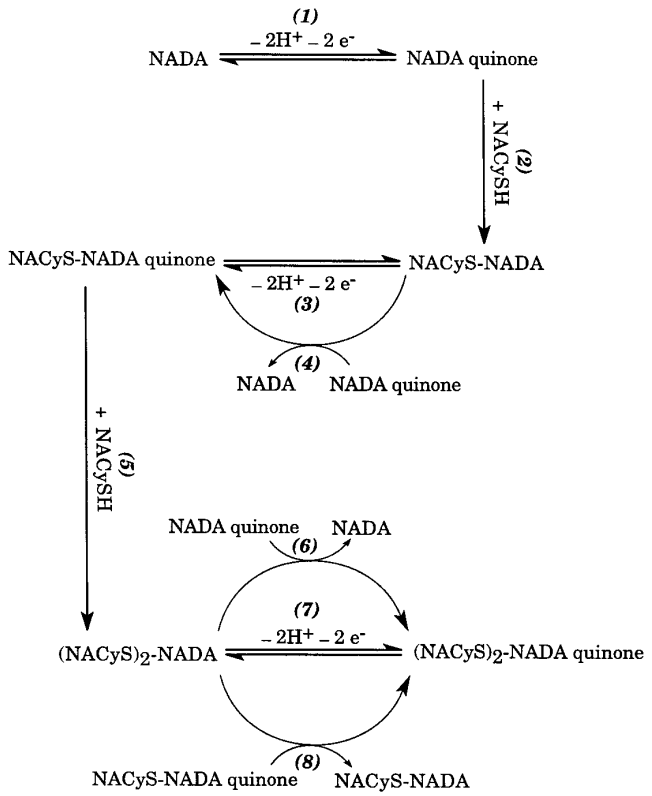


FIG. 5. Cyclic voltammogram of (A) 6-TU-NADA and (B) 6-TU-NBAD in 0.1 M phosphate buffer at pH 7.0. The scan was initiated in the positive-going direction from −0.2 V at a rate of 200 mV/s.

(reaction 1). NADA quinone, after diffusing to the bulk solution, is subject to nucleophilic addition by NACySH to form a monoaddition adduct (reaction 2). The adduct formed is oxidized to its corresponding quinone either electrochemically at the electrode surface (reaction 3) or chemically in the bulk solution by NADA quinone (reaction 4). A second nucleophilic addition of NACySH occurs with the quinone of the monoaddition adduct (reaction 5). The diaddition adduct thus produced is oxidized to its corresponding quinone by three possible routes, one of which is electrochemical at the electrode surface (reaction 7), whereas the remaining two involve homogeneous electron transfer with either NADA quinone (reaction 6) or the quinone of the monoaddition adduct (reaction 8). The homogeneous redox reactions are facile, because the oxidation potentials of NADA, its monoaddition NACySH adduct, and its diaddition NACySH adduct decrease in order of increasing substitution.

The substantial decrease of the cathodic peak during cyclic voltammetry of NADA in the presence of large excess NACySH is the result of quinones being consumed rapidly on the time scale of the experiment. It is possible that this might also be due to homogeneous chemical oxidation of NACySH by the product *o*-qui-



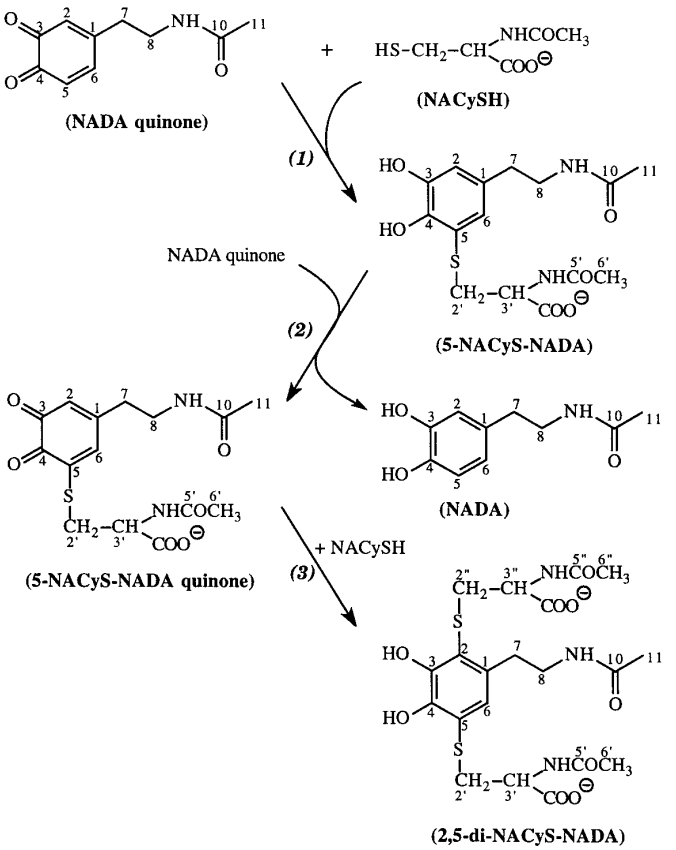
SCHEME 1. Proposed reaction pathways for the electrooxidation of NADA in the presence of NACySH.

nones. While this possibility cannot be ruled out unequivocally, NACySH was shown to be electroinactive in this potential range and, thus, unlikely to be oxidized by any adduct *o*-quinone.

The increase of anodic current in the presence of NACySH is due to the relatively rapid reactions of the nucleophile with NADA quinone and 5-NACyS-NADA quinone and the oxidation of the adducts at the applied potential. Because the oxidations of NADA and its adducts to their corresponding quinones involve two electrons per molecule, the increase of the anodic current by a factor of 3 indicates that the stepwise additions of two molecules of nucleophile for each NADA molecule that is originally present result in six electrons overall being transferred. Because NBAD and NADA have a similar cyclic voltammetric behavior in the presence of NACySH, the pathway proposed in Scheme 1 also applies to the reactions of NBAD with NACySH during cyclic voltammetry.

When compared with the pathway for reactions that occur during cyclic voltammetric oxidation of NADA or NBAD in the presence of NACySH, the pathway for reactions of the pregenerated catecholamine quinone with a large excess of NACySH under nonelectrolyzing conditions is somewhat less complicated. The nucleophilic addition and homogeneous redox reactions of Scheme 1 (reactions 2, 4, and 5) are the principal reactions under these conditions. The LC results for a product mixture from incubating NADA quinone with NACySH at a 1:5 ratio show that (1) the predominant species in the product mixture is the C5 monoaddition adduct, (2) only a small amount of NADA is produced, and (3) the chromatographic peak area of 2,5-di-NACySH NADA is approximately equal to that of NADA (Fig. 2). On the basis of these results, a reaction pathway is proposed for the quinone with a large excess of nucleophile (Scheme 2). First, the nucleophile attacks the quinone principally at C5 on the aromatic ring to form 5-NACyS-NADA (reaction 1). A small portion of the adduct is oxidized subsequently by unreacted NADA quinone, producing 5-NACyS-NADA quinone and generating NADA (reaction 2). 5-NACyS-NADA quinone is attacked subsequently by a second molecule of nucleophile at C2 on the ring to form 2,5-di-NACySH-NADA (reaction 3). According to the proposed scheme, the amount of the diadduct found should equal the amount of NADA that is recovered. The LC results are consistent with this expectation.

The NADA/NACySH system is quite similar to the DA/NACySH system, which was studied previously by



SCHEME 2. Adduct formation from reactions of NADA quinone with NACySH.

our group (31), in terms of both cyclic voltammetric behavior of the catecholamines in the presence of alarge excess of NACySH and LC chromatographic behavior of the product mixtures obtained from the reactions of catecholamine quinones with NACySH. In both systems, the predominant product is the C5 monoaddition adduct, and the second major product is the C2,5 diaddition adduct. Another monoaddition adduct (C2 adduct) was detected in the DA quinone/NACySH reaction system. Based on a comparison of the LC chromatographic patterns, the minor product giving rise to a small peak at 17.2 min in Fig. 2 for NADA quinone/NACySH reaction system, which was not identified because of the limited amount formed, might be analogous to the C2 monoaddition adduct in the reaction system of DA quinone/NACySH.

A C2 adduct of DA, 2-cysteinylDA, also was identified when DA and cysteine were incubated in the presence of H_2O_2 with either Fe^{2+}–EDTA or peroxidase (4). C2 adducts of other catechols, 2-cysteinylDOPA and 2-glutathionylDOPA, also have been obtained from the tyrosinase-catalyzed oxidation of DOPA and conjugation of DOPA quinone with cysteine and glutathione, respectively (9, 10). 2-CysteinylDOPA and 5-cysteinylDOPA also were detected in ratios ranging from approximately 1:6 to 1:4 in acid-hydrolyzed reaction products obtained after DOPA and several proteins were incubated with tyrosinase (13). However, no C2 adducts were identified in cuticular phenoloxidase-catalyzed reactions of NACySH with either 4-methylcatechol or NADA (34) or in apple phenoloxidase-catalyzed reactions of cysteine with 4-methylcatechol (35).

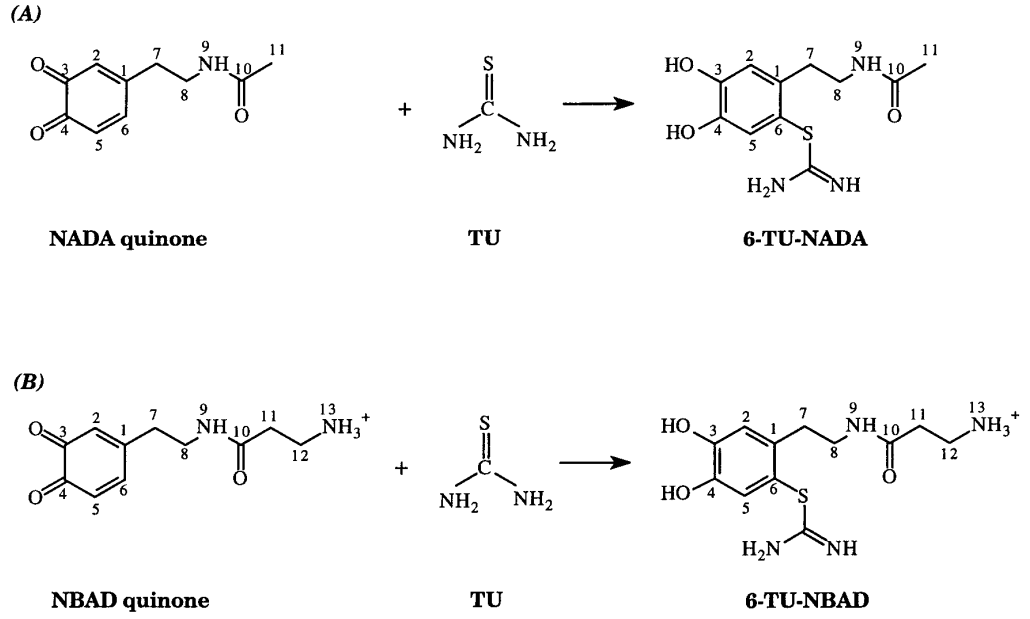
Although all unsubstituted sites on the ring of the quinones of DA, NADA, and NBAD could be attacked by a nucleophile, only two successive additions account for the results under our reaction conditions. This is evidenced by (1) the observation from cyclic voltammetry that the anodic current increases by a factor of 3 when the sulfur-centered nucleophile is added in excess, (2) the results from LC showing that the diaddition adduct is produced at a significant level, and (3) the lack of a LC peak where a triaddition product would be expected. Although the possible formation of a triaddition adduct cannot be precluded, our results clearly show that the third nucleophilic attack must occur much more slowly. To our knowledge, a tri-cysteine addition product of dopamine is produced only when DA undergoes exhaustive electrolysis in the presence of excess cysteine (18, 32).

No C6 adduct was detected in the reaction system of NADA quinone with NACySH, and no C6 monoadduct has been reported in any of the following systems at pH ~7: DA quinone with NACySH (31), DA electrolyzed in the presence of cysteine (18), cuticular phenoloxidase-catalyzed reaction of 4-methylcatechol or NADA with NACySH (34), apple polyphenoloxidase-catalyzed reaction of 4-methylcatechol with cysteine (35), and DA and cysteine incubated in presence of H_2O_2 and either Fe^{2+}–EDTA or peroxidase (4). On the other hand, small amounts of C6 adducts have been obtained from the tyrosinase-catalyzed oxidation of DOPA and conjugation of DOPA quinone with cysteine and glutathione, at relative percentages of only approximately 1 and 5%, respectively (9, 10). In the reaction of 4-methylcatechol quinone and thioacetic acid, a C6 adduct was formed predominantly when the reaction was carried out under basic conditions, whereas the C5 adduct was the predominant product when the reaction occurred under acidic or neutral conditions (36).

The C2,5 diaddition adduct is the second major product resulting from the reactions of NADA quinone and NACySH. No diaddition adduct of NADA has been reported previously to our knowledge. When NADA and NACySH were incubated in the presence of an insect cuticular phenoloxidase, only the C5 monoadduct of NADA was detected (34). However, C2,5 diaddition adducts of DA and other catechols have been reported *in vitro* (31). For example, the C2,5 diaddition NACySH adduct of 4-methylcatechol was identified when 4-methylcatechol and NACySH were incubated in the presence of an insect cuticular phenoloxidase preparation (34). The C2,5 diaddition cysteinyl adduct of DOPA was formed when DOPA and cysteine were incubated in the presence of mushroom tyrosinase (9).

As shown above, 5-NACyS-NADA is the predominant product resulting from the reaction of NADA quinone and NACySH. In other studies (4, 9, 10, 13, 18, 19, 31, 34, 35), similar C5 thio-adducts of various catechols are either the sole or the predominant products identified. On the basis of these results, we can conclude that nucleophiles containing a sulfhydryl group, such as NACySH, cysteine, glutathione, and proteins containing cysteinyl residues, predominantly attack catecholamine quinones at C5 of the aromatic ring under physiological conditions.

Reactions of catecholamines that involve other types of sulfur-centered nucleophiles, such as compounds that contain the thioureylene moiety, are not as well documented as those involving compounds containing the sulfhydryl group. Our cyclic voltammetric results of NADA and NBAD in the presence of TU show that reactions of NADA and NBAD quinones with TU are relatively slow on the time scale of cyclic voltammetry. Because TU adducts have more positive oxidation potentials than the corresponding catecholamines, homogenous redox reactions involving the oxidation of TU adducts by the catecholamine quinones occur much more slowly. This is consistent with our chromatographic results of the product mixture from the reaction of NADA quinone or NBAD quinone with a large excess of TU under nonelectrolyzing conditions. No diaddition adduct was detected in either system.



SCHEME 3. Adduct formation from reactions of NADA quinone (A) and NBAD quinone (B) with thiourea.

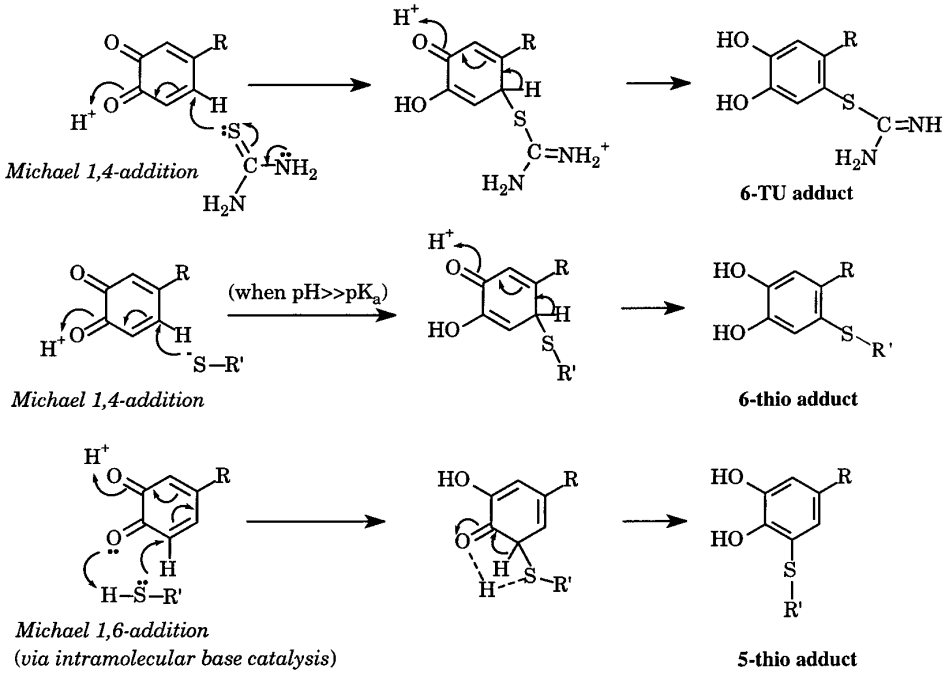
The pathway for adduct formation from reactions of either quinone with TU is shown in Scheme 3. TU attacks NADA and NBAD quinones at the C6 position of the aromatic ring, forming the corresponding adducts. Reactions between 2-thiouracil and DOPA quinone also result in formation of the C6 adduct, 6-thiouracil-DOPA (4). To our knowledge, no reactions of NADA and NBAD quinones with thioureylene-containing nucleophiles have been studied previously.

Comparison of reactivity between the two sulfur-centered nucleophiles, NACySH and TU, with NADA and NBAD quinones shows notable differences. Based on qualitative data from the cyclic voltammetric studies, nucleophilic addition of NACySH occurs several orders of magnitude faster than TU. Thus, NACySH is a substantially better trapping agent for quinones than is TU.

The redox behaviors of the catecholamine adducts of NACySH and TU also differ: NACySH adducts of NADA and NBAD are oxidized at less positive potentials than the corresponding catecholamines and their oxidations are chemically reversible, whereas oxidation of TU adducts occurs irreversibly and at more positive potentials than the corresponding catecholamines. Therefore, NACySH adducts readily undergo homogeneous redox reactions with quinones of corresponding parent catecholamines, whereas TU adducts are much less likely to do so. As a consequence, the pathway for reactions of quinones with NACySH is more complex than that for quinones with TU.

An interesting difference in the reactivities of sulfur-centered nucleophiles is the regioselective outcome of nucleophilic attack by NACySH and TU. The former nucleophile predominantly attacked the C5 position of the aromatic ring of catecholamine quinones and related compounds, whereas the latter attacked the C6 position. The mechanism responsible for the regioselectivity of the ring adducts has remained unclear, although several hypotheses have been proposed (34, 36). For nucleophilic addition reactions of 4-methylcatechol quinone (36), the regioselective outcomes of sulfur-centered nucleophiles were rationalized in terms of the characteristics of the nucleophiles: anionic nucleophiles, which are highly reactive, attack the more electropositive C6 position of the quinone ring, whereas neutral nucleophiles, which are less reactive, attack the less sterically hindered C5 position. In the explanation proposed by Sugumaran *et al.* (34) for the formation of 5-cysteinylDOPA from the reaction of DOPA quinone with cysteine, cysteine was considered a better reductant than a nucleophile, and adduct formation was hypothesized to be the result of a radical reaction.

We propose an intramolecular base-catalyzed mechanism to explain the regioselective outcomes (Scheme 4). Because C6 is the most electropositive site on the *o*-quinone ring, nucleophilic attack normally should occur at the C6 position in a 1,4-Michael addition reaction. This is the case for the addition reactions of thioureylene-containing nucleophiles, such as TU. However, when the nucleophilic center carries a proton that must be removed during nucleophilic attack, the carbonyl group at the C4 position of the *o*-quinone ring can form a hydrogen bond with the nucleophile as the nucleophilic center attacks at the C5 position. Intramo-



SCHEME 4. Mechanism for regioselective nucleophilic additions of *o*-quinones.

lecular base catalysis increases the nucleophilicity of the nucleophile and facilitates formation of the C5 adduct. This is the case for the addition reactions of SH–containing nucleophiles, such as NACySH.

The mechanism proposed here for the regioselectivities of the addition of nucleophiles to aromatic rings of *o*-quinones is consistent with related observations in the literature. For example, C5 adducts have been obtained as predominant products from addition reactions of various catechol quinones with SH-containing nucleophiles such as cysteine, NACySH, and glutathione at physiological pH (4, 9, 10, 18, 31, 34, 35). On the other hand, a C6 adduct was obtained from the reaction of DOPA quinone with 2-thiouracil, which does not have a proton on its nucleophilic center (12).

The influence of experimental conditions on the regioselectivity of adduct formation also can be explained by our proposed intramolecular base-catalyzed mechanism. Different adducts were obtained under different conditions when thioacetic acid reacted with 4-methylcatechol quinone (36). For example, only the C6 adduct was formed in neutral or basic aqueous solutions, whereas only the C5 adduct was obtained in acidic aqueous solutions or in an organic solvent (CH_2Cl_2). According to our proposed mechanism, because thioacetic acid is present predominantly as its conjugate base in neutral or basic conditions, it attacks the more electropositive position, C6. Under acidic conditions, however, thioacetic acid is predominantly protonated, and, therefore, removal of the proton from the SH group by intramolecular base catalysis involving the carbonyl group at C4 would greatly increase the rate of addition of the nucleophile to the C5 position. Likewise, the formation of disubstituted products of NACySH and quinones, the major products of which are the 2,5-adducts, is consistent with an intramolecular base-catalyzed mechanism in which the proton on the second thiol nucleophile forms a hydrogen bond with the C3 carbonyl group of the quinone.

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REFERENCES

1. Rosengren, E., Linder-Eliasson, E., and Carlsson, A. (1985) *J. Neural Transm.* **63,** 247–261.

2. Fornstedt, B., Rosengren, E., and Carlsson, A. (1986) *Neuropharmacology* **25,** 451–454.
3. Fornstedt, B., Bergh, I., Rosengren, E., and Carlsson, A. (1990) *J. Neurochem.* **54,** 578–586.
4. Palumbo, A., d'Ischia, M., Misuraca, G., Martino, L. D., and Prota, G. (1995) *Biochim. Biophys. Acta* **1245,** 255–261.
5. Cheng, F. C., Kuo, J. S., Chia, L. G., and Dryhurst, G. (1996) *J. Neural Transm.* **103,** 433–446.
6. Carstam, R., Brinck, C., and Hindemith-Augustsson, A. (1991) *Biochim. Biophys. Acta* **1097,** 152–160.
7. Zecca, L., Mecacci, C., and Seraglia, R. (1992) *Biochim. Biophy. Acta* **1138,** 6–10.
8. Odh, G., Carstam, R., and Paulson, J. (1994) *J. Neurochem.* **62,** 2030–2037.
9. Ito, S., and Prota, G. (1977) *Experientia* **33,** 1118–1119.
10. Ito, S., Palumbo, A., and Prota, G. (1985) *Experientia* **41,** 960–961.
11. Prota, G. (1992) Melanins and Melanogenesis, Academic Press, San Diego.
12. Napolitano, A., Palumbo, A., d'Ischia, M., and Prota, G. (1996) *J. Med. Chem.* **39,** 5192–5201.
13. Kato, T., Ito, S., and Fujita, K. (1986) *Biochim. Biophys. Acta* **881,** 415–421.
14. Hastings, T. G., and Zigmond, M. J. (1994) *J. Neurochem.* **63,** 1126–1132.
15. Jimenez, F., Garcia-Canovas, F., Garcia-Carmona, F., Iborra, J. L., and Lozando, J. A. (1986) *Int. J. Biochem.* **18,** 161–166.
16. Hirsch, E., Graybiel, A. M., and Agid, Y. A. (1988) *Nature* **334,** 345–348.
17. Kastner, A., Hirsch, E. C., Lejeune, O., Javoy-Agid, F., Rascol, O., and Agid, Y. (1992) *J. Neurochem.* **59,** 1080–1089.
18. Zhang, F., and Dryhurst, G. (1994) *J. Med. Chem.* **37,** 1084–1098.
19. Zhang, F., and Dryhurst, G. (1995) *J. Electroanal. Chem.* **398,** 117–128.
20. Shen, X. M., and Dryhurst, G. (1996) *Chem. Res. Toxicol.* **9,** 751–763.
21. Shen, X. M., Zhang, F., and Dryhurst, G. (1997) *Chem. Res. Toxicol.* **10,** 147–155.
22. Napolitano, A., Memoli, S., Nappi, A., d'Ischia, M., and Prota, G. (1996) *Biochim. Biophys. Acta* **1291,** 75–82.
23. Mars, U., and Larson, B. S. (1995) *Pigm. Cell Res.* **8,** 194–201.
24. Hopkins, T. L., and Kramer, K. J. (1992) *Annu. Rev. Entomol.* **37,** 273–302.
25. Murdock, L. L., and Omar, D. (1981) *Insect Biochem.* **11,** 161–166.
26. Krueger, R. A., Kramer, K. J., Hopkins, T. L., and Speirs, R. D. (1990) *Insect Biochem.* **20,** 605–610.
27. Hopkins, T. L., Morgan, T. D., Mueller, D. D., Tomer, K. B., and Kramer, K. J. (1995) *Insect Biochem. Mol. Biol.* **25,** 29–37.
28. Elchisak, M. A., and Hausner, E. A. (1984) *Life Sci.* **35,** 2561–2569.
29. Graham, D. G., Tiffany, S. M., Bell, W. R., and Gutknecht, W. F. (1978) *Mol. Pharmacol.* **14,** 644–653.
30. Wick, M. M., and Mui, A. (1981) *J. Natl. Cancer Inst.* **66,** 351–354.
31. Xu, R., Huang, X., Kramer, K. J., and Hawley, M. D. (1996) *Bioorg. Chem.* **24,** 110–126.
32. Shen, X. M., Xia, B., Wrona, M. Z., and Dryhurst, G. (1996) *Chem. Res. Toxicol.* **9,** 1117–1126.
33. Huang, X., Xu, R., Hawley, M. D., and Kramer, K. J. (1997) *Bioorg. Chem.* **25,** 179–202.
34. Sugumaran, M., Dali, H., and Semensi, V. (1989) *Arch. Insect Biochem. Physiol.* **11,** 127–137.
35. Richard, F. C., Goupy, P. M., Nicolas, J. J., Lacombe, J-M., and Pavia, A. A. (1991) *J. Agric. Food Chem.* **39,** 841–847.
36. Chavdarian, C. G., and Castagnoli, N., Jr. (1979) *J. Med. Chem.* **22,** 1317–1322.