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Characterization of Products from the Reactions of Dopamine Quinone with *N*-Acetylcysteine

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The reactions between electrochemically prepared dopamine (DA) quinone and *N*-acetylcysteine (NACySH), a protein model nucleophile, have been investigated at pH 7 and pH 2. Major products were purified by semipreparative reversed-phase liquid chromatography and identified by mass spectrometry and nuclear magnetic resonance spectroscopy to be nucleophilic addition products of the quinone with NACySH. The principal product is the monoaddition adduct of the quinone at C(5) of the ring, 5-*S*-(*N*-acetylcysteinyl)dopamine. The diaddition adduct of the quinone at C(2) and C(5) of the ring, 2,5-*S*,*S*′-di-(*N*-acetylcysteinyl)dopamine, and the monoaddition product at C(2) of the ring, 2-*S*-(*N*-acetylcysteinyl)dopamine, are produced to lesser extents. The relative molar ratio of 2-*S*-(*N*-acetylcysteinyl)dopamine, 5-*S*-(*N*-acetylcysteinyl)dopamine, and 2,5-*S*,*S*′-di-(*N*-acetylcysteinyl)dopamine produced at pH 7 is approximately 8:59:33, whereas at pH 2 the ratio is 10:89:1. The uv/vis spectroscopic analysis shows that the two monoaddition products have maxima at 258 and 294 nm, whereas the diaddition product has a maximum at 276 nm and a shoulder at 302 nm. Cyclic voltammetry and chronoamperometry demonstrate that these adducts are oxidized more readily than DA, which causes the anodic current for the oxidation of DA in the presence of NACySH to be kinetically controlled by the rates of the addition reactions. A reaction scheme is proposed for the formation of these products.

INTRODUCTION

Metabolism of dopamine (DA),[2] which is a neurotransmitter in the central nervous system of humans and other vertebrate animals, normally involves the formation of 3-methoxytyramine via *O*-methylation, 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic (homovanillic) acid via oxidative deamination, and norepinephrine via β-hydroxylation (*1*). In insects, DA is a neurotransmitter and is *N*-acylated to *N*-acetyldopamine and *N*-β-alanyldopamine, the main precur-

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[2] Abbreviations used: DA, dopamine; NACySH, *N*-acetylcysteine; LC, liquid chromatography; FAB–MS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; CV, cyclic voltammetry; CA, chronoamperometry; EC, electrochemical detector; DEPT, distortionless enhancement by polarization transfer; 5-NACyS-DA, 5-*S*-(*N*-acetylcysteinyl)dopamine; 2-NACyS-DA, 2-*S*-(*N*-acetylcysteinyl)dopamine; 2,5-di-NACyS-DA, 2,5-*S*,*S*′-di-(*N*-acetylcysteinyl)dopamine.

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sors for quinonoid metabolites involved in sclerotization reactions that lead to adduct and cross-link formation with proteins (*2*). In addition, oxidation of DA to DA quinone is often observed (*3*). In insects at physiological pH, oxidation of DA can involve an intramolecular cyclization reaction that ultimately leads to the formation of a black, insoluble melanin polymer for pigmentation of the cuticle (*2*). Dopamine oxidation and melanin formation might also be associated with Parkinson's disease, where DA-containing cells in the nervous system are susceptible to degeneration (*4*, *5*). DA quinone can also react with nucleophiles such as glutathione, cysteine (*6*, *7*), and sulfhydryl groups in proteins (*8*, *9*). Some of these reactions occur physiologically; for example, the nucleophilic addition product, 5-*S*-cysteinyldopamine, has been detected in striatal tissue from various species (*10*).

The reactions of DA quinone with L-cysteine at physiological pH have been studied previously by continuously electrolyzing a mixture of DA and excess L-cysteine (*11*). Since the initial cysteinyl adducts are oxidized to their corresponding quinones at the applied potential, and since the amine moiety of the cysteinyl adduct also functions as a nucleophile, numerous products were formed, many of which were not identified.

The goal of our work was to clarify the initial steps of the oxidation reaction pathway of DA when a model protein nucleophile is present in excess. To determine the initial site(s) of nucleophilic attack on DA quinone, DA was first oxidized in the absence of a nucleophile to produce DA quinone. The electrolysis was terminated before mixing DA quinone with a nucleophile. This procedure minimizes the generation of additional oxidation products that would be formed if excess chemical oxidant were used to oxidize DA or if exhaustive electrolysis of DA were carried out in the presence of a nucleophile. Since the amino group in cysteine and cysteinyl adducts can act as a nucleophile (*11*), the amino group was acetylated in order to render it unreactive.

Products from the reactions of DA quinone with NACySH were analyzed using reversed-phase liquid chromatography (LC). The major products were purified using semipreparative LC and characterized by fast atom bombardment mass spectrometry (FAB–MS), nuclear magnetic resonance spectroscopy (NMR), uv/vis spectroscopy, and cyclic voltammetry (CV). CV was used to characterize the electrochemical behavior of this system. Rate constants for the addition reactions were estimated using chronoamperometry (CA).

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from commercial sources and used as received: DA and NACySH (Sigma Chemical Co., St. Louis, MO);[3] formic acid, ammonium formate and disodium ethylenediaminetetraacetate (EDTA) (Fisher Scientific Co., Pittsburgh, PA); methanol (uv cutoff: 204 nm) (Baxter Healthcare

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

Corporation, Burdick & Jackson Division, Muskegon, MI); KCl (Mallinckrodt Specialty Chemicals, Chesterfield, MO); and HCl (J. T. Baker Chemical Co., Phillipsburg, NJ).

Electrochemical Preparation of DA Quinone

DA was oxidized in 0.01 M HCl and 0.09 M KCl (pH 2.0) in a custom-made coulometric cell. A platinum gauze working electrode and a Ag/AgCl (saturated KCl) reference electrode were placed in the sample compartment. A platinum auxiliary electrode was separated from this compartment by means of a 0.45-μm nylon 66 membrane so that electrolysis could be conducted efficiently and without mixing of the anode and cathode reaction products. A custom-built three-electrode potentiostat was used to apply a constant potential and to give instantaneous analog current and digital coulomb readouts (*12*). The potential for coulometric oxidation of DA was controlled at 700 mV vs Ag/AgCl (saturated KCl). Electrolysis was terminated when the current-time curve reached a plateau value, indicating that DA oxidation was complete. Electrolysis for 3 min was sufficient for complete oxidation (>99%) of 0.5 ml of 1 mM DA. DA quinone was found to be relatively stable at pH 2.0 in a separate uv spectrophotometric experiment with the decrease in absorbance at 390 nm being less than 0.75% after 3 min (Huang *et al.,* unpublished data).

Analytical LC and Electrochemical Studies of the Reactions of DA Quinone with NACySH

The compositions of reaction mixtures that resulted from mixing 0.4 ml 1 mM DA quinone with 0.2 ml 10 mM NACySH at either pH 7.4 or pH 2.0 were analyzed by LC. The LC system consisted of a Beckman (Berkeley, CA) Model 332 gradient liquid chromatography system equipped with two Model 110A pumps and a Model 420 controller, a Hewlett–Packard (Palo Alto, CA) HP 8452A diode array spectrophotometer equipped with a 1-cm quartz flow cell (Pyrocell Manufacturing Co., Inc., Westwood, NJ), and a Bioanalytical Systems (West Lafayette, IN) LC-4B dual amperometric detector connected to a Hewlett–Packard HPLC ChemStation via a Hewlett–Packard 35900 multichannel interface. The uv/vis spectra of the LC effluent were recorded every 10 s for the duration of the experiment within the 220- to 800-nm wavelength range. The dual amperometric detector that was used for electrochemical detection of the LC effluent consisted of two glassy carbon working electrodes, a Ag/AgCl (3 M KCl) reference electrode, and a stainless steel auxiliary electrode. Electrode potentials were 800 and −100 mV. The former potential is sufficient to oxidize DA and all *N*-acetylcysteinyl adducts of DA, whereas the latter potential is sufficient to reduce DA quinone and all adduct quinones. The two working electrodes were arranged in a parallel configuration so that both electrochemically oxidizable species and reducible species in the LC effluent could be detected simultaneously. Two chromatograms were recorded from the electrochemical (EC) detector and are referred to as LC–EC (oxidation) and LC–EC (reduction) chromatograms. Separation was achieved on a Microsorb-MV C18 column (5 μm, 4.6 × 250 mm) (Rainin Instrument Co., Inc., Woburn, MA) with a binary mobile phase system in which solvents A and B were used. Solvent

A was 150 mM formic acid, 30 mM ammonium formate, and 0.1 mM EDTA (pH 3.0), and solvent B was 50% methanol, 180 mM formic acid, 8 mM ammonium formate, and 0.1 mM EDTA (pH 3.0). The mobile phase gradient was 0 min, 95% solvent A and 5% solvent B; 0–30 min, linear gradient from 5% solvent B to 30% solvent B; 30–40 min, linear gradient from 30% solvent B to 100% solvent B. The flow rate was at 1 ml/min; a 180-μl sample injector loop was used.

A Bioanalytical Systems BAS-100W electrochemical system was used to perform CV and CA. A BAS glassy carbon electrode with an area of 7.1 mm^2 was used as the working electrode. The reference and auxiliary electrodes were a Ag/AgCl (saturated KCl) electrode and a Pt wire electrode, respectively. A small electrochemical cell with a capacity of 1 ml was used. CV of 0.3 mM DA in the presence of 0, 0.3, or 1.5 mM NACySH in 0.1 M phosphate buffer (pH 7.0) and 0.3 mM DA in the presence of 0 or 3.0 mM NACySH in 0.01 M HCl and 0.09 M KCl (pH 2.0) was carried out. The scan rate was 200 mV/s. CA data were acquired for 0.3 mM DA in the absence and presence of 3.0 mM NACySH. The potential was stepped from −0.2 to 0.6 V when in 0.1 M phosphate buffer at pH 7.0 and from 0 to 0.8 V when in 0.01 M HCl and 0.09 M KCl at pH 2.0. These oxidation potentials were sufficiently positive to oxidize DA and all *N*-acetylcysteinyl adducts under these experimental conditions.

Synthesis and Purification of Products from Reaction of DA Quinone with NACySH

A DA quinone solution, prepared by the electrolysis of 10 ml 15 mM DA in 0.01 M HCl and 0.09 M KCl, was added dropwise to 5 ml 150 mM NACySH in 0.5 M phosphate buffer (pH 7.4). The final reaction mixture was colorless and its pH remained at 7.4. The reaction mixture was lyophilized to reduce the volume, and the concentrated solution was filtered and subjected to a semipreparative LC separation. The LC system described above was used, but with the electrochemical detector disconnected. Separation was achieved on a Phenomenex (Torrance, CA) C18 semipreparative column (10 μm, 250 × 10 mm). The mobile phase gradient employed was 0 min, 95% solvent A and 5% solvent B; 0–40 min, linear gradient from 5% solvent B to 40% solvent B. The flow rate was 4 ml/min; a 5-ml sample loop was used. Each product effluent was collected and subjected to a desalting step using the semipreparative LC column. After a product solution was injected onto the column, the salt components were eluted first using distilled water as the mobile phase; the product was then eluted using 25% methanol. The effluent containing the product was subsequently lyophilized to dryness.

Characterization of Products

FAB–MS was performed on a Hewlett–Packard HP 5989A MS Engine. Proton NMR and distortionless enhancement by polarization transfer (DEPT) NMR were performed with a Varian (Palo Alto, CA) UNITY*plus* 400 MHz spectrometer. Products were dissolved in 0.8 ml D_2O. The uv/vis spectroscopic measurements of the reaction products in 0.01 M HCl were performed using the HP 8452A diode-array spectrophotometer with a 1.0 × 0.4-cm quartz cuvette. Cyclic voltammograms

of reaction products in 0.1 M phosphate buffer (pH 7.0) were obtained using the BAS-100W electrochemical system. The scan rate was 200 mV/s.

RESULTS

LC Analysis of Reaction Products of DA Quinone with NACySH

The products obtained from mixing 0.4 ml 1 mM DA quinone with 0.2 ml 10 mM NACySH at either pH 7.4 or pH 2.0 were separated by analytical LC. No discernible peaks were observed in the LC–EC (reduction) chromatograms (data not shown). Four major peaks and several minor peaks were obtained in the LC–EC (oxidation) chromatogram for the reaction conducted at pH 7.4 (Fig. 1A). The peak at 7.26 min is due to DA (**1,** see Scheme 1), while the peaks at 11.23, 21.24, and 25.62 min are due to three major reaction products, identified here as compounds **3, 4,** and **7,** respectively. The same products are observed at pH 2.0, but two of the products, **1** and **7,** are formed in much lower yields than at pH 7.4 (Fig. 1B). The minor peak at 9.1 min is due to NACySH, whereas the minor peak at 8.2 min is due to an impurity in the starting material. The other minor peaks are unidentified.

NMR and MS Identification of Reaction Products

Products **3, 4,** and **7** were first characterized by FAB–MS. The results showed that **3** and **4** both have MH^+ at m/z=315 and that **7** has MH^+ at m/z=476 (spectra not shown). These results strongly suggest that **3** and **4** are both monoaddition adducts (theoretical m/z: 315) of NACySH with DA quinone, whereas **7** is a diaddition adduct of two NACySH molecules with DA quinone (theoretical m/z: 476).

The structures of these adducts were elucidated by NMR spectroscopy (Table 1). The ^{1}H NMR spectrum of **4** shows that the molecule contains two aromatic protons that give *meta*-coupling (J = 2.0 Hz) (Fig. 2A). Because this result indicates that the *N*-acetylcysteinyl moiety is bound to C(5) of the aromatic ring of DA, **4** is identified as 5-*S*-(*N*-acetylcysteinyl)dopamine (5-NACyS-DA). The ^{1}H NMR spectrum of **3** shows that the molecule contains two aromatic protons that display *ortho*-coupling (J = 8.2 Hz), indicating that the *N*-acetylcysteinyl moiety is bound to C(2) of the aromatic ring of DA (Fig. 2B). Therefore, **3** is identified as 2-*S*-(*N*-acetylcysteinyl)dopamine (2-NACyS-DA).

The ^{1}H NMR spectrum of the diaddition adduct **7** shows only a singlet in the aromatic region (Fig. 2C), which suggests that both of the *N*-acetylcysteinyl moieties are attached to the aromatic ring of DA. Since the signals of the DA side chain protons overlap with the signals of the protons in the *N*-acetylcysteinyl moiety in this ^{1}H NMR spectrum, a DEPT NMR experiment was conducted to confirm that the *N*-acetylcysteinyl moieties are not attached to the DA side chain carbons (Fig. 3). The DEPT NMR spectrum exhibits four 180° out-of-phase signals, demonstrating that four $-CH_2-$ groups are present in the molecule, two of which are in the two *N*-acetylcysteinyl moieties and two of which are in the DA side chain. This confirms that both *N*-acetylcysteinyl moieties are bound to the aromatic ring of DA. Based on the fact that there is little or no 6-monoaddition product present in the reaction

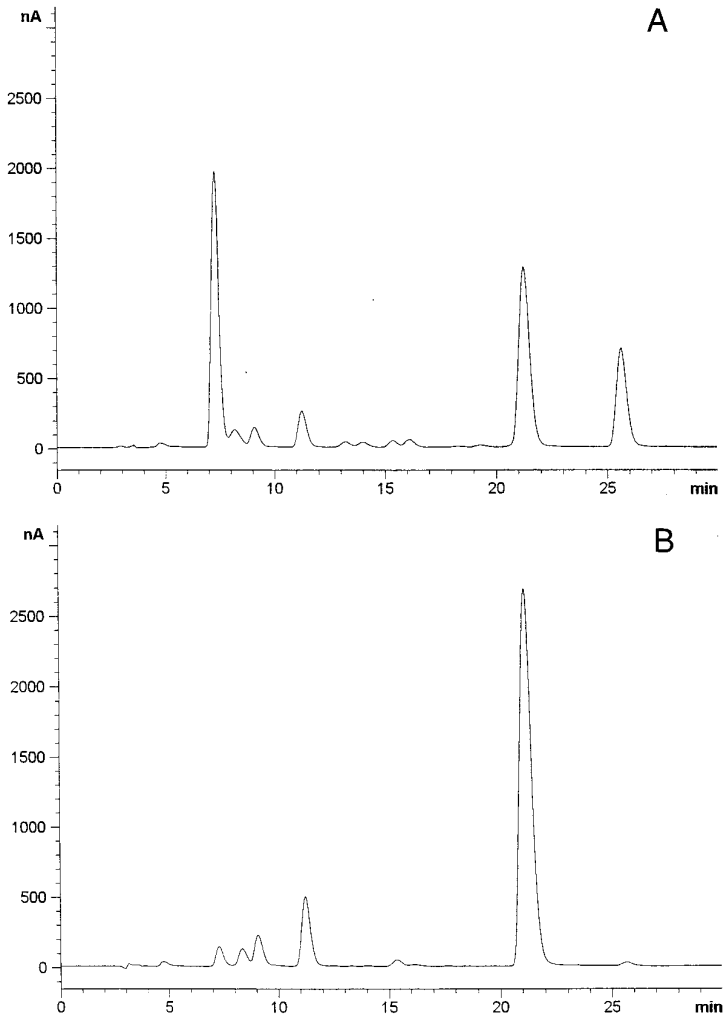
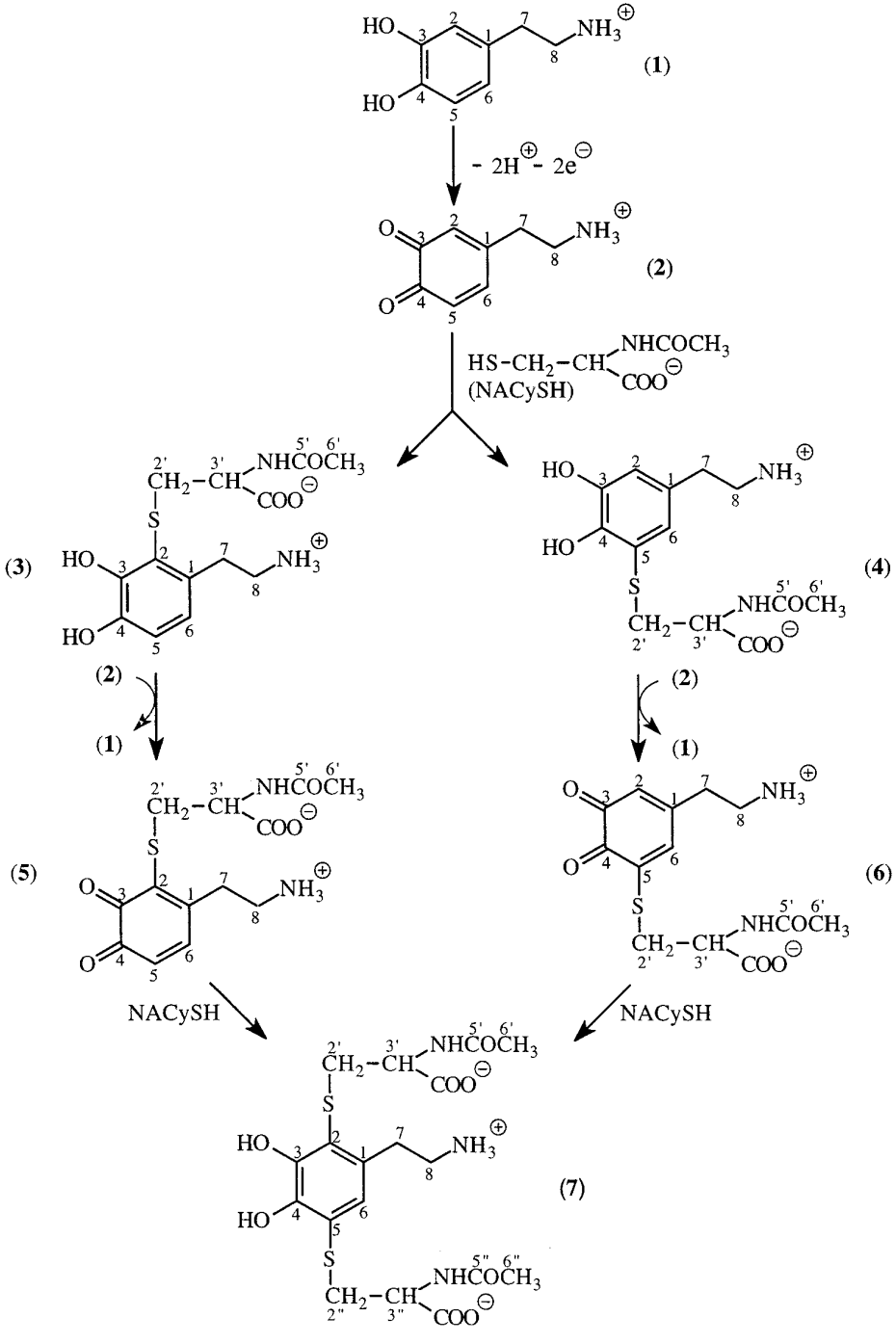


FIG. 1. LC–EC (oxidation) chromatograms of the reaction products of DA quinone and NACySH at (A) pH 7.4 and (B) pH 2.0.

mixture, C(6) of the DA quinone ring is presumed to be the site that is least reactive with NACySH. Accordingly, because assignments where two *N*-acetylcysteinyl moieties are bound to the 2,6-carbons or 5,6-carbons of the DA aromatic ring are excluded, the two *N*-acetylcysteinyl moieties must be bound to C(2) and C(5) of the DA aromatic ring in adduct **7.** This species is identified as 2,5-*S*,*S*′-di-(*N*-acetylcysteinyl)dopamine (2,5-di-NACyS-DA).

The uv/vis and Electrochemical Characterization of the Reaction Products

The adducts were also characterized using uv/vis spectrometry and CV. The uv/vis spectra show that, whereas both monoaddition adducts (**3** and **4**) have the



SCHEME 1. Proposed mechanism for formation of adducts from reactions of DA quinone with NACySH.

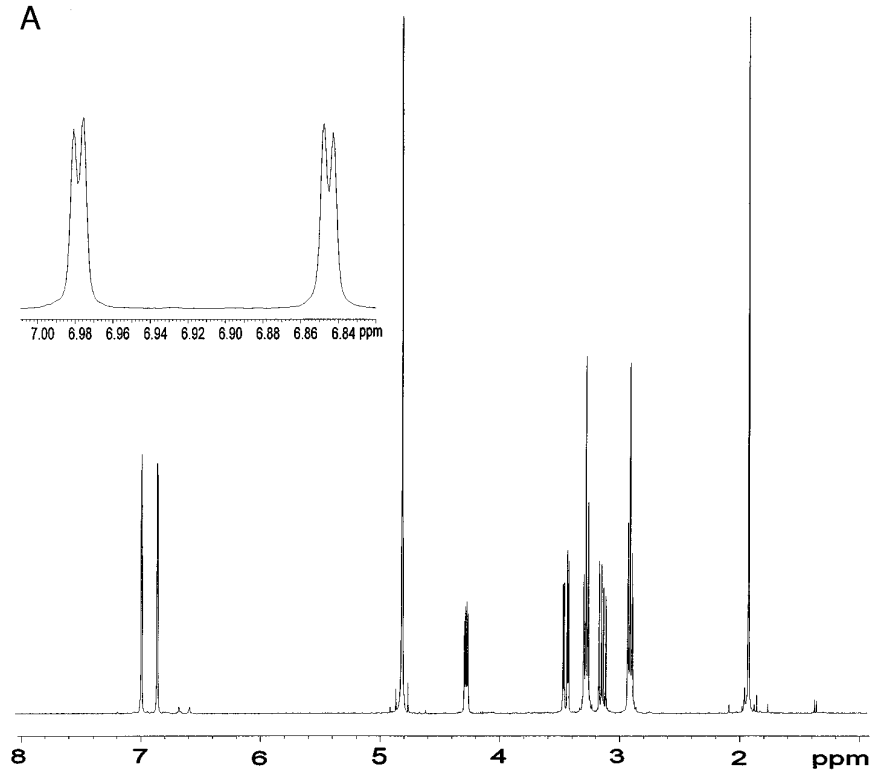


FIG. 2. ^{1}H NMR spectra of addition products from reaction of DA quinone with NACySH: (A) monoaddition product 5-NACyS-DA (**4**), (B) monoaddition product 2-NACyS-DA (**3**), and (C) diaddition product 2,5-di-NACyS-DA (**7**).

same λ_{max} values, 258 and 294 nm, they have different molar absorption coefficients at each λ_{max} (Table 2). In contrast, the diaddition adduct (**7**) has only one λ_{max} at 276 nm and a shoulder at 302 nm. CV data show that all three adducts have slightly less positive redox potentials than DA at pH 7.0, indicating that they would be readily oxidized by any unreacted DA quinone in the reaction mixture (Table 2). Other characteristic data of these adducts are also presented in Table 2.

To estimate the relative molar ratios of adducts **3, 4,** and **7** formed at pH 7.4 and pH 2.0, LC–UV chromatograms (absorbance at λ_{max} versus time) of the reaction mixtures were obtained (not shown). Based on the peak areas and respective molar absorption coefficients, the relative molar ratios of adducts **3, 4,** and **7** are estimated to be 8:59:33 at pH 7.4 and 10:89:1 at pH 2.0.

Electrochemical Studies of DA in the Presence of NACySH

The cyclic voltammogram of DA at pH 7.0 in the absence of NACySH consists of a single anodic peak near 0.26 V for the two-electron oxidation of DA to its corresponding quinone and a single cathodic peak near 0.13 V for the reduction