ANALYTICAL BIOCHEMISTRY 231, 72–81 (1995)

On-Column Reduction of Catecholamine Quinones in Stainless Steel Columns during Liquid Chromatography

Rongda Xu,* Xin Huang,* Karl J. Kramer,† and M. Dale Hawley*

*Department of Chemistry, Kansas State University, Manhattan, Kansas 66506; and †U.S. Grain Marketing Research Laboratory, Agricultural Research Services, United States Department of Agriculture,[1] Manhattan, Kansas 66502

Received April 20, 1995

The chromatographic behavior of quinones derived from the oxidation of dopamine and *N*-acetyldopamine has been studied using liquid chromatography (LC) with both a diode array detector and an electrochemical detector that has parallel dual working electrodes. When stainless steel columns are used, an anodic peak for the oxidation of the catecholamine is observed at the same retention time as a cathodic peak for the reduction of the catecholamine quinone. In addition, the anodic peak exhibits a tail that extends to a second anodic peak for the catecholamine. The latter peak occurs at the normal retention time of the catecholamine. The origin of this phenomenon has been studied and metallic iron in the stainless steel components of the LC system has been found to reduce the quinones to their corresponding catecholamines. The simultaneous appearance of a cathodic peak for the reduction of catecholamine quinone and an anodic peak for the oxidation of the corresponding catecholamine occurs when metallic iron in the exit frit reduces some of the quinones as the latter exits the column. This phenomenon is designated as the "concurrent anodic–cathodic response." It is also observed for quinones of 3,4-dihydroxyphenylalanine, 2,4,5-trihydroxyphenylalanine, 3,4-dihydroxyphenylpropenoic acid, and 3,4-dihydroxybenzoic acid and probably occurs with *o*- or *p*-quinones of other dihydroxyphenyl compounds. The use of nonferrous components in LC systems is recommended to eliminate possible on-column reduction of quinones.

Oxidation of various dihydroxyphenyl compounds to quinones is an important reaction in many natural systems. For example, during sclerotization of insect cuticle, the catecholamines *N*-acetyldopamine (NADA)[2] and *N*-β-alanyldopamine are oxidatively incorporated with cuticular proteins (1,2). The oxidative browning of polyphenols in plant-derived foods and beverages results in loss of nutritional and aesthetic value (3,4). The oxidation of dopamine (DA), a neurotransmitter, is associated with Parkinson's disease (5), and its hydroxylation product, 2,4,5-trihydroxyphenethylamine (6-hydroxydopamine, 6-OH-DA), is highly toxic to the central nervous system (6). Studies of oxidative pathways involving these dihydroxyphenyl compounds via the analysis of quinones, other intermediates, and products are therefore of considerable interest.

Liquid chromatography (LC), coupled with various on-line detectors, such as diode array, electrochemical, and mass spectrometric detectors, is a powerful tool used to purify and characterize complex mixtures of reaction products. However, since many quinones are unstable and subject to degradation, such as by reduction (7) or by nucleophilic attack from either water (4) or buffer components, care must be taken when one interprets a chromatogram and identifies products from the oxidation of dihydroxyphenyl compounds using LC analysis.

In a recent paper (6), the products of DA and NADA oxidation mediated by $NaIO_4$ or mushroom tyrosinase were analyzed by LC using a C18 reversed-phase LC column, an acidic mobile phase, and a single electrochemical detector that operated in either the oxidative or reductive mode. An interesting phenomenon was observed in that an unknown electrochemically oxidizable species eluted at the same retention time as the reducible product, the quinone. In one of the experiments, a reaction mixture consisting of equal volumes

[1] The Agricultural Research Service, USDA is an equal opportunity/affirmative action employer and all agency services are available without discrimination.

[2] Abbreviations used: NADA, *N*-acetyldopamine; DA, dopamine; 6-OH-DA, 6-hydroxydopamine; LC, liquid chromatography; DAD, diode array detector; EC, electrochemical detector; SOS, sodium octyl sulfate; PEEK, polyetheretherketone.

0003-2697/95 $12.00

of 1 mM DA in 24 mM HCl and 2 mM $NaIO_4$ was injected into the column. When the electrochemical detector was used in the reductive mode, an electrochemically reducible species, DA quinone, was detected. When the electrochemical detector was used in the oxidation mode, however, two anodic peaks were observed. One of the peaks occurred at the normal retention time of DA and was attributed to the oxidation of unreacted DA. The other peak had the same retention time as the cathodic peak for DA quinone. It was postulated that this electrochemically oxidizable species was DA semiquinone that was formed either by a one-electron oxidation of DA or from the comproportionation of DA and DA quinone.

Under acidic conditions the probability of DA semiquinone forming during the oxidation of DA does not appear to be high. Although semiquinones derived from catechols and catecholamines have been reported to occur in various systems (8–11), they have been mainly detected by electron spin resonance under neutral or basic conditions. Semiquinones would not likely be present when using acidic reaction buffers or an acidic mobile phase during LC. Furthermore, because the oxidizing agent is present in excess of the amount needed to fully oxidize the catecholamines to their quinones, it is unlikely that the oxidizable species that elutes at the same retention time as the quinone is the semiquinone.

In this study experiments have been conducted to study the identities and the origins of the electrochemically oxidizable species that elute at the same retention times as the quinones during reversed-phase LC analysis of DA quinone and NADA quinone. The LC system used in this study was equipped with both a diode array detector (DAD) and an electrochemical detector (EC) in series. The electrochemical detector was configured with parallel dual working electrodes, which allowed for a more thorough investigation of the phenomenon where both oxidative and reductive processes occur concurrently at the same retention time. This phenomenon will be referred to as the "concurrent anodic–cathodic response" in this paper.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from commercial sources and used as received: DA and NADA (Sigma Chemical Co., St. Louis, MO)[3]; $NaIO_4$ (Aldrich, Milwaukee, WI); ammonium formate, disodium ethylenediaminetetraacetate (EDTA), and citric acid (Fisher Scientific Co., Fair Lawn, NJ); sodium octyl sulfate (SOS) (Eastman Kodak Co., Rochester, NY); methanol (uv cutoff, 204 nm) and acetonitrile (uv cutoff, 189 nm) (Baxter Healthcare Corp., Burdick & Jackson Division, Muskegon, MI); KCl and iron wire (Mallinckrodt Specialty Chemicals, Chesterfield, MO).

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

Oxidations of Catecholamines

For chemical oxidation of DA and NADA, a procedure similar to that of Li and Christensen (6) using excess $NaIO_4$ was performed. Oxidation was effected by mixing 2 mM $NaIO_4$ in 0.01 M HCl with an equal volume of either 1 mM DA or 1 mM NADA in 0.01 M HCl and incubating for 40 s.

For electrochemical oxidation of DA and NADA, a custom-made coulometric microcell with a platinum gauze working electrode, a Ag/AgCl (saturated KCl) reference electrode, and a platinum auxiliary electrode was used (unpublished data). The working electrode and the reference electrode were placed in one compartment. The 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 the instantaneous digital coulomb readout (12). A Hewlett–Packard (Palo Alto, CA) HP 3390A integrator was used to monitor the electrolysis current. The potential for coulometric oxidation was controlled at 800 mV vs Ag/AgCl (saturated KCl). Electrolysis was stopped when the current–time curve monitored by the integrator reached a plateau value, which indicated that the oxidation of DA or NADA was complete. Three minutes of electrolysis was sufficient for oxidation of 0.5 ml of 1 mM DA or NADA in 0.01 M HCl and 0.09 M KCl. Electrolysis of the catecholamines was determined in a separate study to be quantitative (> 99%) (unpublished data).

LC Analysis

Two LC systems were used to perform reversed-phase LC-DAD-EC analysis. System I 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 HP 8452A diode array spectrophotometer equipped with a 1-cm quartz flow cell (Pyrocell Manufacturing Co., Inc., Westwood, NJ), and a Bioanalytical System (West Lafayette, IN) LC-4B dual amperometric detector connected to a Hewlett–Packard HPLC ChemStation via a Hewlett–Packard 35900 multichannel interface. Together with the same electrochemical detector and the multichannel interface that were used in system I, system II consisted of a Hewlett–Packard 1050 Series HPLC quaternary pump, on-line degasser, and DAD controlled by a Hewlett–Packard HPLC[3D]

ChemStation. Separations were achieved on Microsorb-MV C18 stainless steel columns (5 μm, 4.6 × 250 mm) (Rainin Instrument Co., Inc., Woburn, MA) unless indicated otherwise. In some experiments, an α-chrom C18 PEEK (polyetheretherketone) column (5 μm, 2 × 150 mm) (Upchurch Scientific, Oak Harbor, WA) was used. The flow rates were 1 ml/min for the 4.6-mm-diameter columns, unless otherwise indicated, and 0.25 ml/min for the 2-mm-diameter column.

Four mobile phase systems, referred to as mobile phases I to IV, were used in the course of these studies. Mobile phase I was a binary system in which 80% solvent A (15 mM citric acid, 0.2 mM SOS, and 0.7 mM EDTA, adjusted to pH 3.0 with NaOH) and 20% solvent B (50% acetonitrile) were employed. Mobile phase II consisted of 15 mM citric acid, 0.2 mM SOS, 0.7 mM EDTA, and 10% acetonitrile (adjusted to pH 3.0 with NaOH). Mobile phase III was also a binary system in which 85% solvent C (150 mM formic acid, 30 mM ammonium formate, and 0.1 mM EDTA, pH 3.0) and 15% solvent D (100% methanol) were employed. Mobile phase IV consisted of solvent C only.

During LC analysis, uv/vis spectra were recorded either every 10 s on the diode array spectrophotometer within the wavelength range of 220 to 800 nm with system I or every 0.375 s on the DAD within the wavelength range of 220 to 600 nm with system II. Two LC-DAD chromatograms, uv absorbance at fixed wavelengths vs time, were also obtained. One of the fixed wavelengths was 280 nm, which is the characteristic λ_{max} of most 3,4-dihydroxyphenyl compounds, and the second was 390 nm, which is the characteristic λ_{max} or a wavelength close to the λ_{max} of their *o*-quinones. The chromatograms are referred to as LC-DAD (280 nm) and LC-DAD (390 nm). Electrochemical detection of the LC effluent, after passing through the spectrophotometric detector, was achieved on the dual amperometric detector, which consisted of two glassy carbon working electrodes, a Ag/AgCl (3 M KCl) reference electrode, and a stainless steel auxiliary electrode. The potentials applied to the two working electrodes were 800 and −100 mV. The 800-mV potential is sufficient to oxidize hydroxyphenyl compounds, whereas the −100 mV potential is sufficient to reduce their quinones. The two working electrodes were arranged in a parallel configuration so that both electrochemically oxidizable species and reducible species in the LC effluent were detected simultaneously. Two corresponding LC-EC chromatograms, referred to as LC-EC (oxidation) and LC-EC (reduction), were recorded via the multichannel interface by the HPLC ChemStation or HPLC^{3D} ChemStation. Thus, for a single injection, four LC chromatograms (280 nm, 390 nm, oxidation, and reduction) and many uv/vis spectra were obtained. In some of the figures shown in this paper, the baselines of the chromatograms have been offset in order to clarify data presentation.

For some experiments, a Direct Connect Universal Prefilter (Alltech, Deerfield, IL) was attached to the end of the PEEK column for the LC analysis. This filter contains a 0.5-μm stainless steel filter element.

Spectroscopic Analysis

Ultraviolet/visible measurements were performed using the HP 8452A diode array spectrophotometer with either a 1.0 × 0.4-cm or a 1.0 × 0.8-cm quartz cuvette. In a spectral study of the stabilities of the electrochemically prepared DA quinone and NADA quinone in LC mobile phase II, uv/vis measurements of quinone solutions resulting from the electrolysis of 0.25 mM DA or NADA were initiated 20 s after electrolysis was terminated and performed for 960 s at 10-s intervals at a temperature of approximately 22°C.

In a spectral study of the reaction of NADA quinone with iron metal in mobile phase II, a small magnetic stir bar was placed in a cuvette seated in a cuvette holder with a built-in magnetic stirrer. The stirring speed was regulated at 460 rpm and the temperature of the cuvette was controlled at 25°C by a Hewlett–Packard HP 89090A Peltier temperature controller. Ultraviolet/visible measurements were started when 15 mg of the iron metal was introduced into the cuvette containing 0.25 mM electrochemically prepared NADA quinone in mobile phase II. Spectra in the wavelength range from 220 to 800 nm were recorded at 10-s intervals for 4 min. Spectra of iron alone in mobile phase II were also recorded in the same way and subtracted from the spectra of the quinone in the presence of iron to yield difference spectra.

For computer simulation of uv/vis spectra, a Microsoft Excel Macro program was edited to simulate the spectrum that best fit the experimental spectrum for the effluent from the LC column. Based on the uv/vis spectrum the effluent was suspected to contain a mixture of both the quinone and its corresponding catecholamine. First, uv/vis spectra of the standard DA or NADA and its corresponding electrochemically prepared quinone at the same concentration in the LC mobile phase were recorded using the HP 8452A diode array spectrophotometer. The spectral data were then imported into the Microsoft Excel worksheet. The experimental spectrum recorded at a retention time of interest during the LC-DAD-EC analysis was selected and also sent from the HPLC^{3D} ChemStation to the Microsoft Excel worksheet via Dynamic Data Exchange. A simulated spectrum was then obtained by using the expression

$$A_{\text{sim},i} = k(\alpha A_{1,i} + \beta A_{2,i}),$$

where $A_{1,i}$ and $A_{2,i}$ are the ith absorbance data points

in the standard spectra of the catecholamine and the quinone, respectively; coefficients α and β represent the fractions of the catecholamine and the quinone, respectively, in the mixture ($\alpha + \beta = 1$); and k is a factor compensating for concentration differences between the standard spectra and the experimental spectrum. Factor k and coefficients α and β were adjusted until the simulated spectrum matched the experimental spectrum.

RESULTS

Spectroscopic Analysis and Stability of Catecholamine Quinones

A spectral study of the stabilities of electrochemically prepared DA quinone and NADA quinone in LC mobile phase II was performed. Little spectral change occurred over the 16-min duration of the uv/vis measurements from 220 to 600 nm (data not shown). The results demonstrate that the quinones are relatively stable in this mobile phase. The spectrum of DA quinone has a shoulder at 258 nm and a maximum at 390 nm, whereas that of NADA quinone has maxima at 256 and 396 nm. The spectra of DA and NADA in mobile phase II exhibit a single maximum at 280 nm.

LC Analysis of Catecholamine Quinones

The mobile phase used by Li and Christensen (6) was modified slightly in order to improve separation obtained with our LC systems. Analysis of the reaction mixture of NADA with $NaIO_4$ using LC system II gave rise to four chromatograms (Fig. 1). There are two chemically significant peaks in the LC-DAD (280 nm) chromatogram (denoted as peaks I_{280} and II_{280} in Fig. 1A), one peak in the LC-DAD (390 nm) chromatogram (peak I_{390} in Fig. 1B), two peaks in the LC-EC (oxidation) chromatogram (peaks I_{ox} and II_{ox} in Fig. 1C), and one peak in the LC-EC (reduction) chromatogram (peak I_{rd} in Fig. 1D). Because peaks I_{ox}, I_{rd}, I_{280}, and I_{390} have the same retention time, they sometimes will be denoted collectively as peak I, and, similarly, peaks II_{280} and II_{ox} sometimes will be denoted collectively as peak II. Analogous results were obtained for the LC analysis of DA quinone (data not shown). These LC-EC chromatograms are similar to those obtained by Li and Christensen (6).

The effluent for peak I affords both anodic (I_{ox}) and cathodic (I_{rd}) responses. This phenomenon is designated as the "concurrent anodic–cathodic response." In addition, two other features are evident. First, I_{rd} and I_{390} are slightly narrower than I_{ox} and I_{280}, respectively. Second, both I_{ox} and I_{280} exhibit tailing, which appears as an elevated baseline that extends to peaks II_{ox} and II_{280}, respectively. Peak II has the same retention time as that of either the DA or the NADA standard (Fig. 1). Furthermore, the corresponding uv/vis spectrum, which has a maximum at 280 nm, is identical to that of the catecholamine standard. Hence, the species giving rise to peak II must be the catecholamine.

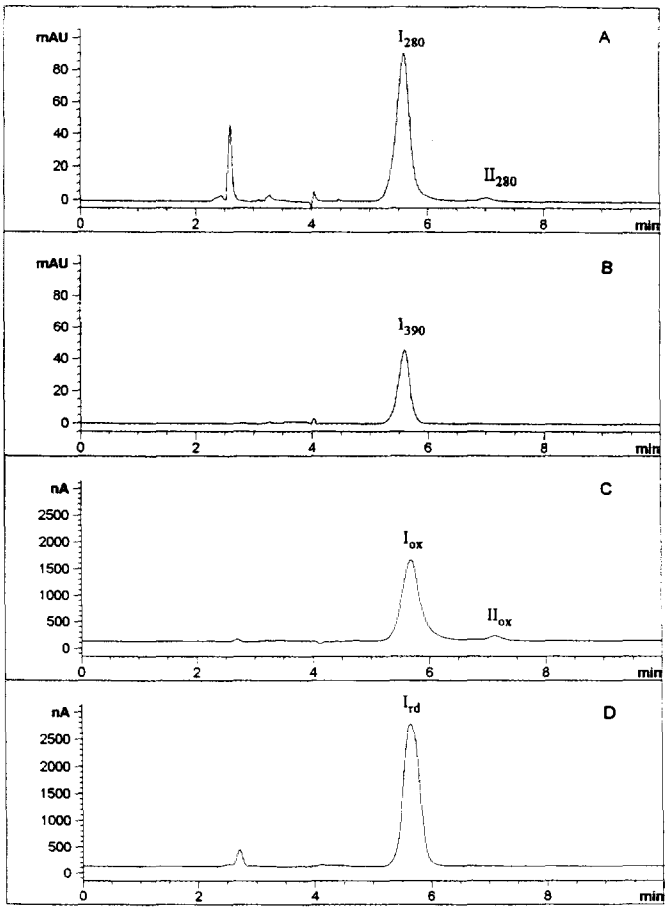

FIG. 1. LC-DAD-EC analysis of NADA quinone using a stainless steel column. A 50-μl aliquot of 0.5 mM chemically prepared NADA quinone was analyzed using LC system II and mobile phase I. (A) LC-DAD (280 nm) chromatogram, (B) LC-DAD (390 nm) chromatogram, (C) LC-EC (oxidation) chromatogram, (D) LC-EC (reduction) chromatogram.

Electrochemically prepared DA quinone and NADA quinone yielded similar chromatographic results as those prepared chemically. Furthermore, a concurrent anodic–cathodic response was observed using different mobile phases as well as with quinone samples derived from the oxidations of 3,4-dihydroxyphenylalanine, 2,4,5-trihydroxyphenylalanine, 3,4-dihydroxyphenylpropenoic acid, and 3,4-dihydroxybenzoic acid (data not shown).

Effects of flow rate and sample volume. When the mobile phase flow rate was decreased from 1.0 to 0.5 ml/min, the ratio of the anodic to the cathodic currents for the concurrent anodic–cathodic response was in-

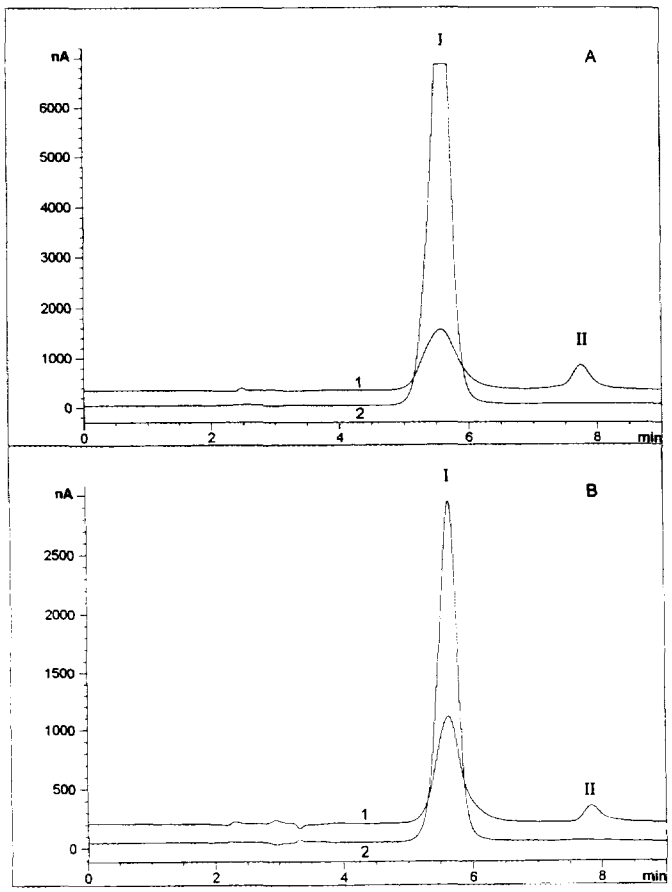

FIG. 2. Effect of sample volume of NADA quinone on the concurrent anodic–cathodic response during LC analysis using a stainless steel column. An aliquot of 0.5 mM electrochemically prepared NADA quinone (A, 180 μl and B, 20 μl) was analyzed using LC system I and mobile phase II. Only LC-EC chromatograms (1, oxidation and 2, reduction) are shown.

creased (data not shown). This result demonstrates that the amounts of the oxidizable species that coelute with the quinones increase with increasing residence times of the samples on the LC column.

The effect of sample volume was also examined. The LC chromatograms obtained from analysis of 180 and 20 μl of 0.5 mM NADA quinone using LC system I and mobile phase II show that the ratio of the anodic to the cathodic currents of the concurrent anodic–cathodic response decreases with increasing volume of sample injected (Fig. 2).

Effect of LC column. In a direct injection experiment, electrochemically prepared quinones were injected into the LC system in which the column had been removed. The result showed that only a small amount of oxidizable species was present (data not shown), indicating that the oxidizable species is produced principally when the quinones are exposed to the LC column.

LC-DAD-EC analyses using different LC columns were also performed. Figure 3 presents LC-EC chromatograms of NADA quinone obtained using three different columns, LC system II, and mobile phase III. The results show that the oxidizable components of peaks I and II are substantial when using either one of the two stainless steel C18 columns (Figs. 3A and B). However, the ratio of the anodic to the cathodic currents for peak I varies from one stainless steel column to the other, even though they are the same type of column from the same manufacturer. The uv/vis spectra recorded for peak I have λ_{max} values at 280 and 396 nm. The height of peak II, which is due to oxidation of NADA, varies as well. In contrast to the results obtained using stainless steel columns, the oxidizable components of peaks I and II, as well as the concurrent anodic–cathodic response phenomenon, are almost negligible when using a PEEK column (Fig. 3C). The uv/vis spectrum recorded for peak I has λ_{max} values at 256 and 396 nm and is identical to that of the NADA quinone standard. Similar results were obtained with DA quinone (data not shown). These results indicate that the concurrent anodic–cathodic response is

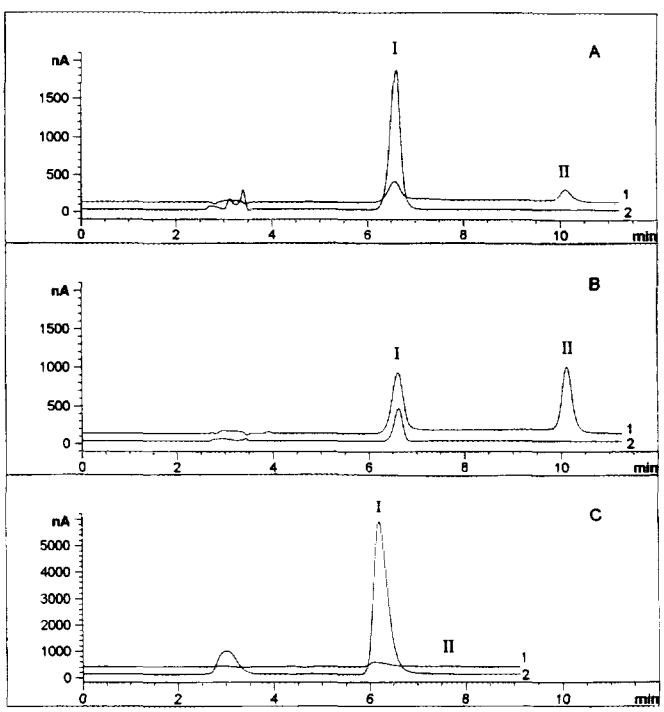

FIG. 3. LC analyses of NADA quinone using different columns. A 20-μl aliquot of 0.5 mM chemically prepared NADA quinone was analyzed using LC system I, mobile phase III, and different columns. Only LC-EC chromatograms (1, oxidation and 2, reduction) are shown. (A) and (B) were obtained on C18 stainless steel columns (5 μm, 4.6 × 250 mm) using a 1.0 ml/min flow rate, whereas (C) was obtained on a C18 PEEK column (5 μm, 2 × 150 mm) using a 0.25 ml/min flow rate.

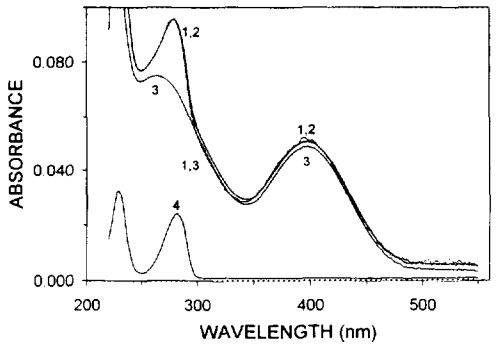

FIG. 4. Simulation of the uv/vis spectrum at the apex of the peak corresponding to the concurrent anodic–cathodic response shown in Fig. 1. Spectrum 1 is the experimental spectrum. Spectrum 2 is the simulated spectrum, which is a summation of weighted standard spectra of NADA quinone (spectrum 3) and NADA (spectrum 4).

caused by exposure of the quinones to stainless steel columns.

Characterization of the Concurrent Anodic–Cathodic Response Phenomenon

The uv/vis spectrum that was acquired at the apex of peak I during LC analysis of DA quinone or NADA quinone has only two absorption maxima at 280 and either 390 or 396 nm, respectively. A separate experiment using LC system I demonstrated that there is no absorption at wavelengths from 500 to 800 nm for the effluent at peak I (data not shown). The cathodic peak (I_{rd}) of the concurrent anodic–cathodic response and the absorption maximum at either 390 or 396 nm are consistent with the presence of DA quinone or NADA quinone. However, since quinones cannot be oxidized at the applied potential of the anode (0.8 V) and since quinones do not have λ_{max} at 280 nm, the anodic peak (I_{ox}) must be caused by some other component that has a λ_{max} at 280 nm.

Since both DA and NADA have λ_{max} at 280 nm, the possibility that the absorption maximum at 280 nm of the concurrent anodic–cathodic response effluent is due to DA or NADA was examined. If DA or NADA was one of the components that gave rise to the concurrent anodic–cathodic response, the uv/vis spectra of peak I would be a summation of the catecholamine and catecholamine quinone spectra. Figure 4 shows a simulated spectrum for a mixture of NADA quinone (83%) and NADA (17%), which matches the experimental spectrum obtained at the apex of the concurrent anodic–cathodic response peak I in Fig. 3. This result is consistent with the oxidizable component of the concurrent anodic–cathodic response being the corresponding catecholamine and the reducible component being the quinone.

Whether DA or NADA is the oxidizable component of the concurrent anodic–cathodic response effluent was further examined by a collection–reinjection experiment. LC-DAD-EC analysis of a 150-μl aliquot of 0.5 mM NADA quinone was performed on a stainless steel column using LC system II (Fig. 5A). The chromatograms show a significant concurrent anodic–cathodic response at a retention time of 6.8 min (peak I) as well as an anodic response at 10.2 min (peak II), which is due to NADA. A similar experiment was performed on the same column using LC system I, but this time with the EC detector disconnected. The effluent at the apex of the peak at 6.8 min was collected and a 150-μl aliquot was immediately subjected to LC analysis using the PEEK column and LC system II. Since the electrochemical detector was disconnected when the effluent was collected, the effluent could not undergo electrochemical oxidation or reduction during LC-DAD-EC analysis. The chromatograms thus obtained after reinjection reveal that the concurrent anodic–cathodic response effluent contains the redox couple NADA quinone and NADA (Fig. 5B). There is no evidence for the presence of another species.

Variation of Quinone to Catecholamine Ratio within the Concurrent Anodic–Cathodic Response Peak

Further examination of uv/vis spectra at different elution times within the concurrent anodic–cathodic response peak of Fig. 1 reveals that the uv/vis spectra are not the same throughout the peak profile (Fig. 6). It was noted above that peak I_{390} is slightly narrower than peak I_{280}. At both ends of peak I_{280} (elution times

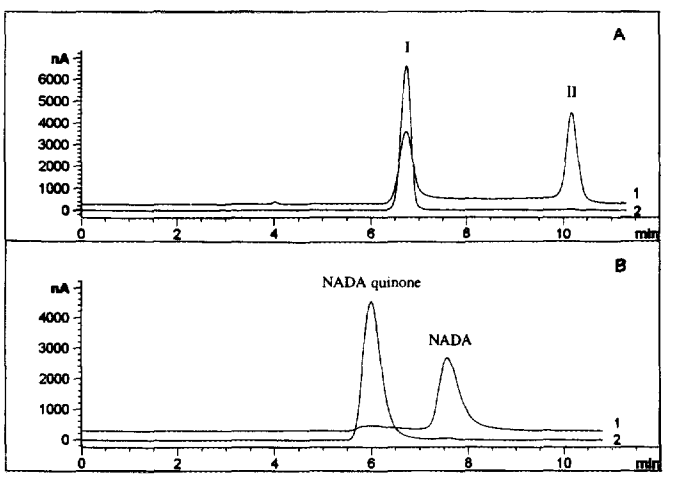

FIG. 5. A collection–reinjection experiment of the concurrent anodic–cathodic response effluent using LC-DAD-EC analysis. Only LC-EC chromatograms (1, oxidation and 2, reduction) are shown. (A) A 150-μl aliquot of 0.5 mM electrochemically prepared NADA quinone was analyzed using LC system II, mobile phase III, and a stainless steel column. (B) A 150-μl aliquot of the effluent collected at 6.8 min from an injection similar to (A) using LC system I with the electrochemical detector disconnected was analyzed using LC system II, mobile phase III, and the PEEK column.

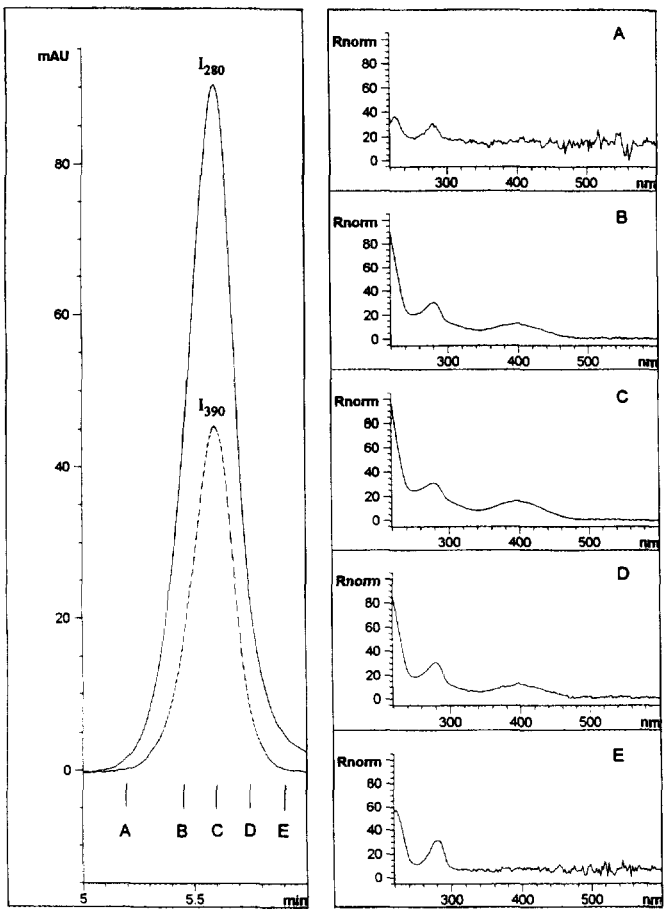

FIG. 6. Ultraviolet/visible spectra recorded at different elution times within peaks I_{280} and I_{390} during LC-DAD-EC analysis of the chemically prepared NADA quinone shown in Fig. 1. Peaks I_{280} and I_{390} in Fig. 1 are shown (left) with the positions marked that correspond to spectra A to E (right). Rnorm = normalization based on absorbance at 280 nm.

A and E in Fig. 6, which are outside of peak I_{390}), each spectrum has only the absorption maximum at 280 nm, indicating that only NADA is present. At elution times B to D (Fig. 6), the spectra all have two maxima at 280 and 396 nm, indicating that both NADA and NADA quinone are present. Simulations of spectra show that the ratios of NADA quinone to NADA vary throughout peak I and that the maximal ratio occurs at the apex of peak I. For elution times A to E in Fig. 6, the ratios of NADA quinone to NADA are 0:100, 70:30, 83:17, 63:37, and 0:100, respectively. Similarly, simulations for the concurrent anodic–cathodic response for a DA quinone sample show that the ratios of DA quinone to DA vary within peak I and that the maximal ratio also occurs at the apex (data not shown).

Characterization of Elevated Baseline

The elevated baseline that extends from the concurrent anodic–cathodic response peak (peak I) to the catecholamine peak (peak II) has been noted previously. Spectral examination of the effluent at all retention times throughout this region reveals the presence of a species that has a spectrum identical to that of the catecholamine (data not shown). This result shows that a low level of the catecholamine is continuously eluted from the column between the retention times of the concurrent anodic–cathodic response peak and the catecholamine peak.

Cause of Catecholamine Formation during LC Using a Stainless Steel Column

Reduction of NADA quinone by iron metal. The difference between a stainless steel C18 column and a PEEK C18 column is the chemical composition of the frits and tubing of the columns. The former is made of stainless steel, whereas the latter is made of PEEK, a chemically inert polymer. To study the possibility that quinone reduction is caused by iron metal from the LC stainless steel column, the stability of the quinone in the presence of iron metal was monitored spectroscopically. Figure 7 shows several uv/vis spectra of NADA quinone in the presence of iron in LC mobile phase II (Fig. 7A), as well as spectra of the same amount of iron alone in the LC mobile phase (Fig. 7B). In Fig. 7B an absorption band with a maximum at 258 nm that increases with time is observed. This band is identical to that of ferrous ion (data not shown). To compensate for the contribution of Fe^{2+} to the spectra of Fig. 7A, each spectrum in Fig. 7B was subtracted from the corresponding spectrum in Fig. 7A. The difference spectra are shown in Fig. 7C. Since NADA quinone is stable in the mobile phase, the difference spectra in Fig. 7C reflect the effect of iron on the stability of NADA quinone. In approximately 4 min, the quinone is almost completely consumed and a species with a λ_{max} at 280 nm is formed. This species was analyzed by LC-DAD-EC and was found to have the same retention time and uv spectrum as the NADA standard (data not shown). Although quantitative significance cannot be attached to these data, they nevertheless clearly demonstrate that the quinone is reduced to the corresponding catecholamine by iron.

The possibility that either ferrous ion or its EDTA complex reduces the quinone was examined in other experiments. It was found that neither ferrous ion nor ferrous EDTA had any effect on the quinone under these conditions. These results, combined with those described earlier that indicate the occurrence of on-column reduction of quinones, strongly suggest that iron metal from the stainless steel LC column causes reduction of the quinones.

Reduction of quinones by stainless steel frit. Coelution of significant amounts of catecholamines with quinones requires substantial amounts of quinones to be

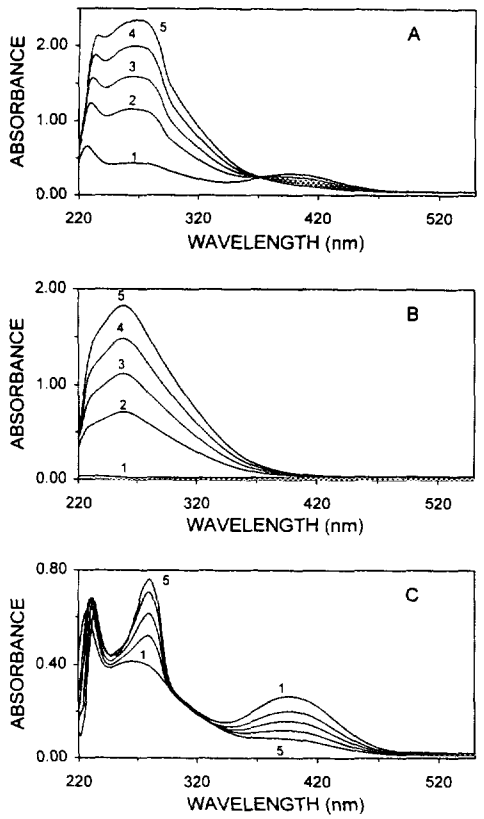

FIG. 7. Ultraviolet/visible spectral study of the reaction of NADA quinone with iron metal. Spectra 1 to 5 were taken at 0, 1, 2, 3, and 4 min, respectively, after addition of iron metal. (A) Spectra of 0.25 mM electrochemically prepared NADA quinone in the presence of stirring iron metal in mobile phase II. (B) Spectra of iron metal in mobile phase II. (C) Difference spectra obtained by subtracting each spectrum of (B) from each corresponding spectrum of (A).

reduced to their catecholamines after eluting from the stationary phase. Examination of the structure of the stainless steel column revealed that the stainless steel frit at the exit end of the column may be the major cause of the reduction. To determine if this exit frit is responsible for the concurrent anodic–cathodic response, a filter (Alltech Direct Connect Universal Prefilter) was connected to the exit end of the PEEK column to mimic the function of the exit frit in the stainless steel column. This filter contains a 0.5-μm stainless steel filter element, which is similar in physical and chemical properties to the frit in a stainless steel column. It should be emphasized that the metal-free PEEK column does not reduce quinones to their catecholamines (Fig. 3C). After installation of the stainless steel filter to the exit end of the PEEK column, however, a significant amount of NADA was detected at the same retention time as that of NADA quinone. For example, when 10 μl of 0.5 mM NADA quinone was injected repeatedly, the fraction of the NADA component in the concurrent anodic–cathodic response effluent ranged from 0.87 in the first injection to 0.57 in the seventh injection (data not shown). The reducing capacity of the frit decreases substantially from the first to the second injection and nearly reaches a steady state after the third injection.

The effect of sample volume of 0.5 mM NADA quinone injected upon the fraction of NADA quinone converted to NADA was also studied using the PEEK column with the stainless steel frit connected to its exit end. The fraction of quinone reduced to catecholamine was much greater when injecting a smaller amount of NADA quinone than when injecting a larger amount. The fraction of quinone reduced ranged from 0.89 when 1 μl was injected to approximately 0.25 when 25 μl or more was injected. This result is consistent with earlier data on the effect of sample volume that was obtained using the stainless steel column (Fig. 4). Moreover, all of these results from studies of the dependence of reduction on quinone amount are consistent with a heterogenous reaction occurring between the quinone and the iron metal surface of the exit frit of the stainless steel columns.

DISCUSSION

LC-DAD-EC analysis of DA quinone or NADA quinone using stainless steel columns shows that an electrochemically oxidizable component is eluted at the same retention time as the electrochemically reducible quinone, causing a concurrent anodic–cathodic response. Data from the collection–reinjection experiment and the computer simulation of spectra show that this electrochemically oxidizable component is the corresponding catecholamine and that the concurrent anodic–cathodic response effluent is not composed of any species other than the quinone and its corresponding catecholamine. Our results are consistent with the following interpretation: peak I_{ox} in the LC-EC (oxidation) chromatogram is due to the oxidation of the catecholamine component, peak I_{rd} in the LC-EC (reduction) chromatogram is due to reduction of the quinone component, peak I_{280} in the LC-DAD (280 nm) chromatogram arises from absorption of both catecholamine and catecholamine–quinone at 280 nm, and peak I_{390} in the LC-DAD (390 nm) chromatogram is due to absorption of the quinone component only. These results are inconsistent with the presence of (i) a semiquinone, which would not only have different absorption maxima at shorter wavelengths, but also an absorption maximum at longer wavelengths (e.g., 600–650 nm) (13,14); (ii) a quinhydrone-type dimeric species (15), which would have continued to exhibit a concurrent anodic–cathodic response after reinjection of the effluent; and (iii) a nucleophilic addition product, which should have a different spectrum and/or a different retention time.

The spectroscopic study of the stabilities of the qui-

nones, the direct injection experiment of the quinones into the flowing system with the column removed, and the LC analysis of the quinones using the PEEK column demonstrate that quinones are relatively stable under these conditions. However, when a stainless steel column is used for LC, reduction of quinones occurs and the fraction reduced increases with decreasing flow rate and decreasing volume of sample injected. Furthermore, reduction of the quinones is observed in the spectroscopic study of quinones in the presence of iron and in the LC analysis using the PEEK column with a stainless steel filter connected to its exit end. All of these results demonstrate the occurrence of an on-column heterogenous reduction of quinones by iron present in the stainless steel column and in other components of the LC system.

The on-column reduction of the quinone is manifested in the following chromatographic behavior. First, a portion of the quinone is reduced by the stainless steel sample loop, the prefilter, and the entrance frit of the column. Since the quinone is still relatively concentrated at this point, only a small fraction of the quinone is reduced. The catecholamine thus produced is separated by the stationary phase and elutes at the normal retention time of the catecholamine (peak II). Second, after entering the column, a portion of the quinone interacts with the stainless steel inner surface of the column tubing and undergoes reduction to the catecholamine. Because the catecholamine is formed continuously during the passage of the quinone through the column, the resulting response at both the anode of the electrochemical detector and the spectroscopic detector monitoring at 280 nm is an elevated baseline that extends from the more rapidly eluting quinone peak (peak I) to the more slowly eluting catecholamine peak (peak II). Third, after exiting the stationary phase, a portion of the quinone is reduced by the stainless steel frit at the exit end of the column. Since the quinone concentration decreases as the quinone flows through the column, and since the frit (pore size 0.5–2 μm) has a relatively large total surface area, a relatively large proportion of the remaining quinone is reduced at this point. The catecholamine thus produced will elute at the same retention time as the quinone, thereby giving rise to the anodic peak of the concurrent anodic–cathodic response, whereas the unreduced quinone gives rise to the corresponding cathodic peak. In addition, the stainless steel tubing between the column and the detectors also contributes slightly to quinone reduction, which is why, when the PEEK column is used, a barely discernible anodic response is observed using our LC systems (Fig. 5C).

The observation that peak I_{rd} is slightly narrower than peak I_{ox} (Fig. 1) can be explained by the dependence of a heterogeneous reaction on reactant amount. The small quantities of quinone at the leading and trailing edges of the quinone band are completely reduced by the exit frit and hence are detected as catecholamine. This reduction results in a slight narrowing of the cathodic peak of the quinone relative to the anodic peak of the catecholamine. The quinone at other locations of the band is reduced to different extents, depending on the specific amount of the quinone. The greater the amount of quinone present, the smaller the fraction of quinone reduced. This phenomenon results in detection of the highest ratio of quinone to catecholamine at the apex of the concurrent anodic–cathodic response.

The concurrent anodic–cathodic response phenomenon is apparently not uncommon in studies of catecholamine quinones employing LC-EC analysis using stainless steel columns. One example is a study where the anodic peak of the concurrent anodic–cathodic response was suggested to be a semiquinone produced during the oxidation of DA and NADA by excess periodate (6). Another example is a study of the oxidation of 6-OH-DA at physiological pH in potassium phosphate buffer (16). The oxidizable species that coeluted with the reducible 6-OH-DA quinone was proposed to be some kind of oxidation product, but the oxidizable species is very likely 6-OH-DA that was produced by the on-column reduction of the 6-OH-DA quinone.

The mechanism proposed here for the on-column reduction of catecholamine quinones by iron metal can also explain the on-column reduction of other related compounds. During the ion-exclusion LC analysis of *p*-benzoquinone, *p*-benzoquinone was reduced to *p*-hydroquinone and two peaks that exhibited the spectra of hydroquinone were obtained (7). The peak that elutes at the normal retention time of hydroquinone is probably hydroquinone produced by reduction of benzoquinone in the sample loop, tubing, prefilter, and entrance frit of the LC column, whereas the second peak is likely hydroquinone produced at the exit frit of the column. The elevated baseline between the two peaks is probably due to hydroquinone produced at the inner surface of the column tubing.

In summary, this study has characterized the cause of reduction of catecholamine quinones during LC analyses using a stainless steel column and other stainless steel components. On the basis of the results reported here, the iron present at the inner surfaces of the stainless steel column tubing and in the frits at both ends of the column is the major reducing agent that leads to the on-column reduction of quinones during LC analysis. LC analysis of the quinones and other intermediates from the oxidation of dihydroxyphenyl compounds is a popular experimental method, but caution must be exercised to prevent unwanted reactions of intermediates and metabolites during the analysis. The use of PEEK or other nonsteel columns would minimize this complication.

ACKNOWLEDGMENTS

We are grateful to T. L. Hopkins and T. Morgan for assistance with this study and for reviewing the manuscript. We also thank C. Duda (Bioanalytical Systems, West Lafayete, IN), M. Gattrell (National Research Council, Ottawa, Canada), and A. G. Schell (Upchurch Scientific, Oak Harbor, WA) for critical comments about the manuscript. This research was supported in part by National Science Foundation Grants DCB 90-19400, MCB-9418129, and CHE 87-14657.

REFERENCES

1. Hopkins, T. L., and Kramer, K. J. (1992) *Annu. Rev. Entomol.* **37,** 273–302.
2. Andersen, S. O., Peter, M. G., and Roepstorff, P. (1992) *Insect Biochem. Mol. Biol.* **22,** 459–469.
3. Cilliers, J. J. L., and Singleton, V. L. (1991) *J. Agric. Food Chem.* **39,** 1298–1303.
4. Fulcrand, H., Cheminat, A., Brouillard, R., and Cheynier, V. (1994) *Phytochemistry* **35,** 499–505.
5. Zhang, F., and Dryhurst, G. (1994) *J. Med. Chem.* **37,** 1084–1098.
6. Li, J., and Christensen, B. M. (1994) *J. Electroanal. Chem.* **375,** 219–231.
7. Gattrell, M. A., and Kirk, D. W. (1987) *J. Chromatogr.* **409,** 404–408.
8. Peter, M. G., Stegmann, H. B., Dao-Ba, H., and Scheffler, K. (1985) *Z. Naturforsch.* **40c,** 535–538.
9. Korytowski, W., Sarna, T., Kalyanaraman, B., and Sealy, R. C. (1987) *Biochim. Biophys. Acta* **924,** 383–392.
10. Kalyanaraman, B., Premovics, P. I., and Sealy, R. C. (1987) *J. Biol. Chem.* **262,** 11080–11087.
11. Kalyanaraman, B., Sealy, R. C., and Liehr, J. G. (1989) *J. Biol. Chem.* **264,** 11009–11019.
12. Van Swaay, M. (1978) *J. Chem. Ed.* **55,** A7–A12.
13. Massey, V., and Palmer, G. (1966) *Biochemistry* **5,** 3181–3189.
14. Chin, D., Jones, S. E., Leon, L. E., Bosserman, P., Stallings, M. D., and Sawyer, D. T. (1982) *in* Electrochemical and Spectrochemical Studies of Biological Redox Components (Kadish, K. M., Ed.), pp. 675–707, Am. Chem. Soc., Washington, DC.
15. Michaelis, L., and Granick, S. (1943) *J. Am. Chem. Soc.* **70,** 624–627.
16. Nappi, A. J., and Vass, E. (1994) *Biochim. Biophy. Acta* **1201,** 498–504.