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Mechanistic Studies of the Electrocatalytic Oxidation of NADH and Ascorbate at Glassy Carbon Electrodes Modified with Electrodeposited Films Derived from 3,4-Dihydroxybenzaldehyde

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Studies of the electrocatalytic oxidation of *β*-nicotinamide adenine dinucleotide (NADH) at glassy carbon rotated disk electrodes modified with electrodeposited films derived from 3,4-dihydroxybenzaldehyde (3,4-DHB) indicate that the mechanism of such electrooxidation proceeds via the formation of an intermediate complex. The reaction also appears to be strongly influenced by the presence of Ca^{2+} and Mg^{2+} ions as well as by pH. Ascorbate can also be electrocatalytically oxidized at these modified electrodes, giving rise to an electrochemical response very similar to that obtained for NADH. Due to this similarity, the presence of ascorbate in NADH determinations presents a severe interference that cannot be mitigated on the basis of electrochemical responses alone. However, this interference effect can be virtually suppressed by the presence of ascorbate oxidase in solution or immobilized on a nylon mesh which, in turn, is in contact with the electrode modified with the film of 3,4-DHB. Using this approach, we describe the construction of an alcohol biosensor based on alcohol dehydrogenase and which is, furthermore, free from interference effects due to ascorbate.

The ubiquitous use, in living cells, of *β*-nicotinamide adenine dinucleotide (NADH) and its oxidized form (NAD^+) as coenzymes by a great number of dehydrogenases has generated numerous studies directed at understanding the factors that may control the redox activity of these biological cofactors. Although the reversible potential for NADH oxidation is estimated to be −0.32 V vs NHE,[1] the direct oxidation of NADH at bare electrodes, in general, requires high overpotentials that can be as large as 1.0 V.[2,3] At carbon electrodes, this high overpotential can be decreased by pretreatment, although these surface-modified electrodes are rapidly deactivated.[4] Oxidation of NADH at lower potentials can be achieved through redox mediators present in the solution or confined at the electrode surface. A number of electrode modifications have been reported for this purpose using different functionalities, such as thionine,[5] phenazine,[6] and phenoxazine[7,8] derivatives adsorbed onto carbon electrodes or as electrodeposited films.[9] It is also generally accepted that *o*-quinones can be quite active in the electrocatalytic oxidation of NADH, and, as a result, numerous derivatives incorporating such a group have been employed.[10]

In these types of systems, the NADH is oxidized by the surface-confined mediator (M_{ox}) in a chemical step defined by a rate constant k_1 (M^{-1} s^{-1}). NAD^+ and the reduced form of the mediator (M_{red}) are the products of this reaction:

$$\mathrm{NADH} + \mathrm{M_{ox}} \xrightarrow{k_1} \mathrm{NAD^+} + \mathrm{M_{red}} \qquad (1)$$

The mediator is subsequently reoxidized at the electrode surface according to eq 2:

$$\mathrm{M_{red}} \overset{k_s}{\rightleftharpoons} \mathrm{M_{ox}} + 2\mathrm{e^-} + x\mathrm{H^+} \qquad (2)$$

where the number of protons involved in the process, *x*, depends on the quinone structure and pH. The decrease in the overpotential is given by the potential difference in the direct electrochemical oxidation of NADH at the bare electrode and the formal potential of the mediator, $E^{\circ\prime}_M$. At pH 7.0, typical values for $E^{\circ\prime}_M$ from around −0.2 to +0.1 V have been reported for electrodes modified with a number of quinone functionalities.[10] In addition, at pH 7.0, the direct oxidation of NADH at unmodified electrodes typically takes place at about +0.70 V, so that the use of electrodes modified with quinone functionalities for the electrooxidation of NADH typically gives rise to significant diminutions in the overpotential.

We recently[11] reported that the electrooxidation of 3,4-dihydroxybenzaldehyde (3,4-DHB) on glassy carbon electrodes gives

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rise to stable redox-active films which exhibit very high and persistent electrocatalytic activity for the oxidation of NADH, including enzymatically generated NADH. Combining the electrocatalytic activity toward NADH of such modified electrodes with the enzymatic activity of aldehyde dehydrogenase immobilized on a nylon mesh, we developed an aldehyde biosensor[12] that exhibited great stability and sensitivity in analytical determinations for a wide variety of aldehydes. In addition, in this type of biosensor, the applied potential used in analytical determinations was sufficiently low to preclude interference by acetaminophen or uric acid. However, the electrodeposited films of 3,4-DHB also catalyzed the electrooxidation of ascorbate, which is one of the most important interferences present in biological fluids.

In the present work, the mechanism of electrooxidation of NADH at glassy carbon (GC) electrodes modified with films derived from 3,4-DHB and the effects of pH and divalent cations on this catalytic process have been studied. In addition, the electrooxidation of ascorbate at such modified electrodes and its interference effects on the determination of NADH have also been investigated. Finally, we describe the design and characterization of an ethanol biosensor based on alcohol dehydrogenase and which is free of interference effects due to ascorbate present at physiologically relevant concentrations.

EXPERIMENTAL SECTION

Materials. Alcohol dehydrogenase (ADH, EC 1.1.1) from Baker's yeast (340 units/mg solid) and ascorbate oxidase (AOX, EC 1.10.3.3) from *Cucurbita* species (163 units/mg solid) were obtained from Sigma and stored as received at 4 and −20 °C, respectively. 3,4-Dihydroxybenaldehyde (3,4-DHB, 97% purity) from Aldrich Chemical Co. was recrystallized twice from water using activated charcoal. Ascorbate and NADH (grade III) were obtained from Sigma Chemical Co. (St. Louis, MO) and were used as received. Tris(hydroxymethyl)aminomethane (Tris) from Sigma, sodium phosphate, and sodium acetate (Carlo Erba) were used in the preparation of buffer solutions (see text). Stock solutions of ADH were prepared by dissolving 1.0 mg (340 units) of enzyme preparation in 15 μL of 0.1 M Tris/HCl (pH 7.0) buffer containing 50% (v/v) of glycerol. AOX stock solutions were prepared by dissolving 0.67 mg (110 units) of enzyme preparation in the same buffer containing glycerol. These stock solutions were aliquoted and stored at −20 °C until use. Under these conditions, the enzyme activity remained stable for several months. 3,4-DHB stock solutions (typically 25−50 mM) were prepared in water or absolute ethanol immediately prior to use. All other chemicals employed were of at least reagent grade and were used without further purification. Water was purified with a Millipore Milli-Q system. All other solutions were prepared just prior to use.

Instrumentation. Cyclic voltammetric studies were carried out using a BAS CV-27 potentiostat and a Linseys $X-Y$ recorder in three-compartment (separated by medium-porosity sintered glass disks) electrochemical cells. All joints were standard taper, so that all compartments could be hermetically sealed with Teflon adapters. Teflon-shrouded glassy carbon (GC) electrodes (area, 0.071 cm^2) were used as working electrodes, and a coiled platinum wire served as the auxiliary electrode. For rotated disk electrode experiments, a Pine Instruments rotator and a RDE-3 bipotentiostat were employed. In this case, a GC electrode from Pine (geometric area, 0.28 cm^2) was used as the working electrode. All measurements were carried at room temperature (22 ± 2 °C), and all potentials are reported against a sodium-saturated calomel electrode (SSCE) without regard for the liquid junction.

Procedures. *Electrode Activation and Modification.* Prior to each experiment, glassy carbon electrodes were polished with 1.0 μm diamond paste (Buehler) and rinsed with water and acetone. They were subsequently activated by placing them in a 1.0 M NaOH solution and holding the potential at +1.20 V for 5 min, followed by potential cycling from −0.20 to +1.0 V in 0.1 M phosphate buffer (pH 7.0) for 5 min. After rinsing, the electrodes were subsequently modified.

Modification with 3,4-DHB was carried out as described previously[11,12] in phosphate buffer (pH 7.0). The modified electrodes were rinsed thoroughly with water and placed in fresh buffer solution, where the potential was cycled between −0.20 and +0.25 V at 100 mV/s for 5 min. Afterward, a cyclic voltammogram was recorded, and the surface coverage for the modified electrode was determined from the integrated charge under the wave centered at around 0.0 V and assuming an n value of 2. Although the surfaces of these electrodes are microscopically rough, it is difficult to estimate the roughness factor, so in all calculations we have employed the geometric area. Thus, the values reported represent upper limits. Typical coverage values were found to be (1.3−2.0) × 10^{-10} mol cm^{-2}, very close to one monolayer.[11]

Electrochemical Measurements of NADH. Prior to NADH measurements, the modified electrodes were placed in 4.0 mL of thoroughly degassed buffer solution (oxygen can influence the linearity of the response), and the potential was cycled between −0.20 and +0.25 V at a rate of 10 mV/s. NADH measurements were performed by subsequently adding aliquots (10 and 20 μL) of NADH stock solutions (typically 0.025 or 0.0025 M) with a gastight syringe. The current due to the electrocatalytic oxidation of NADH was recorded after each addition. Rotating disk electrode experiments in the presence of NADH were made either at a fixed potential or with a very slow potential sweep (5−10 mV/s).

Enzyme Immobilization and Biosensor Construction. Enzyme activities were immobilized on 6 mm diameter nylon mesh disks, closely following the procedure described previously.[12] The following solutions were added to each disk: 2.0 μL of glutaraldehyde (2.5% v/v), 2.0 μL of bovine serum albumin (BSA, 10% w/v), and 1.5 μL of the respective enzyme stock solution. The mixture was carefully homogenized on the surface of the disk and allowed to dry at room temperature for 30 min. Prior to use, the unreacted carboxaldehyde groups in glutaraldehyde were inactivated by immersing the disks in 25.0 mL of 0.1 M phosphate buffer (pH 7.0) containing 0.10 M glycine for 15−20 min at room temperature. When not in use, the membranes were stored in 0.1 M phosphate buffer solution (pH 7.0) at 4 °C.

The biosensor was assembled by placing the enzyme-modified nylon mesh over a glassy carbon electrode previously modified with 3,4-DHB, and the assembly was secured with a holed cap. The biosensor was placed in buffer solution for about 5 min prior to use to ensure solvent equilibration. For ascorbate interference studies and ethanol determinations, the biosensor was placed in 3.0 mL of buffer solution. The amperometric measurements of ethanol were carried out in 0.1 M phosphate buffer (pH 8.0) at an applied potential of +0.18 V. After the background current had decayed to a steady state value, aliquots of a stock solution

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of ethanol in buffer were added. The solution was stirred for 30 s and allowed to stand for 1 min for equilibration. The steady state current (achieved in less than 60 s) in the unstirred solution was subsequently recorded.

Kinetic Models and Calculations. In rotated disk electrode experiments, and for a catalyzed reaction mediated by a redox couple confined to the electrode surface, when the surface coverage (Γ) is at or below a monolayer and the applied potential is at least 120 mV beyond $E^{\circ\prime}$ of the mediator, the general equation for the catalytic current (i_c) as a function of the rate of rotation is[7,13]

$$i_c = \frac{nFA\Gamma k_1 C^* \omega^{1/2}}{1.61\nu^{1/6}D^{-2/3}k_1\Gamma + \omega^{1/2}} \tag{3}$$

where C^* is the bulk concentration of substrate (NADH in this case, mol cm^{-3}), ω is the rate of rotation (rad s^{-1}), ν is the kinematic viscosity (cm^2 s^{-1}), and the other parameters have their usual meaning. The reciprocal of eq 3 gives a Koutecky−Levich-like equation:

$$\frac{1}{i_c} = \frac{1}{nFA\Gamma k_1 C^*} + \frac{1}{0.62nFA\nu^{-1/6}D^{2/3}C^*}\frac{1}{\omega^{1/2}} \tag{4}$$

For large values of $\omega^{1/2}$ ($\omega \rightarrow \infty$), the current is controlled by the kinetic process and reaches an asymptotic maximum value, $i_{c,max}$:

$$i_{c,max} = nFA\Gamma k_1 C^* \tag{5}$$

The value of $i_{c,max}$ can be calculated directly by fitting the experimental data to eq 3 or, more commonly, from the intercept of the Koutecky−Levich plot (eq 4), i.e., from a plot of $1/i_{lim}$ vs $1/\omega^{1/2}$. In our studies, we have employed Koutecky−Levich plots for diagnostic purposes, and values of the rate constants have been obtained from nonlinear least-squares fitting of eqs 3 and 12 (vide infra). The rate constant, k_1, can be obtained from eq 5. However, one of the main sources of error in calculating the value of this constant is the determination of the electrode area. This complication can be avoided by using the value of the coulometric charge, q, obtained experimentally from integration of the cyclic voltammetric wave. Since $q = nFA\Gamma$, eq 5 can be expressed in terms of experimentally measurable parameters, allowing a more accurate determination of the value of k_1:

$$i_{c,max} = qk_1 C^* \tag{6}$$

For a catalyzed reaction (involving NADH in the present case) that proceeds via an intermediate complex and following the model proposed by Gorton et al.,[7,14] one obtains

$$\mathrm{NADH} + \mathrm{M_{ox}} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [\mathrm{NADH\text{-}M]} \xrightarrow{k_{+2}} \mathrm{NAD^+} + \mathrm{M_{red}} \tag{7}$$

Combining rate constants, as in the Michaelis−Menten kinetic model, it is possible to obtain an expression for K_M:

$$K_M = \frac{k_{-1} + k_{+2}}{k_{+1}} \tag{8}$$

Equating the velocities as given by the Michaelis−Menten expression and the current, one can obtain k_1 as a function of K_M:

$$v = [\mathrm{NADH}]\Gamma k_1 = \frac{k_{+2}[\mathrm{NADH}]\Gamma}{K_M + [\mathrm{NADH}]} \tag{9}$$

$$k_1 = \frac{k_{+2}}{K_M + C^*} \tag{10}$$

Inversion of eq 10 gives

$$\frac{1}{k_1} = \frac{K_M}{k_{+2}} + \frac{C^*}{k_{+2}} \tag{11}$$

Equation 11 indicates that a plot of $1/k_1$ vs C^* will be a straight line if a complex is involved in the reaction between NADH and the mediator. Thus, such as plot can be used as a diagnostic test of the kinetic model proposed in eq 7. By combining eqs 6 and 10 and rearranging, it is possible to obtain a relationship between the catalytic current, obtained under kinetic control, and the bulk concentration of NADH:

$$i_{c,max} = \frac{k_{+2}qC^*}{K_M + C^*} \tag{12}$$

Equation 12 has the same form as the Michaelis−Menten equation and indicates that $i_{c,max}$ is proportional to the initial rate for the reaction between NADH and the mediator. Inversion of eq 12 gives

$$\frac{1}{i_{c,max}} = \frac{1}{qk_{+2}} + \frac{K_M}{qk_{+2}}\frac{1}{C^*} \tag{13}$$

The values of K_M and k_{+2} can be obtained by fitting the calculated values of $i_{c,max}$ obtained for different concentrations of NADH to eq 12 or by using the double-reciprocal plot presented in eq 13. The linearity of such a plot can also be used as a further diagnostic probe for the kinetic model.

Substitution of k_1 (eq 10) into eq 4 gives a general expression for the catalytic current as a function of the rate of rotation:[15]

$$\frac{1}{i_c} = \frac{1}{nFAk_{+2}\Gamma} + \left(\frac{K_M}{nFAk_{+2}\Gamma} + \frac{1.61\nu^{1/6}}{nFAD^{2/3}\omega^{1/2}}\right)\frac{1}{C^*} \tag{14}$$

If the assumption of an intermediate complex applies, plots of $1/i_c$ against $1/C^*$ obtained at different rates of rotation, should result in straight lines. Moreover, the intercept of these plots should be independent of both ω and C^* (as indicated in eqs 13 and 14).

Finally, and as was done earlier by substituting q for $nFA\Gamma$, one can obtain

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$$\frac{1}{i_c} = \frac{1}{qk_{+2}} + \left(\frac{K_M}{qk_{+2}} + \frac{1.61\nu^{1/6}}{nFAD^{2/3}\omega^{1/2}}\right)\frac{1}{C^*} \qquad (15)$$

RESULTS AND DISCUSSION

Modification of Glassy Carbon Electrodes with Electrodeposited Films of 3,4-DHB. As described previously, dihydroxybenzaldehyde (DHB) isomers can be oxidatively electrodeposited under controlled potential conditions onto GC carbon electrodes previously activated in alkali solutions. The mechanism of electrodeposition appears to involve oxidation of the hydroquinone, followed by nucleophilic attack by active groups present on the electrode surface.[16] This process can be influenced by the presence of nucleophilic species in the electrolyte capable of competing with the reactive groups present at the electrode surface. As a result, the electrodeposition process and the electrochemical behavior of the modified electrodes are strongly dependent on pH. At neutral pH values (~7.0), electrodeposited films derived from 3,4-DHB exhibited stable and well-defined reversible waves, and the coverages were consistently in the neighborhood of one monolayer. Based on these previous results, all the studies presented here were on films electrodeposited at neutral pH (0.10 M, pH 7.0 phosphate buffer), since they consistently gave high and reproducible coverages and exhibited excellent stability.

The electrochemical responses of electrodeposited films derived from 3,4-DHB and 2,3-DHB were those anticipated for a surface-confined redox couple.[17] The peak currents were directly proportional to the scan rate for sweep rates below 500 mV/s, and the peak-to-peak potential separation (ΔE_p) was small, although not zero. In addition, the formal potential ($E^{\circ\prime}$) was independent of the potential scan rate, suggesting that the transfer coefficient (α) was close to 0.5. However, and as mentioned earlier, for sweep rates above 500 mV/s, the ΔE_p increased significantly, suggesting limitations in the kinetics of charge transfer.

The heterogeneous charge transfer rate constant, k_s, of a surface-confined redox couple can be evaluated from cyclic voltammetric experiments from the variation of the oxidation and reduction peak potentials with the sweep rate following the treatment developed by Laviron.[18] This peak separation will be close to zero when the electron transfer rate is fast relative to the sweep rate and will increase when such a condition is not met. However, non-zero ΔE_p values can also arise as a result of other factors,[19] including uncompensated resistance and others. However, given the experimental conditions and the relatively low coverages involved, we believe that ohmic drops represent, at most, a small contribution. As a result, this method typically provides a lower limit estimate of the value of k_s. Although in the present case the values of k_s were pH dependent, at pH 7.0, values of 25 and 17 s^{-1} were obtained for electrodeposited films of 3,4-DHB and 2,3-DHB, respectively.[16] These are of the same order of magnitude as values reported for adsorbed phenoxazines[7,20] and adsorbed quinones[10] at the same pH.

As mentioned above, there have been numerous reports of the electrocatalytic activity toward NADH of adsorbed or electrodeposited mediators including quinones, various dye-stuffs, and others. In cases where the kinetics of the reaction between NADH and quinones, used as mediators, have been measured, the values obtained for the rate constant have typically been about an order of magnitude smaller than the corresponding values for the electron transfer processes. Under these conditions, one can generally assume that the electron transfer process depicted in eq 2 is fast relative to the chemical reaction between NADH and the mediator shown in eq 1.

Electrocatalytic Oxidation of NADH at 3,4-DHB-Modified Electrodes. The electrocatalytic oxidation of NADH at a GC electrode modified with an electrodeposited film of 3,4-DHB is shown in Figure 1A, where cyclic voltammograms at 10 mV/s of the modified electrode in 0.1 M Tris/acetate buffer (pH 7.0) in the absence (a) and in the presence (b) of 0.5 mM NADH are shown. Under the same experimental conditions, the direct oxidation of NADH at an activated but unmodified GC electrode shows an irreversible wave with a peak potential around +400 mV (not shown). However, it should also be mentioned that such electrodes rapidly become deactivated, and the oxidation wave shifts to significantly more positive values (~+0.70 V). At the modified electrode, the oxidation of NADH gives rise to a typical electrocatalytic response with an anodic peak current that is greatly enhanced over that observed for the modified electrode alone and with virtually no current on the reverse (cathodic) sweep. The peak potential value of +150 mV is very close to that of the surface-confined mediator in the absence of NADH.

Although the rate constant for the chemical reaction between NADH and the surface-confined mediator (k_1) can be evaluated by cyclic voltammetry according to the method proposed by Andrieux and Saveant,[21] the use of rotated disk electrodes[22] typically provides more accurate results, since experiments can be done at a fixed potential and it is much easier to subtract background currents. Thus, the rotating disk electrode method was used for evaluating the kinetics of the reaction between NADH and electrodeposited films of 3,4-DHB.

Figure 1B presents Levich plots (i_c vs $\omega^{1/2}$) for the electrocatalytic oxidation of NADH (at different concentrations from 0.05 to 0.5 mM) at a 3,4-DHB-modified GC electrode, and the clear lack of linearity immediately suggests that the reaction is limited by kinetics and not by transport. In such cases, kinetic information can be obtained from a so-called Koutecky−Levich plot ($1/i_c$ vs $1/\omega^{1/2}$). Figure 1C presents such plots of the data in Figure 1B, and, in this case, well-behaved linear responses are obtained.

In these experiments, the surface coverages of the mediator were close to one monolayer, low enough to ensure ideal behavior in the mediator film. It also allows one to rule out diffusion of NADH through the electrodeposited film as being rate-limiting. Restricted access of NADH to the catalyst could be rate-limiting in the case of thick or multiple-layer films or catalyst networks. However, it is very unlikely that this type of diffusional hindrance will be present in this case, since the film thickness was close to one monolayer. In addition, and as mentioned earlier, slow electron transfer between the electrodeposited 3,4-DHB and the electrode can also be discarded as rate-limiting. Thus, as

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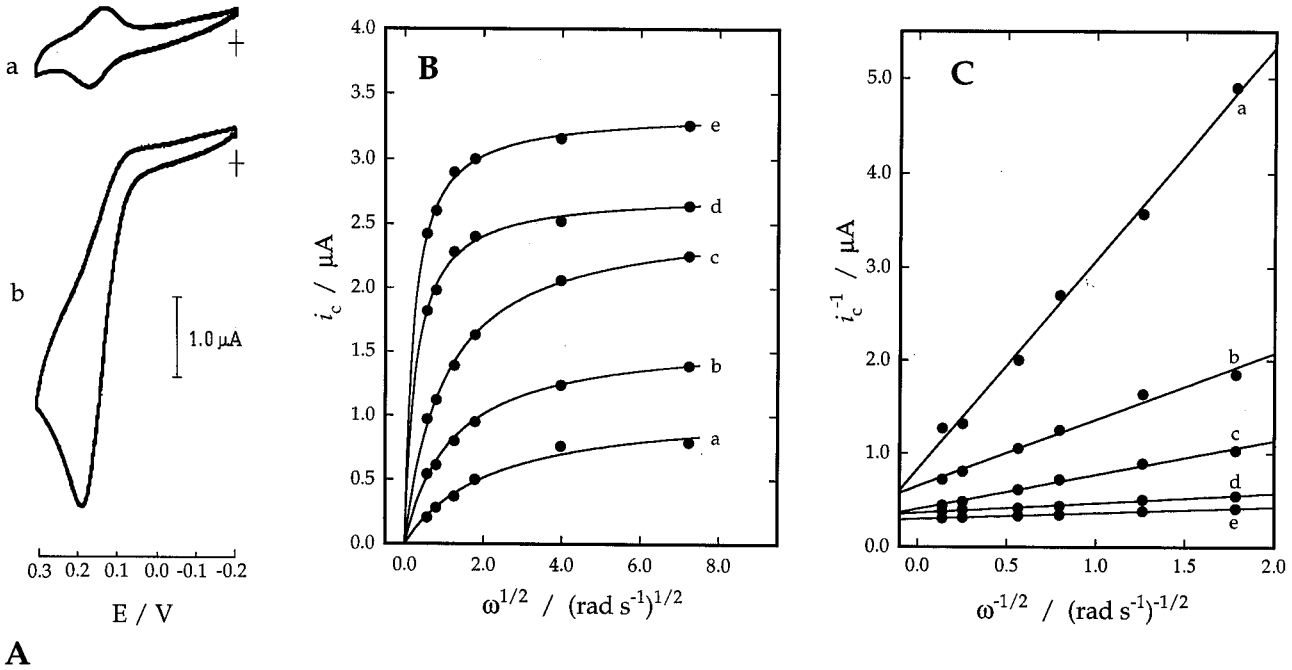

Figure 1. (A) Cyclic voltammogram at 10 mV/s in 0.1 M Tris/0.1 M acetate buffer (pH 7.0) for a glassy carbon electrode modified with a film derived from 3,4-DHB in the absence (a) and in the presence (b) of 0.5 mM NADH. (B) Plot of the electrocatalytic current, i_c, for NADH oxidation at +0.20 V at a glassy carbon electrode modified with a film derived from 3,4-DHB as a function of $\omega^{1/2}$. The NADH concentrations were 0.05 (a), 0.10 (b), 0.20 (c), 0.40 (d), and 0.50 (e) mM. (C) Double-reciprocal plots of the data in part B. In parts B and C, the points are the data, and lines are least-squares fits.

mentioned earlier, we again conclude that the reaction between NADH and the mediator (k_1, eq 1) is the sole rate-limiting step.[23]

Effect of [NADH] and pH on the Electrocatalytic Oxidation of NADH at GC Electrodes Modified with Electrodeposited Films of 3,4-DHB. The effect of [NADH] and pH on the electrocatalytic oxidation of NADH at GC electrodes modified with 3,4-DHB was studied by determining the value of k_1 using the procedures described in the Experimental Section (eq 6). At pH 7, k_1 was found to decrease significantly with increasing bulk concentration of NADH (Figure 2A). A similar observation has been previously reported for the electrooxidation of NADH at electrodes modified with different mediators.[7,24,25] This observation also provides additional evidence against restricted access of the substrate, NADH in this case, as the rate-limiting step. In order to rationalize such a concentration dependence of the rate constant, several authors have proposed that an NADH−mediator complex is formed as an intermediate following a reaction scheme similar to the Michaelis−Menten (MM) model (eq 7).

As described in the Experimental Section, various plots can be used as diagnostic criteria for such a model. One of these, shown in Figure 3A, is a plot of $i_{c,max}$ against the concentration of NADH, which shows the characteristic MM shape. (It should be mentioned that the values of $i_{c,max}$ used in Figure 3A were obtained from nonlinear least-squares fitting of the data in Figure 1B). A double-reciprocal plot of these data (Figure 3B) results in a straight line, as predicted by eq 13. In addition, a plot of the reciprocal of the reaction rate (k_1^{-1}) versus the concentration of NADH (Figure 2B) also results in a straight line, in accordance with eq 11.

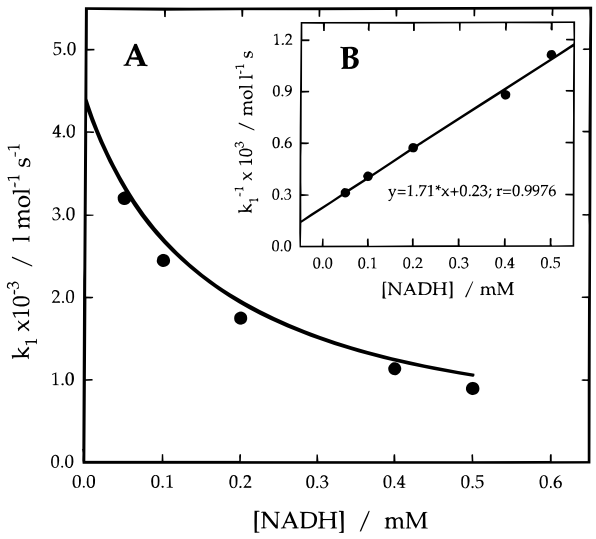

Figure 2. Plot of k_1 (A) and k_1^{-1} (B) vs concentration of NADH for the electrooxidation of NADH at 3,4-DHB-modified electrodes in 0.1 M Tris/0.1 M acetate buffer (pH 7.0). Points are the data, and lines are least-squares fits.

Values of the constants k_{+2} and K_M were obtained using eqs 10−13, and these were subsequently inserted into eqs 10 and 12 to recalculate the theoretical values of k_1 and $i_{c,max}$ at different concentrations of NADH. These values were then plotted against the concentration of NADH, and the results are presented in Figures 2A and 3A, respectively, as solid lines. The excellent correlation between the solid lines and the data points allows us to conclude that the electrooxidation of NADH at electrodes modified with 3,4-DHB proceeds via an intermediate complex.

Similar conclusions have been reported by several authors for the electrooxidation of NADH and analogous molecules in the

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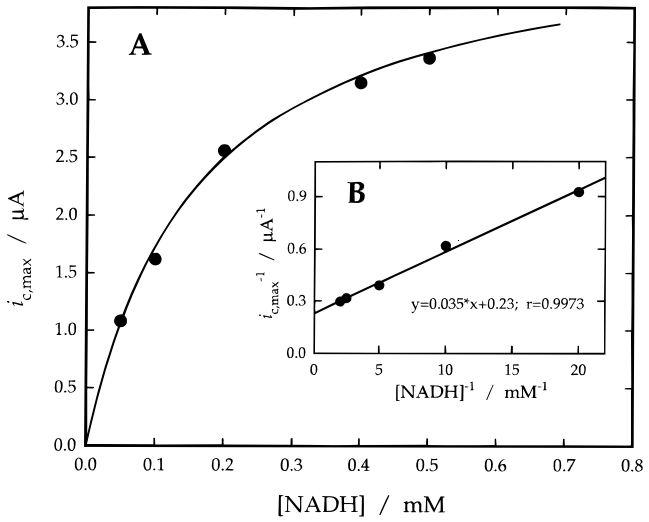

Figure 3. Plot of $i_{c,max}$ vs concentration of NADH (A) and double-reciprocal plot (B) of the same data for the electrooxidation of NADH at 3,4-DHB-modified electrodes in 0.1 M Tris/0.1 M acetate buffer (pH 7.0). Points are the data, and lines are least-squares fits.

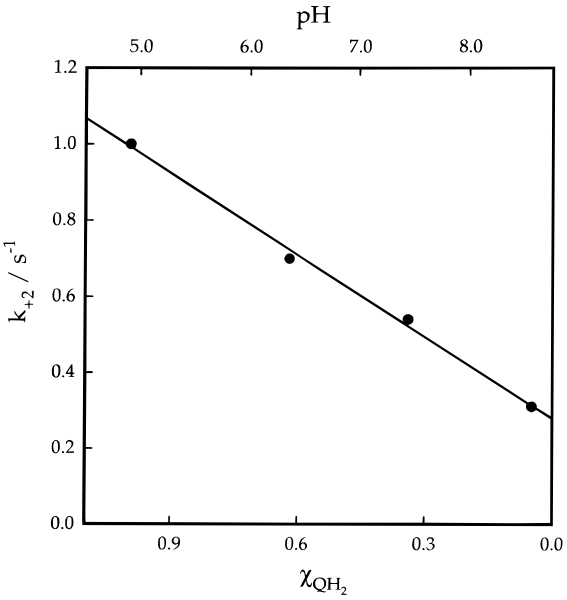

Figure 4. Plot of k_{+2} vs the mole fraction of the protonated form of the mediator (χ_{QH_2}) for the electrooxidation of NADH at 3,4-DHB-modified electrodes in 0.1 M Tris/0.1 M acetate buffer (pH 7.0).

Table 1. Variation of the Rate Constants with pH in the Electrocatalytic Oxidation of NADH at 3,4-DHB-Modified Electrodes

pH[a]	E_{app}[b] (V)	$k_{1(C=0)} \times 10^{-3}$ (L mol^{-1} s^{-1})	k_{+2} (s^{-1})	K_M (mM)
5.0	0.40	14.8	1.00	0.06
7.0	0.25	4.3	0.70	0.14
7.5	0.27	2.8	0.54	0.19
8.5	0.10	1.3	0.31	0.20

[a] 0.1 M Tris/0.1 sodium acetate. [b] Applied potential. Since the formal potential of the surface-immobilized quinone is pH dependent, the applied potential was varied so as to have a constant driving force in all cases.

presence of both one- and two-electron acceptors.[26] The stability of the intermediate complex depends on the polarity of the solvent, and the reaction is strongly influenced by the concentration of protons.[15] In order to test for the effects of pH on the different kinetic constants, GC electrodes were modified with 3,4-DHB in 0.1 M phosphate (pH 7.0), and, afterward, the modified electrodes were immersed in 0.1 M Tris/0.1 M acetate buffer solutions, of different pH, containing NADH. The values of the different kinetic constants obtained in these experiments are summarized in Table 1. For comparison, the overall rate constant, k_1, was calculated for zero concentration of NADH ($k_{1(C=0)}$), since $k_1 = k_{+2}/K_M$ when $C^* \rightarrow 0$ (eq 10). As can be ascertained from Table 1, both the overall rate constant, k_1, and k_{+2} decreased with an increase in pH. On the other hand, the values obtained for K_M increased with increasing pH. The increase of K_M can be interpreted as being due to a diminution in the affinity of the mediator for NADH when the pH increases. This could be due, at least in part, to a change in the polarity of the electrodeposited 3,4-DHB films with pH (the pK_a for 3,4-DHB has been reported[27] to be 7.21). In addition, the mole fractions of the protonated (χ_{QH_2}) and deprotonated (χ_{QH^-}) forms of the mediator in the electrodeposited film will clearly be pH dependent, particularly over the range of pK_a ± 2. Based on this, one would anticipate that the value of k_{+2} would be dependent on the mole fraction of the protonated mediator (χ_{QH_2}), as was, indeed, found (Figure 4). This suggests that the dissociation of the intermediate complex between NADH and QH_2 is faster than that between NADH and QH^-, and, consequently, the catalytic process is more efficient when the mediator is fully protonated (i.e., present as QH_2).

Effect of the Presence of Divalent Cations. According to the results described above, there appears to be a relationship between the presence of a negative charge on the mediator (in the above-mentioned case due to deprotonation of the quinone) and the decrease in the catalytic efficiency of the electrooxidation of NADH at electrodes modified with 3,4-DHB. This would also suggest that such effects could, in principle, be mitigated, at least in part, if one could screen the charge. Katz et al. recently[28] reported that the presence of Ca^{2+} in the electrolyte gave rise to a considerable enhancement in the catalytic electrooxidation of NADH at PQQ-modified electrodes (PQQ = pyrroloquinoline quinone). These authors suggested the formation of a ternary complex between the Ca^{+2} ions, the immobilized PQQ, and NADH that could be more efficient in the electrocatalytic reaction than PQQ and NADH alone. Given our results presented above, and in an attempt to increase the electrocatalytic efficiency of the oxidation of NADH at electrodes modified with 3,4-DHB, we carried out a series of experiments where a variety of cations were deliberately added to the solution.

Curves a and b in Figure 5A show respectively the cyclic voltammograms at 10 mV/s of a 3,4-DHB-modified electrode in Tris/HCl buffer at pH 8.0 in the absence (a) and presence (b) of 1.0 mM NADH. Curves c−e show the effects, on the electrocatalytic current, of having 5.0 mM of NaCl, $MgCl_2$, and $CaCl_2$, respectively, present in solution. As can be ascertained, a significant enhancement in the electrocatalytic current was obtained in the presence of Ca^{2+} and Mg^{2+} ions. In order to compare results from different electrodes obtained in the presence

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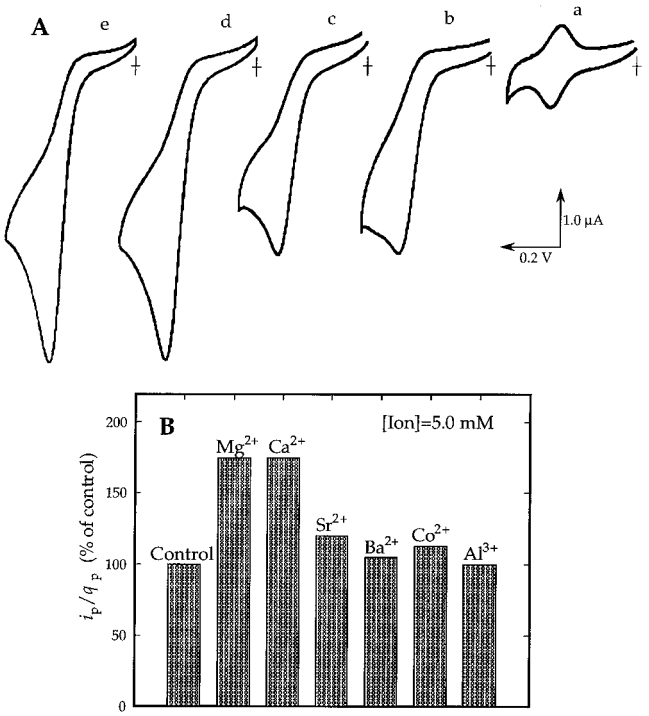

Figure 5. (A) Cyclic voltammograms at 10 mV/s of 3,4-DHB-modified electrodes in 0.1 M Tris/0.1 M acetate buffer (pH 8.0) (a) and in the presence of 1.0 mM NADH (b), 1.0 mM NADH + 0.1 M NaCl (c), 1.0 mM NADH + 5.0 mM $MgCl_2$ (d), 1.0 mM NADH + 5.0 mM $CaCl_2$ (d). Initial potential in all cases was −0.20 V. (B) Charge-normalized peak currents (i_p/q_p) for the electrooxidation of 1.0 mM NADH at 3,4-DHB-modified electrodes in the presence of 5.0 mM of several ions in 0.1 M Tris/0.1 M acetate buffer (pH 8.0). The control represents the value obtained for the charge-normalized peak current in the absence of deliberately added ion.

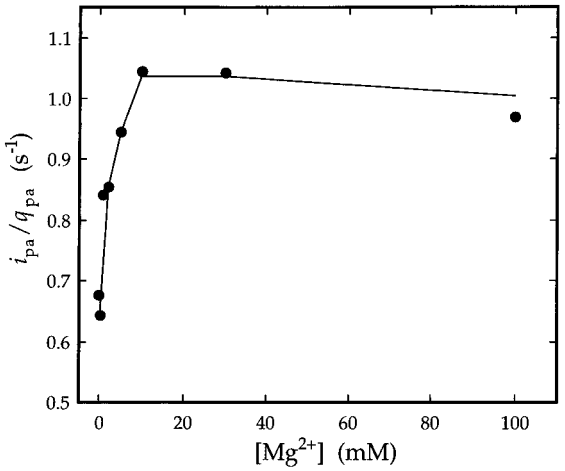

Figure 6. Charge-normalized peak current for the electrooxidation of 1.0 mM NADH at 3,4-DHB-modified electrodes in 0.1 M Tris/0.1 M acetate buffer (pH 8.0) as a function of the concentration of $MgCl_2$.

Table 2. Variation of the Rate Constants with pH in the Electrocatalytic Oxidation of NADH at 3,4-DHB-Modified Electrodes in the Presence of 20 mM Mg^{2+}

pH[a]	E_{app}[b] (V)	$k_{1(C=0)} \times 10^{-3}$ (L mol^{-1} s^{-1})	k_{+2} (s^{-1})	K_M (mM)
5.0	0.40	13.8	1.10	0.08
7.0	0.25	6.2	0.87	0.14
7.5	0.27	9.8	0.59	0.06
8.5	0.10	10.2	0.61	0.06

[a] 0.1 M Tris/0.1 sodium acetate. [b] Applied potential. Since the formal potential of the surface-immobilized quinone is pH dependent, the applied potential was varied so as to have a constant driving force in all cases.

and absence of the different ions, the peak current was normalized to the charge of the modified electrode alone (i_p/q_p). This was then expressed as a percentage of the value obtained in the absence of deliberately added ions (control). The results are presented in Figure 5B, where it is clear that the most significant enhancements were in the presence of Ca^{2+} and Mg^{2+}, both giving rise to an enhancement of about 75%. The presence of Ca^{2+} or Mg^{2+} ions during the electrodeposition of 3,4-DHB had no effect on the electrode modification process. In addition, the electrocatalytic oxidation of NADH in the absence of Ca^{2+} and Mg^{2+} ions in solution, but at electrodes modified in the presence of Ca^{2+} and Mg^{2+}, was virtually identical to that obtained in control experiments where neither Ca^{2+} nor Mg^{2+} was present during the electrodeposition process. Based on these observations, we can rule out nonspecific charge effects as well as the formation of a complex between the ion and the mediator as being responsible for the catalytic enhancement. These facts suggest the possibility of a ternary complex (mediator−NADH−ion) during the charge transfer process as being responsible for the enhancement. As mentioned above, this would be analogous to the observations made by Katz et al.[28] in a related system.

The enhancement effect on the electrocatalytic current for NADH oxidation due to the presence of Ca^{2+} and Mg^{2+} ions was concentration dependent. As shown in Figure 6, in the case of Mg^{2+}, the maximum effects were obtained at concentrations above 15.0 mM. Similar effects were observed for Ca^{2+}. Thus, in all further experiments where Ca^{2+} and Mg^{2+} ions were deliberately added, a concentration of 20 mM was employed.

It has been previously reported that, in numerous cases, modified electrodes used in the electrocatalytic oxidation of NADH exhibit a significant diminution in the electrocatalytic activity with successive scans.[10,15] Although not as marked, we have also observed a decrease in electrocatalytic activity with cycling, although the loss became quite small after about five cycles.[11] However, in the presence of 20.0 mM Mg^{2+}, the decrease in electrode activity during potential cycling was much smaller than that in its absence (data not shown). Thus, these ions give rise to an enhancement of not only the electrocatalytic current but also the stability.

The values of the rate constants for the reaction of NADH at 3,4-DHB-modified electrodes were obtained in 0.1 M Tris/0.1 M acetate buffer at different pH values in the presence of 20.0 mM Mg^{2+}, and the results are presented in Table 2. At pH 5.0, where the predominant form of the mediator is QH_2, the presence of Mg^{2+} ions had virtually no effect on the values of the kinetic constants K_1 and k_{+2} nor on the value of K_M when compared to results obtained in the absence of Mg^{2+}. At pH values greater than or equal to 7.0, the presence of Mg^{2+} ions gave rise to significant increases in the values of k_1 and k_{+2} when compared to results obtained in the absence of Mg^{2+} ions. For example, at pH 8.5, there was a 10-fold increase in the value of k_1 and a 2-fold increase in k_{+2}. In addition, although up to pH 7.0 there were no significant variations in the value of K_M in the presence or absence of Mg^{2+}, at higher pH the values of K_M in the presence of Mg^{2+} were lower than those in its absence. These results suggest that

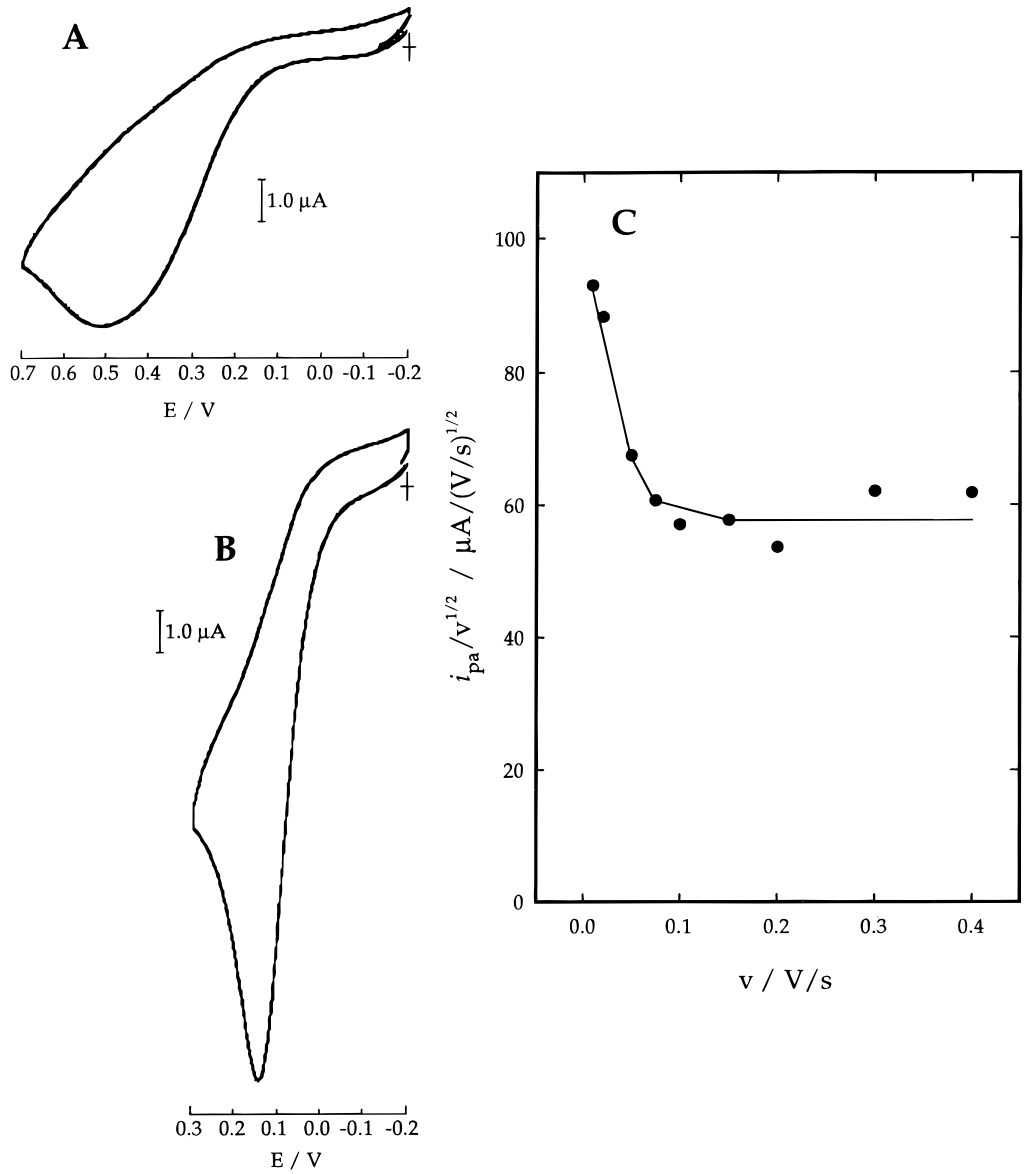

Figure 7. Cyclic voltammogram for the electrooxidation of 1.0 mM ascorbate at a bare GC electrode (A) and a 3,4-DHB-modified electrode (B) at 20 mV/s in 0.1 M phosphate buffer (pH 7.0). (C) Plot of the sweep rate-normalized peak current vs sweep rate for the electrooxidation of 1.0 mM ascorbate at the modified electrode.

the presence of Mg^{2+} favors the interaction between the deprotonated form of the mediator (QH^-) and the NADH in the intermediate complex, which could help explain the pH effect of the enhancement of the electrocatalytic oxidation of NADH in the presence of Mg^{2+} ions.

Electrocatalytic Oxidation of Ascorbate at GC Electrodes Modified with Electrodeposited Films of 3,4-DHB. It has been reported[29] that electrodes modified with catechol and aminophenol functionalities are capable of catalyzing the electrooxidation of ascorbate in a fashion similar to the electrocatalytic oxidation of NADH. If this were true for electrodes modified with films derived from 3,4-DHB, the presence of ascorbate would constitute a very serious interferent in the determination of NADH, particularly if the determinations were carried out in biological matrices, where the concentration of ascorbate can be in the millimolar regime.

In order to ascertain this possibility, the electrochemistry of ascorbate was studied at GC electrodes modified with films derived from 3,4-DHB, and the behavior contrasted with that obtained at unmodified electrodes. The results are depicted in Figure 7. At pH 7.0, ascorbate exhibits a broad and chemically irreversible anodic wave (Figure 7A), with a peak potential of +0.51 V. A plot of the sweep rate-normalized current ($i/v^{1/2}$) vs sweep rate (not shown) exhibited a constant response typical of a diffusion-controlled process. When the oxidation of ascorbate is carried out at an electrode modified with an electrodeposited film of 3,4-DHB, the cyclic voltammetric response exhibits a sharp catalytic wave (Figure 7B) with an anodic peak potential at +0.15 V. This peak potential is the same as that obtained for the 3,4-DHB-modified electrode in the absence of ascorbate. Under these conditions, the decrease in the overpotential for the electrooxidation of ascorbate was around 350 mV. In addition, a plot of the sweep rate-normalized current vs sweep rate exhibits the characteristic shape typical of an EC_{cat} process, as depicted in Figure 7C.

In a previous work,[16] we studied the interference effects of ascorbate in the electrocatalytic oxidation of NADH at glassy carbon electrodes modified with electrodeposited films derived

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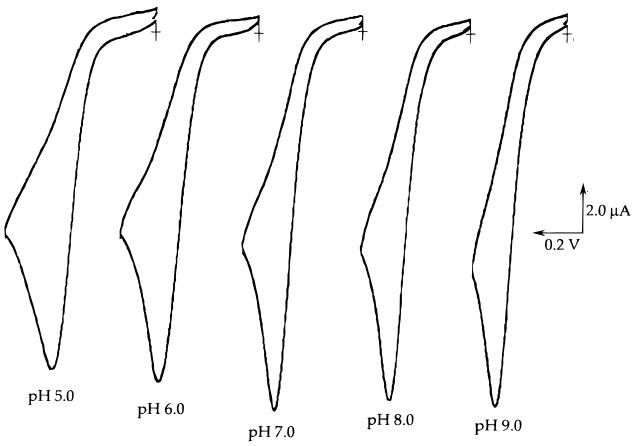

Figure 8. Cyclic voltammograms at 10 mV/s for the electrooxidation of 1.0 mM ascorbate at 3,4-DHB-modified electrodes in 0.1 M phosphate buffers of varying pH. Initial potential in all cases was −0.20 V.

from $[Fe(phen\text{-}dione)_3](PF6)_2$ (phen-dione = 1,10-phenanthroline-5,6-dione) and related materials. In that case, the electrocatalytic oxidation of NADH was achieved at −0.065 V, a value that was about 50 mV more negative than that for ascorbate. This potential difference allowed for the determination of NADH with no significant interference due to ascorbate. However, in the present case of electrodes modified with films of 3,4-DHB, the peak potentials for the electrocatalysis of both analytes are virtually the same, so that an alternate approach was required.

Given the above-mentioned results for NADH oxidation at modified electrodes in the presence of divalent ions, we studied the electrooxidation of ascorbate at 3,4-DHB-modified electrodes in the presence of 20 mM Ca^{2+} or Mg^{2+}. Contrary to NADH, in the case of ascorbate oxidation, no significant differences in the electrocatalytic current were obtained in the presence or absence of Ca^{2+} or Mg^{2+}. This suggests the presence of a specific effect of these ions in the electrocatalytic oxidation of NADH at 3,4-DHB-modified electrodes and, furthermore, that such an interaction is absent in the case of ascorbate.

We also investigated the effect of pH on the electrocatalytic oxidation of ascorbate. Figure 8 presents the electrooxidation of ascorbate at electrodes modified with films derived from 3,4-DHB in 0.1 M phosphate buffer at pH values ranging from 5 to 9. From the voltammetric responses, it is apparent that pH has, at best, a very small effect, with the catalytic current exhibiting a small increase over this pH range. Whereas over this entire pH range the ascorbate is negatively charged (pK_1 for ascorbate is reported to be 4.19), the mole fraction of the deprotonated mediator (χ_{QH^-}) increased with pH. These results indicate that the presence of a negative charge on the molecules involved does not exert a negative influence on the overall catalytic process and suggest a different mechanism from that observed for the electrooxidation of NADH. A series of experiments on the electrooxidation of ascorbate at 3,4-DHB-modified electrodes was carried out with the RDE at pH 7.0 and 9.0. Because the resulting diagnostic plots of $1/i_{c,max}$ vs $1/C^*$ and $1/k_1$ vs C^* were not completely linear over the concentration range of 0.05−2.5 mM, we could not unambiguously state that the electrocatalytic oxidation of ascorbate followed a mechanism similar to that of NADH or whether a different mechanism was operative.

Interference of Ascorbate in NADH Measurements. As shown above, the electrooxidation of ascorbate at GC electrodes modified with 3,4-DHB takes place at a potential that is very similar to that observed for NADH. Figure 9A shows the cyclic voltammetric response of such a modified electrode in solutions containing 1.0 mM NADH, at pH 8.0, after increasing amounts of ascorbate were subsequently added. In all cases, the catalytic waves exhibited a single and well-defined wave at a peak potential of +0.08 V. (Note: The shift in potential to +0.08 V is due to the difference in pH relative to the previously discussed results.) The charge-normalized peak current shows a linear dependence with the concentration of ascorbate (Figure 9B) over the range from 0 to 1.0 mM. In addition, the electrochemical response obtained

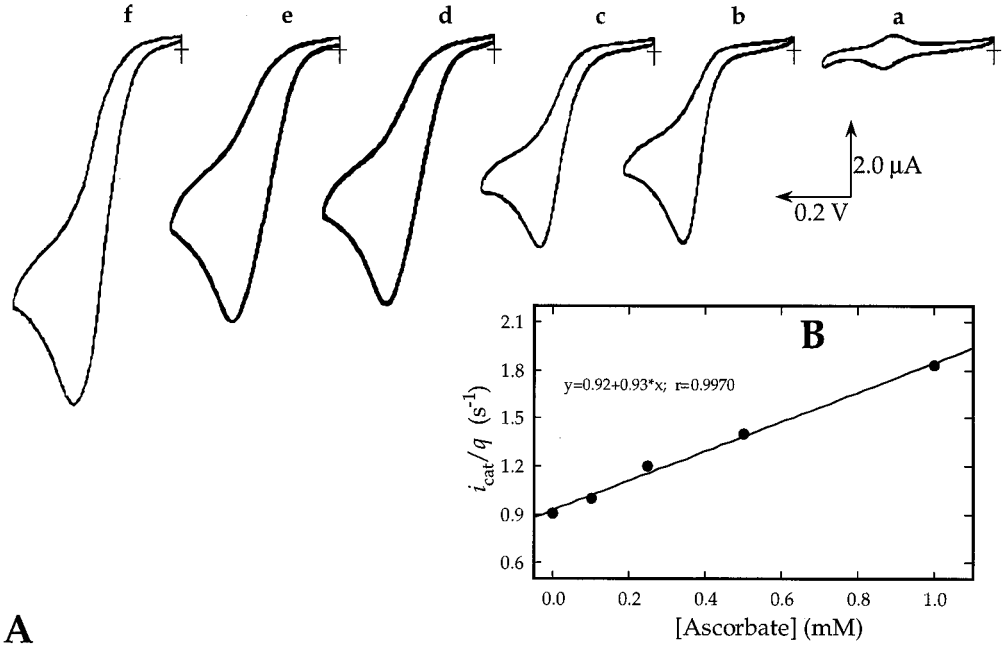

Figure 9. Cyclic voltammograms at 10 mV/s of glassy carbon electrodes modified with films derived from 3,4-DHB in 0.1 M phosphate buffer (pH 8.0) in the absence (a) or in the presence of (b) 1.0 mM NADH, (c) 1.0 mM NADH + 0.1 mM ascorbate, (d) 1.0 mM NADH + 0.25 mM ascorbate, (e) 1.0 mM NADH + 0.5 mM ascorbate, and (f) 1.0 mM NADH + 1.0 mM ascorbate. Initial potential in all cases was −0.20 V. (B) Plot of the charge-normalized electrocatalytic peak current vs concentration of ascorbate.

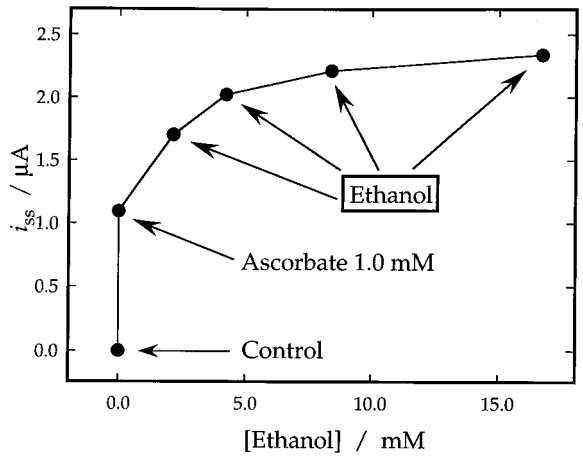

Figure 10. Effect of 1.0 mM ascorbate on the chronoamperometric response of an ethanol biosensor based on ADH in 0.1 M phosphate buffer (pH 8.0) containing 10.0 mM NAD^+. The steady state current at +0.18 V was measured after 30 s.

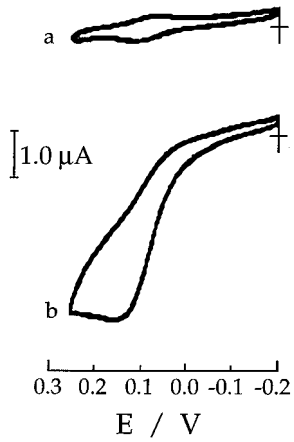

Figure 11. Cyclic voltammogram at 10 mV/s of a GC electrode modified with a film derived from 3,4-DHB and with a nylon mesh containing 1.0 unit of immobilized AOX in 0.1 M phosphate buffer (pH 7.0) containing 1.0 mM ascorbate and in the absence (a) and in the presence (b) of 1.0 mM NADH.

when both analytes were present at the same concentration (1.0 mM) was practically double that obtained when only 1.0 mM NADH was present in the solution. This suggests noncompetitive effects between both analytes for the active mediator sites, at least over this concentration range.

Since ascorbate shows a strong interference effect in the electrocatalytic determination of NADH at 3,4-DHB-modified electrodes, it is clear that such an effect will be evident in the determination of enzymatically generated NADH. In order to demonstrate this, we investigated the effect of ascorbate (1.0 mM) on the electrochemical response of an amperometric ethanol biosensor based on alcohol dehydrogenase (ADH) immobilized on a nylon mesh as described previously.[12] The results of such an experiment are shown in Figure 10, where the steady state current, i_{ss}, obtained in the absence of ethanol and ascorbate (but in the presence of 10 mM NAD^+) was taken as the zero point of the experiment (control). Addition of 1.0 mM of ascorbate when no ethanol was present in solution gave rise to a significant increase in the current, indicating a strong electrocatalytic effect as well as effective transport (diffusion) of ascorbate through the

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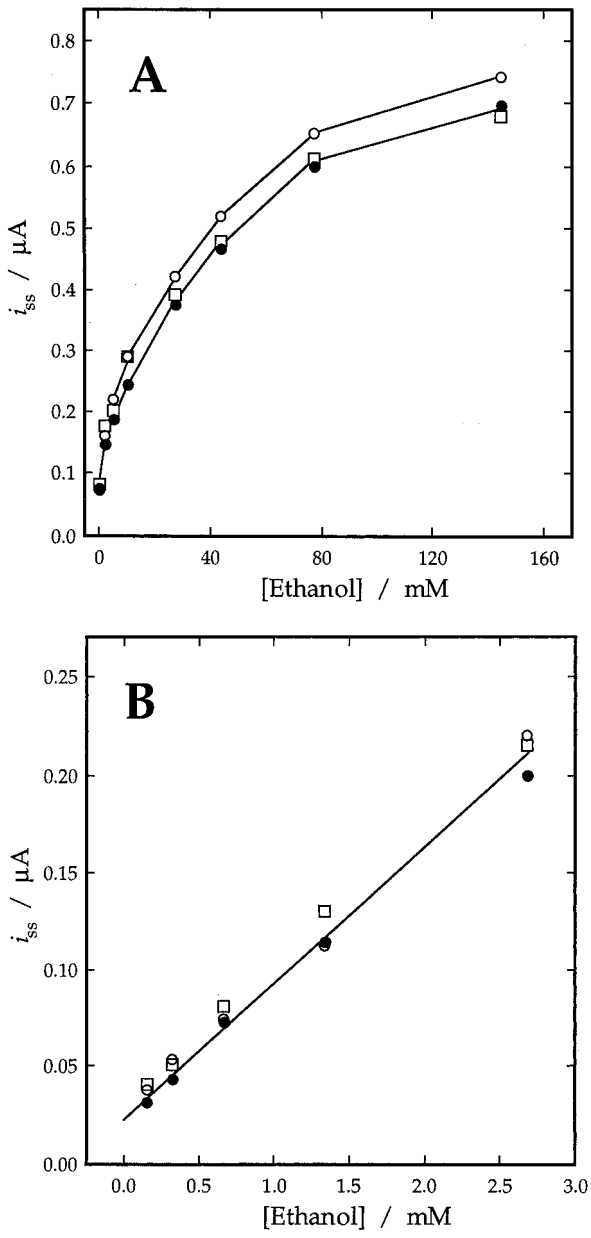

Figure 12. (A) Chronoamperometric calibration curves (i_{ss} vs [ethanol]) obtained at (○) ADH-based biosensor in the absence of ascorbate, (□) ADH/AOX biosensor in the absence of ascorbate, and (●) ADH/AOX biosensor in the presence of 1.0 mM ascorbate. 10 mM NAD^+ was present in all solutions. The steady state current was measured at +0.18 V after 30 s. (B) Linear range of the chronoamperometric responses for part A.

biosensor nylon mesh. Subsequent additions of substrate (ethanol) gave rise to an increase in the electrocatalytic current, which depended on concentration. These increased currents are due to the electrocatalytic oxidation of the enzymatically generated NADH (from the NAD^+ present).

When the concentration of ethanol was 1.0 mM, the steady state current was not double that obtained in the presence of 1.0 mM of ascorbate when no ethanol was present in solution. This fact suggests that the enzymatic generation of NADH is slower than the diffusion and subsequent electrooxidation of ascorbate and NADH at the mediator monolayer. Therefore, the biosensor appears to be controlled by the enzymatic kinetics.

Suppression of Interference Effects Due to Ascorbate. According to the above results, it is not possible to discriminate

between the electrooxidation of ascorbate and NADH by their voltammetric responses at electrodes modified with films derived from 3,4-DHB when both species are present in solution. This can strongly affect the selectivity of biosensors based on enzymatic activities involving NADH measurements in the presence of ascorbate.

Ascorbate oxidase (AOX) is an oxidoreductase enzyme capable of selectively oxidizing L-ascorbate to dehydroascorbate in the presence of molecular oxygen. Preparations of this enzyme with high specific activity (1000−3000 units/mg of protein) are commercially available. In addition, these preparations remain active and stable after immobilization on a nylon mesh using the procedures described earlier. Wightman et al.[30] recently reported that the use of AOX co-immobilized with glucose oxidase gave rise to a virtually complete suppression of interferences due to oxidation of ascorbate in a glucose biosensor. Although, in our case, the interference due to ascorbate is via an electrocatalytic oxidation in the presence of the mediator, we carried out similar experiments in order to ascertain if the presence of AOX could eliminate this interference in electrodes modified with films derived from 3,4-DHB.

Figure 11 shows the cyclic voltammetric response obtained when a 3,4-DHB/AOX biosensor was used in solutions containing 1.0 mM of ascorbate and in the absence (a) or presence (b) of NADH. In the absence of NADH (Figure 11a), the catalytic wave observed for the electrooxidation of ascorbate at the 3,4-DHB-modified electrode is absent, and only the response associated with the surface-immobilized 3,4-DHB is evident. The addition of NADH (1.0 mM) to the solution gives rise to an electrocatalytic wave due to the electrooxidation of NADH at the 3,4-DHB monolayer. In this case, the immobilized AOX does not affect the transport of NADH that diffuses easily through the biosensor and is electrocatalytically oxidized at the 3,4-DHB-modified electrode surface (Figure 11b).

The above results suggest that the co-immobilization of AOX along with a dehydrogenase activity could be a very effective way of determining enzymatically generated NADH in the presence of ascorbate. We tested this possibility by constructing an ascorbate-insensitive (interference-free) ethanol biosensor based on the combination of alcohol dehydrogenase (ADH) and ascorbate oxidase (AOX). The electrochemical response to increasing amounts of ethanol of such a bienzyme biosensor when 1.0 mM ascorbate was present in solution (Figure 12A, ●) was quite similar to that obtained when no ascorbate was added (Figure 12A, □). In addition, this response was very similar to that obtained when an ADH standard biosensor was used in the absence of ascorbate (Figure 12A, ○).

The linear response range and the ethanol detection limit for this ADH/AOX biosensor (Figure 12B) were also similar to those obtained for the standard ADH biosensor in the absence of ascorbate and of the same order of magnitude as that previously reported by us[31] for GC electrodes modified with [Fe(phendione)$_3$](PF6)$_2$ complexes, with the advantage of having a complete suppression of interference effects due to ascorbate. In addition, our level of detection was of the same order as that reported by Park et al.[32] but somewhat higher than that reported by Gorton et al.[33] However, in both of these cases, the interference effects due to the presence of ascorbate would be significant.

CONCLUDING REMARKS

The electrocatalytic oxidation of NADH and ascorbate at glassy carbon electrodes modified with electrodeposited films derived from 3,4-DHB has been studied. In the case of NADH, the process appears to take place via formation of an intermediate complex as derived from RDE studies as a function of NADH concentration.

Because the electrocatalytic responses obtained for both ascorbate and NADH are very similar, it is not possible to discriminate between them on the basis of their electrochemical responses alone. Placing a nylon mesh with immobilized AOX on a GC electrode modified with 3,4-DHB quickly and effectively depletes the ascorbate present at the electrode surface by oxidizing it to dehydroascorbate. This enzymatic depletion of ascorbate does not interfere with the electrocatalytic oxidation of NADH on the electrode surface. An ascorbate-insensitive (interference-free) ethanol biosensor based on the use of both ADH and AOX and a 3,4-DHB-modified GC electrode has been constructed.

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