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Rate-Limiting Steps of Tyrosinase-Modified Electrodes for the Detection of Catechol

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The response currents obtained for tyrosinase-modified Teflon/graphite, carbon paste, and solid graphite electrodes in the presence of catechol are analyzed primarily using rotating disk electrode experiments. The rate-limiting steps, such as the electrochemical reduction of *o*-quinones and the enzymatic reduction of oxygen as well as the enzymatic oxidation of catechol, are theoretically considered and experimentally demonstrated for the different electrode configurations.

The environmental control of organic pollutants such as phenolic compounds in industrial waste effluents has initiated the development of analytical techniques for fast, sensitive, and selective monitoring of hazardous compounds.[1] This is of particular interest for phenols, since they have been shown to be organic carcinogens and mutagens.[2] Phenols can be determined amperometrically through direct electrochemical oxidation.[3] However, direct oxidation suffers a number of drawbacks. Due to the high overvoltage, a high anodic potential needs to be applied, opening up the detection system for interfering reactions. The high applied voltage is also followed by an increase in the background current and noise level. Moreover, direct electrochemical oxidation of phenols is coupled with fouling reactions.[3] The utility of enzyme-based amperometric biosensors to solve these obstacles for the determination of phenols has been experimentally demonstrated in a number of works, where enzymes such as tyrosinase,[4−17] laccase,[18,19] and peroxidase[20] have been successfully integrated with electrochemical transducers. These biosensors operate in a potential window (around 0 V), where they are much less subjected to interfering reactions. Moreover, the sensitivity and detection limits achieved with biosensors are much improved compared to those obtained by direct oxidation. The latter is due to the amplification of the response current as a result of recycling of substrate at the surface of the bioelectrode.

Tyrosinase-modified electrodes have been used for the detection of monophenols and *o*-diphenols, and in this respect they are often experimentally studied. Tyrosinase immobilization procedures, electrode materials, stability, and sensitivity are among the scope of these studies.[4−17] To illustrate achievements in this field, the sensitivity and the detection limits of some of these electrodes for the determination of catechol are presented in Table 1. As can be seen, the highest sensitivity was observed with carbon paste electrodes doped with ruthenium and solid graphite chemically modified with tetracyanoquinodimethane. Ruthenium on carbon,[4] Meldola blue in carbon paste,[5] and chemical modification of solid graphite with tetracyanoquinodimethane[6] and detergents in the buffer solution[7] have been experimentally demonstrated to positively influence the sensitivity and the stability of tyrosinase-based biosensors. The best detection limits were demonstrated with the tyrosinase electrochemically copolymerized in polypyrrole or covalently cross-linked with glutaraldehyde on top of a solid graphite electrode. The detection limits for most of the tyrosinase-modified electrodes still have to be improved in order to measure concentrations of phenolics in environmental waters lower than 5 nM. In some respects, the success of future developments of the tyrosinase-modified electrodes depends on the mechanistic studies of their characteristics.

The aim of the present study is to investigate the mechanism and the rate-limiting steps of some different configurations of tyrosinase-modified electrodes, specifically (i) solid graphite with cross-linked tyrosinase on the surface, (ii) bulk modified Teflon/graphite, and (iii) modified carbon paste electrodes. These biosensor configurations have been used for the detection of phenolic compounds in combination with tyrosinase.[4−17] The

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Table 1. Comparison of the Tyrosinase-Modified Electrodes for the Detection of Catechol in Aqueous Solution

electrode	enzyme immobilization procedure	analysis mode	sensitivity/A M^{-1} cm^{-2}	lower detection limit/nM	ref
glassy carbon	tyrosinase electropolymerized in polypyrrole layer	steady-state	1.5	2.0	9
solid graphite	covalently cross-linked tyrosinase on the carbodiimide-activated graphite	flow injection	2.2	2.3	7
carbon paste	tyrosinase adsorbed on the surface of the electrode	steady-state	1.8	10	10
graphite/epoxy resin composite	lyophilized enzyme powder mixed with composite	flow injection	0.4	40	11
graphite/Teflon composite	lyophilized enzyme powder mixed with composite	flow injection	0.01	200	13
solid graphite	tyrosinase immobilized on the surface of chemically modified electrode	steady-state	3.4	100−300	6
carbon paste	5% ruthenium on carbon admixed with mineral oil, octadecylamine, and enzyme	steady-state	3.1	500	4
carbon paste	enzyme powder admixed during paste preparation	flow injection	0.8	900	7
carbon paste	mixture of graphite powder, tissue from eggplant, and mineral oil	steady-state	0.1	1000	12

results presented in this paper are mainly based on rotating disk electrode (RDE) experiments.

EXPERIMENTAL SECTION

Preparation of Solid Graphite Electrodes with Cross-Linked Tyrosinase on the Surface. Tyrosinase from mushroom with an activity of 3900 units mg^{-1} (Cat. No. T-7755, Sigma, St. Louis, MO) was immobilized on the surface of carbodiimide-activated graphite.[8] Rods of spectrographic graphite (RW 001, Ringsdorff-Werke GmbH, Bonn-Bad, Godesberg, Germany) with a diameter of 0.305 cm were cut, polished on wet emery paper, and washed with deionized water. After polishing, the graphite rods were heated at 700 °C for 90 s in a Muffle furnace and kept for 2 h at room temperature in 0.1 M *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (Cat. No. 03451, Fluka, Buchs, Switzerland) solution, dissolved in 0.05 M acetate buffer at pH 4.8. Later, the rods were rinsed with the same acetate buffer and placed into a solution with 5.6 mg cm^{-3} of tyrosinase concentration in 0.1 M phosphate buffer (pH 6.0) also containing 1.25% (v/v) glutaraldehyde (25% in water, Fluka). The enzyme immobilization reaction was allowed to proceed for 14 h at 4 °C. Finally, the enzyme electrodes were rinsed with 0.1 M phosphate buffer (pH 6.0) and stored in the same buffer at 4 °C until further use.

Preparation of Tyrosinase-Modified Teflon/Graphite Electrodes. Graphite powder (0.08 g) (Cat. No. 50870, Fluka), pretreated Teflon/graphite (0.62 g) (Radiometer A/S, Copenhagen, Denmark), and mushroom tyrosinase (4 mg) were placed in a glass beaker with a glass cover, and the contents were shaken for 30 min. Next, the mixture was pressed within a 1.3 cm diameter pellet press at 7000 kg cm^{-2}. The resulting pellet was cut and press-fitted into a Teflon holder to fit the RDE. The surface of this electrode was initially polished with emery paper and further with an aluminum oxide suspension (Struers, Copenhagen, Denmark) and thoroughly rinsed with water. The geometric surface area of the electrode was equal to 0.073 cm^2.

Preparation of Tyrosinase-Modified Carbon Paste Electrodes. Carbon paste was prepared using the same materials and procedures as reported previously.[4] Graphite powder (40 mg) (Fluka), paraffin oil (60 mg) (Cat. No. 76235, Fluka), and octadecylamine (5 mg) (Cat. No. S-9273, Sigma) were dissolved in chloroform and mixed in an agate mortar. The solvent was allowed to evaporate overnight, and 5 mg of tyrosinase was added to the carbon/oil composite and hand-mixed for 30 min in the mortar. The paste was packed into the end of the Teflon holder and polished on white filter paper. The geometric surface area of the paste electrode was 0.073 cm^2.

Rotating Disk Electrode Experiments. Before starting the RDE experiments, each tyrosinase-modified electrode was allowed to rotate in 0.25 M phosphate buffer solution (pH 6.0) for 20 min to remove weakly adsorbed enzyme. The rotating disk electrode (Tacussel, France; Model EDI) was then placed into a three-electrode electrochemical cell containing 25 cm^3 of a 0.25 M phosphate buffer at pH 6.0. All experiments were performed at a stationary potential, and the current values were collected with a computer-controlled BAS electrochemical analyzer (Bioanalytical System, West Lafayette, IN; Model 100W). After the background current of the electrode became stable, catechol was injected into the electrochemical cell, and the reduction current was recorded at different rotation speeds. Catechol concentrations used in the experimental studies were chosen as low as possible to avoid occurrence of uncontrolled side reactions. The applied potential of the electrode was −0.05 V versus the reference saturated calomel electrode (SCE, Radiometer, Copenhagen, Denmark; Model K-401), fitted to the cell with a Lugging capillary. A platinum wire electrode was used as the counter electrode. This three-electrode setup was used in all electrochemical experiments. All experiments were made at room temperature, and all potentials are referred to the SCE.

Cyclic Voltammetry. CV was performed using the BAS equipment in the potential range from −0.2 to +0.6 V at sweep rates between 0.005 and 0.05 V s^{-1}. CV at the solid graphite electrode containing cross-linked tyrosinase on the surface was carried out after the enzyme was inactivated by keeping the electrodes in HCl solution at pH 2.5 for 3 h. Other experimental conditions were the same as for the RDE experiments. The apparent heterogeneous rate constant for catechol, k_o^{app}, was evaluated from the peak separation in cyclic voltammograms according to the method of Nicholson.[21] The formal redox potential, $E^{\circ\prime}$, of catechol/*o*-quinone was calculated from the midpoint between the anodic and cathodic peak potentials of the cyclic voltammograms.

Chronoamperometry and Rotating Ring Electrode Experiments. These experiments, using a platinum ring electrode, were performed to estimate the diffusion coefficient for catechol. The inner and outer diameters of the ring were 0.332 and 0.358 cm, respectively. In both chronoamperometry and rotating ring electrode experiments, rotating ring disk electrode equipment was used (Oxford Electrodes, Abingdon, Oxon, UK). The electrode was connected to the BAS electrochemical analyzer. In chronoamperometry, the electrode potential was stepped from 0.1 to 0.7

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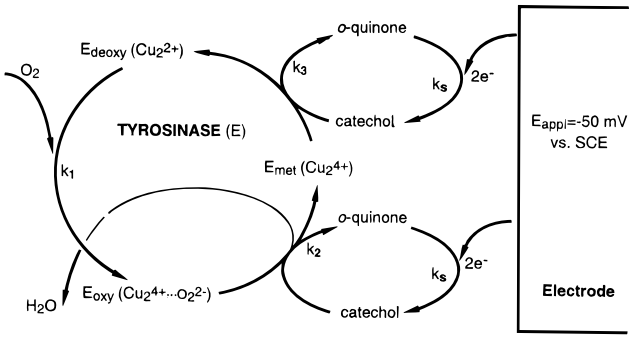

Figure 1. Mechanism of tyrosinase-modified electrode. E_{met}, E_{deoxy}, and E_{oxy} are the resting, reduced, and oxy forms of tyrosinase. k_1, k_2, and k_3 are bimolecular rate constants. k_s is the rate constant of the electrochemical reduction of *o*-quinone.

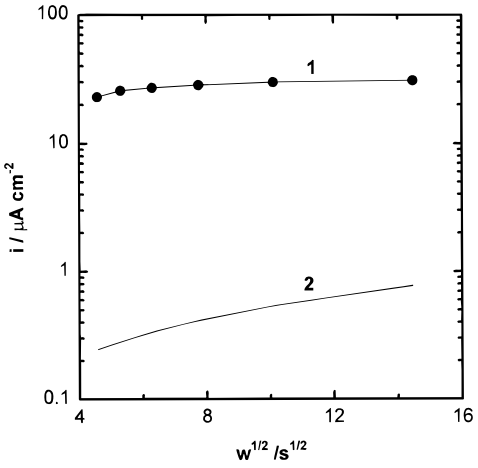

Figure 2. Dependence of reduction current on electrode rotation velocity. Solid graphite electrode modified with cross-linked tyrosinase on the surface. (1) Experimentally observed and (2) theoretically calculated diffusion-limited current values at 0.5 μM of catechol; 0.25 M phosphate buffer solution, pH 6.0; applied potential, −0.05 V vs SCE.

or 0.8 V, when 0.25 M phosphate buffer (pH 6.0) or 0.25 M succinate buffer (pH 5.0) was used as the supporting electrolyte, respectively. Catechol oxidation currents were recorded for 10 s. During rotating ring electrode experiments, the dependence of the catechol oxidation current on the rotation velocity was recorded at 0.5 V in 0.25 M phosphate buffer solution (pH 6.0). When 0.25 M succinate buffer solution (pH 5.0) was used, the potential was held at 0.7 V.

Solutions. A stock solution of 1.0 mM of catechol (Cat. No. C-9510, Sigma) was prepared daily by dissolving an appropriate amount of catechol in acetonitrile. Phosphate buffer was prepared from NaH_2PO_4 (Cat. No. 6346, Merck, Darstadt, Germany), acetate buffer from $CH_3COONa{\cdot}3H_2O$ (Cat. No. 6267, Merck), and succinate buffer from succinic acid (Cat. No. S-0141, Sigma). The pH was adjusted by addition of concentrated sodium hydroxide. All chemicals were of analytical grade. HPLC-grade water was produced in a Milli-RO4 water purification system (Millipore, Bedford, MA) and was used throughout this work.

RESULTS AND DISCUSSION

Mechanism of Tyrosinase-Modified Electrodes. The catalytic cycle of tyrosinase[22] with catechol as substrate and subsequent electrochemical reduction of *o*-quinones occurring at the solution−electrode interface are presented in Figure 1. Within one enzyme turnover two catechol molecules participate with the production of *o*-quinone molecules. This step is followed by an electrochemical reduction of the *o*-quinone to form catechol. Here, cycling of catechol between the enzyme and the electrode is assumed. The validity of this model (Figure 1) was analyzed by comparing the experimentally obtained reduction current density with that theoretically limited by the diffusion of catechol alone. This was done using RDE experiments with a graphite electrode containing cross-linked tyrosinase on the surface. Since the model is assumed to be general for all types of electrode configurations, experiments were performed only with the graphite electrode. Experimental results are shown and discussed below.

The highest current density (j) that can be obtained without any recycling of catechol is illustrated in Figure 2 (curve 2). This current density is determined by the flux of catechol to the electrode and can be calculated using the Levich equation,[23] eq 1, where $n = 2$ is the number of electrons transferred upon

$$j = nFk_{D,M}[M] \qquad (1)$$

electrochemical redox conversion of *o*-quinone/catechol, F is the Faraday constant, $k_{D,M}$ is the mass transport rate constant[23] ($k_{D,M} = 0.62D_M^{2/3}\omega^{1/2}\nu^{-1/6}$), and ω and ν are the rotation velocity of the electrode and the kinematic viscosity of water (0.01 $cm^2\ s^{-1}$), respectively. The diffusion coefficient, D_M, for catechol was assumed to be equal to 8.5 10^{-6} $cm^2\ s^{-1}$ (procedure of the determination is discussed in the Appendix). [M] is the catechol concentration in the bulk solution. The experimentally obtained response currents at different rotation velocities of the electrode are plotted in Figure 2 (curve 1) and are found to range from 94 to 40 times higher than those theoretically calculated (Figure 2, curve 2) at the lowest (20.9 rad s^{-1}) to the highest (209 rad s^{-1}) rotation velocities, respectively. The fact that the observed response currents for the solid graphite electrodes with cross-linked tyrosinase on the surface are higher than the theoretically calculated values, where no recycling of catechol was considered, confirms the mechanism depicted in Figure 1. Moreover, the ratio of the experimental current response to the current theoretically limited by the diffusion of catechol can be regarded as the amplification of the signal (amplification factor, AF) due to the recycling of catechol.

Evaluation of the Recycling of Catechol at a Graphite Electrode with Cross-Linked Tyrosinase on the Surface. The upper limit for the amplification of the signal, AF_{max}, at a tyrosinase-modified electrode can be reached when the enzymatic reactions are relatively fast (not rate-limiting), and the current response is determined by the mass transfer or the electrochemical reduction rate of *o*-quinone at the solution/electrode interface, or both. In this case, the AF_{max} value depends obviously on the rate of two opposing processes: (i) the faster the mass transport (at high rotation velocity), the more the *o*-quinone molecules are flushed away from the electrode surface, resulting in a lower AF_{max}, and (ii) the faster the electrochemical reduction of *o*-quinones, the lower is their loss, causing an increase in the AF_{max}. According to this, the AF_{max} at steady-state conditions can be determined by eq 2. As indicated (eq 2), the AF_{max} value depends only on the

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$$AF_{max} = (k_s + k_{D,M})/k_{D,M} \quad (2)$$

heterogeneous electron transfer (k_s) and the mass transfer rate constants ($k_{D,M}$). The value of $k_{D,M}$ is defined at each rotation velocity, and the value of k_s can be determined experimentally by cyclic voltammetry of catechol. Cyclic voltammograms of catechol were recorded with this electrode and found to be quasi-reversible for the following potential sweep rates: 0.005, 0.01, 0.02, and 0.05 V s^{-1}. The standard heterogeneous electron transfer constant, k_o^{app}, was found to be equal to $0.4(\pm 0.2) \times 10^{-3}$ cm s^{-1}. Thereafter, the heterogeneous electron transfer constant for *o*-quinone reduction was calculated using eq 3,[23] where the

$$k_s = k_o^{app} \exp\left(-\frac{\alpha n F(E_{appl} - E^{\circ\prime})}{RT}\right) \quad (3)$$

electron transfer coefficient, α, and the number of electrons, *n*, involved in the electrochemical redox conversion of *o*-quinone/catechol were assumed to be 0.5 and 2, respectively. The applied potential, E_{appl}, and formal potential, $E^{\circ\prime}$, of the catechol/*o*-quinone redox couple were −0.05 and 0.212 V vs SCE, respectively. *R* and *T* are the universal gas constant and absolute temperature ($T = 298$ K), respectively.

The calculated k_s value was found to be $10(\pm 4.7)$ cm s^{-1} for the cross-linked tyrosinase electrode. According to eq 2, the AF_{max} for catechol is estimated to range from 4040 to 1260 at 20.9−209 rad s^{-1} (between the lowest and highest rotation velocity used in the experiments). These values would allow 43 to 32 times higher amplification factors of the responses, respectively, when compared with the actual amplification factors found for the same electrode (from 94 to 40 at the same rotation velocities).

Despite the theoretically possible maximal signal amplification, which is fulfilled if the enzymatic reactions are fast, the AF_{max} value has additional meaning concerning a single catechol molecule. Using the same considerations (i) and (ii) presented above when discussing AF_{max}, it can be concluded that the AF_{max} value is equal to the number of times the same catechol molecule passes the enzymatic and electrochemical reaction cycle (Figure 1). Thus, the AF_{max} value is equal to the recycling number, RN, of catechol at the electrode. It is important to emphasize, that RN does not depend on the activity of enzyme (eq 2). While the number of catechol molecules oxidized per unit of time does depend on enzyme activity, the number of times the same catechol molecule participates in the electrochemical and the enzymatic reaction cycle depends solely on the rates of heterogeneous electron transfer and the mass transfer of *o*-quinone (eq 2). From the $AF_{max} = RN$, it follows that a single catechol molecule is recycled from 1260 to 4040 times at the cross-linked tyrosinase electrode at rotation velocities between 209 and 20.9 rad s^{-1}.

Theoretical Consideration of the Rate-Limiting Steps of Tyrosinase-Modified Electrodes. The general mechanism presented in Figure 1 points out several reactions that can limit the electrode responses. Two of them, oxidation of catechol and reduction of oxygen, are related to the enzyme activity. The reduction of *o*-quinones is an electrochemical reaction. The dependence of the response on the electrode rotation velocity under the limitation of these three reactions is discussed below.

When the reduction of o-quinone is the rate-limiting step for the response of the tyrosinase-modified electrodes, the following can be taken for granted. The mass transport of oxygen and its reaction with the enzyme are not rate-limiting. Consequently, this simplifies the enzyme kinetics and actually excludes oxygen as a "necessary" reagent for the oxidation of catechol. Further, simple Michaelis−Menten enzyme kinetics with catechol is assumed. Equations 4a−c, based on the equilibrium of the flux, should be fulfilled at steady-state conditions. The concentration of catechol

$$\frac{V_{max}}{K_{m,M}}[M] = k_{D,M}([M] - [M]_o) + k_s[Q]_o \quad (4a)$$

$$k_{D,M}([M] - [M]_o) = k_{D,M}[Q]_o \quad (4b)$$

$$j = nFk_s[Q]_o \quad (4c)$$

is assumed to be much lower than the Michaelis−Menten constant,[24] $K_{m,M} = 0.19$ mM, of mushroom tyrosinase for this substrate. In eqs 4a−c, *j* and V_{max} are the current density of *o*-quinone reduction at the tyrosinase electrode and the maximal value of the enzymatic reaction rate, respectively. [M] and $[M]_o$ are the catechol concentrations in the bulk and at the electrode surface, respectively. $[Q]_o$ is the *o*-quinone concentration at the electrode surface (the concentration of *o*-quinone in the bulk is assumed to be zero). $k_{D,M}$ and k_s are the mass transport and heterogeneous electron transfer (for the electrochemical reduction of *o*-quinones at −0.05 V vs SCE) rate constants, respectively. The diffusion coefficients are assumed to be the same for catechol and *o*-quinone in the solution. Other symbols have their usual meaning (see eq 1). Manipulation of eqs 4a−c leads to the analytical dependence of the current density on the rotation velocity of disk electrode (eq 5). According to eq 5, the current

$$\frac{1}{j} = \frac{1}{nF[M]}\left(\frac{0.62 D_M^{2/3} \omega^{1/2} \nu^{-1/6} K_{m,M}}{k_s V_{max}} + \frac{1}{k_s} + \frac{K_{m,M}}{V_{max}}\right) \quad (5)$$

density should decrease with increasing rotation velocity of a disk electrode. This relationship (eq 5) is valid for unsaturated enzyme kinetics accounted in eq 4a. A similar $j-\omega$ behavior was previously predicted[25] and experimentally demonstrated[26] when the enzyme was saturated with substrate. This leads to the conclusion that, at any $[M]/K_{m,M}$ ratio the response should decrease with increasing rotation velocity of the electrode if the electrochemical reduction of *o*-quinone is the rate-limiting reaction.

When the enzyme kinetics is thought to be the rate-limiting step, the assumption that the tyrosinase is saturated with oxygen should be examined first. The K_m for this substrate has been reported to be 0.14 mM.[24] Less than 0.25 mM concentrations of oxygen can be dissolved in the buffer solutions in equilibrium with air.[27] If the electrochemical conversion of *o*-quinone into catechol is relatively fast, then this compound behaves as an efficient mediator for oxygen reduction at the tyrosinase-modified electrode. Steady-state analysis of mediated amperometric biosensors has been

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comprehensively described[28] and is mainly based on the assumption that the concentration of the mediator is in excess. Saturation of an enzyme with a mediator allows neglecting the consideration that the reaction of the mediator with the enzyme can be the rate-limiting step. However, this can rarely be met for the tyrosinase electrode because of the low concentration of phenols in the environmental samples. For further analysis of the mediated reduction of oxygen at the tyrosinase electrode, it is assumed that the electrochemical reduction of *o*-quinones is fast, and according to this, at steady-state conditions, it should follow that (i) there is no diffusion layer for catechol and (ii) the concentration of *o*-quinones is infinitely low at the electrode surface. The same (i and ii) were experimentally demonstrated for horseradish peroxidase-modified graphite electrodes,[29] the performance of which can be described by a similar mechanism. From the reaction sequence presented in Figure 1, the rate of the enzymatic reaction, v, can be defined by eq 6, leading to a mathematical rate expression of the Michaelis−Menten type, eq 7, where $[M]_o$ and

$$v = k_2[E_{oxy}][M]_o + k_3[E_{met}][M]_o \quad (6)$$

$$v = V_{max}c_o/(K_m^{app} + c_o) \quad (7)$$

c_o are the concentrations of catechol and oxygen at the surface, respectively. V_{max} is equal to $2[E_t][M]_o k_2k_3/(k_2 + k_3)$, where $[E_t]$ is the total enzyme concentration on the surface of the electrode and k_1, k_2, and k_3 are the bimolecular rate constants for the appropriate enzyme−substrate reactions presented in Figure 1. According to the mechanism (Figure 1), the apparent Michaelis−Menten constant for oxygen, K_m^{app}, is defined as $[M]_o k_2k_3/(k_1(k_2 + k_3))$. At steady state, the flux of oxygen, $j = k_D(c^* - c_o)$, is equal to the rate of the enzyme reaction. k_D and c^* are the mass transfer constant and the concentration of oxygen in the bulk solution, respectively. Further, the electrode response will be analyzed separately at three K_m^{app}/c_o ratios, (1) $K_m^{app} \gg c_o$, (2) $K_m^{app} \approx c_o$, and (3) $K_m^{app} \ll c_o$, because the general analytical expression for the current of a tyrosinase electrode based on the equilibrium of the oxygen flux and the rate of the enzyme reaction is complicated.

Enzymatic reduction of oxygen is the rate-limiting step when $K_m^{app} \gg c_o$. This case is met at a low concentration of oxygen, or/and a high activity of tyrosinase, or/and when the catechol concentration is relatively high. The rate of the enzyme reaction is controlled by the rate of the oxygen reaction with tyrosinase, $v = 2k_1[E_t]c_o$, and the dependence of the response on the rotation velocity of the electrode can be linearized in Koutecky−Levich coordinates according to eq 8.[30] It should be noted, however, that

$$\frac{1}{j} = \frac{1}{nFk_Dc^*} + \frac{1}{nF2k_1[E_t]c^*} \quad (8)$$

for practical purposes, tyrosinase-modified electrodes as well as any other biosensor for the detection of phenols in environmental waters are usually directed to determine very low concentrations of the phenolic compounds; therefore, the conditions when $K_m^{app} \gg c_o$ should hardly be fulfilled.

In the case where $K_m^{app} \approx c_o$, the Michaelis−Menten expression, eq 7, cannot be simplified. The combination of the oxygen flux with eq 7 does not lead to a linear current density−rotation velocity relationship (eqs 9 and 10). However, eq 10 can be directly fitted to the experimental data to evaluate the kinetic constants of the enzyme electrodes.

$$J^2 - (k_DK_m^{app} + k_Dc^* + J_{max})J + k_DJ_{max}c^* = 0 \quad (9)$$

$$J = (k_DK_m^{app} + k_Dc^* + J_{max})/2 - \{(k_DK_m^{app} + k_Dc^* + J_{max})^2 - 4k_DJ_{max}c^*\}^{1/2} \quad (10)$$

In summary, it can be stated that the electrode current will increase at higher rotation velocities if the oxygen mass transport or/and the reaction of its enzymatic reduction are the rate-limiting steps for the response of a tyrosinase-modified electrode.

Enzymatic oxidation of catechol is the rate-limiting step when the enzyme is saturated with oxygen ($K_m^{app} \ll c_o$ in eq 7). This situation would be fulfilled if there is a low concentration of catechol (mediator) in the solution or/and a low enzyme activity is obtained (or retained) during the immobilization procedure. In this case, the rate of the enzymatic reaction is controlled by the rate of the reaction of catechol with the enzyme and is practically equal to V_{max} (eq 7) for each catechol concentration. From this, it can be concluded that the electrode current density (eq 11) does not depend on the rotation speed.

$$j = nF2[E_t][M]\ k_2k_3/(k_2 + k_3) \quad (11)$$

Practically Observed Rate-Limiting Steps of Tyrosinase-Modified Electrodes. Below follow the experimental results obtained with the three electrode configurations, and the conclusions about rate-limiting steps for electrode responses are based upon the theoretical aspects presented above.

The decrease of the response with increasing rotation velocity is clearly seen for the *tyrosinase-modified carbon paste electrode* (Figure 3). It can thus be concluded that the response of this electrode is limited by the rate of the electrochemical reduction of *o*-quinones (eq 3). Previously, to enhance the rate of the electrochemical redox reaction for this kind of electrode, ruthenium was doped into the carbon paste, resulting in an about 100 times increase in sensitivity of paste electrodes for catechol.[4] However, ruthenium had no positive effect for Teflon/graphite-[13] as well as epoxy/graphite-based electrodes,[31] because their responses were not rate-limited by the electrochemical reduction of *o*-quinone.

Independence of current on rotation velocity is practically observed for the *Teflon/graphite electrodes* (Figure 4). That behavior is frequently found for other tyrosinase electrodes at low concentration of phenols.[9] Enzymatic oxidation of catechol as the rate-limiting step has to be concluded for these electrode preparations. Electrochemical reduction of *o*-quinone might probably be accelerated by the adsorbed aluminum oxide after polishing the Teflon/graphite electrodes with this suspension.[32] Moreover, the pressure (up to 7000 kg cm^{-2}) to prepare this type of electrode

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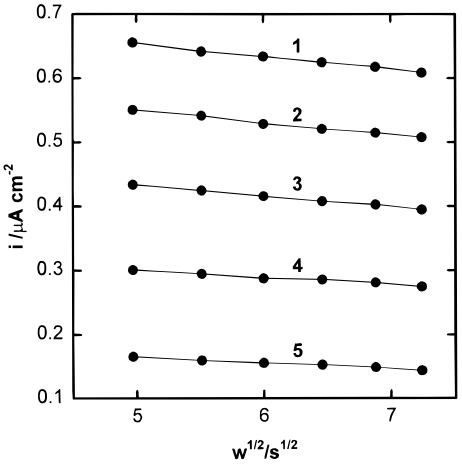

Figure 3. Dependence of reduction current on electrode rotation velocity. Carbon paste electrode modified with tyrosinase. Catechol concentration: (1) 15, (2) 12, (3) 9, (4) 6, and (5) 3 μM in 0.1 M phosphate buffer solution at pH 6.8; applied potential, −0.05 V vs SCE.

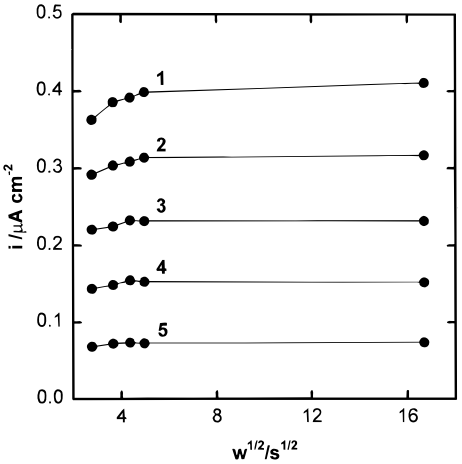

Figure 4. Dependence of reduction current on electrode rotation velocity. Teflon/graphite electrode modified with tyrosinase. Catechol concentration: (1) 0.5, (2) 0.4, (3) 0.3, (4) 0.2, and (5) 0.1 μM in 0.1 M phosphate buffer solution at pH 6.8; applied potential, −0.05 V vs SCE.

or the polishing procedure seems to result in an inactivation of tyrosinase. This conclusion is based on the comparison of sensitivities of carbon paste and Teflon/graphite electrodes containing the same percentage of tyrosinase. The sensitivity of Teflon/graphite electrodes was always found to be much lower (>10) compared with that of the carbon paste electrodes.[13]

In the case of slow enzymatic catechol oxidation, it follows (eq 11) that the responses are linearly dependent on the concentration of catechol. Linear calibrations over a broad concentration interval have been demonstrated previously for a number of tyrosinase-modified electrodes.[7,9,33] Moreover, in this case (eq 11), the sensitivity of the electrode is proportional to the activity of the enzyme, leading to a relative instability of the tyrosinase-modified electrodes. Stabilization of tyrosinase in composite electrodes has to be considered as being important when developing tyrosinase-modified electrodes for the detection of phenolic compounds such as phenol, *p*-cresol, *p*-chlorophenol, etc.[5,19]

A nonlinear increase of j^{-1} versus $\omega^{-1/2}$ was experimentally found for *solid graphite electrodes modified with cross-linked tyrosinase on the surface* (data in Figure 5, but in other coordinates). This actually indicates that the response of this type of electrode is limited by the oxygen concentration. Solid graphite electrodes with tyrosinase cross-linked on the surface exhibited a very high sensitivity for catechol up to 45−70 A M^{-1} cm^{-2}, which resulted in a high rate of oxygen consumption. The statement that the oxygen reduction step is the rate-limiting factor for the response of these electrodes can be additionally supported by a more pronounced j vs $\omega^{1/2}$ dependence at higher concentrations of catechol in the solution (Figure 5). The same fact was experimentally observed previously for tyrosinase copolymerized in polypyrrole[9] deposited on the electrode surface and can be explained by the oxygen reduction step being rate-limiting. It has to be noted that, when analyzing the cross-linked tyrosinase electrode, the enzyme layer is assumed to be very thin. For more exact characterization of the electrode performance, the thickness of the enzyme layer has to be considered, and more complicated experimental setups have to be used.[34]

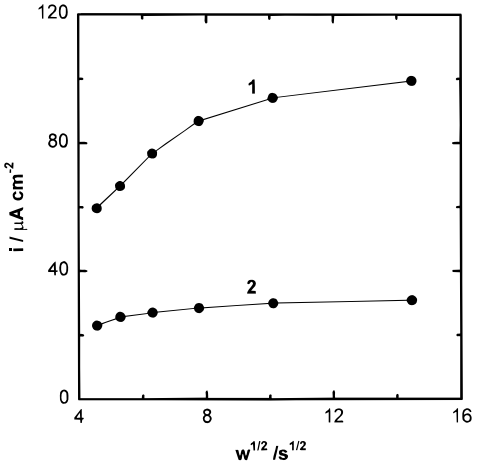

Figure 5. Dependence of reduction current on electrode rotation velocity. Solid graphite electrode modified with tyrosinase cross-linked using glutaraldehyde. Catechol concentration: (1) 2.1 and (2) 0.5 μM in 0.25 M phosphate buffer solution at pH 6.0; applied potential, −0.05 V vs SCE.

From the above calculated values of AF_{max} and the obtained current versus rotation velocity dependence (Figure 5) for solid graphite electrode with cross-linked tyrosinase on the surface, it can be stated that the reaction of the electrochemical *o*-quinone reduction at this type of electrode can be excluded from being considered as rate-limiting step. For the carbon paste- and Teflon-based electrodes, however, a real possibility for the response to be limited by the rate of electrochemical reduction of *o*-quinones exists when enzyme activity is high and/or rates of electron transfer are low. It has been shown previously that insulation of the electrode surface by the organic oil or Teflon in the composite electrode configurations can reduce rates of the heterogeneous electron transfer by a factor of 10 or more.[35,36] Above, the electrochemical rate limitation was concluded for the carbon paste electrode containing a high amount of oil (60%) and confirms the considerations presented here.

In any case when the enzymatic and electrochemical reactions are fast, the current density of the tyrosinase-modified electrode

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is limited by the diffusion of oxygen. According to this, some conclusions about the sensitivity of tyrosinase-modified electrodes can be drawn. The current limited by oxygen diffusion can be calculated using eq 1 with $n = 4$ and a diffusion coefficient and concentration for oxygen[27] equal to 1.9×10^{-5} cm^2 s^{-1} and 0.25 mM, respectively. If it is considered that the electrode response at 40 rad s^{-1} (380 rpm, usual moderate mixing) has to be 10 times lower than that limited by the diffusion of oxygen (577 μA cm^{-2}/10), a sensitivity of 5.77 A M^{-1} cm^{-2} should be given as the upper limit in order to determine catechol concentrations up to 10 μM. A higher sensitivity will cause a nonlinear calibration dependence, and the response will strongly depend on the oxygen concentration in the sample. From a comparison of this sensitivity with the sensitivities presented in Table 1, it can be concluded that the most sensitive electrodes are very close to the above estimated limit.

CONCLUSION

The rate-limiting factors for the response to catechol for tyrosinase-modified electrodes can be (i) the rate of the electrochemical reduction of *o*-quinones, (ii) the rate of oxygen mass transfer to the electrode and its reaction rate with enzyme, and (iii) the enzymatic oxidation of catechol. These limitations are practically met for different electrode configurations. The response of the solid graphite electrode with cross-linked tyrosinase on the surface was found to be limited by oxygen mass transfer and the rate of its reduction by the enzyme. According to the mechanism of tyrosinase-modified electrodes, it was calculated that a single catechol molecule is recycled 1260−4040 times, depending on the electrode rotation velocity. At the same rotation velocities, the signal amplification was in the range from 40 to 94 for the same cross-linked tyrosinase electrode. The high rate of the electrochemical reduction of the enzyme product, *o*-quinone, practically excludes this heterogeneous reaction to be the rate-limiting step at the solid graphite-based electrodes. However, at carbon paste electrodes containing a high amount of oil, the rate of the electrochemical reaction is highly decreased, leading to the electrochemistry of *o*-quinone being the rate-limiting step. For Teflon/graphite electrodes, the enzymatic oxidation of catechol is found to be the rate-limiting reaction.

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APPENDIX: DETERMINATION OF THE DIFFUSION COEFFICIENT FOR CATECHOL

The diffusion coefficient frequently used for catechol is thought to be equal to that determined for 1,4-benzoquinone ($D_M = 2.3 \times 10^{-6}$ cm^2 s^{-1}) in a H_2SO_4/EtOH solution by polarography.[37] However, the very first investigation of the mechanism of the tyrosinase-modified electrode using the rotating ring disk technique showed that ∼4−6 times higher ring currents were observed than what could be expected theoretically due to a diffusion-limited mass transfer of substrate (results not presented). The catechol was always prepared fresh. Additional purification by sublimation of the commercial preparation gave no difference in the electrode currents. Thus, it was necessary to reevaluate the diffusion coefficient of catechol in our experimental conditions. Catechol can probably be oxidized to other products than *o*-quinone at a platinum electrode, and this may lead to the fact that more than two electrons per catechol molecule could be withdrawn in the electrochemical oxidation step. To eliminate the error arising from this uncertainty in the number of electrons transferred during the electrochemical oxidation of catechol, chronoamperometry and rotating ring disk experiments were performed at the same platinum ring electrode, and then the ratio between the slopes obtained from chronoamperometry ($i_{Cottrell}$ vs $t^{1/2}$(slope^{Cottrell})) and rotating ring experiments (i_{Levich} vs $\omega^{1/2}$(slope^{Levich})) was calculated. The theoretical ratio between the slopes of the Cottrell and Levich currents[23] at a ring electrode is defined as

$$\frac{\text{slope}^{\text{Cottrell}}_{\text{theor}}}{\text{slope}^{\text{Levich}}_{\text{theor}}} = \frac{AD_M^{-1/6}}{0.620\pi^{3/2}(r_3^3 - r_2^3)^{2/3}\nu^{-1/6}} = 0.169D_M^{-1/6} \quad (12)$$

where r_3 and r_2 refer to the outer radius and the inner radius of the ring electrode, respectively. The geometric surface area of the ring electrode, A, was 0.054 cm^2. The kinetic viscosity, ν, of the solution is assumed to be 0.01 cm^2 s^{-1}. The experimental ratio between the Cottrell and Levich experiments was found to be equal to 1.09(±0.06) cm$^{-1/3}$ s$^{1/6}$ in 0.25 M phosphate buffer at pH 6.0, and 1.19(±0.22) cm$^{-1/3}$ s$^{1/6}$ in 0.25 M succinate buffer at pH 5.0. From this, the diffusion coefficients, D_M, and the numbers of electrons, n, involved in the oxidation process were found respectively to be equal to 13.5(±4.5) $\times$ 10^{-6} cm^2 s^{-1} and 1.3 ± 0.6 at pH 6.0, and 8.5(±9.5) $\times$ 10^{-6} cm^2 s^{-1} and 2.1 ± 2.8 at pH 5.0. The diffusion coefficients found in this study gave higher values compared with data found in the literature for different catecholamines[38] ranging from 6.2×10^{-6} to 7.8×10^{-6} cm^2 s^{-1}. Nowinski and Anjo[38] have done the experiments at pH 1 (0.1M sulfuric acid), where the different catechols and their *o*-quinone counterparts are probably more stable. The diffusion coefficient used for the calculations in our study was 8.5×10^{-6} cm^2 s^{-1}. Some overestimation of the diffusion coefficient still has to be concluded by a comparison with the diffusion coefficient of 6.5×10^{-6} cm^2 s^{-1} determined for 4-methylcatechol in 0.1 M phosphate buffer.[39] The high standard deviation obtained with our approach does not reveal itself to be an optimal choice of the methods used for the reevaluation of the diffusion coefficient for catechol, because the relatively low standard deviation in the ratio of slopes gave very high standard deviations for the diffusion coefficient and the number of electrons.

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