#### Table V. Comparisons of Present Method with Two Other Methods

sample	method 1 <sup>a</sup>	method 2 <sup>b</sup>	present method
tomato	12.4	15.2	14.1
(mg/100 mL) Citrus unshiu (mg/100 mL)	36.7	36.5	40.5
lemon	44.5	48.0	47.7
(mg/100 mL) strawberry (mg/100 mL)		93.2	86.8

<sup>a</sup> Method 1: 2,6-dichlorophenolindophenol method. <sup>b</sup> Method 2: 2,4-dinitrophenylhydrazine method.

2,4-dinitrophenylhydrazine method except Citrus unshiu.

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# Catalytic Oxidation of Reduced Nicotinamide Adenine Dinucleotide by Graphite Electrodes Modified with Adsorbed Aromatics Containing Catechol Functionalities

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4-[2-(2-Naphthyl)vinyl]catechol (NSCH2) and 4-[2-(9,10ethanoanthracen-9-yi)vinyl]catechol (ASCH<sub>2</sub>) were adsorbed on graphite electrodes. The naphthalene and the ethaneanthracene ring systems were used as anchors to the graphite. The catechol group is free to move out into the electrolyte solution. The electron transfer between the 1,2hydroquinone functionality and the graphite was fast. The surface coverage was at most 9 imes 10<sup>-9</sup> mol cm<sup>-2</sup>. The coenzyme reduced nicotinamide adenine dinucleotide (NADH) could be catalytically oxidized by the immobilized mediating groups. The overvoltage of the NADH oxidation decreased from 410 mV vs. SCE at the unmodified graphite electrode to 185 mV at the NSCH<sub>2</sub> covered electrode at pH 7.0. The reaction rate of the ASCH<sub>2</sub> covered electrode was lower than that of the NSCH<sub>2</sub> electrode. After 30 min of continuous electrochemical cycling of pH 7.0, 30% of the original coverage remained for the NSCH<sub>2</sub> electrode.

The coenzyme reduced nicotinamide adenine dinucleotide (NADH) can only be oxidized electrochemically at high overvoltages (1). The overvoltage at pH 7 is about 1.1 V at carbon (2) and 1.3 V at platinum electrodes (3). If the electrode has been pretreated and if the NADH concentration is at most 0.1 mM, the product at platinum electrodes is enzymatically active NAD $^+$  (4). Electrodes modified so that NADH could be oxidized more easily should open up a new field of applications both in analysis and in biotechnology.

About 300 hydrogenases are known which are dependent on NADH or NADPH as redox transfer agents.

Oxidation of NADH to enzymatically active NAD<sup>+</sup> in homogeneous solution can only be made with a few oxidizing agents, often called mediators.

Surface modifications have been tried as a means to reduce the overvoltage of the electrochemical oxidation. The strategy has been to immobilize functional groups with known mediating properties on the surface. Blaedel and Jenkins (5) could reduce the overpotential with 0.2 V to 0.45 V vs. SCE by an electrochemical pretreatment of the electrode. They presumed that hydroxyl, carbonyl, and quinone functionalities were produced. Tse and Kuwana (6) immobilized 1,2hydroquinones via covalent bonds to the surface of pyrolytic graphite and showed that the functionalities had catalytic activity. The overpotential decreased further about 0.2 V compared to Blaedel and Jenkins. The work demonstrated the feasibility of making catalytic electrodes by the immobilization of mediating functional groups.

Degrand and Miller (7) made a polymer from poly(methacryloyl chloride) and dopamine which adsorbed on the surface of vitreous carbon. The decrease in overvoltage was about 0.25 V. The surface coverage and lifetime were improved compared to the electrode made by Tse and Kuwana.

It has been observed that a number of polycyclic aromatic compounds adsorb strongly to graphite surfaces. Anson and co-workers studied the electrochemistry of 9,10phenanthrenequinone (8, 9) and [1-(9-phenanthrene)-2-(4pyridine)ethene] (10) adsorbed on graphite. Laviron et al.

(11) reported on the electrochemistry of benzo[c]cinnoline adsorbed on graphite. Coenzymes from the flavine group were studied in our laboratory (12, 13). The flavines adsorbed strongly to graphite, and the electron transfer between the functional group and the graphite was quite rapid.

As it is known that some 1,2-quinones can be mediators in homogeneous solution there is a possibility that the adsorbed 1,2-quinones should be able to mediate electron transfer from NADH. No such effect could be seen for the 9,10phenanthrenequinone (6) or for a greater number of condensed aromatic quinones studied preliminarily in our laboratory. The position of the adsorbed quinone functionality, which probably lies flat along the surface, seems to be unfavorable for electron exchange with NADH. Utilization of this mediator seems to be possible only if the functional group can be raised somewhat from the surface. The success reported by other types of immobilization (6, 7) supports this belief.

This paper reports on the adsorption of two types of compounds which have a 1,2-hydroquinone functionality in a side ring connected to a larger aromatic ring system. The larger aromatic ring systems anchor the compounds to the graphite surface and the smaller catechol acts as a mediator. The anchoring aromatic ring also provides a fast electron exchange between the graphite electrode and the adsorbed functionality.

A voltage facilitatetd immobilization of phenazine methosulfate mediator on graphite has simultaneously been made in our laboratory (14). These electrodes also showed catalytic activity for the NADH oxidation.

### **EXPERIMENTAL SECTION**

Syntheses. 4-[2-(2-Naphthyl)vinyl]catechol (I) was synthesized



from 2-naphthalene aldehyde and 3,4-dimethoxybenzyl alcohol. The dimethoxybenzyl alcohol was treated with  $PCl_5$  in dry ether at 0 °C according to Carman and Shaw (15) to produce the acid chloride. The triphenylphosphonium salt was made by boiling the chloride with triphenylphosphine in dry toluene at 110 °C for 48 h (16). The formed precipitate was washed with dry cold ether. A Wittig reaction was made between a slight excess of the phosphonium salt and 2-naphthalene aldehyde dissolved in methylene chloride by addition of 50% aqueous sodium hydroxide. The mixture was agitated vigorously for 20 min (17). The organic layer was separated and the solvent was evaporated.

The product was purified by passing a solution of the product in petroleum ether-ethyl acetate 50:50 through a column of silica gel 60 as described by Still et al. (18). Only a small amount of *cis*-stilbene is formed due to steric hindrance. Furthermore the cis and trans isomers elute in separate fractions from the column. Only the trans form was collected. The ether groups were cleaved by treatment with trimethylsilyl chloride and sodium iodide in dry acetonitrile (19). The necessary reaction time was considerably longer than the one reported (19), probably due to sterical hindrance between the ortho silyl ethers formed prior to hydrolysis.

The 4-[2-(9,10-ethanoanthracen-9-yl)vinyl]catechol (II) was



made from anthracene, acrolein, and dimethoxybenzyl alcohol.



**Figure 1.** Cyclic voltammogram of NSCH<sub>2</sub> adsorbed on graphite in 0.1 M phosphate buffer, pH 7.0, surface coverage 0.7  $\times$  10<sup>-9</sup> mol cm<sup>-2</sup>, starting potential –100 mV vs. SCE, and sweep rate (a) 200, (b) 100, and (c) 50 mV s<sup>-1</sup>.

In the first step 9,10-ethaneanthracene carboxaldehyde was synthesized by a Diels-Alder reaction between anthracene and acrolein in toluene during 16 h at 150 °C in a steel bomb (20). The reaction mixture was filtered and the solvent of the filtrate was evaporated. The aldehyde was dissolved in methanol and any residual anthracene was removed by filtration. The aldehyde monohydrate was formed by adding water and the precipitate was filtered off. The monohydrate was transformed back to the aldehyde at 100 °C under vacuum. The steps of the synthesis and the purification were the same as described above for the compound I. The products, I and II, were identified by IR, NMR, mass spectrometry, and elemental analysis. **Measurements.** The electrodes were spectrographic graphite,

Measurements. The electrodes were spectrographic graphite, 6.1 mm o.d. (RW 003 Ringsdorff-Werke, GMBH). They were polished with emery paper (Tufbak Durite Waterproof Paper Closecote 600-A Type 1) put on a rotating disk polishing machine. The polishing was performed under a continuous flow of deionized water for 5 min. After that, the electrodes were dried in air. Before mounting the electrodes in the holder for the electrochemical experiment, a rubber tube (6.0 mm i.d., isoversinic, Kebo Grave) was slipped over in order to avoid electrical contact between the sides of the electrodes and the solution. This was done from the opposite end of the polished surface in order to avoid affecting the surface mechanically or chemically. The edge of the tube was put just in line with the electrode surface.

The electrochemical surface area was identical with the geometric one when measured in a 2 mM ferrocyanide solution using both a stationary and a rotating disk arrangement. The rubber tubing as well as the sides of the electrodes were quite hydrophobic and no creeping of solution between the electrode and the rubber tubing was detected. Measurements were made with either triangular sweep voltammetry or with a rotating disk assembly (Tacussel EDI) using the SCE as a reference electrode.

All measurements were made in buffers made from 0.1 M pyrophosphate (pH 5.0, 6.0, and 9.0) or 0.1 M phosphate (pH 2.0, 3.0, 4.0, 7.0, 8.0, and 11.0).

The synthesized products were first dissolved in methanolethanol 80:20. An equal volume of aqueous 0.1 M hydrochloride acid was added when the dissolution was complete. The resulting solution was about 0.5 mM. The graphite rods were dipped into this solution under slow agitation for 3–10 min depending on the desired coverage.

The purity of the synthesized products was checked by HPLC on Nucleosil-CN,  $\cdot$ NH<sub>2</sub>, and  $\cdot$ C8, Hypersil ODS-C18 (all 5  $\mu$ m), and  $\mu$ -Porasil-SiOH (10  $\mu$ m) columns using several different methanol-ethanol mixtures as well as acetonitrile as solvents.

## **RESULTS AND DISCUSSION**

**Electrochemistry of Adsorbed NSCH<sub>2</sub>.** Figure 1 shows cyclic voltammograms of the NSCH<sub>2</sub> covered electrode, obtained in a buffer solution with no soluble NSCH<sub>2</sub> present. The adsorption of NSCH<sub>2</sub> had taken place during 3 min from the solution specified in the Experimental Section. After that the electrode had been washed thoroughly with deionized water. The figure demonstrates the properties of the adsorbed



**Figure 2.** Desorption of NSCH<sub>2</sub> vs. time in an unstirred solution: ( $\blacktriangle$ ) pH 5.0; ( $\bigcirc$ ) pH 7.0; ( $\blacksquare$ ) pH 9.0 and 11.0.

layer. Two peaks are observed. The peak current is linearly proportional to the sweep rate over a range of  $25-600 \text{ mV s}^{-1}$ indicating a diffusionless system. The separations between the first anodic and the corresponding cathodic peaks are 40, 50, and 60 mV for the three sweep rates. The corresponding peak separations for the second peaks are 20, 25, and 35 mV. For a fully reversible reaction in which no reactants from the solution take place, the peak separation should be zero (20). The relatively small peak separations indicate fairly fast electrochemical reaction rates (21). If the coverage of adsorbed species is decreased even smaller peak separations are obtained (22). The apparent rate constants, calculated according to these references, were  $3-6 \text{ s}^{-1}$ , about the same for both steps. The two peaks on both the anodic and cathodic sweeps indicate that the reactions occur in two one-electron steps. The intermediate should be the semiguinone, resonance stabilized by the double bond to the naphthalene ring. A further indication that the reaction proceeds with a one-electron mechanism is that the peak widths at half-height are 90-95 mV (theory 90.6/n mV (23)). A plot of the peak current over the charge vs. the sweep rate also gives evidence of two separate electron steps, as n values close to 1 are obtained from the slopes (8). The two electrochemical peaks cannot be caused by two different species as these would have been separated by at least one of the HPLC columns mentioned in the Experimental Section.

The electron transport can take place through different mechanisms. Electrons can be transferred through the double bond resonance structure between the catechol and the anchoring naphthalene ring. The catechol is free to interact directly with the surface so that the electron transfer might also take place through a flip-flop movement. Even an electron tunneling mechanism seems probable.

A plot of  $E_{1/2}$  vs. pH from pH 1 to 9 gives a slope of 59.4 mV/pH unit at 25.0 °C. It can therefore be concluded that an equal number of electrons and protons is involved in the two electrode reactions. The  $E_{1/2}$  at pH 7.0 for the two waves were +95 and 190 mV, respectively.

The amount of NSCH<sub>2</sub> on the electrode increased asymptotically with the time spent in the adsorption solution. There was a leveling off after about 60 min. The highest coverage was around  $9 \times 10^{-9}$  mol cm<sup>-2</sup>. The shape of the cyclic voltammograms changes with coverage. The two peaks merge at high coverage and the separation between the anodic and cathodic peaks increases (23).

**Stability.** Figure 2 shows the rate of desorption of NSCH<sub>2</sub> from the graphite at four different pH values. The measurements were made in unstirred solutions with a continuous electrochemical scanning back and forth. There is a marked increase of the desorption rate with pH. The  $pK_{a_1}$  for catechol itself is 9.85 and the solubility of the reduced form will therefore be higher at higher pH due to deprotonation. No change in stability is observed between pH 9 and 11. The rate of desorption increases somewhat when the solution is stirred. The electrode is stable when stored in air.

Catalytic Oxidation of NADH at the NSCH<sub>2</sub> Electrode. Cyclic Voltammetry. Figure 3, curve a, shows a cyclic



**Figure 3.** Catalytic oxidation of NADH mediated by adsorbed NSCH<sub>2</sub> (curve a), 2.3 mM NADH, pH 7.0, sweep rate 50 mV s<sup>-1</sup>. Curve b shows oxidation at a naked graphite electrode in the same solution. Curve c shows NSCH<sub>2</sub> modified electrode in a buffer without NADH. Starting potential was ~100 mV vs. SCE.

voltammogram obtained with the NSCH<sub>2</sub> covered electrode in a solution which was 2.3 mM in NADH. It can be seen that there is a great increase in the anodic current compared to a scan in a buffer without NADH (curve c). The reason is that NADH diffuses up to the electrode surface and reduces the NSC to NSCH<sub>2</sub>. As NSCH<sub>2</sub> is regenerated by NADH during the sweep, there will be an increase in the current. For the same reason the cathodic current is smaller in the presence of NADH as NSCH<sub>2</sub> is produced simultaneously from both an electrochemical and a chemical step. The overall reaction thus proceeds as shown in eq 1–3. The rate-determining step

$$NSC + NADH + H^{+} \xrightarrow{n_{1}} NSCH_{2} + NAD^{+}$$
(1)

$$NSCH_2 \rightleftharpoons NSC + 2H^+ + 2e^-$$
(3)

$$NADH \rightarrow NAD^+ + H^+ + 2e^-$$
(3)

is given in eq 1 with a rate constant  $k_1$ . Thus the rate of the reaction in eq 2 can be considered fast compared to the one in eq 1. The overall oxidation of NADH by the electrode is given in eq 3.

The catalytic effect can be seen directly when curve a is compared with curve b in Figure 3. Curve b is obtained with an untreated graphite electrode. The decrease in overvoltage is 225 mV at pH 7.0 and 315 mV at pH 9.0.

Succeeding scans with stirring between them result in only minor decreases of the peak height of curve a. The decrease follows the course given by Figure 2 which indicates that a repeated recycling can take place without loss of activity from other sources than desorption of NSCH<sub>2</sub>. The peak current was proportional to the NADH concentration in the range tested 0.7-7 mM. Andrieux and Saveant (24) derived a relation between the peak current and the concentration for the case when  $k_1$  in eq 1 is infinitely fast.

$$i_{\rm p} = 0.496 n F A D_{\rm R}^{1/2} v^{1/2} C_{\rm R}^* (n F / RT)^{1/2} \tag{4}$$

The symbols have their usual significances. Low values of  $k_1$  result in lower values of the constant (0.496). This constant was found to be 0.30 for a NSCH<sub>2</sub> electrode with a coverage of  $\Gamma = 1.3 \times 10^{-9}$  mol cm<sup>-2</sup>. The rate constant  $k_1$  in eq 1 associated with this value could then be calculated to  $\sim 2 \times 10^{6}$  M<sup>-1</sup> s<sup>-1</sup>.

**Rotating Disk Experiments.** Experimental results with the NSCH<sub>2</sub> covered electrode mounted in a rotating disk assembly are shown in Figure 4. A Levich line, assuming a very fast chemical reaction, was calculated (curve a) by using a diffusion coefficient of  $2.4 \times 10^{-6}$  cm<sup>2</sup> s<sup>-1</sup> (25) for NADH.

The currents obtained in NADH solutions are shown by curves b-d. The potential was kept at +275 mV vs. SCE, i.e., just on the plateau obtained when the voltage was scanned



Figure 4. Anodic oxidation of NADH on a rotated disk graphite-NSC electrode. Curve a shows the theoretical line for a very fast reaction. Experimentally obtained curves are shown for a surface coverage of (b)  $1.3 \times 10^{-9}$ , (c)  $1.5 \times 10^{-9}$ , and (d)  $0.5 \times 10^{-9}$  mol cm<sup>-2</sup>. Applied voltage was +275 mV vs. SCE, pH 7.0. Concentrations for a, b, and d were 1.90 mM and for c was 1.76 mM NADH.

slowly during rotation. The rate of rotation was increased stepwise. Following each step the current stabilized in a few seconds. When the rotation rate was returned to the original slower value, the current had decreased according to the desorption in Figure 2. An unmodified electrode gave no oxidation current at this voltage. The NADH concentrations in b and c were 1.90 mM and in d, 1.76 mM. Curve b was obtained with an electrode with a surface coverage of  $1.3 \times$  $10^{-9}$  mol cm<sup>-2</sup>. The coverages for curves c and d were 1.5 and  $0.5 \times 10^{-9}$  mol cm<sup>-1</sup>, respectively.

As seen in Figure 4 the current reaches a maximum level as the rotation speed is increased. This is expected in a rotating disk experiment, when the current is completely under kinetic control. Andrieux et al. (26) derived the following equation for surface modified catalytic electrodes when the current has reached its maximum value.

$$i = nFAk_1 \Gamma_{\text{Ox}} C_{\text{R}}^* \tag{5}$$

The symbols have their usual significance and  $k_1$  is the rate constant of eq 1. At the potential used the rate of the electrochemical reaction, eq 2, can be considered infinite and the reaction in eq 1 is the rate-determining step. In this way the curves shown in Figure 4 give  $k_1$  values of 1.2, 1.0, and  $1.4 \times$  $10^{6}$  M<sup>-1</sup> s<sup>-1</sup> which agree well with the one evaluated from the cyclic voltammetrical experiments.

Electrochemistry of Adsorbed ASCH2. Cyclic voltammograms of ASCH<sub>2</sub> adsorbed on graphite electrodes were generally very similar to those of the adsorbed NSCH<sub>2</sub> except that the electrochemical reaction rate was somewhat smaller. The apparent rate constant was about 0.8 s<sup>-1</sup>. The  $E_{1/2}$  and its pH dependence were the same and there were also two peaks indicating separate one-electron steps.

The intramolecular conduction of the compound II should be smaller than that of compound I, and it was found that the electrochemical rate is at least 3 times slower than that for the naphthalene. The original coverage was about the same but the desorption was faster. The desorption was in fact too fast to make experiments with a rotating disk electrode with a constant and well-defined coverage.

Catalytic Oxidation of NADH on the ASCH<sub>2</sub> Electrode. Figure 5 shows a cyclic voltammogram obtained with ASCH<sub>2</sub> adsorbed on a graphite electrode. Curve c shows the voltammogram in a buffer without other reagents. The surface coverage was  $0.7 \times 10^{-9}$  mol cm<sup>-2</sup>. In the presence of 2.4 mM NADH a catalytic oxidation occurs at (a) in the upper curve. At more anodic voltages, (b) in Figure 5, NADH is oxidized directly without involvement of the attached mediating groups.



Figure 5. Catalytic oxidation of NADH mediated by adsorbed ASCH<sub>2</sub> (a) as well as direct oxidation of NADH (b). Surface coverage was  $0.7 \times 10^{-9}$  mol cm<sup>-2</sup> with 2.4 mM NADH, pH 9.0, and sweep rate 50 mV

Curve c was obtained under the same conditions except that no NADH was present. Starting potential was -200 mV vs. SCE.

A comparison between the catalytic oxidation of the NSCH<sub>2</sub> and the  $ASCH_2$  electrodes (Figures 3 and 5) shows that the reaction between NADH and the mediating group is much slower for the ASCH<sub>2</sub> electrode. The overvoltage was decreased less effectively than on the NSCH<sub>2</sub> electrode. As a matter of fact the ASCH<sub>2</sub> species was synthesized in the belief that the rate of the chemical step would be favored by the more elevated position of the catechol group.

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