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# Renewable Alcohol Biosensors Based on Alcohol-Dehydrogenase/Nicotinamide-Adenine-Dinucleotide Graphite Epoxy Electrodes

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## ABSTRACT

Alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) have been incorporated within rigid graphite epoxy matrices to yield renewable (by polishing) alcohol sensors. The enzyme and cofactor retain their bioactivity on confinement in the graphite epoxy, with the oxidation current of the NADH formed serving as the response. Because of the intimate contact between the biocatalytic and sensing sites, the reagentless sensor produces steady-state signals within 15–30 s. The influence of various experimental variables is explored for optimum analytical performance. Common polishing procedures are used to regenerate fresh bioactive surfaces.

**KEY WORDS:** Alcohols, biosensors, graphite epoxy.

## INTRODUCTION

Durable and reusable biosensors are highly desired for reliable assays of different clinical, environmental or industrial samples. Recent studies have illustrated that the incorporation of biocomponents into a graphite epoxy matrix can result in renewable amperometric devices [1–3]. The bulk of these robust bioelectrodes serves as a source for the biological activity, and “fresh” biosurfaces can be easily obtained by polishing. Successful performance has been reported for the incorporation of glucose oxidase, tyrosinase, bilirubin oxidase, and horseradish peroxidase within rigid graphite epoxy electrodes. Because of their reusable nature and mechanical stability (compared to their nonrenewable, membrane-based counterparts), bulk-modified bioelectrodes hold a great promise for routine biosensing applications.

The objective of the present study was to expand the concept of renewable graphite-epoxy bioelectrodes towards dehydrogenase enzymes and their nicotinamide cofactors. This group of oxidoreductases is of special interest because it consists of several hundred enzymes [4]. More than 250 of these depend on the nicotinamide adenine dinucleotide (NAD) system. The following sections thus describe the fabrication, characterization, and performance of a polishable biosensor for alcohols based on the coimmobilization of alcohol dehydrogenase (ADH) and  $\text{NAD}^+$  within graphite epoxy matrices.

## EXPERIMENTAL

### Apparatus

Experiments were performed in a Bioanalytical Systems (BAS) Model VC-2 10-ml electrochemical cell. The cell was joined to the working electrode, reference electrode ( $\text{Ag}/\text{AgCl}$ , Model RE-1, BAS), and platinum wire auxiliary electrode through holes in its Teflon cover. The three electrodes were connected to the EG&G PAR Model 364 polarographic analyzer, the output of which was displayed on a Houston Omniscribe strip-chart recorder. A magnetic stirrer and stirring bar provided the convective transport. Surface images were obtained with a Nanoscope 2 STM (Digital Instruments).

### Electrode Preparation

The modified electrode was prepared from an epoxy-bonded graphite (grade RX, Dylon Inc., Cleveland) in a manner reported for other biocomponents [1]. ADH and  $\text{NAD}^+$  were added to the 1:1 resin/accelerator mixture (at 5–7.5 and 10% w/w, respectively). A thorough mixing proceeded for 15 min. A portion of this mixture was packed into the end of a 3-mm i.d. glass tube. Electrical contact was established via a copper wire. The electrode was then cured at room temperature for 50 hours. The surface was polished with a 600-grit silicon carbide paper, followed by a 0.05  $\mu\text{m}$  alumina slurry. Residual polishing material was removed by thoroughly rinsing with doubly distilled water. This polishing procedure

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was usually repeated before each experiment. The bioelectrodes were stored at 4°C.

### Reagents

All solutions were prepared with deionized water. Methanol, 1-propanol, 1-butanol (Baker), allyl alcohol (Eastman), 2-propanol (Fisher), and ethanol (U.S. Industrial Chemicals) were used without further purification. The supporting electrolyte was a 0.05 M phosphate buffer solution (pH 7.4). Stock solutions of the alcohols (0.34 M) were prepared daily in the electrolyte solution. Yeast alcohol dehydrogenase (E.C.1.1.1.1., 350 U/mg) and  $\beta$ -nicotinamide adenine dinucleotide were obtained from Sigma.

### Procedure

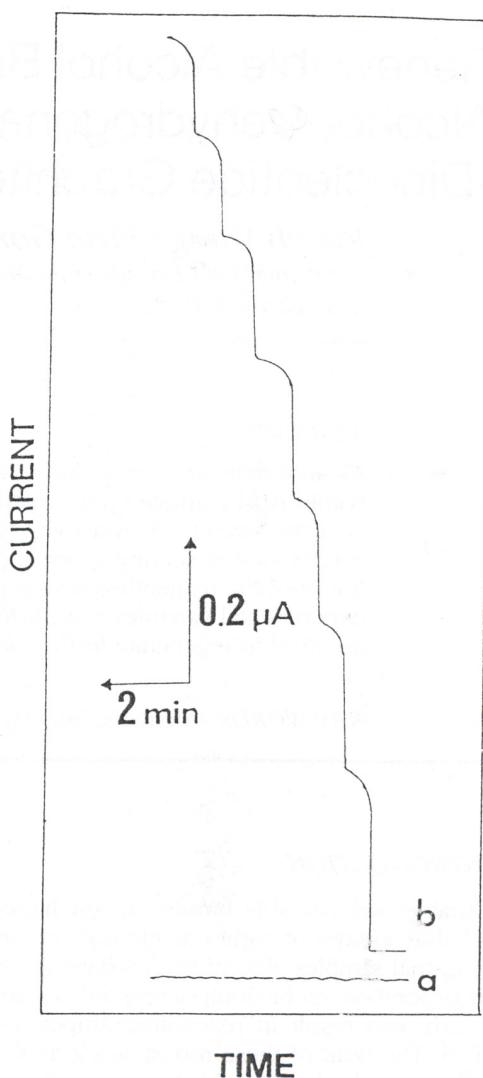
The freshly polished electrode was immersed in the blank solution. The operating potential (+0.70 V) was applied and transient current were allowed to decay for 10 min to a steady-state value. Aliquots of the substrate stock solutions were then added to the stirred solution, and the resulting currents were recorded. All measurements were made at room temperature.

### RESULTS AND DISCUSSION

Preliminary experiments were undertaken first to illustrate that NAD<sup>+</sup> can be incorporated, function, and regenerated within the graphite-epoxy matrix. Figure 1 compares the response of a 15% (w/w) NAD<sup>+</sup>-graphite epoxy electrode, in the absence (**a**) and presence (**b**) of dissolved ADH, to successive standard additions of ethanol. As expected, in the absence of the enzyme the cofactor electrode is not responsive to the additions of ethanol. In contrast, the same electrode responds very rapidly to ethanol, when ADH is present in the solution, producing a steady-state response within ca. 45s. The fact that NAD<sup>+</sup> can be actively incorporated within the graphite epoxy matrix, led us to its coimmobilization with ADH. Further work was thus performed with the resulting ADH/NAD<sup>+</sup>-electrodes.

Scanning tunneling microscopy (STM) was employed to gain insights into the complex nature of ADH/NAD<sup>+</sup>-surfaces. Figure 2 shows a STM image of a typical surface region of the bioelectrode. Such an image offers a unique visualization of the conductive graphite region. Since STM is restricted to electrically conductive phases, the tunneling disappears abruptly as the tip passes over the insulating region (of the epoxy, enzyme, and cofactor). As a result, the graphite region (shown as "mountains") can be easily distinguished from the electrically isolated portion (shown as flat "valleys"). Analogous observations of an unmodified graphite epoxy surface yielded a different spatial variation of the conductive and insulating regions (not shown), as expected from the absence of nonconductive modifiers. A similar STM investigation of the microdistribution of conductive/insulating regions has been reported for carbon paste electrodes [5].

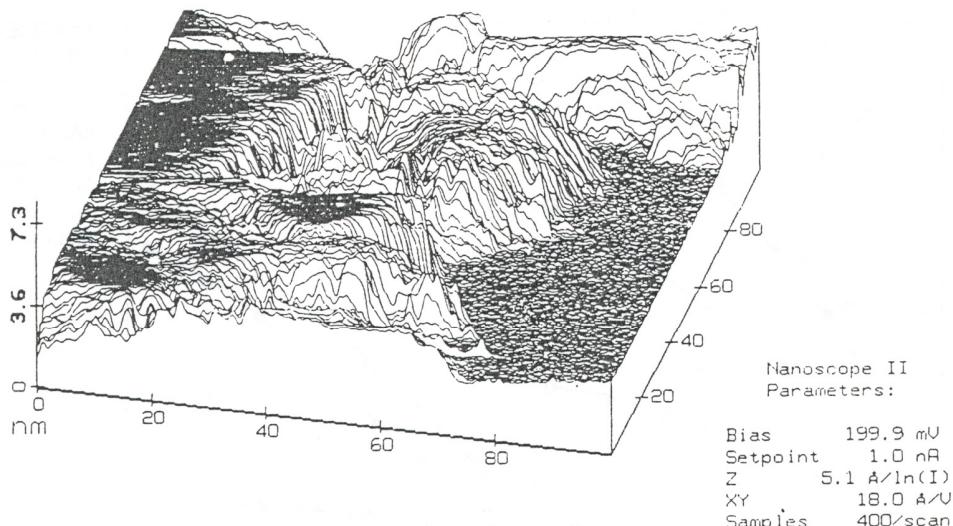
Figure 3 shows typical response to additions of allyl



**FIGURE 1.** Current-time recording obtained at the 15% (w/w) NAD<sup>+</sup>-containing graphite epoxy electrode on increasing the ethanol concentration in  $4 \times 10^{-2}$  M steps. Solution, 0.05 M phosphate buffer (pH 7-4) without (**a**) and with (**b**) 75 U/ml ADH- Operating potential, +0.7 V; stirring rate, 300 rpm.

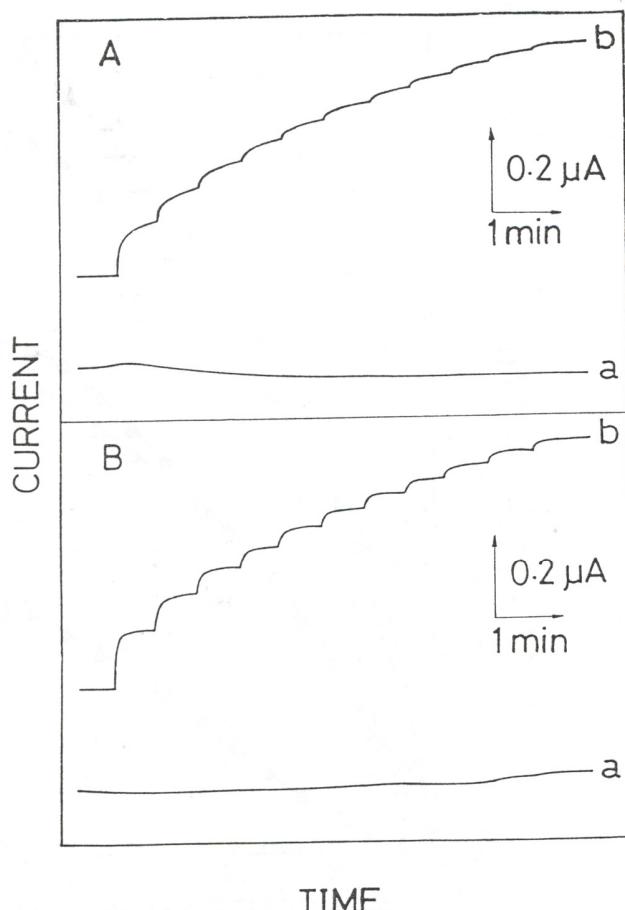
alcohol (**A**) and ethanol (**B**) at unmodified (**a**) and ADH/NAD<sup>+</sup>-modified (**b**) graphite epoxy electrodes. Notice the absence of response at the plain graphite-epoxy surface. In contrast, the biologically modified electrode responds very rapidly to the change in the substrate concentrations, producing steady-state currents within 15–30s. Such fast response is attributed to the close proximity of the enzyme and its cofactor, coupled to their intimate contact to the graphite sensing sites, and the absence of external (membraneous) barriers for the substrate transport. Indeed, such alcohol biosensors are based on a unique integration of the biorecognition and transduction elements in one robust unit.

The graphite-epoxy approach allows controlled loading of the biological modifier. The effects of the ADH and NAD<sup>+</sup> contents were evaluated from calibra-



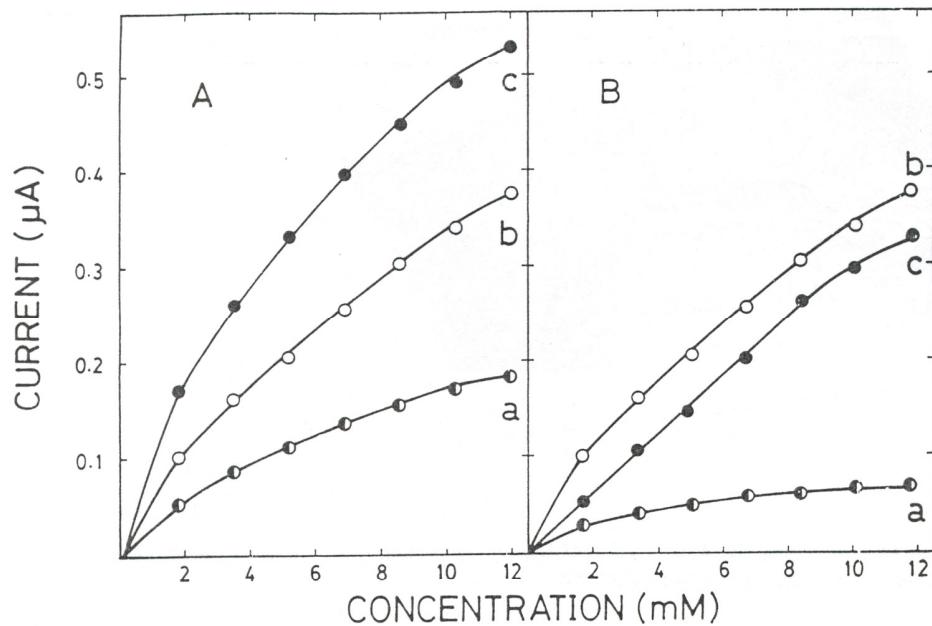
**FIGURE 2.** STM image of a 5% ADH/10% NAD<sup>+</sup>(w/w)-containing graphite epoxy electrode. Tunneling current, 1 nA; tip bias voltage, 200 mV.

**FIGURE 3.** Current-time recording obtained at (a) unmodified and (b) 5% ADH/10% NAD<sup>+</sup>(w/w)-containing graphite-epoxy electrode on increasing the concentration of allyl alcohol (**A**) and ethanol (**B**) in  $3.4 \times 10^{-3}$  M steps. Solution, 0.05 M phosphate buffer (pH 7.4). Other conditions, as in Figure 1.



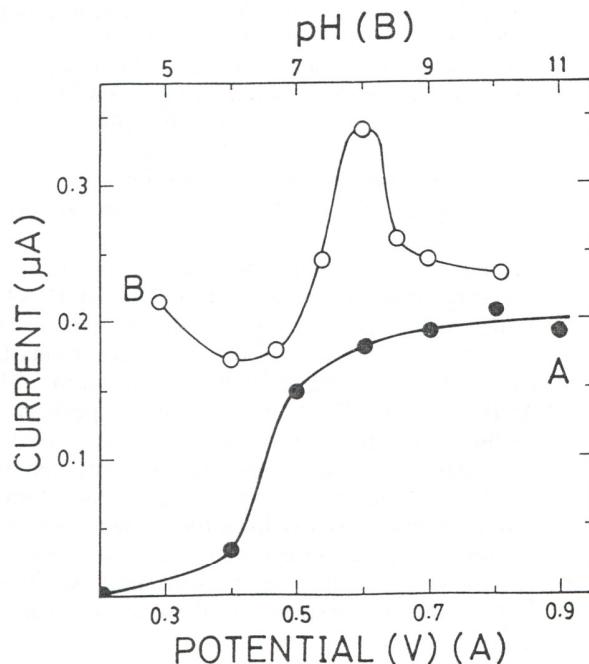
tion graphs for ethanol (Figure 4A and B, respectively). The response increased (nonlinearly) with the increase in the enzyme loading (e.g., 170, 330, and 490 nA at 2.5, 5, and 10% (w/w) ADH for 10 mM ethanol). In contrast, the ethanol current increased substantially by increasing the NAD<sup>+</sup> content between 5 and 10% (w/w), after which, at 15% (w/w) it decreased slightly. Such behavior is attributed to the low ADH loading (5% (w/w)). Other experimental variables affecting the response were evaluated (Figure 5). For example, the current rapidly increases with the operating potential between 0.2 to 0.5 V, after which it starts to level off (**A**). Such a profile is expected for the detection of the NADH product. The relatively low detection potentials compare favorably with those common at other carbon surfaces. The steady-state current depends strongly upon the solution pH [8]. While a slight decrease in the response is observed between pH 4.9 and 6.8, the current increases sharply over the pH range 6.8–8.0. Higher pH values yielded a sharp decrease in the response. The maximum sensitivity at pH 8.0 is in excellent agreement with the optimum pH reported for yeast ADH [6].

Figure 6 shows the dependence of the steady-state amperometric response of the rigid ADH/NAD<sup>+</sup> electrode on the concentration of six different alcohols. As expected for biocatalytic reactions, deviations from linearity are observed at high concentrations. The yeast ADH is known [7] to readily oxidize primary alcohols (with the exception of methanol), and to slowly oxidize secondary ones. The trend in sensitivity (ethanol > allyl alcohol > 1-butanol > 1-propanol > 2-propanol > methanol), however, differs in part from the known biospecificity of the solution-phase enzyme [8]. The exact reason for this change is not clear at the present time. Additional characteristics of the response to these alcohols are given in Table 1.

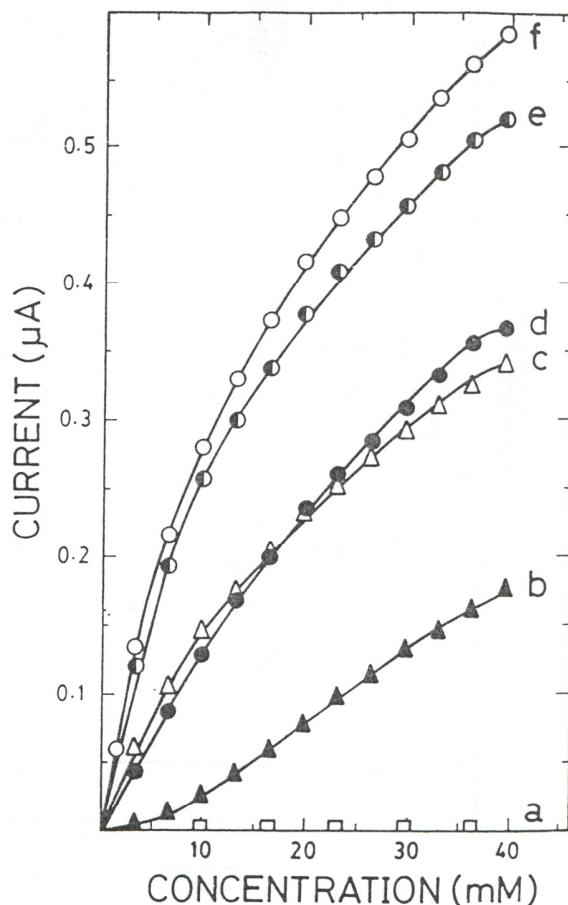


**FIGURE 4.** Effect of (A) ADH and (B) NAD<sup>+</sup> loading in the graphite epoxy on the ethanol response. ADH loadings (A), 2.5 (a), 5 (b), and 10 (c)% (w/w) (using 10% (w/w) NAD<sup>+</sup>); NAD<sup>+</sup> loadings (B), 5 (a), 10 (b) and 15 (c)% (w/w) (using 5% (w/w) ADH). Other conditions, as in Figure 1.

**FIGURE 5.** Dependence of the  $3.4 \times 10^{-3}$  M ethanol and allyl alcohol response on the operating potential (A) and solution pH (B) respectively. Conditions, as in Figure 1, except that 5% ADH/10% NAD<sup>+</sup> and 7.5% ADH/10% NAD<sup>+</sup> (w/w) were used in A and B, respectively.



**FIGURE 6.** Dependence of the steady-state current on the concentration of methanol (a), 2-propanol (b), 1-propanol (c), 1-butanol (d), allyl alcohol (e), and ethanol (f). Conditions, as in Figure 3b.



**TABLE 1** Response of the Graphite-Epoxy Alcohol Sensor<sup>a</sup>

Alcohol	Linear range, mM	Sensitivity, <sup>b</sup> nA/mM	Response relative to ethanol <sup>c</sup>	$K_m$ , app. <sup>d</sup> mM
Ethanol	0–4.4	40	1.0	22.7
Allyl alcohol	0–5.8	35	0.80	24.7
1 Propanol	0–8.2	17	0.58	33.3
1 Butanol	0–11.7	13	0.62	59.9
2 Propanol	12–32	4	0.26	200.0
Methanol	0	0	—	—

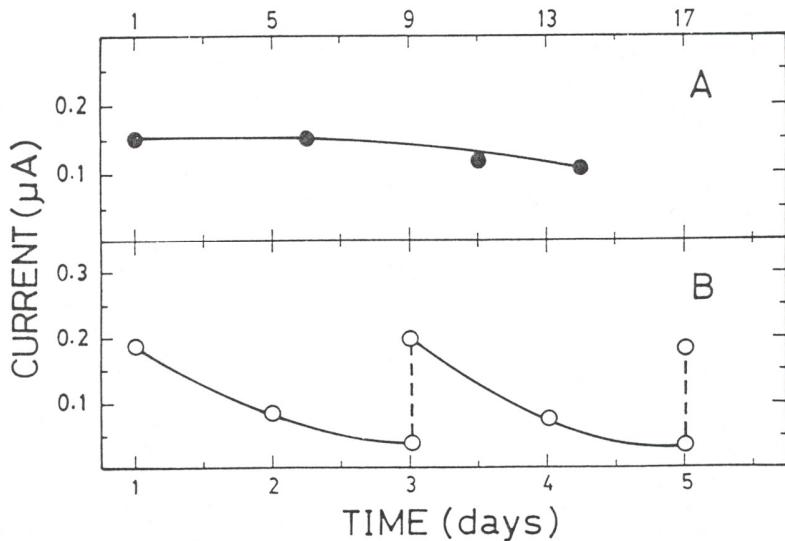
<sup>a</sup>Based on the data of Figure 6<sup>b</sup>Slope of linear portion<sup>c</sup>At 30 mM<sup>d</sup>From corresponding Lineweaver-Burke plots

Figure 7 demonstrates the ability to polish the surface of the alcohol sensor to reproducibly renew its surface. Profile A illustrates the stability of the reagentless sensor over a two-week period, with a freshly polished surface before each run. A very slow decrease of the response to ethanol is observed during this prolonged operation. A faster decay (with up to 75% depression within 3 days) is observed with unpolished surfaces (Profile B). The exact reason for this loss in activity is not clear at the moment. Fouling of the graphite sites by adsorbed product or a slow leaching of one of the modifiers from the outer surface may account for this failure. Nevertheless, subsequent polishing immediately restored the surface bioactivity and hence the response to its original value. Satisfactory reproducibility is observed for the individual surfaces. A series of eight successive measurements of 3.4 mM ethanol, each recorded with a freshly polished surface, yielded a mean peak current of 151 nA, with a range of 137–175

nA, and a relative standard deviation of 10%. Overall, the stability and reproducibility data clearly indicate that ADH and NAD<sup>+</sup> retain their bioactivity on confinement in the graphite epoxy, and that they are uniformly dispersed within this matrix.

In summary, the experiments described above illustrate an attractive avenue for the fabrication of reagentless alcohol sensors. The polishable (renewable) nature and fast response of graphite-epoxy bioelectrodes make them favorable for many practical applications (e.g., assays of beverages, monitoring of fermentation processes). The regeneration and speed advantages are coupled with the ease and low cost of fabrication. Additional advantages may be achieved via the coimmobilization of a redox mediator (to facilitate the oxidation of NADH). The fast response should be attractive for dynamic flow measurements (as required for automated clinical assays or process control). Even though the concept is presented in terms of ADH, it could be extended

**FIGURE 7.** Stability of the response to 3.4 mM ethanol. (A) The surface was polished prior to each daily use. (B) The surface was polished periodically (after the first recording in days 3 and 5). Other conditions, as in Figure 3b, except that a 10% ADH/7.5% NAD<sup>+</sup> (w/w) composition was employed in B.



to many other NAD<sup>+</sup>-dependent dehydrogenases. Hence, the versatile graphite-epoxy biosensor fabrication scheme holds a great promise for the monitoring of a wide variety of bioanalytes.

#### ACKNOWLEDGMENT

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#### REFERENCES

1. J. Wang and K. Varughese, *Anal. Chem.* 62 (1990) 318.
2. J. Wang and M. Ozsoz, *Electroanalysis*, 2 (1990) 647.
3. U. Wollenberger, J. Wang, M. Ozsoz, E. Gonzalez-Romero, and F. Scheller, *Bioelectrochem Bioenerg.*, 26 (1991) 287.
4. L. Gorton, *J. Chem. Soc. Faraday Trans.*, 82 (1986) 1245.
5. J. Wang, T. Martinez, D. Yaniv, and L. McCormick, *J. Electroanal Chem.* 286 (1990) 265.
6. G. G. Guilbault, *Handbook of Enzymatic Methods of Analysis*, M. Dekker, New York, 1976, p. 64.
7. T. E. Barman, *Enzyme Handbook*, Vol. 1, Springer-Verlag, Berlin, 1969, p. 23.
8. T. Bicsak, L. Kann, A. Reiter, and T. Chase, *Arch. Biochem. Biophys.* 216 (1982) 1982.