Anal. Chem. **2000,** *72,* 520−527

Electrocatalytic Detection of NADH and Glycerol by NAD^+-Modified Carbon Electrodes

M. Isabel Álvarez-González, Silvana B. Saidman,† M. Jesús Lobo-Castañón, Arturo J. Miranda-Ordieres, and Paulino Tuñón-Blanco*

Departamento de Química Física y Analítica, Universidad de Oviedo, 33006 Oviedo, Asturias, Spain, and Departamento de Química e Ingeniería Química, Instituto de Ingeniería Electroquímica y Corrosión (INIEC), Universidad Nacional del Sur, 8000 Bahia Blanca, Argentina

The electrochemical oxidation of the adenine moiety in NAD^+ and other adenine nucleotides at carbon paste electrodes gives rise to redox-active products which strongly adsorb on the electrode surface. Carbon paste electrodes modified with the oxidation products of NAD^+ show excellent electrocatalytic activity toward NADH oxidation, reducing its overpotential by about 400 mV. The rate constant for the catalytic oxidation of NADH, determined by rotating disk electrode measurements and extrapolation to zero concentration of NADH, was found to be 2.5×10^5 M^{-1} s^{-1}. The catalytic oxidation current allows the amperometric detection of NADH at an applied potential of +50 mV (Ag/AgCl) with a detection limit of 4.0×10^{-7} M and linear response up to 1.0×10^{-5} M NADH. These modified electrodes can be used as amperometric transducers in the design of biosensors based on coupled dehydrogenase enzymes and, in fact, we have designed an amperometric biosensor for glycerol based on the glycerol dehydrogenase (GlDH) system. The enzyme GlDH and its cofactor NAD^+ were co-immobilized in a carbon paste electrode using an electropolymerized layer of nonconducting poly(*o*-phenylenediamine) (PPD). After partial oxidation of the immobilized NAD^+, the modified electrode allows the amperometric detection of the NADH enzymatically obtained at applied potential above 0 V (Ag/AgCl). The resulting biosensor shows a fast and linear response to glycerol within the concentration range of 1.0×10^{-6}−1.0×10^{-4} M with a detection limit of 4.3×10^{-7} M. The amperometric response remains stable for at least 3 days. The biosensor was applied to the determination of glycerol in a plant-extract syrup, with results in good agreement with those for the standard spectrophotometric method.

It is well-known that the adenine derivatives, nicotinamide adenine dinucleotide (NAD^+) and its reduced form, dihydronicotinamide adenine dinucleotide (NADH), are the key central charge carriers in living cells. NAD^+ is a ubiquitous cofactor, used by more than 300 dehydrogenase enzymes. In this molecule, the nicotinamide ring is the site of the reversible redox process. It accepts two electrons and a proton from a substrate (SH_2) in the presence of a suitable enzyme (E) to form NADH, the oxidized form of the substrate (S), and a proton, in the general enzymatic reaction (1).

$$SH_2 + NAD^+ \overset{E}{\rightleftharpoons} S + NADH + H^+ \qquad (1)$$

The NADH enzymatically produced can be electrochemically monitored through its reoxidation to NAD^+. In consequence, a considerable amount of analytical research in the past decades has been concerned with the electrochemistry of the NAD^+/NADH redox couple at conventional electrode materials. At anodic potentials higher than 0.4 V (Ag/AgCl) on carbon paste electrodes, the nicotinamide ring of the NADH undergoes a two-electron oxidation in aqueous solution to produce NAD^+. Although the reversible formal potential for the couple NAD^+/NADH is −0.32 (NHE)[1] at pH 7, high applied potentials are required to achieve an appreciable electron-transfer rate owing to the slow heterogeneous kinetics at unmodified metal and carbon surfaces.[2,3] The adsorption of NAD^+ and the occurrence of uncontrolled side reactions (due to the ECE mechanism of NADH oxidation) may also cause important interferences.[4,5]

A way to decrease the high overpotentials and minimize the side reactions is to use an appropriate mediator that can improve electron-transfer kinetics. In this way, much effort has been dedicated in the last few decades to identifying materials which can effectively overcome the kinetic barriers for the electrochemical regeneration of NAD^+. Therefore, there is a great deal of interest in the development of new materials or new immobilization strategies for already known electron-transfer mediators as is shown by the number of publications about the subject.[6−35]

* To whom correspondence should be addressed. (Fax) 34−985−103125; (e-mail) ptb@fluor.quimica.uniovi.es.

† Universidad Nacional del Sur.

(1) Clark, W. M. *Oxidation−reduction potentials of organic systems*; William and Wilkins: Baltimore, MD, 1960.
(2) Moiroux, J.; Elving, J. P. *Anal. Chem.* **1978**, *50*, 1056.
(3) Jaegfeldt, H. *J. Electroanal. Chem.* **1980**, *110*, 295.
(4) Moiroux, J.; Elving, J. P. *J. Am. Chem. Soc.* **1980**, *102*, 6533.
(5) Jaegfeldt, H. *Bioelectrochem. Bioenerg.* **1981**, *8*, 355.
(6) Pariente, F.; Tobalina, F.; Moreno, G.; Hernández, L.; Abruña, H. D. *Anal. Chem.* **1997**, *69*, 4065.
(7) Pariente, F.; Tobalina, F.; Darder, M.; Lorenzo, E.; Abruña, H. D. *Anal. Chem.* **1996**, *68*, 3135.
(8) Pariente, F.; Lorenzo, E.; Tobalina, F.; Abruña, H. D. *Anal. Chem.* **1995**, *67*, 3936.
(9) Pariente, F.; Lorenzo, E.; Abruña, H. D. *Anal. Chem.* **1994**, *66*, 4337.
(10) Cai, C. X.; Xue, K. H. *J. Electroanal. Chem.* **1997**, *427*, 147.

10.1021/ac9908344 CCC: $19.00
Published on Web 01/04/2000

Thus, the most recent efforts to prepare modified electrodes that show electrocatalytic activity toward NADH oxidation have focused on the use of electropolymerized films as new materials, either redox polymers such as poly (3,4-dihydroxybenzaldehyde),[6−9] poly (azure I),[10] poly(nile blue A),[11] poly(methylene blue),[12] and poly-(methylene green)[13] or conducting polymers such as poly(*o*-aminophenol),[14] poly(*o*-phenylenediamine),[14] and poly(aniline).[15] Electrodes modified with electrogenerated[16] or dip-coated[17,18] polymers containing inorganic metal complexes have also been described. Among the best-known structures, which can be active in the electrocatalytic oxidation of NADH are found *o*-quinones,[19,20,21] *p*-quinones,[22] and phenothiazine[23−25] and phenoxazine[26−29] derivatives. Recent work has been mostly focused on new immobilization strategies such as dip-coated ion-exchanger polymers loaded with catalysts such as toluidine blue O[23] and meldola blue,[26] inclusion in self-assembled monolayers on platinum[19] and gold[20,24,25] electrodes, or inclusion in composite[28] and sol−gel[29] electrodes. Structural effects of the electrode on the electrocatalysis have also been recognized, and a decrease in the overpotential needed for NADH oxidation can be achieved by using single-crystal[30] or thick-film[31] gold electrodes or by electrochemical activation of carbon fiber electrode surfaces.[32,33] In addition, enzymatic regeneration of NAD^+ has been described using different schemes such as diaphorase,[34] lipoamide dehydrogenase,[35] or tyrosinase and salicylate hydroxylase.[36] Recent reviews of work in this area can be found[37,38] in the literature.

During the development of different biosensors for substrates of dehydrogenase enzymes, we have recently found that NAD^+ modified carbon paste electrodes show highly enhanced responses to the corresponding enzymatic substrate after anodic polarization at potentials above 1.2 V. The investigation of this effect allowed us to find a new approach for the electrocatalytic oxidation of NADH. After the electrochemical oxidation of the adenine moiety in NAD^+ molecules at potentials above 1.2 V(Ag/AgCl) in alkaline solutions, an oxidation product is obtained which shows high electrocatalytic activity for NADH oxidation, decreasing the oxidation overpotential by about 400 mV. In the present work, this new electrocatalytic system is described. In addition, we demonstrate the possibility of coupling the new electrocatalytic system to the reaction catalyzed by dehydrogenase enzymes, by using immobilized glycerol dehydrogenase for the preparation of a glycerol biosensor.

The determination of glycerol constitutes an important test in different fields such as clinical diagnosis and the pharmaceutical and food industries. Therefore, a rapid, and at the same time economical, method is desired for its measurement in a variety of samples. Several enzymatic sensors, preferentially based on dehydrogenase enzymes, have been developed for the measurement of glycerol. In these methods, glycerol is quantitatively related to the enzymatically produced NADH which can be monitored spectrophotometrically,[39] fluorimetrically,[40] or electrochemically either directly[41] or using an electron-transfer mediator.[42,43] One important advantage of the enzymatic systems employing electrochemical detection schemes is that no interference from absorbent species and turbidity is usually observed. In this context, we report on the development of a very simple amperometric glycerol sensor in which NAD^+ acts not only as cofactor of the dehydrogenase enzyme but also as precursor of the NADH electrocatalytic system. The feasibility of this approach was demonstrated by its application to the determination of glycerol in a plant-extract syrup.

EXPERIMENTAL SECTION

Reagents and Materials. The enzyme glycerol dehydrogenase (GlDH) (E. C. 1.1.1.6) from *Cellulomonas sp.* (65 units per milligram of protein), the cofactor nicotinamide adenine dinucleotide (reduced, NADH, and oxidized, NAD^+, forms), adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), 2,8-dihydroxyadenine (DHA), parabanic acid, alloxan, allantoin, glycerol, 9-ethyladenine, 6-dimethylaminopurineriboside, 6-benzylaminopurineriboside, 6-dimethylaminopurine, and 6-benzylaminopurine were purchased from Sigma (Spain). The monomer *o*-phenylenediamine and the silicone high vacuum grease were obtained from Merck (Spain). Spectroscopic-grade graphite powder was obtained from Dicoex (Bilbao, Spain). The glycerol test kit used for the determination of this compound in a pharmacological product was purchased

(11) Cai, C. X.; Xue, K. H. *Anal. Chim. Acta* **1997**, *343*, 69.
(12) Silber, A.; Hampp, N.; Schuhmann, W. *Biosens. Bioelectron.* **1996**, *11*, 215.
(13) Zhou, D. M.; Fang, H. Q.; Chen, H. Y.; Ju, H. X.; Wang, Y. *Anal. Chim. Acta* **1996**, *329*, 41.
(14) Lobo, M. J.; Miranda, A. J.; López-Fonseca, J. M.; Tuñón, P. *Anal. Chim. Acta* **1996**, *325*, 33.
(15) Bartlett, P. N.; Birkin, P. R.; Wallace, E. N. K. *J. Chem. Soc., Faraday Trans.* **1997**, *93*, 1951.
(16) Wu, Q.; Maskus, M.; Pariente, F.; Fernández, V. M.; Lorenzo, E.; Abruña, H. D. *Anal. Chem.* **1996**, *68*, 3688.
(17) Ju, H.; Leech, D. *Anal. Chim. Acta* **1997**, *345*, 51.
(18) Popescu, I. C.; Domínguez, E.; Narvaez, A.; Pavlov, V.; Katakis, I. *J. Electroanal. Chem.* **1999**, *464*, 208.
(19) Lorenzo, E.; Sánchez, L.; Pariente, F.; Tirado, J.; Abruña, H. D. *Anal. Chim. Acta* **1995**, *309*, 79.
(20) Sun, J. J.; Xu, J. J.; Fang, H. Q.; Chen, H. Y. *Bioelectrochem. Bioenerg.* **1997**, *44*, 45.
(21) Zare, H. R.; Golabi, S. M. *J. Electroanal. Chem.* **1999**, *464*, 14.
(22) Murthy, A. S. N.; Sharma, J. *Talanta* **1998**, *45*, 951.
(23) Huang, Z. W.; Persson, B.; Gorton, L.; Sahni, S.; Skotheim, T.; Bartlett, P. *Electroanalysis* **1996**, *8*, 575.
(24) Chen, H. Y.; Zhou, D. M.; Xu, J. J.; Fang, H. Q. *J. Electroanal. Chem.* **1997**, *422*, 21.
(25) Ohtani, M.; Kuwabata, S.; Yoneyama, H. *J. Electroanal. Chem.* **1997**, *422*, 45.
(26) Persson, B.; Lee, H. S.; Gorton, L.; Skotheim, T.; Bartlett, P. *Electroanalysis* **1995**, *7*, 935.
(27) Nagy, G.; Kapui, I.; Gorton, L. *Anal. Chim. Acta* **1995**, *305*, 65.
(28) Gründig, B.; Wittstock, G.; Rüdel, U.; Strehlitz, B. *J. Electroanal. Chem.* **1995**, *395*, 143.
(29) Sampath, S.; Lev, O. *J. Electroanal. Chem.* **1998**, *446*, 57.
(30) Xing, X.; Shao, M.; Liu, C. C. *J. Electroanal. Chem.* **1996**, *406*, 83.
(31) Silber, A.; Bräuchle, C.; Hampp, N. *J. Electroanal. Chem.* **1995**, *390*, 83.
(32) Nowall, W. B.; Kuhr, W. G. *Anal. Chem.* **1995**, *67*, 3583.
(33) Hayes, M. A.; Kuhr, W. G. *Anal. Chem.* **1999**, *71*, 1720.
(34) Cosnier, S.; Le Lous, K. *Talanta* **1996**, *43*, 331.
(35) Bu, H. Z.; Mikkelsen, S. R.; English, A. M. *Anal. Chem.* **1998**, *70*, 4320.
(36) Huang, T.; Warsinke, A.; Kuwana, T.; Scheller, F. W. *Anal. Chem.* **1998**, *70*, 991.
(37) Katakis, I.; Domínguez, E. *Mikrochim. Acta* **1997**, *126*, 11.
(38) Lobo-Castañón, M. J.; Miranda-Ordieres, A. J.; Tuñón-Blanco, P. *Electroanalysis* **1997**, *9*, 191.
(39) Pfandl, A.; Mensching, D. *Pharm. Ind.* **1984**, *46*, 403.
(40) Cañizares, P.; Luque de Castro, M. D. *Analyst (Cambridge, U.K.)* **1995**, *114*, 989.
(41) Prodromidis, M. I.; Stalikas, C. D.; Tzouwara-Karayannis, M. I. *Talanta* **1996**, *43*, 27.
(42) Kronkvist, K.; Wallentin, K.; Johansson, G. *Anal. Chim. Acta* **1994**, *290*, 335.
(43) Lauranavicius, V.; Kurtinaitene, B.; Gureviciene, V.; Boguslavsky, L.; Geng, L.; Skotheim, T. *Anal. Chim. Acta* **1996**, *330*, 159.

from Boehringer-Mannheim (Spain). Other chemicals employed were of analytical grade. All solutions were prepared with water purified with a Milli-Q (Millipore) system.

Biosensor Preparation. Unmodified carbon paste was prepared by thoroughly mixing 1 g of spectroscopic-grade graphite powder and 0.323 g of silicone grease. The 7% GlDH-10% NAD^+-modified carbon paste was prepared by mixing the unmodified carbon paste with the corresponding proportions of the lyophilized enzyme preparation (GlDH) and the cofactor (NAD^+). A portion of the resulting paste was then packed firmly into the working-electrode cavity. The electrode surface was smoothed on a paper over a glass surface and then covered with a PPD film obtained by potential scan electropolymerization in aqueous solution. In the electropolymerization step, the electrode potential was cycled between −0.5 and +0.7 V (Ag/AgCl) at 50 mV s^{-1} in an oxygen-free phosphate solution pH 10 containing 5×10^{-4} M *o*-phenylenediamine under nitrogen atmosphere. These PPD-modified electrodes were thoroughly rinsed with phosphate solution and submitted to an oxidative pretreatment in order to generate the NADH electrocatalytic system. The pretreatment consisted of the application of a double potential step from +0.15 to +1.25V with a pulse width of 60 s. The electrodes thus prepared were used for the amperometric measurement of glycerol in a phosphate background solution of pH 10.

Apparatus. Voltammetric and chronoamperometric studies of NAD^+ oxidation were performed using an Autolab Electrochemical analyzer (Eco Chemie B. V., Utrecht, The Netherlands). Rotating disk electrode measurements were carried out with a 663 VA stand system (Metrohm) using a carbon paste electrode of 4 mm in diameter. A Metrohm VA detector was used as an amperometric detector, and its output was recorded using a Metrohm E-586 Labograph stripchart recorder.

All the measurements were made using a conventional three electrode system. A modified carbon paste electrode acted as the working electrode and a platinum wire as the counter electrode. All potentials were referred to a silver/silver chloride/saturated potassium chloride electrode. The static working electrode was constructed with a Teflon body, a stainless steel contact, and a disk-shaped active surface 3.18 mm in diameter. A Shimazu UV-1203 spectrophotometer was used for spectrophotometric measurements. Synchronous fluorescence spectra were recorded using a Perkin-Elmer LS-50B fluorescence spectrometer. IR spectra of the lyophilized desorption products were recorded as KBr pellets using a Perkin-Elmer Paragon 100 spectrometer and fast atom bombardment (FAB) mass spectra were recorded on a VG-AutoSpecE instrument using nitrobenzyl alcohol as the matrix.

Analytical Procedure. The biosensor, prepared as described above, was used for the determination of glycerol in a pharmaceutical product. The accuracy of the biosensor was evaluated by comparing it to standard enzymatic methods using the Boehringer test kit.

One milliliter of the sample was diluted to 250 mL with water. Amperometric measurements were carried out with the biosensor placed in 20 mL of a stirred pH 10 phosphate solution at an applied potential of +0.15 V. When the background current had decayed to a steady-state value, an aliquot (typically 250 μL) of the diluted sample solution was injected and the steady-state current was recorded. After that, four different aliquots (50 μL) of a glycerol stock solution (1×10^{-2} M) were injected into the electrochemical cell, and a new steady-state current was reached with each addition. The results thus obtained were used to construct a standard-addition graph and to calculate the glycerol concentration in the sample. The spectrophotometric measurements were basically conducted according to the procedure outlined by the enzymatic kit supplier.

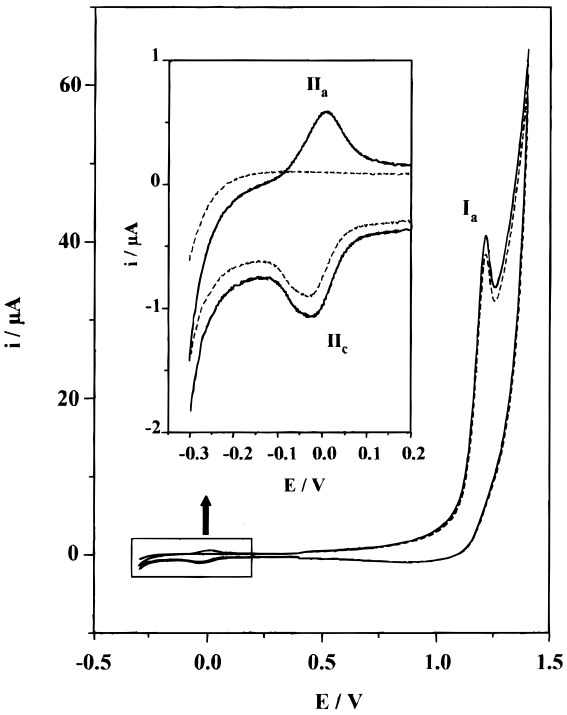


Figure 1. Cyclic voltammograms obtained with an unmodified carbon paste electrode in a 0.1 M phosphate solution pH 10, containing NAD^+ 5×10^{-4} M. Dotted line: first potential scan. Solid line: second scan. Scan rate: 50 mV s^{-1}.

RESULTS AND DISCUSSION

Electrocatalysis of NADH Oxidation at Carbon Paste Electrodes Modified by Adsorbed NAD^+ Oxidation Products. A considerable amount of research about the electrochemistry of the NAD^+/NADH redox couple has been carried out owing to its role as charge carrier in the living cells. These studies are mainly concerned with the oxidation (NADH) or reduction (NAD^+) of the nicotinamide ring of the molecule. Thus, while the basic reduction of NAD^+ has been extensively studied,[44] the electrochemical oxidation of the adenine moiety in NAD^+ has, to our knowledge, not been considered. We have found that NAD^+ in alkaline solutions (phosphate buffer pH 10) is oxidized on carbon paste electrodes at high potentials, as is shown in Figure 1, peak I_a. This process is due to the oxidation of the adenine moiety in the NAD^+ molecule as is indicated by the close agreement between the obtained oxidation peak ($E_p = 1.2$ V (Ag/AgCl)) and that previously described for the oxidation of adenosine at carbon electrodes.[45] One of the oxidation products forms a reversible system at potentials close to 0 V (Figure 1, process II_a/II_c), only

(44) Dryhurst, G. *Electrochemistry of Biological Molecules*; Academic Press: New York, 1977.
(45) Drhyhurst, G.; *Talanta*, **1972**, *19*, 769.

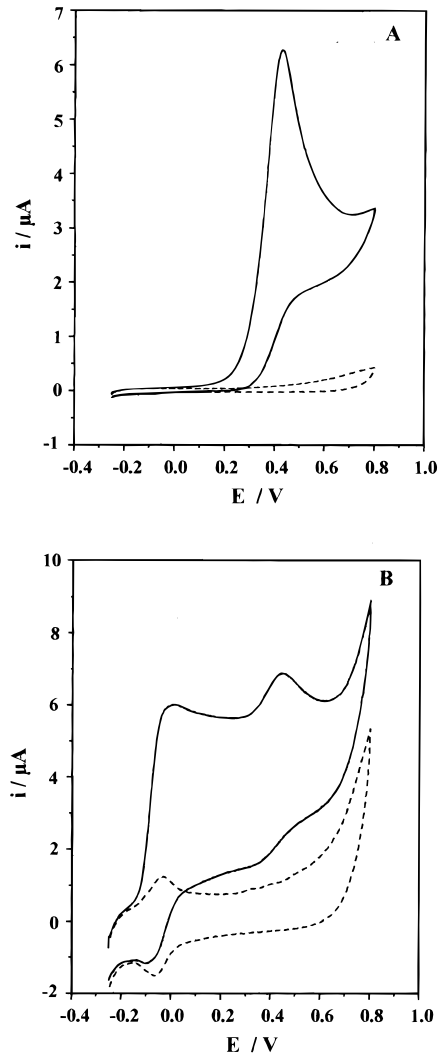


Figure 2. Cyclic voltammograms obtained with a carbon paste electrode after five potential scans in a phosphate solution pH 10, (A) without and (B) with NAD^+ 5×10^{-4} M. Dotted line: Background. Solid line: NADH 5.0×10^{-4} M. Scan rate: 50 mV s^{-1}.

observed when the first anodic scan was performed beyond the oxidation process I_a. This oxidation product is strongly adsorbed on the electrode surface as shown in Figure 2B (dashed line) which presents the voltammograms obtained when the same electrode is subsequently removed from the cell, rinsed with water, and placed in a fresh buffer solution without NAD^+. The wave shape is that expected for a surface-immobilized redox couple with a small ΔE_p value (24 mV at 50 mV s^{-1}). In addition, the anodic and cathodic peak currents of the reversible system obtained after NAD^+ oxidation are directly proportional to the scan rate for sweep rates between 2 and 500 mV s^{-1}, which confirms the adsorption nature of the process.

The process giving rise to the anodic peak I_a was studied in solutions of pH varying from 5 to 12. The adsorbed couple II_a/II_c observed after the oxidation of NAD^+, appears, independently of the pH of the background electrolyte used for the oxidation of NAD^+, in the pH range between 5 and 11. At pH above 11 no reversible system was observed after oxidation of NAD^+ at high potentials. The formal potential of the surface redox couple was pH dependent, with a slope of 59 mV /pH unit ($E^{\circ\prime} = 0.542 - 0.059$ pH, $r = 0.9993$, $n = 7$) in the pH range 5−11, which agrees with the anticipated Nernstian value for a two-electron two-proton process.

Surface coverage for NAD^+ oxidation products was determined from the integrated charge under the cathodic wave, assuming a two-electron process (see below). The electrochemically active surface for a carbon paste electrode 3.18 mm in diameter was estimated to be 0.084 cm^2 by chronocoulometric measurements. Surface coverage between 2×10^{-12} and 2×10^{-10} mol cm^{-2} can be obtained at pH 10 by varying NAD^+ concentration and the number of potential scan cycles applied during the electrode-modification step. In addition, there were no significant changes in the surface coverage over pH values from 8 to 10. For pH values above 10 and below 8, a significant decrease in the surface coverage was observed for a given electrolysis time and NAD^+ concentration. On the basis of these results, all further studies were made at pH 10.

The carbon paste electrode modified with one of the NAD^+ oxidation products shows catalytic activity for NADH oxidation as is demonstrated in Figure 2B (solid line). When cyclic voltammograms for the modified carbon paste electrode were obtained in the presence of NADH (0.5 mM) an enhancement in the oxidation current at ~0 V (Ag/AgCl) and a decrease in the cathodic wave were observed. This behavior is consistent with a very strong electrocatalytic effect. The magnitude of the catalytic current was proportional to the concentration of NADH in the solution. It has been described in the literature that the polarization of carbon paste electrodes at high potentials leads to o-quinone groups which catalyze the NADH oxidation.[46] To rule out this possibility, the cyclic voltammetric responses of unmodified carbon paste electrodes to 0.5 mM NADH were recorded after five cyclic potential scans between −0.2 and 1.4 V at 50 mV s^{-1} were applied to the electrode in phosphate solution pH 10. Results are presented in Figure 2A. As can be observed, no catalytic current for NADH oxidation is obtained and NADH is oxidized at 0.45 V at this activated but unmodified carbon paste electrode. These results exclude any significant contribution of surface quinone groups generated during the potential scan until 1.40 V in the observed catalytic current. Compared with the direct oxidation of NADH at a bare carbon paste electrode, even if subjected to an oxidative pretreatment, the electrode modified with NAD^+ oxidation products diminishes the overpotential needed for NADH oxidation by more than 400 mV.

To identify the products of NAD^+ oxidation responsible for the electrocatalytic oxidation of NADH, FT-IR and mass spectrometry studies were attempted. Adsorbed products, obtained after repeated electrooxidation by cyclic voltammetry between −0.2 and +1.4 V of NAD^+, 10^{-2} M, at pH 10 on several carbon electrodes, were desorbed in an ethanol−water (1:1) solution. This solution was then lyophilized and the solid products used for characterization by FT-IR and mass spectrometry. Unfortunately, it was not possible to obtain relevant information from these techniques, because an insufficient amount of product could be isolated from the electrode surface.

Dryhurst et al. studied the electrochemical oxidation of adenine at graphite electrodes and proposed a mechanism[44,47] which was

(46) Blaedel, W. J.; Jenkis, R. A. *Anal. Chem.* **1975**, *47*, 1337.
(47) Dryhurst, G.; Elving, P. J. *J. Electrochem. Soc.* **1968**, *115*, 1014.

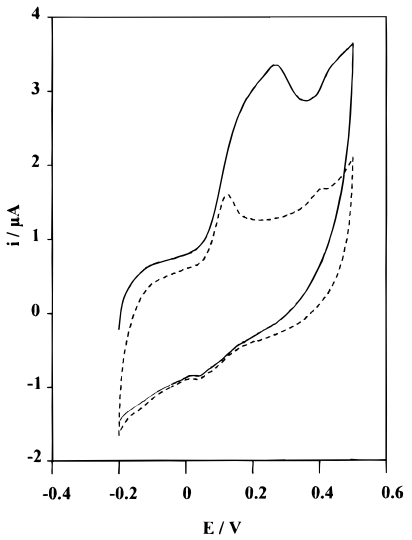


Figure 3. Cyclic voltammogram obtained with an unmodified graphite electrode in a 0.1 M phosphate solution pH 10, containing 2,8-DHA 5×10^{-4} M and Triton X-100 0.01%. Dotted line: without NADH. Solid line: with NADH 2×10^{-4} M. Scan rate: 50 mV s^{-1}.

further confirmed by Goyal et al.[48] These studies reveal that adenine is electrochemically oxidized in two sequential $2e^-/2\ H^+$ reactions to obtain 2,8-dihydroxyadenine, which rapidly oxidizes to give a molecule with a quinone-diimine structure. This diimine can only be detected by fast-sweep voltammetry and will be readily attacked by water to give finally alloxan, parabanic acid, or allantoin as major products. As far as we know no similar study of the oxidation of adenine nucleotides has been made.

As the NADH catalyst described is only obtained after the oxidation of the adenine moiety in the NAD^+ molecule, it seems reasonable to suppose an oxidation mechanism similar to that described for adenine, in such a way that some of the oxidation products could act as an NADH catalytic structure. We have verified that neither parabanic acid nor alloxan or allantoin can act as electrocatalyst for NADH oxidation. This means that from the different oxidation products only the quinone-diimine structure could act as an NADH catalyst. In fact, studies of the electrochemical and electrocatalytic behavior of 2,8-dihydroxyadenine were carried out in pH 10 buffered solutions containing DHA, 5×10^{-4} M, and Triton X 100, 0.01%, in order to increase the limited solubility of this compound. In cyclic voltammetry, as is shown in Figure 3, a well-defined oxidation peak is obtained with a peak potential of 0.12 V at pH 10. This peak was accompanied on the reverse scan by a cathodic peak at 0.047 V. As was previously reported,[48] the anodic process corresponds to the oxidation of 2,8-DHA to the corresponding diimine. This species undergoes a fast chemical reaction which competes with the reduction in the reverse scan. Upon addition of NADH, 2×10^{-4} M, there was an enhancement of the measured anodic peak current. This means that, despite the short life of the diimine obtained, this species can oxidize NADH acting as catalyst for NADH oxidation.

A similar electrochemical and electrocatalytic behavior was observed for the products obtained after the oxidation of other adenine nucleotides, such as adenosine, adenosine monophosphate, and adenosine triphosphate and, in general, for other N9-substituted adenine derivatives such as 9-ethyladenine, 6-benzylaminopurineriboside, and 6-dimethylaminopurineriboside. In all cases, the oxidation of the adenine moiety of the molecule on carbon paste electrodes occurs at potentials about 1.2 V at pH 10 in 5×10^{-4} M solutions of the corresponding parent compound. This process give rises to oxidation products that strongly adsorb on the electrode surface and act as electrocatalysts for NADH oxidation. In the cases of 6-dimethylaminopurineriboside and 6-benzylaminopurineriboside, the adsorbed electrocatalytic redox couple could only be obtained after several potential scans at scan rates above 7 V s^{-1}. This fact probably indicates that the oxidized forms of the catalyst derived from such parent compounds are less stable and can only be stabilized after adsorption on the electrode surface. Table 1 summarizes the electrochemical characteristics of the NADH catalyst obtained after oxidation of different adenine derivatives on carbon paste electrodes and the catalytic current measured in the same conditions for a 1×10^{-4} M NADH solution. It may be significant that not only adenine nucleotides but also all the other N-9 substituted adenines assayed give rise upon oxidation to reversible redox couples with formal potentials close to 0 V (Ag/AgCl) at pH 10 and catalytic activity toward NADH oxidation. On the other hand, neither adenine nor other N9-unsubstituted adenines assayed, like 6-dimethylaminopurine and 6-benzylaminopurine, produces such a catalytic system, even at the highest scan rate tested.

Table 1. Formal Potentials and Surface Coverage (**Γ**) for the NADH Catalyst Obtained after Oxidation on CPE of the Corresponding Parent Compound in a Buffer Solution pH 10.0 (Conditions in the Text)

parent compound	$E^{\circ\prime}$/**V**[a]	Γ/mol cm^{-2}	i_{cat}[b]/**A**
AMP	−0.062	1.4×10^{-10}	1.3×10^{-6}
ADP	−0.055	5.3×10^{-11}	5.7×10^{-7}
ATP	−0.056	8.7×10^{-11}	9.7×10^{-7}
adenosine	−0.055	7.5×10^{-11}	4.8×10^{-7}
9-ethyladenine	−0.065	2.3×10^{-11}	3.0×10^{-7}
6-benzylaminopurineriboside	−0.033	3.1×10^{-12}	9.0×10^{-8}
6-dimethylaminopurineriboside	−0.064	5.6×10^{-12}	6.0×10^{-8}

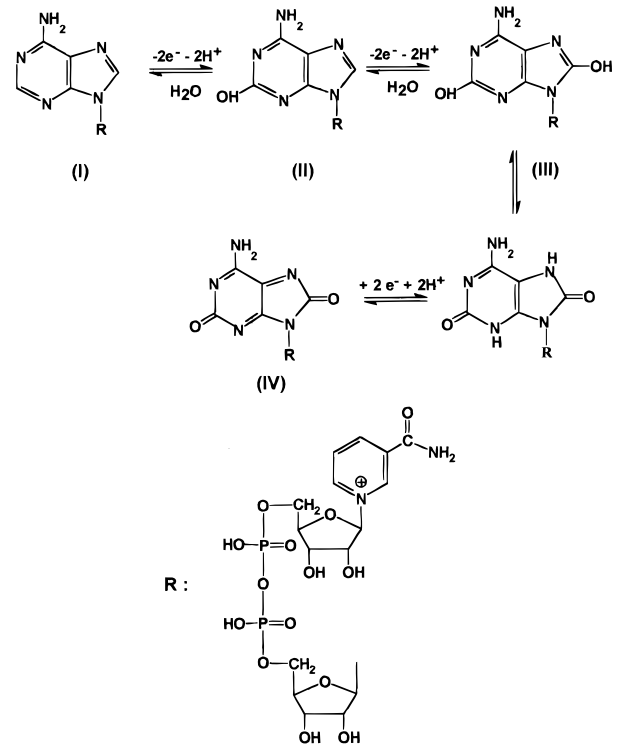
[a] $E^{0\prime}$vs. Ag/AgCl at pH 10.0 [b] The i_{cat} values refer to the catalytic current measured at pH 10 for a NADH concentration 1.0×10^{-4} M.

In addition, studies by synchronous fluorescence spectroscopy, a method that has been used to identify different organic compounds,[49] provided some additional supporting evidence for the formation of a 2,8-dihydroxyadenine derivative. Synchronous fluorescence spectra of water−ethanol (1:1) solutions of 2,8-dihydroxyadenine, 1×10^{-6} M, and NAD^+-oxidation products were recorded by scanning both monochromators simultaneously (scan rate 240 nm min^{-1}) at a 30 nm constant difference. The solution of the NAD^+ oxidation product was obtained after desorption in 5 mL of ethanol−water (1:1), during 30 min, of the products adsorbed on seven different carbon electrodes by six continuous potential scans between −0.2 and +1.4 V in 0.1 M phosphate solution pH 10 containing NAD^+, 10^{-2} M. The synchronous spectra obtained for both 2,8-dihydroxyadenine and the collected NAD^+ oxidation products show a clearly defined peak of maximum intensity at $\lambda = 379$ and 383 nm, respectively.

(48) Goyal, R. N.; Kumar, A.; Mittal, A. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1369.

(49) Warner, I. M.; Soper, S. A.; McGown, L. B. *Anal. Chem.* **1996**, *68*, 73R.

Scheme 1



From the obtained results and by analogy with earlier investigations on purine oxidation, we propose for the electrochemical oxidation of NAD^+ a mechanism (Scheme 1) similar to that previously described by Dryhurst et al.[44,47] for the oxidation of adenine. The oxidation of the adenine moiety in NAD^+ proceeds by two sequential two-electron, two-proton oxidation steps to give the 2-hydroxyadeninedinucleotide (II) and the 2,8-dihydroxyadeninedinucleotide (III). Further removal by a two-electron, two-proton oxidation step results in the formation of the corresponding quinone-diimine (IV) stabilized by adsorption on the electrode surface. Peak II_c (Figure 1) could therefore be due to the reversible reduction of the quinone-diimine back to 2,8-dihydroxyadeninedinucleotide. The electrocatalytic oxidation of NADH, obtained with a carbon paste electrode modified by the adsorbed NAD^+ oxidation product, may be ascribed to this quinone-diimine species since, as is well-known, this kind of structure can act as a NADH electrocatalyst.

Cyclic voltammetry and rotating disk electrode (RDE) techniques were employed to study the kinetics of the reaction between the NAD^+ oxidation product and NADH. In the first case, the rate constant (k) can be obtained according to the method of Andrieux and Saveant[50] for an S-shaped electrocatalytic wave independent of sweep rate by recording the limiting catalytic current as a function of the NADH concentration. Using modified electrodes with a mean coverage of 4.2×10^{-12} mol cm^{-2}, an S-shaped electrocatalytic wave was obtained. In addition, there was no detectable change in the limiting current obtained for a NADH concentration of 5×10^{-4} M at different scan rates, between 2 and 500 mV s^{-1}. The limiting current for NADH oxidation at 25 °C increases linearly with increasing concentration

(50) Andrieux, C. P.; Saveant, J. M. *J. Electroanal. Chem.* **1978**, *93*, 163.

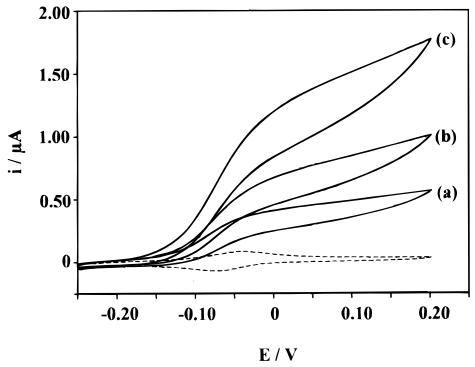


Figure 4. Cyclic voltammograms obtained with a carbon paste electrode modified by cyclic potential scan between −0.2 and +1.2 V (2 scans) in a background electrolyte containing NAD^+ 10^{-3} M. Background electrolyte: 0.1 M phosphate solution pH 10. Dotted line: without NADH. Solid line: with NADH (a) 2.44×10^{-4} M, (b) 4.76×10^{-4} M and (c) 9.1×10^{-4} M. Scan rate: 50 mV s^{-1}.

of the cofactor between 1.0×10^{-4} M and 5.0×10^{-3} M, as can be observed in Figure 4. From the slope of the corresponding linear plot, an average rate constant of $(1.3 \pm 0.2) \times 10^4$ M^{-1} s^{-1} was calculated.

As for RDE measurements, the rate constant can be evaluated from the dependence of the limiting catalytic current (i_{cat}), measured at 0.15 V for several NADH concentrations, on the rate of the rotation (ω) by using the Koutecky-Levich equation. A linear dependence between $1/i_{cat}$ and $1/\omega^{1/2}$ was obtained for modified electrodes with a mean coverage of 3.7×10^{-11} mol cm^{-2} and for NADH concentrations between 4.2×10^{-5} and 5.0×10^{-4} M. From the intercept of the Koutecky-Levich plots obtained for NADH concentration between 1×10^{-4} and 5.0×10^{-4} M, a mean value of $(1.5 \pm 0.3) \times 10^4$ M^{-1} s^{-1} was found for the kinetic constant. This value is close to that determined from cyclic voltammetric measurements and similar to that obtained for the reaction between NADH and other previously described mediators.[37] At NADH concentrations below 1.0×10^{-4} M, the value of k, obtained using the RDE method, increases when the NADH concentration decreases. In this concentration range, a plot of the reciprocal of the reaction rate ($1/k$) versus NADH concentration results in a straight line. This behavior is typical of a catalyzed reaction that proceeds via an intermediate complex following a reaction scheme similar to that of Michaelis-Menten, according to the model proposed by Gorton et al.[51] From the slope and the intercept of this plot, the values of the apparent Michaelis-Menten constant, K_M, and the rate constant for the complex dissociation reaction, k_2, can be evaluated. A K_M of 0.01 mM and a k_2 of 2.9 s^{-1} were obtained. To compare the value of the rate constant for the overall reaction to those previously reported for the NADH oxidation by other mediators, this constant was evaluated at zero reactant concentration. A constant $k_{C=0}$ of 2.5×10^5 M^{-1} s^{-1} was obtained when the plot $1/k$ versus NADH concentration was extrapolated to zero.

Modified carbon paste electrodes, prepared by subjecting unmodified carbon paste electrodes to five cyclic potential scans between −0.3 and 1.4 V in a phosphate buffer pH 10 solution containing NAD^+ 5×10^{-4} M, were used for the amperometric

(51) Gorton, L.; Torstensson, A.; Jaegfeldt, H.; Johansson, G. *J. Electroanal. Chem.* **1984**, *161*, 103.

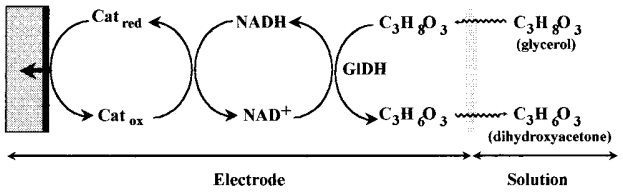


Figure 5. Reaction scheme for the determination of glycerol.

detection of NADH. The amperometric response of this kind of modified carbon paste electrode to increasing concentrations of NADH was investigated using static working electrodes in magnetically stirred solutions at an applied potential of +50 mV. A linear response to the reduced cofactor concentration between 1.0×10^{-6} and 1.0×10^{-5} M was found ($i/\text{nA} = 0.215 + 4.79 \times 10^5$ [NADH]/M; $r = 0.9991$; $n = 7$) with a detection limit, estimated from the peak to-peak noise of the baseline, of 4.0×10^{-7} M. The time required to obtain a steady-state response was 12 s.

Design of the Glycerol Biosensor. To confirm the usefulness of the described electrocatalytic system, a glycerol biosensor was developed. The amperometric biosensor is based on the reaction sequence outlined in Figure 5. Glycerol dehydrogenase catalyzes the oxidation of glycerol to dihydroxyacetone in the presence of nicotinamide adenine dinucleotide (NAD^+). The NADH enzymatically produced is amperometrically detected through its reoxidation to NAD^+ by the catalytic system previously described.

The enzyme glycerol dehydrogenase and the cofactor NAD^+ were dispersed into a carbon paste matrix, as described in the Experimental Section. Since NAD^+ has to act not only as cofactor but also as precursor of the NADH electrocatalytic system, a cofactor proportion in the carbon paste of 10% was chosen. Since the response of the system also depends on the amount of the immobilized enzyme and high biocatalytic activity is needed in order to guarantee high sensitivity and stability, 7% of the enzyme was used in all the experiments. Higher proportions of modifiers cannot be employed, to prevent electrode surface breakup.

The immobilization of enzyme and cofactor dispersed in the carbon paste is achieved by covering the modified carbon paste electrode with a nonconducting PPD film, electropolymerized as described in the Experimental section. Electropolymerization of *o*-phenylenediamine on carbon paste electrodes leads to PPD films with different electrochemical and conducting properties depending on the electrolysis conditions used for its preparation.[14,52−54] When the pH of the electropolymerization medium is higher than 7, electroinactive films, used to prevent interference and electrode fouling, are formed.[14] In our experimental design, the pH of the electropolymerization media was 10, so the resulting polymer is electroinactive and prevents the diffusion of the modifiers to bulk solution.

The generation of the electrocatalytic system was achieved by oxidation of a fraction of the immobilized NAD^+. This stage was reached by application of a double potential step. The pulse high must be such that the NAD^+ is oxidized (the end potential must be higher than +1.2 V) but surface quinone groups are not generated, as was previously indicated. To optimize this parameter, electrodes without modifiers were constructed and covered with PPD. During the oxidative pretreatment all the double potential steps start at +0.15 V, and different pulse amplitudes were studied. It was observed that if the pulse end potential is lower than 1.5 V no significant amount of *o*-quinone groups are generated on the electrode surface and then no appreciable NADH oxidation currents at low applied potentials are observed. The oxidation pretreatment selected was the application of a double potential step to an end potential of 1.25 V which is enough to oxidize NAD^+ and does not generate any significant amount of surface quinone groups. The effect of pulse widths on the following amperometric response to NADH and glycerol at an applied potential of +0.15 V was determined. The amperometric response to NADH increases linearly with the pulse width, whereas the amperometric response to glycerol increases with the time of electrolysis at 1.25 V until 60 s and decreases for wider pulses. This can be due to the fact that in the biosensor design NAD^+ acts not only as precursor of the NADH electrocatalytic system but also as cofactor of the enzyme. Since longer pulse widths produce higher amounts of the electrocatalytic material but also a decrease in the amount of the enzymatically active cofactor immobilized on the electrode surface, a compromise between both effects was taken by selecting electrolysis times no longer than 60 s.

The activity of the immobilized enzyme is strongly influenced by the pH of the working buffer. A set of different modified electrodes was prepared and the current obtained for a glycerol concentration of 1.0×10^{-4} M at +0.15 V in phosphate solutions in the pH range 7−11 was measured. A maximum response was found at pH 10, whereas at pH 11 a decreased response and an increased background are obtained. These results are in accordance with the previously reported optimum pH for this enzyme in solution.[55] It is known that potassium and ammonium function as activators of the enzyme, whereas sodium acts as an inhibitor. To control these effects, a phosphate solution containing 30 mM of each ion was used for further measurements.

The effect of the detection potential on the biosensor sensitivity was examined for applied potentials varying between −100 and +700 mV and a glycerol concentration of 5.0×10^{-5} M. The hydrodynamic voltammetric curve plotted in Figure 6 shows that current increases with the applied potential, reaching a plateau between +50 and +250 mV. At potentials higher than +300 mV, a new increase of current is observed due to the direct uncatalyzed oxidation of the NADH enzymatically generated. The potential selected for further investigations was +150 mV, although amperometric signals useful for glycerol detection can be obtained at applied potentials as low as 0 V.

The amperometric response of the modified carbon paste electrodes, using the optimum conditions, to increasing concentrations of glycerol in stirred solutions was investigated. Figure 7 shows amperograms of the background electrolyte to which glycerol has been added stepwise from 1.00×10^{-5} to 3.68×10^{-4} M. The increase of the amperometric current corresponding to 3.98×10^{-6} M was 0.8 nA. A linear response to glycerol concentration from 9.97×10^{-7} to 1.0×10^{-4} M was found ($i/\text{nA} = 1.62 \times 10^5$ [glycerol]/M + 0.47, $r = 0.9991$, $n = 10$) with a detection limit of 4.3×10^{-7} M.

(52) Barbero, C.; Silber, J. J.; Sereno, L. *J. Electroanal. Chem.* **1989**, *26*, 333.
(53) Oyama, N.; Ohsaka, T. *Synth. Met.* **1987**, *18*, 375.
(54) Emr, S. A.; Yacynych, A. M. *Electroanalysis*, **1995**, *7*, 913.
(55) White, J. S.; White, D. C. *Source book of Enzymes*; CRC Press: Boca Raton, FL, 1997.

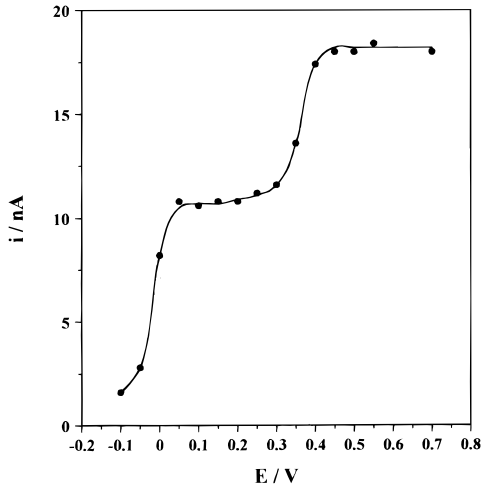


Figure 6. Hydrodynamic voltammogram for a 10% NAD^+−7% GlDH-modified carbon paste electrode covered by PPD and preoxidized. Background electrolyte: 0.1 M phosphate pH 10. Glycerol concentration: 5.0×10^{-5} M.

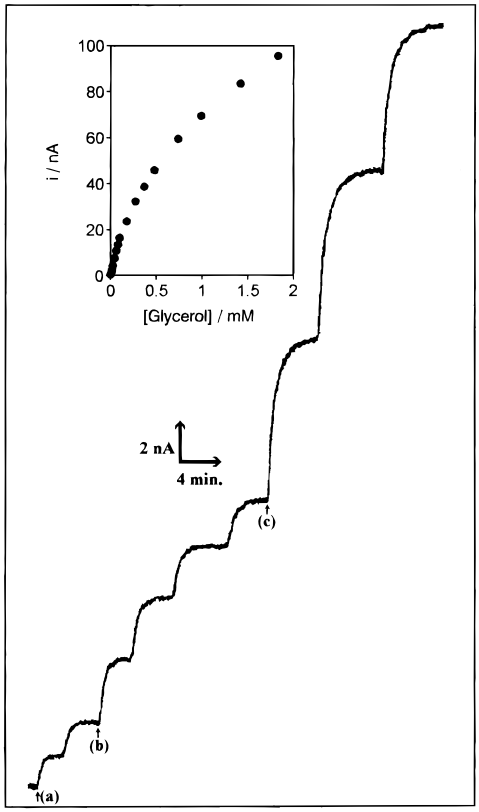


Figure 7. Current−time recordings obtained with the glycerol biosensor for successive injections of glycerol (a) 1×10^{-5} M, (b) 2×10^{-5} M, and (c) 1×10^{-4} M. Applied potential: 0.15 V. Background electrolyte: 0.1 M phosphate, pH 10.

Reproducibility between different electrodes, prepared equally from the same batch of modified carbon paste, was evaluated for a set of seven electrodes. The mean plateau current obtained for a glycerol concentration of 8×10^{-5} M at an applied potential of +150 mV was 14.1 nA, with a relative standard deviation of 2.9%. The response time for 95% of the steady-state current was 75 s.

The useful life of this sensor is at least 3 days. The signal is steady during 2 days, whereas the third day a decrease of about 20% of the initial response is observed. The modified carbon paste was stable at least 1 month under storage conditions protected from moisture and at room temperature. The complete manipulation needed for the construction of the sensor takes no more than 25 min.

Selectivity of the response depends on both enzymatic selectivity and electrochemical detection. Glycerol dehydrogenase suffers from lack of selectivity. Moreover, other species that could be present in the sample may be oxidized at the working electrode potential. The amperometric response of the proposed biosensor to different species was investigated, varying their concentrations from 9.95×10^{-5} M to 9.06×10^{-4} M. There was no interference from alanine, glucose, and triethylene glycol. Citric, tartaric, and malic acids produced amperometric signals at a concentration of 5×10^{-5} M lower than 5% of the corresponding one recorded for glycerol at the same concentration level. Significant interference was observed for 1,2-ethanediol and 1,2-propanediol (equal signal to the corresponding one for glycerol) and ascorbic acid (twice the signal for glycerol).

The biosensor was used for the determination of glycerol in a plant-extract syrup, as indicated in the Experimental Section. The diluted samples yield well-defined amperometric responses similar to those of glycerol standards. A good correlation was found between the results from the biosensor (0.65 ± 0.02 M, $n = 10$) and those from the enzymatic spectrophotometric method (0.64 ± 0.03, $n = 3$).

CONCLUSIONS

A new electrocatalytic system for NADH oxidation, obtained after oxidation of NAD^+ at potentials above 1.2 V, was found. A similar system could be obtained after oxidation of other adenine derivatives such as adenosine, adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate. The oxidation of the adenine group in these molecules gives rise to products that strongly adsorb on the electrode surface. Under the operation conditions, one of the products formed during the adenine oxidation exhibits a high catalytic activity toward NADH oxidation. The electrocatalytic response of carbon paste electrodes modified with these oxidation products shows a linear dependency on the NADH concentration, with a limit of detection of 0.4 μM. The rate constant of the catalytic process, determined by cyclic and RDE voltammetry, is comparable with those previously reported for the faster electron-transfer mediators for NADH oxidation.

The application of the new catalyst described to biosensor development has been demonstrated by the construction of a very simple glycerol biosensor in which NAD^+ acts not only as cofactor but also as precursor of the electron-transfer mediator for NADH oxidation. The proposed biosensor allows fast measurement of glycerol with low cost and rapid preparation, which recommended it to other practical applications (assay of foods, biological samples, pharmaceutical products, etc.). It proved to be suitable for the amperometric detection of glycerol in a plant-extract syrup.

ACKNOWLEDGMENT

The Spanish Ministry of Education and Science, DGICYT project PB 97-1304, supported this work.

Received for review July 27, 1999. Accepted November 4, 1999.

AC9908344