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Fundamental Review

Catalytic Electrooxidation of NADH for Dehydrogenase Amperometric Biosensors

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Abstract. The developments in the techniques of NADH catalytic oxidation relevant for incorporation in amperometric biosensors with dehydrogenase enzymes are reviewed with special emphasis in the years following 1990. The review stresses the direct electrocatalytic methods of NAD^+ recycling as opposed to enzymatic regeneration of the coenzyme. These developments are viewed and evaluated from a mechanistic perspective of recycling of NADH to enzymatically active NAD^+, and from the point of view of development of technologically useful reagentless dehydrogenase biosensors. An effort is made to propose a method for the standardization of evaluation of new mediating and direct coenzyme recycling schemes. A perspective is given for the requirements that have to be met for successful biosensor development incorporating dehydrogenase enzymes that open the analytical possibilities to a number of new analytes. The intrinsic limitations of the system are finally discussed and a view of the future of the field is presented.

Key words: dehydrogenase biosensors, mediators, NADH electrochemical oxidation, enzyme electrodes.

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The redox processes of the nicotinamide coenzyme have been the subject of numerous studies in the past 20 years due to its participation in the enzymatic catalysis of more than 300 dehydrogenases useful both in bioprocesses and analytical applications. Of special interest is the efficient and reversible recycling of the coenzyme so that the costs involved in the use of such enzymes can be lowered.

Setting the scope of this review, it should be first noted that, in what concerns the electrochemical oxidation of the coenzyme, NADH and NAD(P)H, will be treated as equivalent, with distinctions made when necessary. As mentioned, NAD^+ (Fig. 1) is a coenzyme ubiquitously used by more than 300 dehydrogenase enzymes following various mechanisms and containing various cofactors [1]. The specific function of the coenzyme depends on the mechanism of the dehydrogenase, and ranges from the "simple" hydride transfer for enzymes that contain other redox cofactors, to elaborate 3-D structure-function interactions for others [2] and it is not the subject of the present review. As mentioned, part of the high interest in NAD^+/NADH is due to the potential use of dehydrogenases in bioreactors for stereospecific

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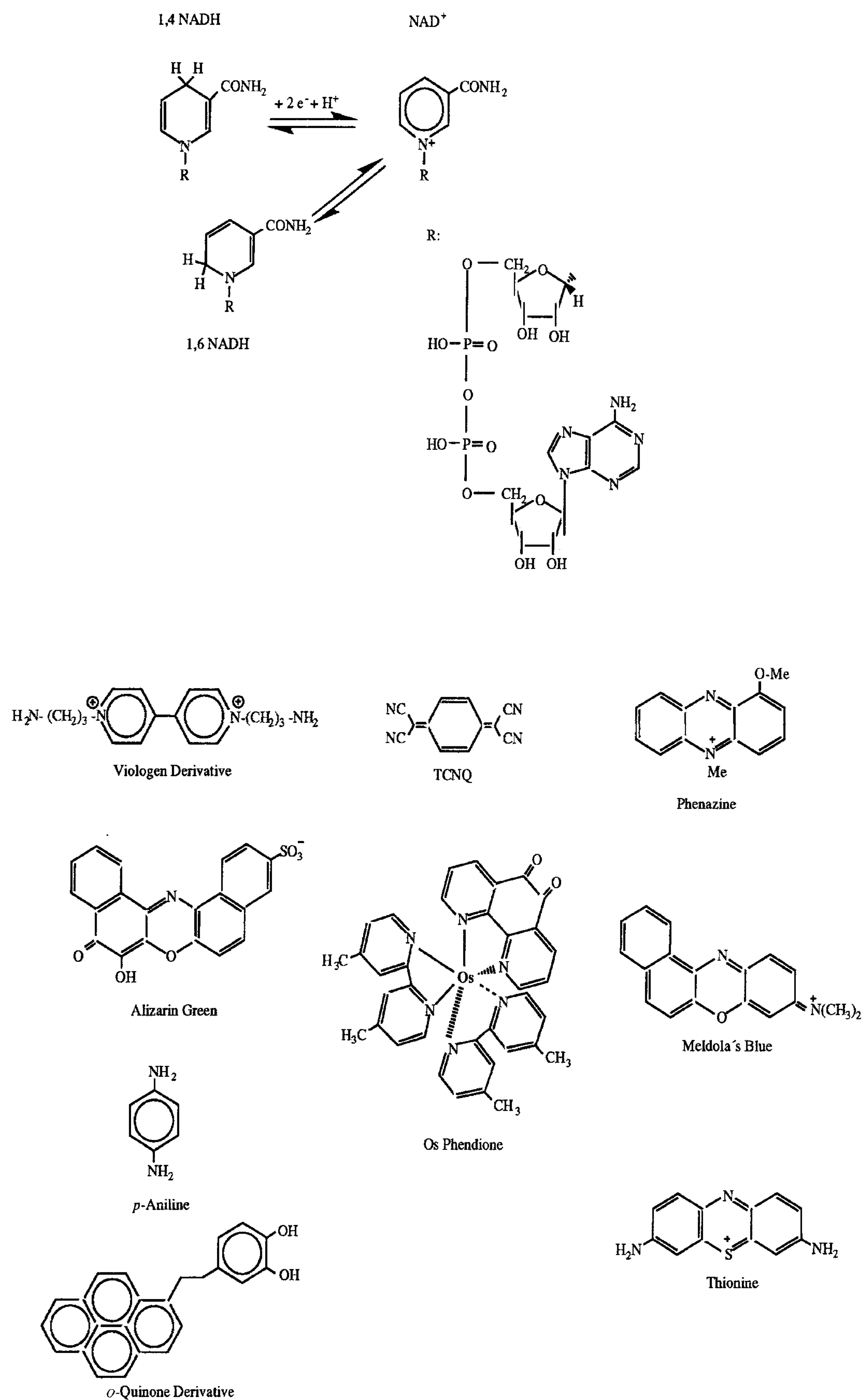
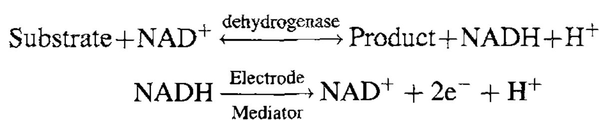


Fig. 1. Structure and redox transformations of NAD^+, and structures of main types of mediators mentioned in this work

synthesis, drug production, etc. [3–5]. Such applications are not the subject of this review either, although at various instances the rich experience from this field will be drawn upon to suggest various alternative systems for NADH oxidation useful in biosensor design, and to elucidate its mechanism. In the late 70's and early 80's an additional interest was shown in NAD^+ modification and immobilization due to its significance in affinity chromatography [6, 7]. The experience gained about its chemical properties in this period, also acted as the incubator for the use of these cofactors in biosensors.

In the 80's with the significant increase in the interest in biosensors, especially in electrochemical ones (devices that use a material of biological origin as a biorecognition element and transform its biorecognition reaction to an electrical signal) the interest in NAD^+/NADH recycling was once more renewed. This was a mixed blessing because while involving more research workers in the study of this complicated system on one hand, it permitted, on the other, gross oversimplifications and an inflation of poorly characterized recycling systems and methods, partly due to the willingness of the scientific community to forgive oversimplifications due to the novelty of biosensor research in those years, and partly due to the justifiable relaxed criteria for NAD^+ recycling necessary for a device such as the "generic" biosensor, without a specific end use and specifications to meet. It is not surprising that the most lasting works appearing during these years, come from groups with an electrochemical or electroanalytical background. In the late 70's and 80's therefore appear the most lasting and cited works in the NAD^+/NADH recycling literature, be it in bioreactor design or biosensor development [8–15].

Today biosensors have evolved beyond the embryonic stage of the "novelty" and both from a fundamental point of view and from a technological application one (incorporation in the technoeconomic process) need a qualitative jump that will permit their incorporation in real life applications and capital generation. The process that we are referring to when talking about biosensors based on dehydrogenases, is depicted in Scheme I and Fig. 2.



$$\text{Substrate} + NAD^+ \xleftrightarrow{\text{dehydrogenase}} \text{Product} + NADH + H^+$$

$$NADH \xrightarrow[\text{Mediator}]{\text{Electrode}} NAD^+ + 2e^- + H^+$$

Scheme I

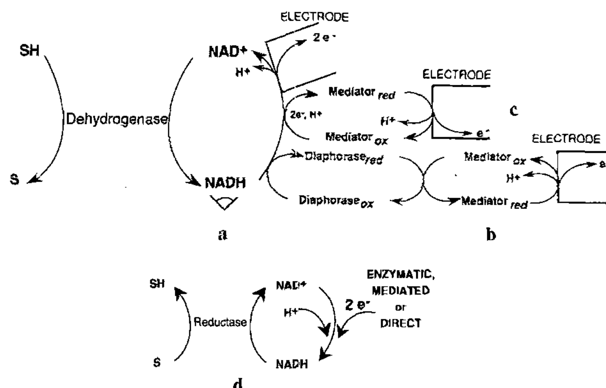


Fig. 2. Schematic diagrams of NAD^+ dehydrogenase sensors: **a** optical detection, **b** enzymatic recycling, **c** mediated electrocatalytic recycling of dehydrogenases. **d** a generic reductase sensor configuration

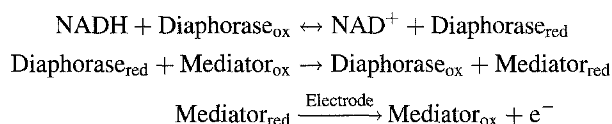
Of course, the reduction of the substrate is also possible, and this opens the question of NAD^+ reduction, which is not the subject of this review, but the interested reader is referred to some works treating the subject [16–22]. A dehydrogenase oxidizes a substrate, while at the same time reduces NAD^+, in a generally accepted hydride transfer reaction. The generated NADH can be directly detected optically by its fluorescence, and most commonly by its absorbance at 340 nm. Such detection is the basis of numerous enzymatic assays and enzymatic analyte determination procedures in solution. NADH being a reductant, its formation can cause color changes of oxidants, which is the basis of some commercialized strip-tests. In such tests, in general, the fate of NADH is not important, and neither is its recycling.

There are various advantages however from an analytical point of view to the reversible recycling of NADH in such a scheme. (We will use here the word "reversible" to signify chemically reversible, and the word "fast" to signify electrochemically reversible behavior). To begin with, a biosensor in order to fully exploit the advantages that can be conferred over alternative analytical methods, has to be reagentless; a biosensor using NAD^+, more so, because of the high costs of the coenzyme. Assuming that a way has been found to immobilize NAD^+, only its reversible recycling can guarantee the reagentless aspect of the biosensor. Secondly, a reversible recycling of NADH, permits the shifting of the equilibrium in Scheme I, a fact that permits analytical devices with higher sensitivity and lower detection limits. Thirdly, with appropriate schemes, signal amplification is possible

with the same results, and fourthly, lifetimes of the sensors are increased.

In amperometric biosensors therefore, the oxidation of NADH can be used both for signal transduction and for NADH recycling to active NAD^+. The redox potential of the $NAD^+/NADH$ couple depends on pH and the type of solvent, but it is generally accepted to be −0.56 V vs. SCE at pH 7.0. The electrochemical oxidation of NADH at an electrode surface can be achieved at potentials between 0.5 and 0.7 V at this pH [8, 22–24], which means an activation overpotential of between 1.1 and 1.3 V. Although there is some controversy on whether the products of this electrooxidation contain enzymatically active NAD^+, today it is generally accepted that they do although it is still debated to what extend. Even so, the electrochemical detection and recycling at these potentials has little analytical utility because various substances could be electrooxidized directly on the electrode surface at these potentials, defeating the purpose of using an enzyme to impart selectivity to the biosensor.

The goal therefore for biosensor development (and to a large extent for the other applications as well) is to diminish this overpotential for reversible and fast NADH recycling. There have been two approaches to achieve this decrease of overpotential. In one, the enzymatic recycling of NADH is opted for, as depicted in Scheme II (see also Fig. 2b).

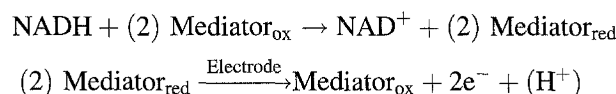


$$\text{NADH} + \text{Diaphorase}_{ox} \leftrightarrow \text{NAD}^+ + \text{Diaphorase}_{red}$$
$$\text{Diaphorase}_{red} + \text{Mediator}_{ox} \rightarrow \text{Diaphorase}_{ox} + \text{Mediator}_{red}$$
$$\text{Mediator}_{red} \xrightarrow{\text{Electrode}} \text{Mediator}_{ox} + e^-$$

Scheme II

This intellectually challenging approach, will be discussed only briefly in this review. It should be noted however, that it presents the "natural recycling way" as the reversibility of NAD^+ formation is guaranteed by the enzymatic catalysis. Traditionally, especially for bioreactor systems, the use of a second enzyme imposed the need of a second substrate of a reductase with the subsequent product purification problems. The use of diaphorases and in recent years NADH oxidases [25–29], has blown new life in such approaches and especially for biosensors, approaches such as that depicted in Scheme II seem promising for the close future when the availability of these enzymes becomes more widespread and of low cost and their stability higher.

The central subject of this review is the approach to decrease the NADH oxidation overpotential according to the strategy depicted in Scheme III and Fig. 2c.



$$\text{NADH} + (2)\ \text{Mediator}_{ox} \rightarrow \text{NAD}^+ + (2)\ \text{Mediator}_{red}$$
$$(2)\ \text{Mediator}_{red} \xrightarrow{\text{Electrode}} \text{Mediator}_{ox} + 2e^- + (\text{H}^+)$$

Scheme III

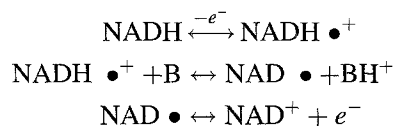
According to this strategy, an electrochemical catalyst is generated or incorporated on an electrode surface permitting the achievement of reversible NADH oxidation at lower overpotentials. This approach is of importance both for the development of electrochemical bioreactors and for that of biosensors. The criteria for the design of these electrochemical catalysts have been approached satisfactorily in the past [30] and our purpose here is not to repeat, but only to critically add the accumulated experience in the last six years in this area.

In doing so, we underline the need to standardize the development and evaluation process of these mediators, as an aid to further discoveries. We start with an overview of the mechanism of heterogeneous electrochemical oxidation of NADH, and continue with a review of the major developments on such materials in these years evaluating the results from the mechanistic point of view. In continuation, we examine some of the electrocatalysis schemes that resulted in reagentless dehydrogenase electrodes within the frame that practically useful biosensors have to meet. We finally suggest an easy-to-follow protocol for the evaluation of new electrocatalysis schemes with special emphasis on the goal to achieve technologically viable biosensors.

Mechanism of Electrochemical Oxidation of NADH

a. Electrooxidation on Electrode Surfaces

Studies on the direct electrochemical oxidation of NADH on electrodes conducted in the 70's and beyond [8–11, 31–33], suggest the following reaction scheme for the oxidation of NADH:



$$\text{NADH} \overset{-e^-}{\longleftrightarrow} \text{NADH}\bullet^+$$
$$\text{NADH}\bullet^+ + \text{B} \leftrightarrow \text{NAD}\bullet + \text{BH}^+$$
$$\text{NAD}\bullet \leftrightarrow \text{NAD}^+ + e^-$$

Scheme IV

where ***B*** is a base. A disproportionation reaction is also possible homogeneously:

$$NAD\bullet + NADH\bullet^{+} \rightarrow NAD^{+} + NADH \quad \text{Reaction 1}$$

A controversy still exists about the rate determining step in this sequence. We think that there is sufficient evidence [32, 34, 35] to suggest that the rate limiting process is the protonation-deprotonation equilibrium of the cation radical. The processes are further complicated due to the adsorption/desorption equilibria of reactants, products and intermediates as discussed below.

Enzymatically, this reaction is generally carried out by reductases in a manner where the radical intermediate is stabilized and an effective hydride transfer occurs fast and reversibly [36–38]. As mentioned, this is not the case with the electrochemical catalysis during which the intermediate radicals formed are of sufficient reactivity to give rise to parallel reactions as the disproportionation depicted in Reaction 1. Usual techniques used to verify this mechanism include electrochemical studies in organic or high pH solvents and spectroscopic or spectroelectrochemical observations with native NADH or its synthetic analogs [9–15, 35, 39–42].

The result of this direct electrooxidation is generally thought to be enzymatically active NAD^{+} [22, 43]. However, fouling of electrodes, activated or not, is observed with time especially when the NADH concentration is higher than 0.5 mM. One reason for this can be the formation of NAD• dimers that are also observed during NAD^{+} reduction on various electrode surfaces [44]. The second possible reason is the formation of stable adducts between surface species and reaction radical intermediates [12]. Other possible side reactions, such as the disproportionation reaction 1, are dependent on the concentration of $NADH\bullet^{+}$. Fast cyclic voltammetry on ultramicroelectrodes [35] suggests that the way to avoid them is to diminish the concentrations of the reactive species in the diffusion layer. On the other hand, the less than 100% yields observed, are attributed to the hydrolysis of the coenzyme that is substantial in aqueous solutions, especially at low pH. We have reported [45] that hydrolysis of the coenzyme plays indeed a very significant role, calculating half life-times of NADH in solution at pH 6.0 of about 9 hours, while other works [7, 22, 46] have calculated it to be 17 hours at pH 7.5. From this data it seems that eventually hydrolysis of NADH (NAD^{+} shows significantly longer half life times on the order of several days) may be the limiting factor for the development of technologically significant biosensors intending coenzyme recycling if no care is taken for its stabilization.

In conclusion it can be said that despite uncertainties that still exist, the mechanism shown in Scheme IV implies a highly activated step for the formation of the radical cation, the subsequent deprotonation being very favorable at most technologically relevant pHs (3–8) due to the low pK of the radical cation, but still the rate limiting step. The second electron transfer is presumably very fast but the existence of the NAD• radical especially in its adsorbed state is the reason for most of the electrode fouling reactions. From the point of view of biosensor development, the mediators used to catalyze NADH oxidation should first lower the activation overpotential while at the same time stabilizing the radical intermediates to avoid side reactions.

b. Mediated Electrooxidation

The first experiments that suggested that electrocatalytic oxidation of NADH was possible were those conducted by Blaedel and Jenkins [15] who showed that pretreatment of electrodes, especially carbon ones, could lower the overpotential of NADH oxidation. This ability was attributed to the formation of quinoid (equivalent to *o*-quinones) or oxygen containing derivatives on the pretreated surface. As a consequence, several ortho quinones and benzoquinones were used to effect NADH oxidation (see Fig. 3) followed by phenoxazine, phenothiazine, and phenazine dyes. These works were summarized by Gorton and his collaborators on several occasions [8, 30, 47] and by Bartlett et al. [48, 49]. The mechanism of NADH oxidation by these mediators has been studied both in solution and with the mediator immobilized on an electrode surface [8, 12, 13, 30, 42, 50–57].

In the case of the redox dyes kinetic and physical evidence [8, 30, 50, 55, 56, 58] suggest the existence of a mediator-NADH complex that is probably the rate limiting step of the reaction. Scheme V summarizes the mechanism with the possible side reactions that they have proposed to explain the pH-dependent behavior of such catalytic systems.

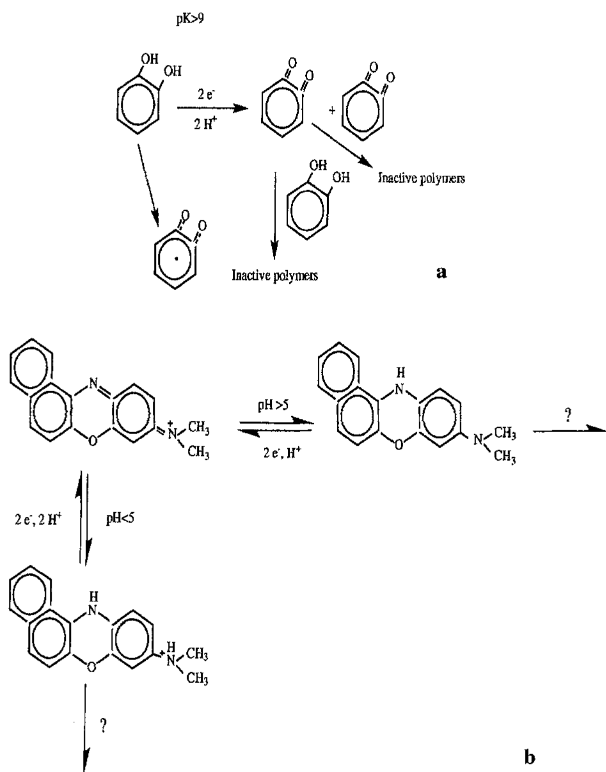
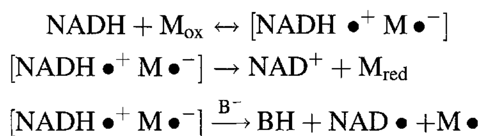


Fig. 3. Possible side reactions during heterogeneous electron transfer of **a** quinoid and **b** polyaromatic mediators used for NADH electrocatalytic oxidation



$$\mathrm{NADH} + \mathrm{M_{ox}} \leftrightarrow [\mathrm{NADH}\bullet^{+}\,\mathrm{M}\bullet^{-}]$$
$$[\mathrm{NADH}\bullet^{+}\,\mathrm{M}\bullet^{-}] \rightarrow \mathrm{NAD^{+}} + \mathrm{M_{red}}$$
$$[\mathrm{NADH}\bullet^{+}\,\mathrm{M}\bullet^{-}] \xrightarrow{B^{-}} \mathrm{BH} + \mathrm{NAD}\bullet + \mathrm{M}\bullet$$

Scheme V

where $\boldsymbol{B}$ is a base, and $\boldsymbol{M}$ the mediator, and the third of these reactions, occurring mostly at high pH, could lead to further side reactions and electrode fouling.

Although the intermolecular complex theory appears to be today generally accepted for NADH oxidation by a variety of fused polyaromatic redox dyes, studies (that involve kinetic isotope effects and acid-base catalysis in addition to electrochemistry) of the reaction of NADH with ortho and para quinones (*Q*) and to some extend with aromatic diamines (see Fig. 1) [12, 13, 37, 42, 51, 53, 54, 59] show that with these oxidants the reaction proceeds through a net hydride transfer although the exact mechanism is most closely represented by Scheme VI.

$$\mathrm{NADH} + \mathrm{Q} \rightarrow [\mathrm{NAD}\bullet\mathrm{QH}\bullet]$$
$$[\mathrm{NAD}\bullet\mathrm{QH}\bullet] \rightarrow \mathrm{NAD^{+}} + \mathrm{QH^{-}}$$
$$\mathrm{QH^{-}} + \mathrm{H^{+}} \rightarrow \mathrm{QH_2}$$

Scheme VI

In this scheme a highly stabilized transition state complex appears to be forming, a fact that gives rise to relatively high reaction rate constants between NADH and quinoid compounds. The inconvenience of this sequence is that a stable side product seems to be forming as described by Reaction 2 which leads to catalyst poisoning.

$$[\mathrm{NAD}\bullet\mathrm{QH}\bullet] \rightarrow \mathrm{NAD{:}QH} \qquad \text{Reaction 2}$$

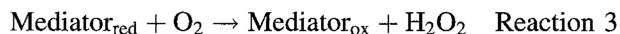
An inconvenience of the schemes presented so far is that the redox potentials of the mediators are pH-dependent. A case where at least the mediator electrochemistry is not pH-dependent, is the use of one-electron mediators (most notably metal complexes) to effect NADH oxidation. There have been various reports [42, 57, 60, 61] on such mediators. The general mechanism of NADH oxidation by such mediators is believed to follow the ECE mechanism of the direct electrode oxidation of the coenzyme. This fact, may give rise to electrode fouling reactions as is the case in the direct electroxidation. It is curious that such mediators should exhibit catalytic properties since they do not seem to form any intermediate stabilizing complex with NADH and they do not possess the ability of proton abstraction.

An interesting novelty that might have technological significance, is the use of polyoxometalates to effect the reaction [62, 63]. A recent report [63] suggests that two one-electron steps are taking place with these compounds as well, with an intervening proton transfer to the solvent, the rate limiting step being the first electron transfer. The instability of these compounds at high pH, and the problem of incorporating them on an electrode surface will have to be solved in order for them to have further impact on NADH biosensor development. Finally, the well known vanadyl-mediated NADH oxidation appears to have little technological significance at least in biosensor applications.

Although the mechanism of NADH oxidation by these mediators has been treated with relative detail, the regeneration of the mediators on electrodes and the reversibility (chemical and electrochemical) of this reaction has not been so extensively studied.

However, it could be a source of various artifacts and additional side reactions that might eventually limit the efficiency and lifetime of the possible NADH sensors and subsequent dehydrogenase biosensors. Compiling various data and observations from the literature we have constructed Fig. 3 that shows the possible mechanisms for the reoxidation of these mediators. It should be noted, that most of these reactions are also pH-dependent and are supposed to be $2e^-$, $(2)H^+$ transfers. At least for the redox dyes, no deterioration of their electrochemical behavior has been reported with time, while for the quinoid mediators the surface-catalyzed side reactions are summarized in Fig. 3a.

A reaction competing with the reoxidation of mediators at electrode surfaces is usually ignored in the relevant literature, but is well known to take place, and it is their reoxidation by dioxygen with the simultaneous production of H_2O_2 which can also be used for NADH detection (Fig. 4).



$$\text{Mediator}_{red} + O_2 \rightarrow \text{Mediator}_{ox} + H_2O_2 \quad \text{Reaction 3}$$

This reaction can pose a serious problem both because it introduces an oxidizing agent close to the surface, and because it makes the NADH oxidation schemes O_2 partial pressure-dependent.

A strategy that we have been following recently [45] and was first demonstrated by Abruña [64], might present a solution to side reaction problems especially those related to *o*-quinones: The complexation of an *o*-quinoid structure with a transition metal (Fig. 1), yields *o*-quinones with higher chemical reversibility in alkaline solvents. This fact, may relieve the electrode fouling problems present with such compounds and may be an interesting alternative for the future of NADH oxidation systems. With such compounds, the mechanism of NADH oxidation is believed to be the same as mentioned before for quinones, but the greater delocalization of the possible radical electrons, seems to be conferring higher stability to them. The work on these compounds is in very preliminary stages.

From this brief study of the mechanism of NADH oxidation, the following conclusions can be drawn:

1. Direct electrochemical oxidation of NADH on electrode surfaces suffers from high overpotentials and uncontrolled side reactions due to the obligatory ECE mechanism.
2. Mediating schemes exploit the ability of certain oxidants to facilitate hydride transfer from NADH to them through charge transfer complex formation. In this respect it is curious that one-electron mediators with no H^+ abstraction capacity have also shown NADH electrooxidation catalysis.
3. From the available mediating schemes it appears that the extended aromatic ring structures (commonly called redox dyes) provide a stable and technologically viable method to construct NADH oxidizing, chemically modified electrodes providing redox stability not only towards NADH oxidation, but also towards the heterogeneous electron transfer to electrodes. The comparison is favorable when made to unsubstituted quinones that although they demonstrate higher reaction rate constants with NADH, they suffer from more side reactions that can be forbidding for viable technological applications of biosensors.
4. The need is detected to study the mechanism of NADH oxidation in a technologically relevant manner. In this effort, fast scan voltammetry at ultramicroelectrodes and spectroelectrochemical techniques seem to have an important role to play in the future.

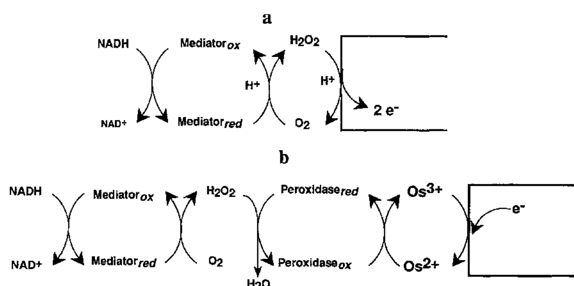


Fig. 4. Dioxygen dependent oxidation competing with the reoxidation of NADH mediators. **a** Schematic of the reaction, **b** NADH sensor constructed based on this principle [107]

Evaluation of NADH Oxidation Schemes

In Table 1 we present the major NADH oxidation mediating schemes on chemically modified electrodes (CMEs) that have been up until now developed. We follow the evaluation scheme followed most commonly in the literature which consists of reporting the reaction rate constants for oxidation of NADH, and the operating potential for said oxidation (usually the overpotential decrease is obtained if this number is substracted from 0.7 V vs SCE that is the average potential for the direct oxidation of NADH). The

Table 1. Electrochemical NADH oxidation configurations

Mediating scheme	E_w V/ref	Immobilization	Rate constant ($M^{-1}s^{-1}$)	Sensitivity $\mu A\ mM^{-1}cm^{-2}$	Stability	Ref.
Phenazine/phenoxazine	0/SCE	composite (graphite-epoxy)	–	–	>60h (FIA)	70
Quinones	0.2/SCE	electropolymerization	–	–	2 h (FIA)	70
TCNQ	0.38/SCE	dip-coating	1.46×10^6	–	–	71
TCNQ	0.4/SCE	electrodeposition	4.8×10^6	230 (SS)	–	72
$Co - FeCN_6$	0.5/SCE	electrodeposition	3.5×10^3	–	–	73
NMP^+	0.01/Ag/AgCl	Reineckates	3.1×10^3	36.4 (SS)	17 days	74
TBO	0.15/Ag/AgCl	carbodiimide	–	–	unstable	75
3-β-naphthoyl TBO	0/Ag/AgCl	adsorption	1.4×10^4	208 (FIA)	–	55
Quinone	0.05/SCE	adsorption	–	115 (CV)	–	76
Meldola Blue polymer	0/SCE	adsorption	–	0.04 (SS)	–	77
3,4-dihydroxybenzaldehyde	0.3/Ag/AgCl	electropolymerization	–	4 (FIA)	> 1 day	85
PQQ	0/SCE	carbodiimide	4.6×10^3	0.32 (SS)	14 h (FIA)	86
TBO	0.2/Ag/AgCl	covalent	–	41 (SS)	–	83
Meldola Blue	0/SCE	adsorption (precondition)	–	300 (RDE)	–	89
Poly (methylene Blue)	0.2/SCE	electropolymerization	–	1 (SS)	120 h	82
Poly (thionine)	0.1/Ag/AgCl	electropolymerization	–	0.42 (FIA)	4 h (FIA)	81
3-β-naphthoyl-Nile Blue	0/SCE	adsorption	5×10^4	214	–	56
TBO Polymer	0.2/SCE	carbon paste	–	150 (FIA)	> 10 days	78
Methylene Green	0.2/SCE	carbon paste	–	22 (FIA)	–	90
Indole-5-carboxylic acid	0.45/SCE	electropolymerization	–	–	–	67
OsPhendione	0.15/Ag/AgCl	carbon paste	–	140	> 1 day	45
Activated	0.145/SCE	gold, thick film	–	–	> 70h (FIA)	93
Activated: (1.2 V, NaOH)	0.8/SCE	glassy carbon	–	–	days (LC)	66
Activated: (sine wave)	0.5/Ag/AgCl	carbon fiber	–	–	1000s of scans	69
Enzymatic: LDH/LOx, H_2O_2 transduction	0.65/SCE	immunodyne membrane	–	0.283	12 days (storage)	98
Enzymatic: diaphorase, Fc-methanol transduction	0.1/SCE	polypyrrole	–	100	17 h(SS)	105
Enzymatic: NADH oxidase, $RuCl_6^{2-}$	0.15/SCE	crosslinking, mediators in solution	–	5000	> 1 wk (storage)	103
Enzymatic: diaphorase	0.3/SCE	no immobilisation	–	–	–	100
Enzymatic: diaphorase, Fc-transduction	0.2/SCE	glutaraldehyde	–	350	–	99
Enzymatic: peroxidase, $Os(bpy)_2Cl(PVP)$ transduction	0/SCE	"wired" peroxidase	–	1000	–	107

Abbreviations: LOx: Lactate oxidase, LDH: Lactate dehydrogenase, TBO: Toluidine Blue, OsPhendione: Os(4,4′-dimethyl, 2,2′-bipyridine)$_2$(1, 10-phenanthroline, 5, 6-dione)$_2$, NMP: N-methylphenazinium, TCNQ: Tetracyanoquinodimethanide, FIA: Flow Injection Analysis, SS: Steady State, CV: Cyclic Voltammetry, LC: Liquid Chromatography, RDE: Rotating Disc Electrode.

stability data whenever available are also included. For works before 1990 the reader is referred to the very comprehensive works by Bartlett et al. [48] and Gorton [8].

When mediators are used, a piece of data that is missing in many works is the reaction rate constant for the heterogeneous electron transfer between the mediator and the electrode. As we mentioned, this may be important from many respects. It is also noted that the stability data are under varied conditions, and in most cases not well documented in the works mentioned. We note that the criteria for a successful mediating scheme are the following:

1. Possibility to immobilize the mediator on an electrode surface

For a technologically useful configuration, the mediator should be possible to be immobilized on an electrode surface yielding a chemically modified surface. Various configurations have appeared in the last few years as we mention later, where mediators and biological molecules are entrapped in an "epoxy" or "biocomposite" electrode or a carbon paste configuration. This technique solves the immobilization problem, but even in this case, leaching of the mediator should be minimal. Most enzyme electrodes

today at the commercial level, are produced by thick film technology, and these configurations are much more amenable to this technology (screen printing) than surface modification techniques. However, for the detailed study of the stability of the mediator, and for the determination of the kinetics of the catalyzed reactions and the heterogeneous electron transfer, surface-immobilized species are much more useful. Furthermore, the work on biosensors, and the impetus that it provides in bioelectrochemistry, should be viewed as a possibility to expand the accumulated experience in the field of bioelectronics. And for this, whatever transduction configuration should be able to take advantage of the latest feature size discrimination levels in microlithography. Therefore, ideally, the mediators should not only be possible to be immobilized, but also selectively so. Of course, for biosensor manufacturing, the ability to remain in a carbon paste or epoxy-type of material is more important.

2. *Stability of the mediator under the operating conditions, in solution and under cycling*

With stability of the mediator, we are referring to its chemical stability which includes resistance to hydrolysis, photolysis, chemical oxidation, nucleophilic or electrophilic attacks, and complex ligand replacement in the case of metal complexes, all under the operating conditions which should in principle be aqueous solvents between pH 4 and 8. It is tempting to assume that stability longer than that of NADH or NAD^+ under the operating conditions is not necessary. This in principle is not true. First, the goal in biosensor development should be to develop robust sensors with stabilities longer than the enzyme or coenzyme lifetimes, and even custom made for a particular application. This means, that if necessary, a biosensor should in principle be able to meet month or year stability requirements, at least under storage, without problem. Secondly, although the half lifetimes of NAD^+ and NADH at pH 7 have been calculated to be about 15 days and 13 hours respectively, these limits are by no means the upper limits of required mediator stability. On one hand, there are applications where continuous NAD^+ injection in a carrier system is an acceptable strategy, and on the other, very easily in carbon paste-like configurations a "pool" of excess NAD^+ can be created. A pool of excess mediator cannot be so easily accomplished because of the kinetic requirements of the system: A successful system should regenerate NAD^+ faster than it can be consumed by the enzyme, to appropriately shift the dehydrogenase equilibrium towards the product side (Scheme I) so that high sensitivities, low limits of detection and high current densities can be obtained. Therefore, the mediator cannot afford to live on "credit" as easily as the NAD^+ even if "pools" of reserve mediator can be formed. This problem has been repeatedly noted in the literature but only the group of Gorton has addressed it specifically with molecular engineering of their preferred types of dyes [30] effecting modifications that stabilize the mediators under slightly alkaline conditions where most of the dehydrogenase equilibria are favorably shifted to the product side. Another effect that can be possible especially in carbon paste configurations, is that in the hydrophobic environment of the paste, the NAD^+ may be additionally stabilized against hydrolysis reactions, a fact that imposes even higher stability requirements on the mediator so that the electrode not be limited by the mediator stability. Even so, mediator stability data are sparse as can be appreciated in Table 1, and not quantitatively documented in most of the appearing works.

It should be noted, that the chemical stability of the mediator is a distinct problem from the stability of the adsorbed layer: The stability of the adsorbed layer is a problem that could in principle be solved by surface modification and incorporation of the mediator in polymers, but its chemical stability is an intrinsic molecular property that can probably only be solved by chemical modification of the molecule and molecular engineering.

3. *Fast heterogeneous electron transfer without competing and side reactions of the mediator*

Even if a mediator is chemically stable under the operating conditions, its stability under repeated redox cycling should be a matter of confirmation as noted above. It should not be forgotten that most of the dyes and quinoid compounds are characterized by $2e^-$, $(2)\,H^+$ redox processes. It is not at all obvious that these processes can occur reversibly in the chemical and electrochemical sense on electrode surfaces especially in water. The exact nature of the redox reactions on electrodes for various of these mediators is still under investigation. For example, in Fig. 3 we

depict some of the possible side reactions that might occur at the electrode surface for *o*-quinone mediators. Similar reactions could be possible for the rest of the dyes used as mediators, although the chemical irreversibility of the reaction does not manifest itself in electrochemical studies. On the other hand, the mediators, due exactly to the complicated electron-proton transfer steps, may show a quasi reversible or even irreversible cyclic voltammetric behavior, indicative of slow heterogeneous kinetics, a fact that eventually may limit the utility of such electrodes making their response limited by the heterogeneous kinetics of the mediator. This problem would of course render the electrodes useless if it is accompanied by chemical or redox cycling instability of the mediator.

The reader is also reminded that Reaction 3 is also interfering with the reoxidation of the mediator at the electrode and a fast heterogeneous electron transfer rate constant also means successful competition with this reaction so that the formation of peroxide which may give rise to additional side reactions is avoided.

4. Fast electron transfer between the mediator and NADH without side reactions for production of enzymatically active NAD^+

The stability of the mediator should also be tested under NADH catalysis conditions. Even a mediator chemically stable and stable under redox cycling, simply presents a mediator with little interactions with itself and with the solvent. This does not mean that the mediator will not interact with the intermediates of the NADH oxidation products, especially since these intermediates may contain (according to Schemes III–VI) free radicals and may require participation of solvent protons. These types of reactions are of the same nature described in Reaction 2 and they may be very difficult to detect simply with electrochemical experiments. This is especially true when these reactions occur at very low rates but may be amplified with higher NADH concentrations as mentioned.

Despite these complications, a relatively straightforward way to detect any faults in the whole redox sequence, is by using bulk electrolysis techniques of NADH with surface-modified electrodes of large area. The products of this electrolysis and their concentrations should be identified and the coulombetric integration of the current, should generally give 2F Coulombs / mol NADH electrolyzed. The identification of the products can be done by HPLC or LC methods [65, 66], and the catalytic activity of the presumed produced NAD^+ checked enzymatically. Although this is a simple method, it has very rarely been used in mediator evaluation studies. Any discrepancy between the mass and charge balances, should be further investigated. Any deactivation of the mediator should in principle be possible to be detected by simple cyclic voltammetry of the adsorbed mediator layer. A note of importance, is that all the results should be corrected for the NADH hydrolysis that is considerable in the time scales needed for the complete electrolysis of the coenzyme.

5. Suitable electrochemical potential of the mediator (between −50 and 50 mV vs. SCE) where the NADH oxidation occurs (a decrease of overpotential between 500 and 900 mV)

Finally, the decrease of the NADH oxidation overpotential by the use of a mediator usually also means the overpotential decrease for the oxidation of a number of interfering substances. For example, it has been reported [67, 68] that ascorbic acid or dopamine oxidation is also facilitated on surface activated electrodes or modified with various polyaromatic dyes, or electroactive polymers and a similar behavior is expected in the cases of almost all the mediators used in the various studies. It has correctly been mentioned [69] that when this overpotential decrease is achieved by a mediator and not by the activation of the surface of an electrode, the discrimination ability of the electrode by means of a potential scan is also lost because all the oxidation electrocatalyses occur at the same potential.

On the other hand, in our experience, oxygen reduction to (presumably) H_2O_2 (mentioned in Reaction 3) is also catalyzed in an equally drastic manner by the mediators, making itself present even at 0 m V vs. SCE. It is for this reason that we limit the useful mediator potentials between −50 and 50 mV, with actually the optimum potential at 0 because neither the ascorbic oxidation nor the O_2 reduction are appreciably activated at this potential. It is of interest to note that various mediators have been observed [67] to show higher electrocatalytic activity (and more substantial overpotential decrease) for the oxidation of interferents like ascorbate than for that of NADH.

In the light of these observations, the data included in Table 1 can be discussed more productively.

Persson [55] and Persson and Gorton [56] have employed a molecular engineering strategy to create mediators based on dyes (phenoxazines, Toluidine Blue, etc.) that exhibited high stability at alkaline pH, less effect of the rate of electrooxidation of NADH on pH, and higher oxidation rate constants. Their results show that modification of the pK and redox potential values of the mediators and avoidance of the formation of imino structures within the mediator, have these beneficial effects. The stability of the new mediators under NADH catalysis and the faster kinetics demonstrated a rational strategy to achieve improvements in mediator designs.

The same group in various collaborations continued their persistent work on fused ring polyaromatics, incorporating the dyes in various poly(siloxane), mostly insoluble in water, backbones in various publications [30, 77, 78] on the steps of the Miller approach [79]. In a recent attempt [77] to develop a model for the redox catalysis on such polymer CMEs, they found that NADH diffusion in the hydrophobic, precipitated film is slowed by 5–6 orders of magnitude, but they report a high stability of the film under repeated cycling (no quantitative data given) which is a marked improvement over similar *o*-quinone based redox films. The strategy seems to be a promising line in NADH oxidation, once the stability of these modifying polymers is investigated, and the swellability of the films by water is improved.

Another way to form stable films of mediators on electrode surfaces, is to use electropolymerization of various dyes like indole derivatives [67], thiophene [80], thionine [81], and methylene blue [82]. These films typically have a 10^{-6}–10^{-8} mol cm^{-2} surface coverage and they present more important swelling problems in aqueous solutions than the previously mentioned deposited redox polymers, or the adsorbed monolayers. These problems are reflected in longer response times, higher detection limits, and lower, in general, sensitivities for NADH detection. A positive effect is that the stability of such adsorbed layers is sometimes observed to be remarkably increased, presumably due to the protection of the film from the solvent. The stability refers both to cycling stability (although cycling in such films takes a special meaning, since the electron "diffusion" properties of such films are very poorly defined, and so is their redox behavior) and longevity of the adsorbed films. It seems to us, that although this approach can potentially lead to manufacturable biosensors (if co immobilization of dehydrogenases and NAD^+ is achieved in them) it is not the best way to go about the development and study of new catalytic materials for NADH oxidation.

In exchange, an alternative way to immobilize mediators on electrode surfaces, seems much more promising because it allows fundamental studies and provides a method for the expansion of this technology to bioelectronic microsystems. This consists of various ways to immobilize monolayers of the mediators. It has been demonstrated that this can be achieved either by covalent attachment to surface species [75, 83], or by derivatizing the surface or the mediator so that self assembled monolayers could be formed [76, 83], or by using electropolymerization of species where the electropolymerizable functionality did not interfere with the redox behavior of the electron transfer moiety [84, 85], a strategy that yielded much better characterized modified electrodes. Various types of mediators were used for these studies, dye-like molecules, *o*-quinones, or metal complexes alike. Katz et al. [86] presented an intellectually challenging work that we think should be used as a model for mediator characterization studies for NADH electrooxidation. The work was interesting because they used a PQQ (a dehydrogenase cofactor, and *o*-quinone) immobilized on an electrode surface in monolayers, and although the configuration may have little practical utility, it demonstrated that such studies can be used even as enzyme mechanism modeling systems, and for the demonstration of bioelectronic devices. From the NADH electrocatalysis point of view, a more careful characterization (and quantitative account) of the NADH bulk electrolysis products, and a spectroelectrochemical characterization of the modified electrodes are the only problems that we see in proposing this work as the model for NADH electrocatalysis studies as we try to propose in this review.

Other approaches along this line (electrochemically well characterized monolayers or multilayers) included "precipitation" on electrode surfaces of Reinecke salts of various dyes or of Co hexacyanoferrate salts [73, 74]. In general, this type of modified electrodes, showed low detection limits, well behaved electrochemistry, fast response times, and high sensitivities to NADH, while being amenable to careful mechanistic studies and valid conclusions for mediator design for NADH electrooxidation. How-

ever, their stability (both of the layers, and under cycling or continuous operation, or even storage) was generally lower than that of the electropolymerized configurations, although the Reineckates [74] showed the highest operational stability quantitatively documented in the literature.

Other configurations for NADH oxidation included some more works on the TCNQ modified electrodes [71, 72] along the lines introduced by Kulys [87] and Alberry and Bartlett [88] in the early 80's. As in previous works, an "excellent" stability of these electrodes is ascertained, but it seems doubtful that quantitative stability studies can be carried out with this configuration. As a technological solution to the construction of NADH sensors, the configuration seems in principle attractive and it has been reported [48] that no fouling reactions occur on these electrodes. Some of the highest sensitivities and bimolecular rate constants were reported with this configuration. The operating potentials are still high and an interesting point arises since TCNQ was previously thought [48] to be unreactive towards NADH oxidation.

Continuously, new mediating schemes appear for the oxidation of NADH, without necessarily being incorporated with electrochemical detection. An example mentioned above is that of polyoxometalates [62, 63], and it is worth mentioning the case of tetrazolium salts that have been used in micellar systems [91] for NADH recycling and enzyme catalysis mimicking. A hydride-transfer mechanism is discussed in this work, or at least a two-electron transfer process, which could make these materials very useful if they were stable under redox cycling on electrode surfaces, since possible side reactions due to the formation of radicals could be excluded. This could certainly be a useful tool for NAD^+/NADH cycling in bioreactors, but its utility in biosensor construction would have to be investigated further. As we mentioned earlier, we are experimenting with a new type of *o*-quinone derivatives [45] where the *o*-quinone is incorporated in a ligand of a central transition metal atom in the hope of achieving extended stability of the mediator and give it improved capacity to form stable adsorbed layers. Studies have also appeared on the determination of rate constants with one-electron redox molecules, especially metal complexes [73, 92] and attempts have been made for the immobilization of these mediators on polymeric materials. In general, these configurations showed low current densities, and their stability has not been assessed but is expected to be low due to fouling as a consequence of the obligatory ECE mechanism followed by this type of mediators. This assertion is invalidated by the results reported by Lowe [61] asserting stability of these electrodes, studies that however did not see any continuation.

Surface activation approaches have also been reported in the last few years. As mentioned earlier, as these electrodes cause electrocatalytic oxidation at lower potentials but not necessarily at those of the mediator, they permit the discrimination between different electroactive species (possible interferences). According to the discussion above about the obligatory ECE mechanism in such surfaces, one would expect electrode fouling to be a consequence of the use of such surfaces. This conclusion as mentioned has been put under question by a series of works by Bourdillon and coworkers, who assigned any low yield of enzymatically active NAD^+ to coenzyme hydrolysis. Remarkable stability and retention of the electrocatalytic activity was observed in cases where carbon fibers were electroactivated and used for NADH detection and discrimination from interferents with fast scan voltammetry [35, 69] or when even higher surface area electrodes were used in flow conditions [66]. The possibility of fast polarization and radial out-diffusion from the microelectrode surface may explain these very positive results. A development with technological importance, has been the ability to produce with thick film techniques gold electrodes that showed decreased overpotentials for NADH oxidation [93]. This approach combined with the previous results may lead to manufacturable NADH sensors.

In the field of technologically important advancements, we have to also include the carbon paste or "biocomposite" electrodes because they provide a way to immobilize the mediators. It is certainly a configuration that should not be used for basic studies or for mediator design conclusions, but in terms of providing a vehicle for producing manufacturable sensors, is certainly worth mentioning and using. The electron transfer process in carbon pastes has not been sufficiently investigated, with the exception of some recent studies [94, 95]. A pinhole mechanism has been suggested for the function of CPs, but many results seem to contradict this simplistic approach. What is certain about CP is that it is an electrochemically sluggish surface, that permits its bulk

modification. In several cases mediators have been incorporated in CP electrodes [78, 90, 45] to produce NADH and dehydrogenase substrate electrodes. The key element for such configurations to have success, is that the mediator has to have a higher affinity for the hydrophobic carbon paste phase than for the aqueous analytical matrix. This has not always been achieved, and mediator leaching could be the limiting stability factor in this case. As mentioned, the creation of a "pool" of mediator and its cycling in a hydrophobic environment, protected by usual side reactions in aqueous environment, can produce an artificial but technologically important long life of the NADH sensors.

A final word on the evaluation of mediated and direct systems for NADH oxidation, should go for the models developed, for the steady state response of such systems [96, 97]. Although in most of the cases they refer to the response of electrodes incorporating enzymes, the comment made here is still valid even when simply NADH oxidation is considered: The existing models ignore the possibility of slow (quasi reversible or irreversible) heterogeneous electron transfer. They also depreciate limitations due to slow electron "diffusion" or proton transport. These considerations are important almost always for most of the redox dyes used as NADH oxidation mediators and they become more important when the films modifying the electrodes are thicker than a few monolayers as in most technologically viable developments. These considerations should always be kept in mind when analyzing and evaluating the kinetics of mediators.

As mentioned earlier, the NADH oxidation schemes are not limited to direct, catalytic ones. Enzymatic regeneration of NAD^+ has been attempted [25, 29, 99, 106] on electrodes using a type of NADH oxidases usually grouped as diaphorases according to the scheme depicted in Fig. 2b. Although not our subject in this review, we mention as extremely interesting the isolation and cloning of several NADH dehydrogenases and oxidases in the last few years, some of which are thermostable and have already been evaluated as electrochemical sensor systems for NADH [26, 28, 98, 100–105]. The obvious presumed advantage of such systems is that they provide the most reversible possible reoxidation of NADH, maintaining thus the stability of the coenzyme for long time periods. This advantage has to be judged against the following factors: i. the complexity of the system when an enzyme is added in the configuration (usually with an equilibrium constant that has to be maintained at all times towards the side of the production of NAD^+), ii. the stability of the enzyme used as the recycling vehicle, and iii. the efficiency of the enzyme-regenerating mediating scheme. It is our view that such enzymatic systems especially when based in thermostable enzymes, can find applications in (electro)bioreactor systems in need of NAD^+ recycling and in biosensors, although much more readily in the former. However, works appearing on them are intellectually challenging and for applications where low detection limits are necessary or for coupling of bioelectronic devices, these configurations show a promising future.

A configuration that underlines the importance of the competing Reaction 3 for the reoxidation of mediators [107] by O_2, has appeared using peroxidase for the detection of the produced peroxide (see Fig. 4b). It should be noted that this configuration showed high sensitivities although its stability and reliability in the presence of interferences are questionable.

In all this, it should not be forgotten that the possibility to reduce reversibly NAD^+ from both the point of view of biosensor (when reductases are used as biorecognition elements as in Fig. 2d) and bioreactor development, and bioelectronics coupling of the coenzyme. In this respect, the electrochemically catalyzed NAD^+ reduction becomes relevant but only tangentially relates to our purposes here. As a general comment, we mention that such electrocatalytic reductions have been attempted either by activated electrode surfaces [108], or by various viologens immobilized in different ways on electrode surfaces but to our knowledge, direct and reversible production of NADH from NAD^+ has not been documented without enzymatic help [17, 20, 21, 109–111] due to the formation of dimers or the enzymatically inactive 1,6-dihydropyridine form of NADH (Fig. 1). The configuration mentioned [108] and the claimed NAD^+ reduction at neutral red [112] or alizarin green [30, 47] CMEs are not sufficiently documented to allow us to comment with confidence on the ability of such systems to produce enzymatically active NADH. It still seems that the most promising non-enzymatic NAD^+ reduction system is based on the capacity of rhodium complexes for simultaneous $2e^-$ transfer [19, 113].

From this brief glance in the electrochemical NADH oxidizing systems developed in the last few years it appears that the most technologically

significant developments that should be explored in the future are the electropolymerized systems, the polymers incorporating dyes and mediators, the carbon paste and the enzymatic configurations based on thermostable NADH oxidizing enzymes. For the former three, possible improvements should come by increasing the swellability and permeability of these configurations to the substrates permitting at the same time more facile electron and proton diffusion while maintaining the stability of the modifying layers. For the enzymatic configurations, the transduction chemistry for the enzymatic reaction should be improved. Once stability of mediators and reversibility of the NADH oxidation are assured, attention should be paid to the operating potentials of the electrodes. We note that only a handful of the entries in Table 1 operate at what we defined as the optimum potential.

Dehydrogenase Electrodes Based on NADH Oxidation Schemes

The various NADH oxidation schemes can be obviously applied in combination with a dehydrogenase enzyme to develop biosensors for the respective dehydrogenase substrates according to Scheme I and Fig. 2. The considerations mentioned in the previous section about the mediators or the creation and maintenance of the electrocatalytic surface are obviously true also when an NADH electrode is combined with a dehydrogenase to produce a biosensor for its substrate, and therefore there is not much to add in this section on these matters. We think that it is technologically mature to limit our consideration to truly reagentless biosensors. In these, a dehydrogenase should be confined on a surface with NAD^+ and the mediator or the NADH-oxidising enzyme, and as the substrate oxidation yields NADH, the mediator or the diaphorase oxidizes it, producing ideally enzymatically active NAD^+ so that continuous addition of the coenzyme in the analytical matrix is not necessary. Some of the configurations developed in recent years are summarized in Table 2 including the electrode characteristics and components. The additional factors to be taken into consideration in this section are:

1. Immobilization of the coenzyme: The coenzyme, preferably in its NAD^+ form (NADH if the sensor is based on a reductase) should be retained on the electrode surface. Since the 1970's several works have dealt with the development of macromolecular NAD^+ adducts that retained their catalytic activity and could be readily immobilized [6, 7]. This technology was thought to be incorporable to biosensor technology. Therefore, NAD^+ modified dehydrogenases and electrode surfaces could seem as a possible approach. However, the mobility necessary for the NAD^+ adduct and the high recycling rate necessary, usually prohibit the use of such schemes in real devices. The coupling of the coenzyme to sepharose [114, 115] and its inclusion in gels covering the electrode surface [25, 116] have been seen as practical solutions. Of course, the easiest solution was to bulk-modify carbon-paste like materials and this approach was quickly adopted for the majority of the studies involving NAD-dependent dehydrogenase electrodes [117–129]. The pros and cons of this configuration have been discussed above but as now the goal is to produce viable electrodes, the advantages far outweigh the disadvantages for usual biosensing applications. Obviously, when response analysis of the sensors for electrode development is needed, the configuration is not the most highly recommended one, since the application of the whatever steady-state model requires well characterized electrode surfaces.

2. Dehydrogenase equilibria: It was alluded to before, that most of the dehydrogenase enzymes react under an equilibrium (see Scheme I) and for a useful sensor with high sensitivities and low detection limits, a shift towards the product side is necessary. For this to happen, the NAD^+ concentration has to be kept the highest possible at all times and it is important for this purpose to use mediators that provide a rate for oxidation of NADH higher than the rate of its enzymatic production. The achievement of an excess of NAD^+ at all times and the fast disappearance of NADH, also solves the other usual problem of dehydrogenases, that of product and sometimes substrate inhibition. In addition, as NAD^+ is much more stable than NADH towards hydrolysis, the disappearance of NADH is essential for the stability of the sensor.

Considering a situation where the NAD^+ reaction with the enzyme is rate limiting for the enzymatic reaction (this is the case where biosensor engineering has something to say) and assuming a density of 1 g mL^{-1} for all components in a hypothetical "film" on an electrode, we permit ourselves to make a simplistic calculation to demonstrate the importance of biosensor and molecular engineering in the construction of

useful biosensors. For the typical 100 kDa dehydrogenase having a turnover number of $100\,s^{-1}$ and a K_m for NAD^+ of $10^{-4}M$, let us assume that a requirement of the relative rates in order to comply with what was said above is that only 1% of the coenzyme should exist in the NADH form (this is important also because the stability of NAD^+ is much higher than that of NADH). In our hypothetical film, the concentration of NAD^+ should be at least 100 times higher than its K_m, to assure that it is not the rate limiting factor to electrode response, and for the same reason at least 1000 times higher than the enzyme concentration. Under these conditions, the rate of enzymatic production of NADH is proportional roughly to the product of the turnover number multiplied by the enzyme concentration. If a concentration of $0.1M$ of NAD^+ is used (a reasonable number that imposes however the requirement of very reversible mediators so that byproducts are not formed), then the rate of NADH production is of $10^{-2}Ms^{-1}$, and about 17% of the film volume is occupied by enzyme and coenzyme. A similar concentration of the mediator in this film (assuming a molecular weight of 300 Da) would lead to a film occupied 20% by the recognition and transduction chemistries, which is also a reasonable assumption. Since the concentration of NADH has to be in our case always $10^{-3}M$ or lower, and the rate of its disappearance about 10 times higher than that of its production so that the electrooxidation is not either the rate limiting step, it follows that a mediator with a rate constant of at least $100\,M^{-1}s^{-1}$ is needed under the film conditions, to have a sensor that may be limited by substrate diffusion. Obviously, as the film construction parameters change, (usually it is found that more enzyme is needed to be added so that its stability is not the factor limiting the stability of the sensor) the mediator rate constants should be higher. It is therefore obvious, that the rate constants for NADH oxidation by the mediator is a property of utmost importance for the maintenance of favorable dehydrogenase equilibria and diffusionally limited operation of the sensors.

3. Stability of the sensors: When the enzyme is incorporated in the electrode configuration, it is necessary to consider its stability as well. One usual technique to depreciate the importance of enzyme deactivation, is to increase the amount of the immobilized enzyme as mentioned above so that its deactivation is not noted (there is always enough enzyme around). In the case of the dehydrogenase electrodes this may have adverse effects on the equilibrium mentioned in (2) above because it increases the rate of production of NADH. A second technique usually employed is to slow down the substrate mass transport in the sensing layer of the electrode so that it becomes the rate limiting process of the response. This is usually achieved by electrode-covering membranes. Apart from manufacturability problems that this strategy may impose, it may not be applicable especially when low sensitivities are dealt with or low detection limits are desirable. As we have mentioned in another work [130] the most useful technological way to achieve substrate mass-transport limitation is not to slow it down, but to make faster all the other processes, and especially the NADH oxidation step (because there is little intervention power in the enzyme kinetics except by microenvironmental changes, that are largely unpredictable, or by genetic engineering). It is for this, that for dehydrogenase biosensors NADH mediator design and optimization are really the key issues. A fast and stable mediator will allow high enzyme and NAD^+ amounts to be coimmobilized creating a "reserve contingent" of these sensor components so that high stabilities can be obtained.

From the data included in Table 2 it can be seen that the reagentless dehydrogenase sensors developed are scarce in the literature. It should be noted, that we do not include in this account any "biosensor" or biosensor system where the NAD^+ coenzyme would have to be injected in a carrier solution and was not immobilized on the electrode surface. Various observations can be made on the data included in Table 2.

The first relates to the types of mediating schemes used for NADH oxidation. It is obvious that the mediators that resulted in higher sensitivities for NADH oxidation in Table 1, also result in higher current densities for the reagentless dehydrogenase biosensors. Of course, for the NADH oxidizing enzyme-based sensors, the important parameter is the mediating scheme for the diaphorase reaction. This, in view of the above discussion, should not be surprising, and simply underlines the importance of rational mediator design.

But even a fast and reversible mediator appears not to be enough to confer the desired characteristics to the dehydrogenase reagentless biosensors; now the electrode configuration seems to be the most impor-

Table 2. Reagentless dehydrogenase biosensors

Dehydrogenase	E_w V/ref	Immobilization	Electrode configuration	Mediat. / Sens. $\mu A\ mM^{-1}cm^{-2}$	Linearity/ Stability	Ref.
Alcohol	0.4/SCE	NAD^+: Schiff base	carbon paste	–/–	0.75 μM/unst.	117
L-Lactate	0.4/SCE	NAD^+: Schiff base	carbon paste	–/–	1 μM/unst.	117
Alcohol	0/Ag/AgCl	dialysis membrane	NMP^+TCNQ^-	NMP^+/–	–	132
Alcohol	0.875/SCE	dialysis membrane	Pt	no/1	0.06 mM/-	133
L-Lactate	0.875/SCE	dialysis membrane	Pt	no/-	0.2 mM/-	133
Malate	0.875/SCE	dialysis membrane	Pt	no/-	0.4 mM/-	133
Glutamate	potentiometric	dialysis membrane	glass	–/–	–/–	25
L-Lactate	potentiometric	dialysis membrane	glass	–/–	–/–	25
Glucose-6-phosphate	−0.15/Ag/AgCl	dialysis membrane	carbon paste	diaphorase/5.5	1.5 mM/–	118
Glycerol	−0.15/Ag/AgCl	dialysis membrane	carbon paste	diaphorase/3.1	4 mM/–	118
L-Lactate	−0.15/Ag/AgCl	dialysis membrane	carbon paste	diaphorase/–	6 mM/–	118
Alcohol	−0.15/Ag/AgCl	dialysis membrane	carbon paste	Diaphorase/–	8.5 mM/–	118
Alcohol	0.7/Ag/AgCl	dialysis membrane	carbon paste	no/–	–/–	119
D-Lactate	0.08/Ag/AgCl	PVA-cellophane	Pt	diaphorase/0.5nA	1 mM/20 d (R.T)	28
D-Lactate	0.1–0.55/$Fe(CN)_6$	cellophane membrane	Pt	NADHox/–	3 mM/sev. days	131
L-Lactate	0.3/Ag/AgCl	polypyrrole	Pt	MeB/1.3	3 mM/–	135
Alcohol	0.05/Ag/AgCl	dialysis membrane	graphite	NMP^+/0.6	1 mM/–	134
Formate	0.05/Ag/AgCl	dialysis membrane	graphite	NMP^+/0.3	2 mM/–	134
Glucose	0/Ag/AgCl	eastman AQ™	carbon paste	cresyl Blue/0.6	Not linear/–	121
Glucose	0.1/Ag/AgCl	eastman AQ™	carbon paste	MB/2.4(no cover)	70 mM (cover)/–	122
Alcohol	0.1/Ag/AgCl	poly(ethyleinimine) in CP	carbon paste	TBO poly/0.2	1 mM/–	123
L-Lactate	0/SCE	nafion/electropolymer.	glassy carbon	MeB/0.18	not linear/–	82
Alcohol	0.2/Ag/AgCl	poly(ethyleinimine) in CP	carbon paste	TCNQ/1.8	4 mM/–	125
L-Lactate	0.2/Ag/AgCl	poly(ethyleinimine) in CP	carbon paste	TCNQ/1.8	2 mM/–	125
L-Lactate	0.7/Ag/AgCl	mixed in composite	composite	no/0.6	20 mM/–	136
D-Lactate	−0.05/Ag/AgCl	poly(ethyleinimine) in CP	carbon paste	TBO poly/1.5	5mM/30% 15 h, FIA	126
L-Lactate	−0.05/SCE	cellulose acetate	thick film	MB/–	20 mM/–	139
Glucose	0/SCE	Eastman AQ™	carbon paste	TBO poly/–	5 mM/–	127
Alcohol	0.7/SCE	mixed in composite	composite	no/–	4.4 mM/–	137
Alcohol	0.7/SCE	mixed in composite	composite	Ru activ/–	17 mM/–	138
Alcohol	0/Ag/AgCl	mixed in paste	carbon paste	Methyl. Green/–	6 mM/–	128
Alcohol	0.58/Ag/AgCl	mixed in paste	carbon paste	fumed silica act/–	not linear/–	129
Glucose	0.15/Ag/AgCl	mixed in paste	carbon paste	OsPhen/25	20mM/10% 16 h, SS	45

Abbreviations: TBO: Toluidine Blue, OsPhen: Os(4, 4′-dimethyl, 2, 2′-bipyridine)$_2$(1, 10-phenanthroline, 5, 6-dione)$_2$, NMP: N-methylphenazinium, MeB: Methylene Blue, MB: Meldola Blue, TCNQ: Tetracyanoquinodimethanide, FIA: Flow Injection Analysis, SS: Steady State operation. Linearity values for alcohol oxidase refer to ethanol as substrate.

tant factor. And this brings us to the second important observation. There can be discriminated three types of electrode configurations: In the first category, a macromolecular adduct of the coenzyme, and the biorecognition and transduction chemistries are all trapped behind a dialysis or *in situ* polymerized membrane [25, 28, 118, 119, 130–134]. In these configurations, when the sensitivities are high (for similar enzymes) the stability of the electrode is low. It is only with the most highly crosslinked *in situ* constructed membranes that the stability is increased, but at the cost of the sensitivity. The second type of configuration attempts to entrap the coenzyme in electropolymerized films [135, 82]. These configurations show poor stability in contrast with the simply NADH oxidizing counterparts in Table 1, and of course low sensitivities for reasons already explained. Finally, the third type of electrode configurations make use of carbon paste or "biocomposite" materials [117–129, 136–139]. These configurations show the best stability especially when covered with some membrane as for example Eastman AQ™, but at the cost of sensitivity. The pattern that emerges is a simple one: The electrode configurations that have the capacity to form a "reserve pool" of the co-enzyme (as are the CPs and the membrane covered ones) show high stability and sensitivity. The cover membranes also slow the substrate in-diffusion, but most impor-

tantly the NAD^+ out-diffusion, both factors that according to our previous analysis should increase electrode lifetimes. The ideal situation would be to construct an electrode with high swellability and ability of retention of the coenzyme at the same time. But the carbon paste configuration as noted may not only provide entrapment capacity, but also a hydrophobic environment that increases the stability of both the mediators and the coenzyme. It is therefore difficult to reconcile the two requirements. With such irreconcilable requirements in mind is that we introduced recently [140, 141] a new type of carbon paste material, that we called "binder paste" so that more flexible electrodic configurations could be constructed. In these materials, desired properties can be given to the electrode playing with the hydrophilicity of the "binder", so electrodes with the desired properties can be easily envisioned for reagentless dehydrogenase biosensors as well.

The use of the polymeric dyes that have been introduced in carbon paste materials and seem to be now the mediating system of choice for dehydrogenase biosensors, results in electrodes with high stability even in the absence of cover membranes. We think that the reason this is so, is because of the structural properties of these mediators; what was a disadvantage in the case when they were immobilized on a glassy carbon surface (low permeability) [77], in reagentless biosensors in CP [123, 124, 126, 127] becomes an advantage entrapping NAD^+ and maintaining it in the electrode, especially when the proton transfer limitations are overcome by the inclusion of poly(ethyleneimine) in the paste. Structural characteristics of the mediator may also be behind the intriguing result of the last entry in Table II, of the glucose biosensor developed in our laboratories [45]. This sensor shows the highest reported sensitivity for this enzyme and a remarkable stability even without cover membranes. Although the work on this configuration and mediator is still in starting stages, it seems that the Osphendione mediator is neither a particularly fast nor a particularly reversible one. However, we have shown its ability to remain for extended periods entrapped in the CP configuration, and maybe its complexation with the coenzyme explains the long life time of this configuration.

In conclusion we can say that for the construction of useful reagentless dehydrogenase biosensors, the important property of the system is the ability to incorporate high enzyme amounts without shifting the dehydrogenase equilibrium, but even more important is the availability of electrode configurations that can retain these amounts of enzyme and the appropriate respective coenzyme and mediator amounts. This makes the advancement of the state of the art in reagentless dehydrogenase biosensors, the subject not only of molecular design, electrochemistry and enzymology as we have seen up to now, but also of materials science.

A Protocol for Configuration Evaluation

In this section, we do not pretend to give new guidelines for the biosensor evaluation procedures, since most of what we suggest has been applied in various of the mentioned in this review excellent works [12, 13, 55, 56, 60, 86]. However, we think that this attempt to systematize, is justified due to the different criteria used in various studies and the incomplete data encountered in our efforts to evaluate different mediating schemes. We think in general three stages should be discerned in NADH catalytic system and biosensor evaluation (here we will strictly limit ourselves to non-enzymatic systems):

1. Demonstration of catalysis: The easiest method to demonstrate catalysis is to poise an electrode with all necessary components immobilized, at a sufficiently oxidizing potential and observe steady state catalytic response to NADH or substrate injections, usually in deaerated solutions, and preferably under well defined hydrodynamic conditions, performing the appropriate control experiments to assure that under the same conditions no direct oxidation occurs. The potential dependence of the possible catalytic current at steady state is also a useful piece of data, accompanied with response curves to different substrate or NADH concentrations. Although this is a quick test to assess the worthiness of further time investment in a configuration, it has almost no value for the classification of mediators and oxidation and recycling systems because the values obtained are characteristics of the individual electrodes and not of the chemistry used. To assess the catalytic efficiency of the mediator used, a cyclic voltammetric study [13, 142] is usually sufficient, in the case of NADH done with fresh solutions and in deoxygenated buffers. From such studies, bimolecular rate constants can be calculated by varying within certain limits the

concentrations of NADH and mediator. The assumption here is that the mediator is a reversible redox couple, and that there is an excess of the reductant species, although different situations have been compensated mathematically [143, 144]. Usually, a 1 mM NADH solution with 0.1 mM mediator complies with these requirements. Reporting the ratio i_c/i_d (the catalytic plateau current divided by the diffusional current) is a useful manner to evaluate the catalytic efficiency of NADH mediators, common values of this ratio ranging between 1.5 and 4 [100, 145]. It should be noted, that another useful quick diagnostic of catalytic efficiency is the persistence of a catalytic wave for the above conditions at least at 10 mV s^{-1}.

2. *Mediator electrochemistry and reaction rate constants calculation*: One way to calculate the reaction rate constants was mentioned in (1) above. This is convenient when the mediator is not adsorbed, but even so, first the electrochemistry of the mediator should be assessed. For mediators not adsorbed, their electrochemical characteristics can be evaluated with standard methods described [146]. In general, it is expected that the NADH oxidation catalysts show a $2e^-$, $(2)H^+$, pH dependent electrochemistry, and for this the electrochemical study should be extended over the entire pH window. These studies are usually chronoamperometric or cyclic voltammetric. Deviations from the ideal behavior should be examined, and the heterogeneous electron transfer rate constants determined, by hydrodynamic or CVmetric methods [146]. The same procedure should be followed in the case that the mediators are adsorbed (pH behavior, study of ideality deviations). The methods in this case are similar and have also been described in the literature [55, 100]

Once the electrochemical properties of the mediators are determined, one can proceed to calculate the reaction rate constants between the NADH and the mediator, either with CVmetry as described above, or (for adsorbed mediators) with hydrodynamic experiments as amply described elsewhere [55, 56] or spectrophotometircally in solutions containing the oxidized mediator and NADH. Only the hydrodynamic results are technologically relevant if reagentless biosensors are to be constructed with the mediator under evaluation. Studies at different pHs and NADH concentrations, or surface coverages yield information also on the mechanism of the NADH electrooxidation [55, 56]. Care should be taken that the surface coverages do not substantially exceed 10^{-10}–10^{-9} mol cm^{-2} (one or a few monolayers) so that the analysis can follow the literature described methodology. If substantially thicker films are formed, their permeability has to be calculated with hydrodynamic experiments, and the conditions should be such that electron or proton diffusion are not rate limiting. In the case of irreversible or quasi reversible electrochemistry of the mediator the necessary corrections should be made. Such situations are usually obvious, not only from the CVmetric behavior of the mediator, but also from non-zero intercepts of the usual Koutecky-Levich plots. These experiments usually yield a lower limit of the reaction rate constants. In cases where the structure of the films and the electrochemical behavior deviates highly from ideality, the calculation of the rate constants requires the assumption of a model, a fact that can be subject of research designs on its own, so if possible, they should be avoided.

3. *Reversibility of NADH oxidation and stability*: Once the basic kinetic parameters of the system have been determined, it is technologically relevant to determine if the NAD^+ produced is enzymatically active and to assess the stability of the catalytic system. The question of stability of the mediator is important for applications of the biosensor. Usually, the factors that affect stability are well known (light, pH, etc.) for every mediator. Quantitative results on such studies should be more often reported. The stability is a concept related to the final use of a biosensor. If it is developed with a specific use in mind, it should be tested accordingly, although such limited studies may be of limited scientific value, and this has always to be assessed. For example an one-use biosensor, should be stable under ambient dry storage conditions, the operational stability being irrelevant. But for a full account of the stability of the mediator, all the factors mentioned in the previous sections should be taken into account. Jaegfeldt et al. [12] have demonstrated some ingenious experimental designs to not only assess stabilities, but also to decipher the deactivation mechanisms of mediators. In general the stability of the mediator should be assessed in the absence and presence of NADH. The application or non-application of potential, changes in pH, solution species, other environmental parameters, cycling, are parameters that should be studied. If the

mediator is immobilized on the surface or adsorbed, differentiation should be made between stability of the adsorbed layer and redox and chemical stability of the mediator. Usually, such studies should not only be electrochemical, but also spectrophotometric (transparent electrodes, thin layer cells, etc.). If operating conditions in the presence of O_2 are required, all stability studies should take into account this factor as well.

Within the stability time parameters of the mediator, bulk electrolysis studies of NADH should be conducted. As mentioned, in a typical such experiment, in the absence of dioxygen a certain amount of NADH is oxidized. The supposedly active NAD^+ produced should be used with a dehydrogenase to drive an oxidation reaction to completion (for example with alcohol oxidase and ethanol as substrate). The enzymatic turnover results should also be compared with the results of equivalent commercial NAD^+ preparations. All results should be corrected for the concurrent NADH hydrolysis. The coulometric, enzymatic and photometric (at 340 nm) mass balances of the bulk electrolysis and active NAD^+ concentrations should agree. If they do not, a catalytic efficiency can be calculated, and possible problems of the catalytic mechanism can be detected. It is for this reason that such studies are of high value.

Such a study is fairly complete and useful to make conclusions and compare catalytic systems. In continuation, what is left for the completion of a reagentless biosensor is the necessity to immobilize all the necessary constituents in a durable, robust, and stable biosensor together with a dehydrogenase. We have seen a few approaches that seem very promising for the future of dehydrogenase-based biosensors and bioelectronic research (carbon pastes and biocomposites, electropolymerized films, polymeric mediators). We hope that this last aspect of biosensor development will never be the subject of a standardization scheme, since it is the area where the individual contributions in this active field of research, will have more impact in the years to come.

Note added in Proof

Our assertions that the promising configurations (those using polyaromatic dyes) may see their realization with the use of polymeric materials that incorporate such dyes as soon as the water swellability of these polymers can be achieved, have shown a justification by the recent work of the group of Lund [147] where they used a poly(ethylenimine) backbone to attach the dyes. This configuration achieved one of the highest reported sensitivities for non-enzymatic NADH oxidation ($300\,\mu A\ cm^{-2}$). However, when reagentless dehydrogenase electrodes were aimed at, the authors still used the "security" of the carbon paste configuration that appears to be the most viable possibility for large scale production of such sensors. It should be noted that to achieve these electrodes, the authors followed the steps of our previous works [78, 123, 124, 130, 140, 141] that proved that hydrophilisation of the carbon paste is the only way to give high performance characteristics to this configuration, and mass transport-limited operation of enzyme electrodes should be always attempted.

Conclusions

In this work the NADH oxidation schemes have been examined and the advances in the last 5 years critically reviewed especially from the point of view of dehydrogenase biosensor development. We noted a necessity for the more technologically relevant study of the mechanism of oxidation of NADH and of that of the heterogeneous electron transfer of mediators, especially of the fused aromatic dyes that seem to be promising NADH oxidation mediators. We saw that polymeric mediators, electropolymerization techniques and NADH oxidizing enzymes provide the most promising bases for dehydrogenase biosensor development and bioelectronics coupling. And when it came to reagentless biosensors we have seen that screen printed materials of which carbon paste configurations are a possible precursor, are the most technologically viable alternative.

We reiterate our belief that biosensors have passed their infancy and should yield useful and viable systems integrable in the socioeconomic process. In our knowledge, the configurations described in the literature and partly reviewed here, are not much less advanced than efforts of the private sector in this area. The point has been reached where products can and should come out of biosensor research and this can occur without compromising the quality of the produced work. In times like these, one can allow oneself the luxury to pose for a moment and think of the ultimate limit in this process. We mentioned that the coenzyme is known to have low stability. In the absence of a stable NAD-mimicking compound,

one cannot help but remember the words of the poet [148]:

Our efforts are of those disaster-prone;
our efforts are like those of Trojan men.
Somehow we get somewhere; somehow
we lift our heads; and we begin
to take some courage and to raise our hopes.
But always something will crop up to stop us short.
Achilles from the trench will ever rise
and jump out with loud yells to frighten us
... sure and certain is our fall.

But if the results presented here, that ascertain half life times of biosensors longer than the hydrolysis half life time of NAD^+ or NADH, do not convince one that the future of dehydrogenase biosensor development can be a bright one, then let it be the poet again who gives the answer:

Honor to those who in their lives
have set themselves to guard Thermopylae....
Let greater honor be to those
who do foresee (and many can foresee)
that Ephialtes will eventually appear,
and that the Medes will in the end get through.

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References

[1] Nomenclature Committe of the International Union of Biochemistry, *Enzyme Nomenclature*, Academic Press, New York, 1984.
[2] K.-S. You, *CRC Critical Reviews in Biochemistry* **1985**, *17*, 313.
[3] S. B. Sobolov, M. D. Leonida, A. Bartoszko-Malik, K. I. Voivodov, F. Mckinney, J. Kim, A. J. Fry, *J. Org. Chem. 1996*, *61*, 2125.
[4] E. C. Stevenson, G. M. McNally, P. L. Spedding, *J. Chem. Tech. Biotechnol. 1995*, *63*, 165.
[5] E. Stevenson, P. G. Ibbotson, P. L. Spedding, *Biotechnol. Bioeng.* **1993**, *42*, 43.
[6] K. Mosbach, *Adv. Enzymol.* **1978**, *46*, 205.
[7] A. F. Bückmann, G. Carrea, in: *Advances in Biochemical Engineering/Biotechnology, Vol. 39* (A. Fiechter, ed.) Springer, Berlin Heidelberg New York, 1989, p. 97.
[8] L. Gorton, *J. Chem. Soc., Faraday Trans. 1*, **1986**, *82*, 1245.
[9] H. Jaegfeldt, *J. Electroanal. Chem.* **1980**, *110*, 295.
[10] Z. Samec, P. J. Elving, *J. Electroanal. Chem.* **1983**, *144*, 217.
[11] J. Moiroux, P. J. Elving, *J. Am. Chem. Soc.* **1980**, *102*, 6533.
[12] H. Jaegfeldt, T. Kuwana, G. Johansson, *J. Am. Chem. Soc.* **1983**, *105*, 1805.
[13] A. Kitani, Y. -H. So, L. L. Miller, *J. Am. Chem. Soc.* **1981**, *103*, 7636.
[14] D. Ch. -S. Tse, T. Kuwana, *Anal. Chem.* **1978**, *50*, 1315.
[15] W. J. Blaedel, R. A. Jenkins, *Anal. Chem.* **1975**, *47*, 1337.
[16] A. J. Fry, S. B. Sobolov, M. D. Leonida, K. I. Voivodov, *Tetrahedron Lett.* **1994**, *35*, 5607.
[17] A. Berger, M. Comtat, *Bioelectrochem. Bioenerg.* **1992**, *27*, 495.
[18] R. Marín, J. M. Rodríguez, *J. Electroanal. Chem.* **1989**, *260*, 101.
[19] E. Steckham, S. Herrmann, R. Ruppert, J. Thömmes, C. Wandrey, *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 388.
[20] S. Chao, M. S. Wrighton, *J. Am. Chem. Soc.* **1987**, *100*, 5886.
[21] A. L. De lacey, M. T. Bes, C. Gómez-Moreno, V. M. Fernández, *J. Electroanal. Chem.* **1995**, *390*, 69.
[22] J. Bonnefoy, J. Moiroux, J.-M. Laval, C. Bourdillon, *J. Chem. Soc., Faraday Trans. 1*, **1988**, *84*, 941.
[23] R. W. Coughlin, M. Aizawa, B. F. Alexander, M. Charles, *Biotecnol. Bioeng.* **1975**, *17*, 515.
[24] H. Jaegfeldt, A. Torstensson, G. Johansson, *Anal. Chim. Acta* **1978**, *97*, 221.
[25] P. Davies, K. Mosbach, *Biochim. Biophys. Acta* **1974**, *370*, 329.
[26] D. Kirstein, F. Scheller, F. Schubert, *Appl. Biochem. Microbiol.* **1983**, *18*, 386.
[27] K. Miki, T. Ikeda, S. Todoriki, M. Senda, *Anal. Sci.* **1989**, *5*, 269.
[28] M. Montagné, J. -L. Marty, *Anal. Chim. Acta* **1995**, *315*, 297.
[29] T. Yagi, *Biochim, Biophys. Acta* **1993**, *1141*, 1.
[30] L. Gorton, B. Persson, P. D. Hale, L. I. Boguslavsky, H. I. Karan, H. S. Lee, T. A. Skotheim, H. L. Lan, Y. Okamoto, in: *ACS Symp. Ser. No. 487* (P. G. Edelman, J. Wang, eds.), ACS, Washington, DC, 1992, p. 56.
[31] R. L. Blankespoor, L. L. Miller, *J. Electroanal. Chem.* **1984**, *171*, 231.
[32] P. Hapiot, J. Moiroux, J. -M. Savéant, *J. Am. Chem. Soc.* **1990**, 112, 1337.
[33] S. Fukusumi, S. Koumitsu, K. Hironaka, T. Tanaka, *J. Am. Chem. Soc.* **1987**, *109*, 305.
[34] A. Anne, P. Hapiot, J. Moiroux, P. Neta, J. -M. Savéant, *J. Phys. Chem.* **1991**, *95*, 2370.
[35] W. G. Kuhr, V. L. Barrett, M. R. Gagnon, P. Hopper, P. Pantano, *Anal. Chem.* **1993**, *65*, 617.
[36] J. W. Verhoeven, W. van Gerresheim, F. M. Martens, S. M. van der Kerk, *Tetrahedron* **1986**, *42,* 975.
[37] S. Fukuzumi, Y. Kando, T. Tanaka, *J. Chem. Soc. Perkin Trans. II* **1984**, *4*, 673.
[38] H. Deng, J. Zheng, D. Sloan, J. Burgner, R. Callender, *Biochemistry* **1989**, *28*, 1525.
[39] Agnes, J. Moiroux, *J. Org. Chem.* **1990**, *55*, 4608.
[40] J.-C. Lepretre, D. Limosin, G. Pierre, P. Chautemps, G. Gellon, J. -L. Pierre, *J. Electroanal. Chem.* **1990**, *286*, 63.
[41] K. Kalyanasundaram, T. Colassis, R. Humphry-Baker, P. Savarino, E. Barni, E. Pelizzetti, M. Grätzel, *J. Am. Chem. Soc.* **1989**, *111*, 3300.
[42] L. L. Miller, J. R. Valentine, *J. Am. Chem. Soc.* **1988**, *110*, 3982.
[43] A. Fassouane, J. -M. Laval, J. Moiroux, C. Bourdillon, *Biotechnol. Bioeng.* **1990**, *35*, 935.
[44] H. Jaegfeldt, *Bioelectrochem. Bioenerg.* **1981**, *8*, 355.
[45] M. Hedenmo, A. Narváez, E. Domínguez, I. Katakis, *Analyst* **1996**, *121*, 1891.
[46] C. H. Wong, G. M. Whitesides, *J. Am. Chem. Soc.* **1981**, *103*, 4890.

[47] L. Gorton, E. Csöregi, E. Domínguez, J. Emneus, G. Jínsson-Pettersson, G. Marko-Varga, B. Persson, *Anal. Chim. Acta* **1991**, *250*, 203.
[48] P. N. Bartlett, P. Tebbutt, R. G. Whitaker, *Prog. Reaction Kinetics* **1991**, *16*, 55.
[49] P. N. Bartlett, R. G. Whitaker, *Biosensors* **1987**, *2*, 359.
[50] L. Gorton, A. Torstensson, H. Jaegfeldt, G. Johansson, *J. Electroanal. Chem.* **1984**, *161*, 103.
[51] B. W. Carlson, L. L. Miller, *J. Am. Chem. Soc.* **1985**, *107*, 479.
[52] A. Kitani, L. L. Miller, J. Am. Chem. Soc. **1981**, *103*, 3595.
[53] C. A. Coleman, J. G. Rose, C. J. Murray, *J. Am. Chem. Soc.* **1992**, *114*, 9755.
[54] S. Fukuzumi, M. Ishikawa, T. Tanaka, *J. Chem. Soc. Perkin Trans. II* **1989**, *11*, 1811.
[55] B. Persson, *J. Electroanal. Chem.* **1990**, *287*, 61.
[56] B. Persson, L. Gorton, *J. Electroanal. Chem.* **1990**, *292*, 115.
[57] B. W. Carlson, L. L. Miller, P. Neta, J. Grodkowski, *J. Am. Chem. Soc.* **1984**, *106*, 7233.
[58] F. Ni, H. Feng, L. Gorton, T. M. Cotton, *Langmuir* **1990**, *6*, 66.
[59] S. Fukuzumi, N. Nishizawa, T. Tanaka, *J. Org. Chem.* **1984**, *49*, 3571.
[60] L. G. Lee, G. M. Whitesides, *J. Am. Chem. Soc.* **1985**, *107*, 6999.
[61] B. F. Y. Y. Hin, C. R. Lowe, *Anal. Chem.* **1987**, *59*, 2111.
[62] K. Essaadi, B. Keita, L. Nadjo, *J. Electroanal. Chem.* **1994**, *367*, 275.
[63] B. Keita, K. Essaadi, L. Nadjo, M. Desmadril, *Chem. Phys. Lett.* **1995**, *237*, 411.
[64] C. A. Coss, H. D. Abruña, *Inorg. Chem.* **1985**, *24*, 4263.
[65] T. Yao, Y. Matsumoto, T. Wasa, *Anal. Chim. Acta* **1989**, *218*, 129.
[66] E. J. Eisenberg, K. C. Cundy, *Anal. Chem.* **1991**, *63*, 845.
[67] M. Somasundrum, J. V. Bannister, *J. Chem. Soc. Chem. Commun.* **1993**, 1629.
[68] C. Ueda, D. Ch. -S. Tse, T. Kuwana, *Anal. Chem.* **1982**, *54*, 850.
[69] W. B. Nowall, W. G. Kuhr, *Anal. Chem.* **1995**, *67*, 3583.
[70] W. Schuhmann, J. Huber, H. Wohlschläger, B. Strehlitz, B. Gründig, *J. Biotechnol.* **1993**, *27*, 129.
[71] A. S. N. Murthy, *Bioelectrochem. Bioenerg.* **1994**, *33*, 71.
[72] A. S. N. Murthy, A. R. L. Gupta, *Anal. Chim. Acta* **1994**, *289*, 43.
[73] Ch. -X. Cai, H. -X. Ju, H. -Y. Chen, *J. Electroanal. Chem.* **1995**, *397*, 185.
[74] B. Gründig, G. Wittstock, U. Rüdel, B. Strehlitz. *J. Electroanal. Chem.* **1995**, *395*, 143.
[75] D. D. Schlereth, E. Katz, H. -L. Schmidt, *Electroanalysis* **1994**, *6*, 725.
[76] E. Lorenzo, L. Sánchez, F. Pariente, J. Tirado, H. D. Abruña, *Anal. Chim. Acta* **1995**, *309*, 79.
[77] B. Persson, H. S. Lee, L. Gorton, T. A. Skotheim, P. Barlett, *Electroanalysis* **1995**, 7, 935.
[78] E. Domínguez, H. L. Lan, Y. Okamoto, P. D. Hale, T. A. Skotheim, L. Gorton, *Biosens. Bioelectron.* **1993**, *8*, 167.
[79] C. Degrand, L. L. Miller, *J. Am. Chem. Soc.* **1980**, *102*, 5728.
[80] N. F. Atta, A. Galal, A. E. Karagözler, H. Zimmer, J. F. Rubinson, H. B. Mark, *J. Chem. Soc., Chem. Commun.* **1990**, 1347.
[81] K. Tanaka, S. Ikeda, N. Oyama, K. Tokuda, T. Ohsaka, *Anal. Sci.* **1993**, *9*, 783.
[82] A. A. Karyakin, E. E. Karyakina, W. Schumann, H. -L. Schmidt, S. D. Varfolomeyev, *Electroanalysis* **1994**, *6*, 821.
[83] D. D. Schlereth, E. Katz, H. -L. Schmidt, *Electroanalysis* **1995**, *7*, 46.
[84] F. Xu, H. Li, S. J. Cross, T. F. Guarr, *J. Electroanal. Chem.* **1994**, *368*, 221.
[85] F. Pariente, E. Lorenzo, H. D. Abruña, *Anal. Chem.* **1994**, *66*, 4337.
[86] E. Katz, T. Lötzbeyer, D. D. Schlereth, W. Schumann, H. -L. Schmidt, *J. Electroanal. Chem.* **1994**, *373*, 189.
[87] J. Kulys, *Enz. Microb. Technol.* **1981**, *3*, 344.
[88] W. J. Albery, P. N. Bartlett, *J. Chem. Soc., Chem. Commun.* **1984**, 234.
[89] G. Nagy, I. Kapui, L. Gorton, *Anal. Chim. Acta* **1995**, *305*, 65.
[90] H. -Y. Chen, A. -M. Yu, J. -L. Han, Y. -Z. Mi, *Anal. Lett.* **1995**, *28*, 1579.
[91] R. M. Uppu, *J. Inorg. Biochem.* **1995**, *58*, 193.
[92] M. Tian, S. Dang. *Electroanalysis* **1995**, *7*, 1063.
[93] A. Silber, C. Bräuchle, N. Hampp, *J. Electroanal. Chem.* **1995**, *390*, 83.
[94] V. R. Taliene, T. Ruzgas, V. Razumas, J. Kulys, *J. Electroanal. Chem.* 1994, *372*, 85.
[95] J. Kulys, L. Gorton, E. Domínguez, J. Emnéus, H. Jarskog, *J. Electroanal. Chem.* **1994**, *372*, 49.
[96] W. J. Albery, A. R. Hillman, *J. Electroanal. Chem.* **1984**, *170*, 27.
[97] K. W. Sim, *Anal. Chim. Acta* **1993**, *273*, 165.
[98] D. A. Scott, A. W. Skillen, *Anal. Chim. Acta* **1992**, *256*, 47.
[99] H. -C. Chang, A. Ueno, H. Yamada, T. Matsue, I. Uchida, *Analyst* **1991**, *116*, 793.
[100] Y. Ogino, K. Takagi, K. Kano, T. Ikeda, *J. Electroanal. Chem.* **1995**, *396*, 517.
[101] T. Matsue, H. Yamada, H. -C. Chang, I. Uchida, K. Nagta, K. Tomita, *Biochim. Biophys. Acta* **1990**, *1038*, 29.
[102] M. Tabata, F. Koushima, M. Totani, *Anal. Chim. Acta* **1994**, *298*, 113.
[103] M. Somasundrum, J. Hall, J. V. Bannister, *Anal. Chim. Acta* **1994**, *295*, 47.
[104] F. Mizutani, S. Yabuki, T. Katsura, *Sensors and Actuators B* **1993**, *13*, 574.
[105] T. Matsue, N. Kasai, M. Narumi, M. Nishizawa, H. Yamada, I. Uchida, *J. Electroanal. Chem.* **1991**, *300*, 111.
[106] Y. Kashiwagi, T. Osa, *Chem. Lett.* **1993**, 677.
[107] M. Vreeke, R. Maidan, A. Heller, *Anal. Chem.* **1992**, *64*, 3084.
[108] S.-E. Yun, M. Taya, S. Tone, *Biotechnol. Lett.* **1994**, *16*, 1053.
[109] S. Aono, I. Okura, *Inorg. Chim. Acta* **1988**, *152*, 55.
[110] V. D. Westerhausen, S. Herrmann, W. Hummel, E. Steckhan, *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1529.
[111] H. Günter, A. S. Paxinos, M. Schulz, C. van Dijk, H. Simon, *Angew. Chem. Int. Ed. Engl.* **1990** *29*, 1053.
[112] A. A. Karyakin, O. A. Bobrova, E. E. Karyakina, *J. Electroanal. Chem.* **1995**, *399*, 179.
[113] K. Umeda, A. Nakamura, F. Toda, *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2260.
[114] H. Ukeda, M. Imabayashi, K. Matsumoto, Y. Osajima, *Agric. Biol. Chem.* **1989**, *53*, 2909.
[115] H. Ukeda, M. Imabayashi, K. Matsumoto, Y. Osajima, *Agric. Biol. Chem.* **1989**, *53*, 2265.
[116] Y. Yamazuki, H. Maeda, *Agric. Biol. Chem.* **1982**, *46*, 1571.
[117] T. Yao, S. Musha, *Anal. Chim. Acta* **1979**, *110*, 203.

[118] K. Miki, T. Ikeda, S. Todoriki, M. Senda, *Anal. Sci.* **1989**, *5*, 269.
[119] M. Senda, T. Ikeda, K. Miki, H. Hiasa, *Anal. Sci.* **1986**, *2*, 501.
[120] U. Bilitewski, R. D. Schmid, in: *Biosensors: Applications in Medicine, Environmental Protection and Process Control* (R. D. Scmid, F. Scheller, eds.), VCH, Weinheim, 1989, p.99.
[121] L. Gorton, G. Bremle, E. Csöregi, G. Jönsson-Pettersson, B. Persson, *Anal. Chim. Acta.* **1991**, *249*, 43.
[122] G. Bremle, B. Persson, L. Gorton, *Electroanalysis* **1991**, *3*, 77.
[123] E. Domínguez, H. L. Lan, Y. Okamoto, P. D. Hale, T. A. Skotheim, L. Gorton, B. Hahn-Hägerdal, *Biosens. Bioelectron.* **1993**, *8*, 229.
[124] E. Domínguez, G. Marko-Varga, H. L. Lan, Y. Okamoto, P. D. Hale, T. A. Skotheim, B. Hahn-Hägerdal, L. Gorton, in: *Enzyme Engineering XI* (D. S. Clark, D. A. Estell, eds.), New York Academy of Sciences, New York, 1992, p. 230.
[125] P. C. Pandey, *Anal. Biochem.* **1994**, *221*, 392.
[126] H.-C. Shu, B. Mattiasson, B. Persson, G. Nagy, L. Gorton, S. Sahni, L. Geng, L. Boguslavsky, T. A. Skotheim, *Biotechnol. Bioeng.* **1995**, *46*, 270.
[127] B. Persson, H. L. Lan, L. Gorton, Y. Okamoto, P. D. Hale, L. I. Boguslavsky, T. A. Skotheim, *Biosens. Bioelectron.* **1993**, *8*, 81.
[128] Q. Chi, S. Dong, *Anal. Chim. Acta* **1994**, *285*, 125.
[129] J. Wang, J. Liu, *Anal. Chim. Acta* **1993** *284*, 385.
[130] I. Katakis, E. Domínguez, *TrAC* **1995**, *14*, 310.
[131] M. Montagné, H. Erdmann, M. Comtat, J. -L. Marty, *Sensors and Actuators B* **1995**, *26* 440.
[132] W. J. Albery, P. N. Bartlett, A. E. G. Cass, K. W. Sim, *J. Electroanal. Chem.* **1987**, *218*, 127.
[133] W. J. Blaedel, R. C. Engstrom, *Anal. Chem.* **1980**, *52*, 1691.
[134] J. J. Kulys, U. Bilitewski, R. D. Schmid, *Anal. Lett.* **1991**, *24*, 181.
[135] Y. Ikariyama, T. Ishizuka, H. Sinohara, M. Aizawa, *Denki Kagaku*, **1990**, *58*, 1097.
[136] J. Wang, Q. Chen, *Electroanalysis* **1994**, *6*, 850.
[137] J. Wang, E. González-Romero, M. Osoz, *Electroanalysis* **1992**, *4*, 539.
[138] J. Wang, E. González-Romero, A. Reviejo, *J. Electroanal. Chem.* **1993**, *353*, 113.
[139] S. D. Sprules, J. P. Hart, S. A. Wring, R. Pittson, *Anal. Chim. Acta* **1995**, *304*, 17.
[140] A. Narváez, J. Parellada, E. Domínguez, I. Katakis, *Quím. Anal.* **1996**, *15*, 75.
[141] J. Parellada, A. Narváez, E. Domínguez, I. Katakis, *Biosens. Bioelectron.* **1997**, in press.
[142] C. P. Andrieux, C. Blocman, J. M. Dumas-Bouchiat, F. M'Halla, J. M. Saveant, *J. Electroanal. Chem.* **1980**, *113*, 19.
[143] M. L. Olmstead, R. S. Nicholson, *Anal. Chem.* **1969**, *41*, 851.
[144] R. S. Nicholson, *Anal. Chem.* **1965**, *37*, 667.
[145] S. Itoh, H. Fukushima, M. Komatsu, Y. Ohshiro, *Chem. Lett.* **1992** 1583.
[146] A. J. Bard, L. R. Faulkner, *Electrochemical Methods Fundamentals and Applications*, Wiley, New York, 1980, Ch. 6.8.
[147] Z. Huan, B. Persson, L. Gorton, S. Sahni, T. Skotheim, P. Bartlett, *Electroanalysis* **1996**, *8*, 575.
[148] C. P. Cavafy, *The Greek Poems of C. P. Cavafy as translated by Memas Kolaitis*, Orpheus, New Rochelle, 1989, pp. 15, 22.

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