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Enzymatic Electrocatalysis: Electrochemical Regeneration of NAD^+ with Immobilized Lactate Dehydrogenase Modified Electrodes

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Abstract: Covalent immobilization of LDH on vitreous carbon provides a surface endowed with both electrochemical and enzymatic activities. The working area of an RVC electrode is large enough to perform experiments involving the two types of reactions, separated or coupled, which results in appreciable bulk concentration changes. The extent of protein coverage of the electrode surface which ranges from 0 to 13 pmol/cm^2 can be measured fluorimetrically after hydrolysis of the immobilized enzyme. Progressive masking of the surface occurs with increasing polymerization time. At the molecular level of enzymatic production of NADH, regeneration of NAD^+ results from the electrochemical oxidation of NADH. A current efficiency of 99.0% for enzymatically active NAD^+ regeneration is obtained during the course of technically simple recycling experiments.

The present paper deals with enzymatic electrocatalysis as defined by the following statement of Murray and co-workers:[1] reactions which, on electrode-immobilized molecules, can undergo repetitive cycling between different oxidation states are pertinent to eventual electrocatalytic activity.

In general, attached electroactive molecules act as fast electron transfer mediators for a substrate dissolved in the bulk solution and which is not (or only slowly) electroactive at the naked electrode.[2] It is to be expected that substantial improvements in particular in reaction specificity can result from the use of more and more sophisticated attached molecules or polymers with specific chemical sites of reaction.[3,4] In complement to this purely chemical approach, it is interesting to immobilize the "pre-manufactured" catalytic sites of enzymes.

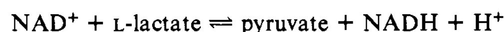
At the present time, almost 1000 oxydoreductases are known to catalyze a wide variety of specific redox reactions. The most exciting possibility is the direct electron transfer between the enzyme native site and the electrode surface. That seems to be feasible with some flavoenzymes,[5-7] but due to the large size of the protein, the electron transfer is very slow.

However, many oxidoreductases, including the important class of dehydrogenases, cannot exchange electrons with the electrode directly since they do not bear built-in prosthetic groups. That is the reason why we have brought an alternative approach into play in order to develop an efficient and general use of oxydoreductases in electrochemistry (Figure 1).

The enzyme is covalently bound to the electrode surface and a small electroactive redox molecule (for example, the cofactor) carries electrons from the active site of the enzyme to the electrode surface. The transformation of substrate into product benefits from both the energy supplied by the electrode and the specific catalytic activity of the enzyme site. Some theoretical and experimental aspects of the resulting kinetic synergy already have been analyzed,[8-11] and it appears that molecular proximity between enzyme and electrode surface is a fundamental key to that synergy effect.

In the present paper, our purpose is twofold: first, to study the effective masking of the electrode surface by the attached proteins, a problem which cannot be investigated when electrodes with small working areas are used since the total amount of attached protein is then too small to be measured correctly; and second, preparative scale experiments need an electrode working area large enough to ensure an electrode area/volume of reactor ratio close to 1 cm^2/cm^3. In order to focus only on the problems related to the increase in the electrode surface, we chose the enzyme/cofactor model which has already been used with disk electrodes.[10] At this stage it was also profitable to work with an electrode whose surface is not microporous since it enabled us to produce a model avoiding the difficulties related to the porosity of electrodes of large working areas. We chose to work with reticulated vitreous carbon (RVC), a new material endowed with interesting properties, some of which already have been reported in the literature.[12-14]

The enzyme, lactate dehydrogenase from rabbit skeletal muscle (L.D.H.), was used as a model of $NADH/NAD^+$ dehydrogenase:



$$NAD^+ + \text{L-lactate} \rightleftharpoons \text{pyruvate} + NADH + H^+$$

The above reaction is shifted toward the right-hand side at pH 9. The electrochemical reaction which regenerates NAD^+ is

$$NADH \xrightarrow{K} NAD^+ + H^+ + 2e$$

Results and Discussion

(1) The RVC Electrode. We used the kind of electrode which Blaedel and Wang designed and called a "rotating RVC disk electrode".[15] Its compact geometry provides a large nonmicroporous working area within a small external volume and allows efficient mass transfer due to the high void volume of RVC. Actually such an electrode looks externally more like a cylinder, and when rotated the hydrodynamics are unfortunately not well elucidated. We performed some preliminary experiments in order to determine whether the mass transfer can be easily modeled. Various electrodes with external cylinders 1 to 8 mm high but

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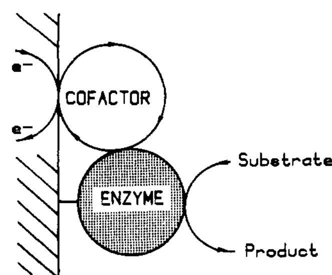

Figure 1. A possible pathway for enzymatic electrocatalysis with electrochemical regeneration of the cofactor in the case of an enzyme without built-in prosthetic groups. We used a dehydrogenase, with L-lactate and pyruvate being the substrate and the product, respectively. The cofactor was the $NADH/NAD^+$ redox system.

having the same diameter (5 mm) were tested with the ferro–ferricyanide redox system. After pretreatment, which is a requisite for reproducibility (see Experimental Section), we examined the dependence of the current at a controlled potential upon the following parameters: ferricyanide concentration C, up to 10^{-3} M, electrode working area, S, from 1 to 9 cm^2, and rotation frequency from 200 to 3000 rpm. The controlled potential was chosen so as to obtain the limiting current i_l which can be expressed by the equation $i_l = nFKSC\omega^{\alpha}$ [15] where n and ω are respectively the number of electrons involved in the electrochemical process and the angular rotation speed. K and α are constants which depend on the flow rate.

In agreement with Blaedel and Wang we found a good proportionality between i_l and C, but the dependence of i_l upon S did not obey a simple law. Values of α as low as 0.48 or as high as 0.63 were obtained depending on both ω and the electrode geometry instead of the value 0.5 corresponding to the rotating-disk electrode. The distribution of these data shows that the mass transfer results from both laminar (α close to 0.5) and turbulent (α close to 0.66[16]) flow. Therefore, there cannot be a simple description of the diffusion layer like the one corresponding to the validity of the Levitch equation, and simulation of the behavior of the enzyme RVC electrode cannot be realized as thoroughly as in the case of the enzyme-disk (or cylinder) electrode.[9,10,17]

(2) NADH Oxidation and Electrode Pretreatment. It is well-known that pretreatment of glassy carbon can alter markedly and specifically its electrochemical activity. There has been increasing interest in optimizing empirically the pretreatment of glassy carbon electrodes, and several procedures have been proposed recently in the literature.[15,18-20]

In our case, it was absolutely essential to preoxidize the electrode surface in a rather drastic manner in order to produce the superficial carboxylic groups which are required for the immobilization of the enzyme (see further in the text). Also, the enzyme loses its activity, once it is bound to the electrode surface, when the electrode potential becomes negative or more positive than 0.9 V SCE. Therefore, we had to cope with these two constraints and the further complication that the various types of RVC available behave differently.

The following preliminary experiments were done with "bare" electrodes, i.e., without immobilized enzymes, and the production of surface carboxylic groups when specified was ascertained by the fact that enzymes could be bound (see next section) to the surface of identical electrodes having had the same history.

When we used "standard RVC" we found this material to be very sensitive to oxidation. For example, chemical preoxidation, by dipping the electrode in the $HNO_3/K_2Cr_2O_7$ solution (see Experimental Section), was sufficient to form surface carboxylic groups, but the current resulting from subsequent electrochemical oxidation of NADH (later called NADH current) was not at all reproducible and decreased dramatically with time (20% per h).

After electrochemical preoxidation, which is more efficient, the NADH current was halved. It could be restored when the preoxidation was followed by an electrochemical pretreatment similar to the one proposed by Blaedel and Wang.[15] However, the time dependence of this anodic current could not be improved. Finally we gave up working with "standard RVC" since we had not been able to obtain reproducible and satisfying results and we chose to use only the "RVC 4" type of material.

It is not possible to form surface carboxylic groups without electrochemical preoxidation of "RVC 4". After preoxidation the NADH current is rather small, but it becomes more than ten times greater and reaches a convenient level after a pretreatment of the Blaedel and Wang type. After such a pretreatment a decrease in NADH current of about 20% per day is still observed, the electrode being kept under working conditions (i.e., oxidizing NADH) or at rest in the background solution. This progressive loss of activity of the electrode surface with time is probably related to the specific behavior of that kind of glassy carbon since it has not been observed with TOKAI or VITRECARB glassy carbon disk electrodes, at least when the surfaces of those electrodes were coated with adsorbed NAD^+.[20,21] The reduction of NAD^+ at "RVC 4" electrodes can be observed only at a potential at which the background current is rather large. Thus only very poorly defined current–potential curves are obtained by means of cyclic voltammetry, the technique which was used for measuring the amount of adsorbed NAD^+ at coated TOKAI and VITRECARB glassy carbon disks. Therefore it has not been possible to carry out a systematic study of the adsorption of NAD^+ at "RVC 4" electrodes which could bring useful information concerning the specific behavior of "RVC 4".

The preceding results show that the surface structure depends markedly on the type of glassy carbon which is used and that the choice of the electrode material is obviously of fundamental importance. The preliminary experiments also led us to attach enzymes to electrode surfaces which were first electrochemically preoxidized and secondly pretreated according to the procedure proposed by Blaedel and Wang.[15]

(3) Enzyme Immobilization. Various procedures have been reported in the literature in order to immobilize enzymes directly on graphite[9,6] or glassy carbon[8,10,14,17,22,23] electrodes. They can be summarized as follows: a surface functional group (carboxyl or hydroxyl) is activated by carbodiimide or cyanuric chloride before reaction with peripheral amino groups of the protein. In the delicate case of dehydrogenase immobilization, we found it necessary to develop an enzyme homopolymer on the electrode surface so as to maintain the tetrameric active structure of the enzyme. This problem was solved by simultaneous activation and immobilization in the presence of a low concentration of carbodiimide.[10]

However, many questions remained unanswered, concerning in particular the optimal size of the immobilized homopolymer with respect to the enzymatic activity, the enzymatic yield of the coupling, and the possible masking of the electrode surface by the attached protein. The use of "RVC 4" electrodes can bring some answers to light.

As the homopolymer molecular weight can be controlled by the time of reaction between the enzyme and the cross-linking reagent,[24] the "RVC 4" electrode was dipped into a mixture of LDH (1 mg/mL) and carbodiimide (1 mg/mL) for six different durations varying from 0 to 15 h. Then a technique was developed to assay the total quantity of immobilized protein. That was not an easy task because such a quantity is very small. For example, one layer of LDH (assumed to be a 6.8 nm diameter sphere, M = 140 000) covering statistically 1 cm^2, the covered fractional area being 0.50,[25] amounts to 2.3 pmol or 0.32 μg of protein per cm^2.

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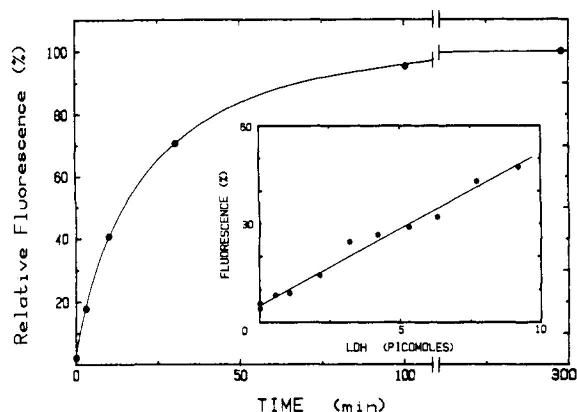

Figure 2. Progress of LDH hydrolysis measured in percent of fluorescence (corrected for blank) as a function of time. The amplification factor is given by the ratio of the fluorescence at t = 300 mn over the initial fluorescence. We found 110. The insert gives the standard curve for fluorimetric assay of hydrolyzed protein, the x axis indicating the amount of LDH (picomoles) for each assay. Hydrolysis was carried out in 6 N HCl at 100 °C.

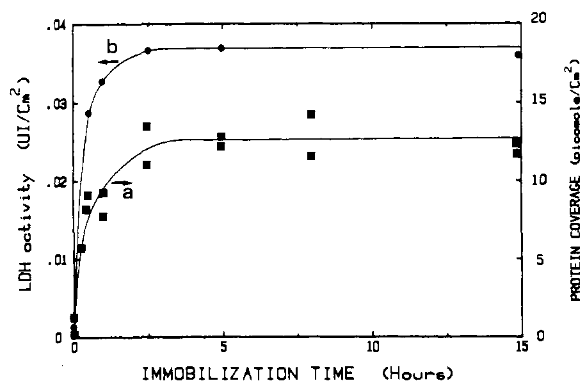

Figure 3. Comparison between the plots of enzymatic activity (curve b) and protein coverage (curve a) of a "RVC 4" electrode vs. the immobilization time. Both carbodiimide and LDH concentrations were 1 mg/mL in 0.02 M phosphate buffer at pH 7.5. The enzymatic activity was measured spectrophotometrically with 5 × 10^{-3} M NAD^+ and 0.1 M lactate in 0.1 M glycine buffer at pH 9.0. The electrode rotation frequency = 2000 rpm.

Wilson and co-workers[9] assayed radioactively labeled (iodinated) glucose oxidase in the 10–100 pmol/cm^2 concentration range. The sensitivity of the technique we developed is about 1–2 pmol/cm^2. The principle of the method lies in performing the hydrolysis of the protein so as to amplify the amount of amino acids which can be titrated. Also, the proteins are released from the electrode surface at the same time as they are hydrolyzed. In tetrameric LDH the total number of amino acids is 1280[26] while there are no more than 20 peripheric lysine residues. Therefore the amplification factor can amount to at least 60. Actually we found more than 100 as can be seen in Figure 2. The assay of amines was performed according to the very sensitive fluorimetric procedure proposed by Udenfriend and co-workers.[27] The maximum coverage is 13 ± 1 pmol of LDH/cm^2. Assuming that this coverage is realized through a stacking of monolayers and that the coverage due to each monolayer still amounts to 2.3 pmol/cm^2 as calculated before in the text, the total thickness of the enzyme layer is ca. 40 nm, i.e., 6 times that of a monolayer. A polymerization degree of 6 is consistent with the immobilized layer structure at maximum coverage, the corresponding molecular weight of the soluble polymer being 8 × 10^5. Of course all the

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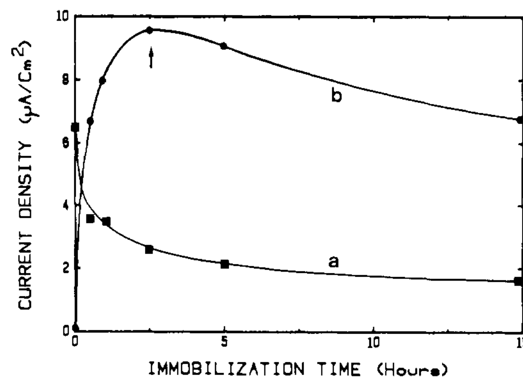

Figure 4. NADH current measured at 0.7 V SCE as a function of immobilization time. Curve a: 4.76 × 10^{-5} M NADH was introduced in the reactor without lactate, i.e., there was no enzymatic reaction occurring. Curve b: 2 × 10^{-2} M lactate and 5 × 10^{-4} M NAD^+ were initially introduced and NADH was enzymatically produced at the interface electrode/solution. Same conditions as in Figure 3 except for the rotation frequency which was 500 rpm. the plotted current is corrected for background (about 1 µA).

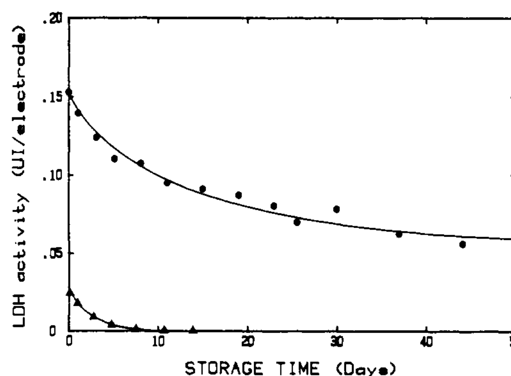

Figure 5. Inactivation curve for the immobilized LDH-RVC electrode (●). Storage conditions: temperature 4 °C, 0.2 M phosphate buffer, pH 7.5. The spectrophotometric determination of activity also provides a means of measurement of the amount of non-covalently immobilized protein which desorbs (see Figure 6). This slight leakage (▲) is observable only within a few days.

preceding values are averages, the true local values depending on the reticulation of the protein layer. Independent molecular weight measurements by means of gel filtration of soluble polymers prepared in similar conditions[10] gave results which are in good agreement since they ranged from 5 × 10^5 to 10^6.

The immobilization yield in catalytically active proteins can be deduced from the knowledge of the enzymatic activity (Figure 3). Experimental conditions for the measurement of enzymatic activity were chosen in such a manner that external diffusional constraints could be neglected, namely both the substrate concentrations and the rotation speed were large. The immobilization yield found to be 15 ± 2% is correct for dehydrogenase.[28] The maximal catalytic flux J_M = 7 × 10^{-10} mol cm^{-2} s^{-1}, a level of activity which is higher than that obtained with the same enzyme immobilized on a glassy carbon disk since a value of 2 × 10^{-10} was determined electrochemically for J_M in the latter case.[10] Once again such results reveal the variations in the vitreous carbon surface. On scanning electron microscopy, the surface of RVC looks very smooth and without holes whereas the surface of a glassy carbon disk is pitted irregularly with holes of diameter around 1 µm. As a result, the preoxidation of RVC is probably more regular, and the number of accessible carboxylic groups per unit surface area is enhanced accordingly. That may explain why a progressive masking of the electrochemical activity by immobilized proteins appears clearly in Figure 4 (curve a) and is not observable in the case of a glassy carbon disk. This masking which

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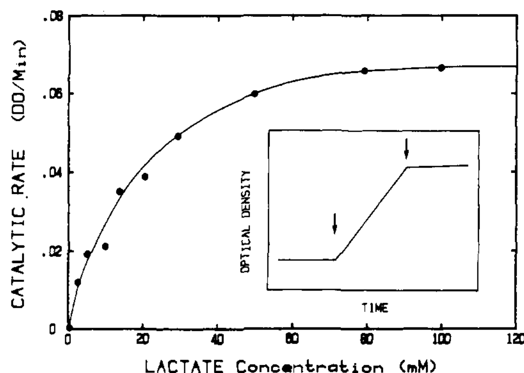

Figure 6. Spectrophotometric determination of LDH kinetics, LDH being immobilized on an RVC electrode. Initial NAD^+ concentration was 5 mM, the volume of the cuvette was 3.5 cm^3, and the electrode area was 3.2 cm^2. The insert shows how an experiment is carried out. First arrow: the rotated electrode is put in the cuvette. Second arrow: the electrode is taken out and possible enzyme leakage is then measured.

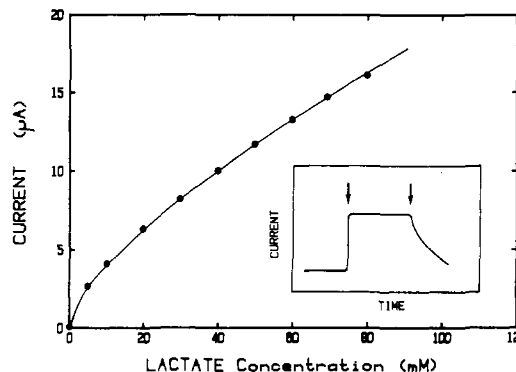

Figure 7. LDH kinetics. Electrochemical measurements were performed with an immobilized LDH–RVC electrode. The electrode potential is +0.7 V SCE, and the electrode area is 2.9 cm^2. The insert reproduces the experimental observation after stabilization of the backgound current in 0.1 M glycine buffer at pH 9, 0.1 M KCl, and 5 mM NAD^+ and lactate is injected (first arrow). The current jump results from the local production of NADH. The second arrow indicates the beginning of rinsing.

causes the decay of the oxidation current of exogeneous NADH becomes logically greater with increasing protein coverage. Unfortunately, it has not been possible to deduce the electrochemical rate constant and the diffusion coefficient of NADH from this kind of measurement due to the lack of understanding of the hydrodynamics, as mentioned earlier.

Curve b in Figure 4 demonstrates that optimization (arrow) can be achieved through a compromise between the electrochemical and the enzymatic activities. The long-term stability of the immobilized enzyme can be monitored by periodic spectrophotometric determination of the activity (Figure 5). The observed half-life of 3 weeks corresponds to the already reported thermal inactivation of immobilized dehydrogenase.[10,28]

(4) Enzyme Kinetics of Immobilized LDH–RVC Electrodes. When the enzyme is immobilized on electrodes of large areas such as RVC electrodes, two independent methods can be used to measure LDH activity. The spectrophotometric method consists of the classical measurement of the initial rate of appearance of NADH in the bulk solution (Figure 6). In this case the electrode hydrodynamics are not important provided that the solution is correctly stirred and the electrode rotation speed high enough to suppress the effects of external diffusional limitation for the substrates.

Experiments show that the initial rate does not depend on the electrode rotation frequency when the latter is greater than 1000 rpm. Under such conditions the kinetic constant found for immobilized LDH is identical with that found when LDH is not immobilized since we determined an apparent Michaelis constant of 1.5×10^{-3} M for lactate, NAD^+ concentration being 5 mM, instead of 1.4×10^{-3} M when the enzyme is not immobilized. Consequently, no appreciable *internal* diffusional limitations occur within the layer of immobilized enzymes. Simple theoretical considerations can justify this result if the enzyme layer behaves like an enzymatic membrane. Then the dimensionless parameter σ, used for evaluating the diffusional constraints inside the membrane, is[29] $\sigma = V_M e^2/K_M D$ where V_M is the maximal enzymatic rate per volume unit of membrane (V_M being ca. 10^{-6} cm) and D is the diffusion coefficient of lactate inside the membrane. D in the protein layer cannot be measured, but even if it is 10 times smaller than in the solution, let us say ca. 10^{-6} cm^2 s^{-1}, σ is still very small (ca. 10^{-3}). This allows diffusional hindrances in the enzymatic layer to be ignored.

The electrochemical oxidation of interfacial NADH is also a potential means to determine the enzymatic activity of immobilized LDH.[10] However, the effects of complex hydrodynamics appear clearly in the results (Figure 7). The current cannot be linked directly to the enzymatic rate since the curve drawn in Figure 7 is not Michaelian. As long as a convenient model for the hydrodynamics of the rotated RVC electrode is not available, it will not be possible to produce a theoretical analysis of the coupling between enzymatic and electrochemical reactions. Such a theoretical study was performed earlier with rotated-disk electrodes.[8-10] In the present work, we were mostly concerned with the mass balance in the bulk solution.

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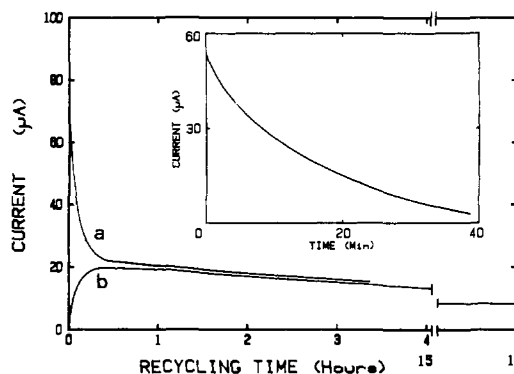

Figure 8. Controlled-potential electrolysis, NADH current–time plot. Curve a: initial injection of NADH. Curve b: initial injection of NAD^+. Experiment b was carried out right after experiment a, with the same electrode. The electrode potential is 0.7 V SCE, the electrode surface is 7.5 cm^2, the J_M measured spectrophotometrically is 3.5×10^{-10} mol cm^2 s^{-1}, the rotation frequency is 500 rpm, the reactor volume is 2.7 cm^3, with 0.1 M L-lactate in 0.1 M glycine buffer at pH 9 and 0.1 M KCl, and the initial concentration of NADH (a) or NAD^+ (b) is 6.85×10^{-5} M. The insert shows the curve obtained under identical conditions but without lactate. All currents are corrected for background.

(5) NAD^+ Regeneration at the Immobilized LDH–RVC Electrode. When the enzymatic reaction does not take place, 90% of NADH is electrochemically oxidized within 40 mn (Figure 8, insert). Classical logarithmic analysis of the current–time curve[30] or Coulometric measurements show that two electrons per molecule of NADH are transferred. With respect to the production of enzymatically active NAD^+, the current efficiency is close to 100% but its determination, following only one electrochemical regeneration, cannot be performed with sufficient accuracy to ascertain that repetitive regeneration (cycling) will give satisfactory yields.[31]

Results of typical experiments with continuous interfacial regeneration of NAD^+ are shown in Figure 8. Each curve (a and b) consists of two parts. For times greater than 0.5 h, curves a and b are superimposed, a steady state like current is obtained, and electrochemical and enzymatic fluxes are equal. Transient

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currents depending on the initial conditions are measured at shorter times. For example, when NAD^+ is introduced alone initially, the rate of the enzymatic production of NADH exceeds that of the electrochemical regeneration of NAD^+ and the interfacial NADH concentration (therefore the NADH current) increases accordingly till the steady state is reached. Simulation of these transient currents is not beyond possibility within the framework of theoretical models of controlled-potential electrolysis with coupled chemical reactions.[30] However, the mathematical developments are rather cumbersome due to the complexicity of the enzymatic rate constant, and they will be presented elsewhere.

The steady-state current should be time independent provided that the three following conditions are fulfilled: the enzymatic rate is constant, i.e., the rate of the backward reaction can be neglected; the electrochemical oxidation of NADH yields 100% enzymatically active NAD^+; and fouling of both the enzyme layer and electrochemical interface does not occur significantly.

Concerning the first point, we chose to operate with a high lactate concentration and a relatively low NAD^+ (or NADH) concentration in order to minimize the effect of pyruvate accumulation on the catalytic rate. After 16 h and $P = 18$ regenerating cycles (experiment b, Figure 8), the pyruvate concentration is 1.2 mM and the lactate concentration is practically unchanged. P can be deduced from the Coulometric measurements since P is given by the equation $P = Q/2\ FVC_i$, where Q is the number of coulombs which have been transferred, C_i the initial NAD^+ concentration, and V is the volume of the reactor. The influence of pyruvate concentration on the initial enzymatic rate has been examined in other experiments with non-immobilized enzyme. The decrease in the catalytic rate was found to be 18% when increasing the pyruvate concentration up to 1.2 mM. Therefore part of the steady-state current decay can be due to the occurrence of the backward enzymatic reaction. However, this phenomenon cannot account for more than one-third of the current decay.

To test the second point, we used an enzymatic method (see Experimental Section) for determining the ratio N_F of the sum of enzymatically active NAD^+ and NADH remaining at the end of the experiment over the quantity of cofactor originally introduced. The yield per regenerating cycle is then $R\ (\%) = 100(P - 1 + N_F)/P$. In the case of experiment b, Figure 8, we found $N_F = 0.78$ after 16 h and thus $R = 98.8\%$. At pH 9 the thermal denaturation of NAD^+ and NADH can be evaluated according to procedures described elsewhere.[32] It amounts to 0.32% per h for NAD^+ and the denaturation of NADH is comparatively insignificant. The time scale of a regenerating cycle is roughly 1 h, and under kinetic control 75% of the cofactor exists in the NAD^+ form. That gives a thermal deactivation of 0.2% per cycle. Consequently, the efficiency of the electrochemical regeneration of NAD^+ is 99.0%. This value is in good agreement with that obtained by Jaegfeld and co-workers,[31] who separated the reactors in which the enzymatic and the electrochemical reactions took place. As pointed out before by several authors,[18,31] the pretreatment of the electrode surface can improve the yield of enzymatically active NAD^+. In the present work the electrode pretreatment was optimized with the sole purpose of attaining high and stable NADH current densities and, of course, our pretreatment may not be the best suited to obtaining a good yield of enzymatically active NAD^+. This point is important because a yield of 99.0% provides the potential for transforming an amount of lactate only 100 times greater than that of cofactor initially introduced in the reactor. The ability to perform 10^4 cycles is a requisite for the economically valuable syntheses of chemical compounds. There is still a great deal to do but the simplicity of our experimental manipulations should permit a significant contribution to be made.

Concerning the third point, aging of the immobilized enzyme electrode is likely since the decrease of the steady-state current cannot be justified only by the occurrence of the backward enzymatic reaction (which can account for only 18% of the decay) and the inactivation of NAD^+ (22%). In the field of enzyme technology, it is well-known now that the stability of the enzyme under operational conditions differs from that observed under conditions of storage.[33,34] Likewise changes of the electrochemical activities of chemically modified electrodes during long-time experiments have already been reported.[35] Further studies will be aimed at improving experimental approaches to these problems.

Conclusion

The RVC electrode with immobilized LDH constitutes a minireactor which is compact and easy to handle. It provides a means of analysis of the parameters conditioning the progressive denaturation of the cofactor during its electrochemical regeneration. An accurate determination of these parameters is a requisite for considering further steps in the elaboration of procedures for the syntheses of complex molecules.

The technique described in this paper is relevant to that purpose for the following reasons: it is possible to perform a number of cycles of regeneration of the cofactor which is large enough to allow a precise determination of the yield of the electrochemical regeneration and, the regeneration occurs in the microenvironment of the enzyme with consequent kinetic advantages.[10]

In addition, the working area/geometrical volume ratio of the electrode used in this work is small but it can be increased considerably (several orders of magnitude) as, for example, in the case of a flow-through electrode, and the immobilization of the enzyme renders the separation procedures easier but once again the determination of the yield of regeneration is crucial since the aim should be to produce inside the flow-through electrode the number of regenerating cycles corresponding to the exhaustion of NAD^+ initially introduced in the reactor (i.e., to its total denaturation).

Our technique can also be applied to other enzyme/cofactor couples, and besides the development of our work concerning the electrochemical regeneration of NAD^+, we intend to generalize the use of such a technique to other oxidoreductases and other cofactors.

Experimental Section

(1) Preparation of RVC Electrodes. Both "standard RVC" and "RVC 4", 100 pores per in., were obtained from Fluorocarbon Process Systems Division of Anaheim, CA. Cylinders 5 mm in diameter and heights ranging from 1 to 9 mm were carved in the RVC block. Each cylinder was cemented with epoxy adhesive to a glassy carbon disk electrode prepared according to a procedure already described.[15] Before use, the electrode was washed thoroughly in an ultrasonic cell and the electric resistance of the contact was checked (less than 10 Ω).

(2) Electrode Preoxidation and Pretreatment. For electrochemical preoxidation the rotated (1000 rpm) electrode was maintained in the nitrochromic solution (HNO_3 10% + $K_2Cr_2O_7$ 2.5%)[8] during 20 s, the controlled anodic current density being 80 mA cm^{-2}. The electrodes were conditioned and pretreated following the procedure of Blaedel and Wang:[15] i.e., three cathodic (−1.25 V SCE) − anodic (+1.25 V SCE) cycle (scan rate 0.1 V s^{-1}) in deaerated 0.2 M phosphate buffer at pH 7.5 plus 0.1 M KCl with 2-min waiting times at each extremity of the potential scan. For the electrochemical oxidations of both NADH and $Fe(CN)_6^{4-}$ the pretreatment ended at positive potentials.[19] Before measurement, the background current was stabilized, typically by waiting 0.5 h.

(3) Measurements of Surface Areas and Hydrodynamics. The determination of the electrode area was performed by means of cyclic voltammetry at the stationary RVC electrode.[36] For the $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ redox couple the separation between the anodic and cathodic peaks is 90 mV (scan rate 100 mV s^{-1}). Thus the electrode area was deduced from the expression of the peak current in the case of irreversible electron transfer, the difference between the anodic peak potential and the potential at half-peak height being used for the determination of the transfer coefficient.[36] Various experiments with solutions of ferrocyanide or ferricyanide in the 10^{-5}–10^{-4} concentration range and scan rates from

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50 to 150 mV s^{-1} gave data with a dispersion of only 2% for the effective area, the diffusion coefficients of ferricyanide and ferrocyanide being taken as 6.93 and 6.56 $cm^2 s^{-1}$, respectively.[37]

The hydrodynamic behavior of the rotated RVC electrode was tested as follows: after pretreatment the rotated electrode was maintained in a large volume of background solution (50 cm^3) at a controlled potential corresponding to the oxidation of ferrocyanide till the background current reached a steady state and then an aliquot of stock solution of ferrocyanide was injected and the current jump rapidly measured in order to avoid ferrocyanide consumption. The electrode rotation frequencies ranged from 200 to 3000 rpm. A variable-speed Tacussel EDI motor with a Tacussel CONTROVIT controller was used to rotate the electrode.

(4) Immobilized Protein Assays. Hydrolysis of native LDH and fluorimetric assays of the amino groups were used as standards. For hydrolysis, 50-μL aliquot of a 4 mg cm^{-3} stock solution were added to 2-cm^3 samples of 6 M hydrochloric acid and the resulting solutions were held at 99 °C for 5 h (or various durations as shown in Figure 2) in closed test tubes. The assay of immobilized LDH is of interest only if the corresponding electrode area is known. Therefore we proceeded as follows: RVC cylinders 7 mm high were weighed before gluing and their areas were determined as described above. A typical result was 450 $cm^2 g^{-1}$. After immobilization the electrode was rinsed thoroughly (final rinsing with water). Cylinders 3 mm high were cut at the bottom of each electrode, dried during one night, and weighed. The RVC areas were deduced from the weights. Hydrolysis of each piece was carried out in 200 μL of 6 M hydrochloric acid under the same conditions as with native LDH. After being cooled the solution was neutralized with 100 μL of 12 M sodium hydroxide; 50 μL of hydrolysate (i.e., between 1 and 10 pmol of LDH) and 1.5 cm^3 of 0.2 M borate buffer pH 9 were introduced in a test tube. After rapid addition of 0.5 cm^3 of fluorescamin solution (14 mg in 50 cm^3 of acetone) and vigorous mixing, the fluorescence was measured.

Fluorimetric assays[27] were performed with a JY 3 JOBIN-YVON spectrofluorimeter (excitation wavelength 366 nm; emission 470 nm). For standardization we used a solution of quinine sulfate (0.1 mg L^{-1}) in 0.1 M sulfuric acid. Due to the high sensitivity of the method, extra precautions were essential for cleaning glassware and filtering solutions (on 0.22-μm MILLIPORE membranes).

(5) Spectrophotometric Measurements. The absorbance due to the production of NADH was measured at 340 nm ($\epsilon_{NADH,340}$ = 6220 $M^{-1} cm^{-1}$) in order to determine the enzymatic activity. Typically the reaction mixtures contained initially 3.8 cm^3 of 0.1 M glycine buffer at pH 9 plus 0.1 M KCl plus 5 mM NAD^+ plus lactate at various concentrations. The rotated electrode dipped only in the top region of the solution. We used a HEWLETT-PACKARD (type 8450 A) absorption spectrophotometer, and the measurements were not altered by light from the surroundings.

In order to assay enzymatically active NAD^+ in the presence of lactate and pyruvate, we had to operate according to a procedure avoiding lactate and pyruvate interferences. Therefore we used a reagent consisting of alcohol dehydrogenase (2 units cm^{-3}) and ethanol (1%) in glycine-buffered background. Under such conditions more than 95% of the enzymatically active NAD^+ is transformed into NADH in 10 min, and the total amount of NADH can then be deduced from the absorbance of the solution at 340 nm.

(6) Electrochemical Measurements. The reference electrode was a saturated KCl calomel electrode and the counter electrode was a platinum foil. Both were put into different compartments communicating with the working electrode compartment through glass frits. Cells with two distinct working electrode compartment volumes were used: 2.7 cm^3 for recycling experiments and 50 cm^3 for other purposes. The water-jacketed cells were thermostated at 25 °C. Electrochemical measurement of immobilized enzyme activity was realized as already described in the case of a disk electrode (Figure 7 and ref 10), but due to the lack of hydrodynamic model, comparisons can be established only at fixed rotation frequency (in general 1700 rpm).

(7) Chemicals. NAD^+ and NADH (grade III), LDH (type XI), alcohol dehydrogenase (A 3263), and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methoxy *p*-toluene sulfonate (C 1011) were obtained from SIGMA. Fluorescamin, l-lactate, and pyruvate were obtained from FLUKA. Other reagent grade chemicals were from CARLO ERBA. All chemicals were used as received.

Registry No. LDH, 9001-60-9; NAD^+, 53-84-9; NADH, 58-68-4.

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Molecular Association and Monolayer Formation of Soluble Phthalocyanine Compounds

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Abstract: This paper reports on synthesis, association in solution, and Langmuir–Blodgett monomolecular layer formation of H_2, Mg, Co, Ni, Cu, Zn, Pd, Pt, Bi, and Pb phthalocyanine compounds with cumylphenoxy peripheral substitution on each benzo ring at either the 2- or 3-position (tetracumylphenoxy-substituted isomer mixture). The degree of association is dependent on the identity of the complexed ion and varies from monomeric for the Pb compound to tetrameric for the Pt phthalocyanine as determined by vapor pressure osmometry measurements on 10^{-2}–10^{-3} M toluene solutions. The phthalocyanine compounds form stable Langmuir–Blodgett monomolecular layers to film pressures greater than 20 mN/m. The force–area curves indicate that the phthalocyanine units are densely packed with a cofacial orientation. Electronic spectra of solutions and X-ray diffraction patterns from bulk solids indicate that the phthalocyanine rings are cofacially oriented within an aggregate structure. The complexed metals (Ni, Pd, Pt) inducing the highest degrees of association in solution also yield correspondingly larger areas per phthalocyanine unit in the monolayer.

Phthalocyanine compounds continue to be the subjet of increasing research activity.[1] As thin films, these compounds display properties of particular current interest such as electrical conductivity,[2] electrical switching between conducting states,[3] photovoltaic effects,[4] oxidative catalytic activity,[5] and electrochromism,[6] which can be modified by interactions with chemical vapors,

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