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Electrochemical investigations of the reaction mechanism and kinetics between NADH and riboflavin immobilised on amorphous zirconium phosphate

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Abstract Electrochemical investigations of the reaction mechanism and kinetics between riboflavin immobilised on zirconium phosphate (ZPRib) in carbon paste and NADH showed results yielding reliable information about aspects on the mechanism of the electron transfer reaction between the flavin and NADH. The formal potential ($E^{\circ\prime}$) of the adsorbed riboflavin was −220 mV versus SCE at pH 7.0. A shift about 250 mV towards a more positive potential compared with its value in solution was assigned to the interaction between the basic nitrogen of riboflavin and the acid groups of ZP. The invariance of the $E^{\circ\prime}$ with the pH of the contacting solution and the effect of different buffer constituents were attributed to the protection effect of ZP over the riboflavin. The electrocatalytic oxidation of NADH at the electrode was investigated using cyclic voltammetry and rotating disk electrode methodology using a potential of −50 mV versus SCE. The heterogeneous electron transfer rate constant, k_{obs}, was 816 M^{-1} s^{-1} and the Michaelis-Menten constant, K_M, was 1.8 mM (confirming a charge transfer complex intermediate in the reaction) for an electrode with a riboflavin coverage of 6.8×10^{-10} mol cm^{-2}. This drastic increase in the reaction rate between NADH and the immobilised riboflavin was assigned to the shift of the $E^{\circ\prime}$. A surprising effect with addition of calcium or magnesium ion to the solution was also observed. The $E^{\circ\prime}$ was shifted to −150 mV versus SCE and the reaction rate for NADH oxidation increased drastically.

Key words Riboflavin · NADH · Zirconium phosphate · Chemically modified carbon paste electrode

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Introduction

Investigations about biological electron transfer reactions have been widely exploited for a long time [1–4]. However, there are still many unanswered questions about these reactions, as the answers previously given are not sufficiently clear until the present day [5]. One of the most essential of all biological electron transfer reactions is the electron transfer reaction between NADH and flavoproteins, e.g., in the mitochondrial electron transfer chain. It is still debated whether the charge from NADH to the flavin is donated as a single hydride or involves separate individual electron transfer steps. Many electrochemical investigations on the reaction mechanism and kinetics in solution or with a flavin modified electrode have been made, but did not reveal sufficiently clear data to explain the mechanisms [6–9]. A major obstacle is that the difference between the $E^{\circ\prime}$ values of NADH and free flavins is not large enough to allow a rapid reaction sufficient to obtain reliable results about the mechanism, at least with electrochemical methods [10]. Several attempts to increase the reaction rate between flavins and NADH have been made, derivatising the flavins [11–13]. Another alternative is the immobilisation of flavins on matrices to change the electronegativity of its surroundings [14].

Through the development of new modified electrodes, various alternative ways have been shown how to change the $E^{\circ\prime}$ of a reactant/catalyst as a result of the immobilisation process [15, 16]. Different electrode materials have been employed such as various carbon electrode materials including carbon paste, gold, platinum, clays, silica, etc. [17–22]. The electrode material many times affects the $E^{\circ\prime}$ (or the midpoint potential, E_m) of the electroactive species [23], and the stability depends on the immobilisation procedure [24]. Many attempts have been made to shift the $E^{\circ\prime}$ of electroactive compounds to fall into ranges denominated as ideal, −100 up to 0 mV for biosensors purposes [23, 25], and to increase the electrocatalytic activity [26].

In a recent publication [27], two phenoxazine and phenothiazine derivatives (Nile blue and methylene blue), commonly used as mediators in bioelectrochemistry, were immobilised on new electrodes materials based on transition metal phosphates. When mixed into carbon paste they revealed drastic shifts in their $E^{\circ\prime}$ values towards more positive values. Moreover, there was no variation of the $E^{\circ\prime}$ in the entire pH range between 1 and 9 and these electrodes showed a high electrocatalytic activity for NADH oxidation. Immobilisation of flavins onto this electrode material was thus performed in order to investigate the effect on the $E^{\circ\prime}$ on this class of organic redox compounds and also the possible use for efficient electrocatalytic oxidation of NADH. A description of the preparation of zirconium phosphate, immobilisation of riboflavin and some electrochemical investigations of the reaction mechanism and kinetics between NADH and riboflavin immobilised on zirconium phosphate are reported.

Experimental

Synthesis of zirconium phosphate

Zirconium phosphate (ZP) was synthesised according to a previously reported method [28]. A solution of 1 M phosphoric acid (Merck, Darmstadt, Germany) was slowly added onto 500 mL of zirconium chloride (Fluka, Buchs, Switzerland) prepared in 0.1 M HCl, until complete precipitation. The gelatinous precipitate was washed with distilled and demineralised water (Milli-Q quality) and dried in an oven at 80 °C. The resulting solid was ground until the particle size became smaller than 0.05 mm, drizzled in a sieve.

Riboflavin immobilisation onto ZP

To 50 mL of an aqueous solution of 0.001% (w/v) riboflavin (Sigma, St. Louis, Mo., USA), 50 mg of ZP were added. The mixture was shaken for 1 h and the resulting strongly yellow coloured solid was filtered and washed 10 times with deionised water. Finally the solid with adsorbed riboflavin was dried at 50 °C for 30 min [27]. This material will be hereafter designated as ZPRib.

Supporting electrolytes

The supporting electrolyte used in the electrochemical cell was in most cases a solution of 0.1 M KCl (Merck). The solution pH was adjusted to a desired value through additions of HCl or KOH. Buffer solutions of Hepes (Sigma), Tris (Aldrich, Milwaukee, Wis., USA), Pipes (Sigma) and phosphate (Merck) in a concentration of 0.1 M were also used to investigate the effect of various buffer constituents.

NADH solution

Solutions of NADH (Sigma) were always freshly prepared before use in the appropriate buffer. The actual concentration was evaluated by monitoring the absorbance of the solution at 340 nm, considering a molar extinction coefficient of 6600 cm M^{-1} [29].

Preparation of modified carbon paste electrode

The carbon paste electrode was prepared through thorough mixing 50 mg of graphite (Fluka), 50 mg of ZPRib, and two drops ($\approx$25 µl) of paraffin oil (Fluka) in a mortar. This paste was put into a cavity of a home-made rotating pyrolytic graphite disk electrode holder mounted to fit a rotating electrode device (Princeton Applied Research, Boston, Mass., USA, model EG/G 636).

Electrochemical measurements

An electrochemical analyser (Bioanalytical Systems, West Lafayette, Ind, USA, model BAS 100 W) connected to a PC for potential control and data collection was used for all electrochemical experiments. All the measurements were carried out using a typical three-electrode electrochemical cell, where a saturated calomel electrode (SCE) served as the reference, a platinum foil as the auxiliary and the modified carbon paste as the working electrode. All measurements were performed after bubbling the solution in the cell with pure nitrogen for 20 min.

Results and discussion

Riboflavin is an important biological compound, mainly due to its role in electron and proton transfer processes in the living system [2]. The structure of flavins has been well known for a long time and many papers have been published on investigations on the mechanism of the catalytic processes involving flavins [2, 3, 5, 10, 11, 13, 30–34]. The electrochemistry of flavins both in solution and when immobilised on electrode surfaces have been reported [2–4, 8, 9, 34]. However, the immobilisation of any flavin on inorganic material such as zirconium phosphate (ZP) has not been reported yet. ZP is well know as a good ionic conducting material and it is strongly acidic [28]. In a previous paper [27], the immobilisation and basic electrochemistry of methylene blue and Nile blue on ZP were described. The immobilisation was regarded as a simple ion exchange process with the proton of the phosphate groups from the ZP surface. However, in the present case, analysing the riboflavin structure presented in Fig. 1, it is hard to consider the immobilisation as a simple charge interaction process. Probably a stronger interaction should be involved in the immobilisation process, because riboflavin did not leach out even in acid solution and the ZPRib kept the same yellowish colour.

Cyclic voltammograms (CVs) obtained for ZP (Fig. 2, curve A) and ZPRib (Fig. 2, curve B) in pure

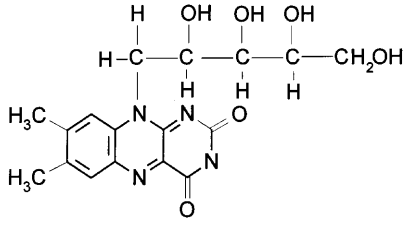

Fig. 1 Structure of riboflavin

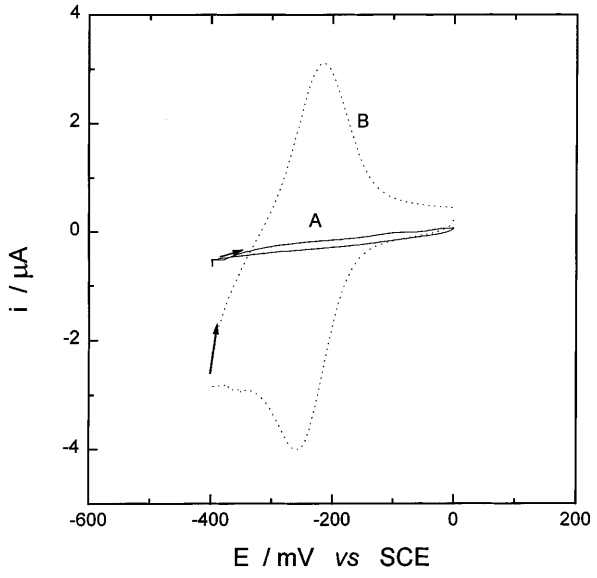

Fig. 2 Cyclic voltammograms obtained for ZP (*A*) and ZPRib (*B*) modified carbon paste electrodes at a scan rate of 20 mV s^{-1} in 0.1 M Hepes buffer, pH 7.0

electrolyte solutions show clearly that riboflavin was immobilised on ZP. The $E^{\circ\prime}$[$E^{\circ\prime}$ = (E_{pc} + E_{pa})/2] [35], where E_{pc} is the cathodic and E_{pa} the anodic peak potential, for riboflavin immobilised on ZP was −220 mV versus SCE, at pH 7.0. In solution at the same pH the $E^{\circ\prime}$ is −470 mV [33], thus reflecting a drastic change when immobilised on ZP. The CVs obtained initially for ZPRib (Fig. 3) suggest that a small quantity of oxygen remains present in the system, interfering with the shape of the expected CV (see below) because of the well-known electrocatalytic activity of flavin to reduce oxygen [34, 36]. A longer deaerating time was tested to eliminate all oxygen, but no significant improvement in the initial CVs was obtained, probably reflecting that oxygen was strongly adsorbed onto the electrode surface. Recently, Wang and coworkers [37] also showed that carbon paste contains considerable amounts of oxygen sufficient to supply an oxidase modified carbon paste with oxygen in a deaerated sensor system for prolonged periods. However, after several successive cycles (about 20) the influence of oxygen could be eliminated, indicating a good electrocatalytic activity for oxygen reduction.

The peak separation (ΔE_p) was about 45 mV at 20 mV s^{-1} and this value did not change considerably even for faster scan rates, indicating a good electron transfer rate between the flavin and the conducting material of the electrode. In Fig. 4 the dependence of the peak potentials with the scan rate is presented as well as the peak separation. The dependence of both the cathodic and anodic peak potentials with the scan rate shows no significant variation of the peak potentials with the scan rate. The small value for the ΔE_p even at 100 mV s^{-1} (≈65 mV), in contrast to those observed for methylene blue and Nile blue (250 and 115 mV at 50 mV s^{-1}, respectively) adsorbed on ZP [27], suggests a better electron transfer in the present case. Although the anodic and cathodic waves are not full images of each other, in contrast to the theoretical case for a immobilised species [35, 38], a good reversibility in the redox process can be observed as shown in Fig. 5. A more linear dependence (Fig. 6) of the peak current with the scan rate, rather than with the square root of the scan

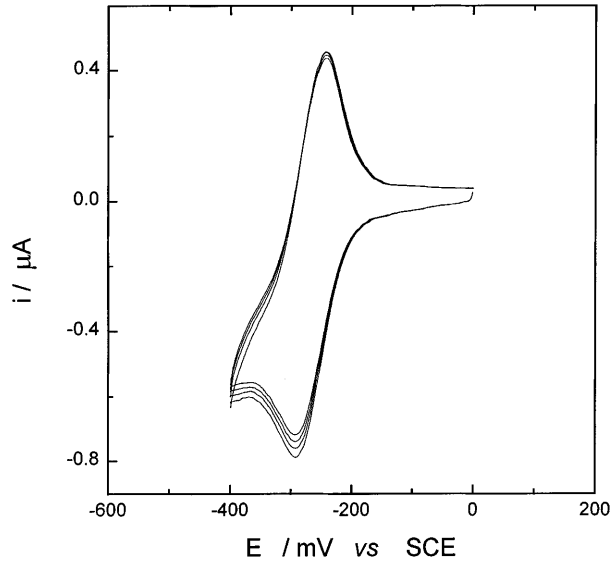

Fig. 3 Successive cyclic voltammograms obtained for a ZPRib modified carbon paste electrode at a scan rate of 20 mV s^{-1} in 0.1 M Hepes buffer, pH 7.0

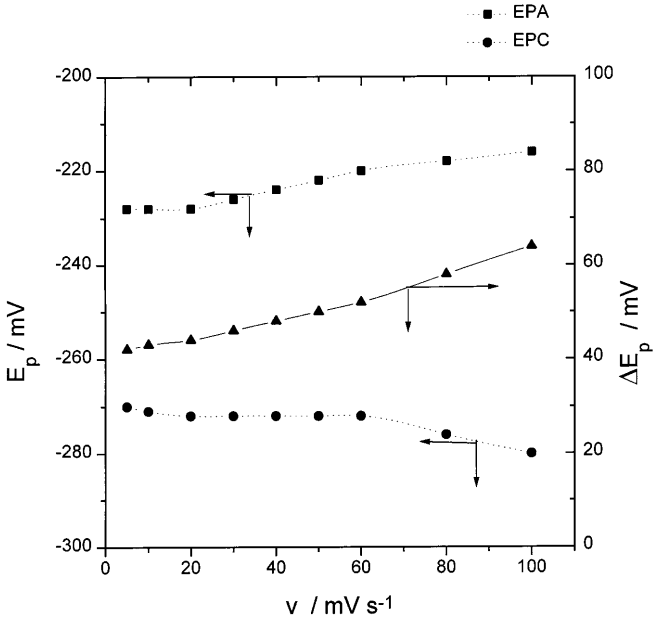

Fig. 4 Dependence of the peak potential (E_p) and peak separation (ΔE_p) of cyclic voltammograms as a function of the scan rate, obtained for a ZPRib modified carbon paste electrode in 0.1 M Hepes buffer, pH 7.0

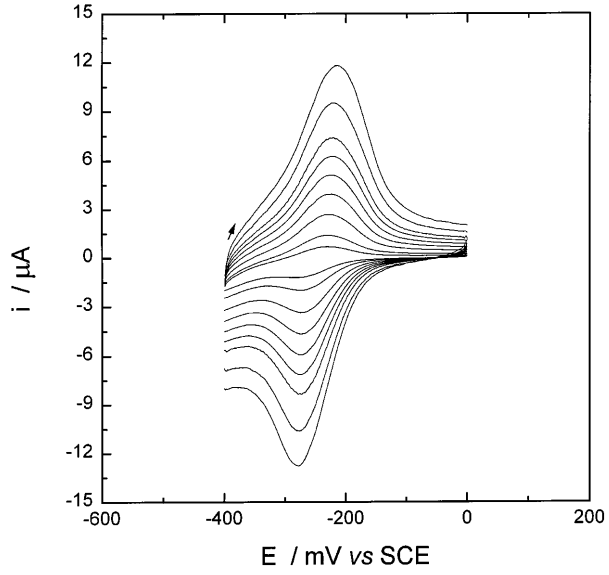

Fig. 5 Cyclic voltammograms obtained for a ZPRib modified carbon paste electrode in 0.1 M Hepes buffer, pH 7.0, at different scan rates in the sequence: 5, 10, 20, 30, 40, 50, 60, 80 and 100 mV s^{-1} from lower to higher peak current. *Arrow* indicates starting potential and sweep direction

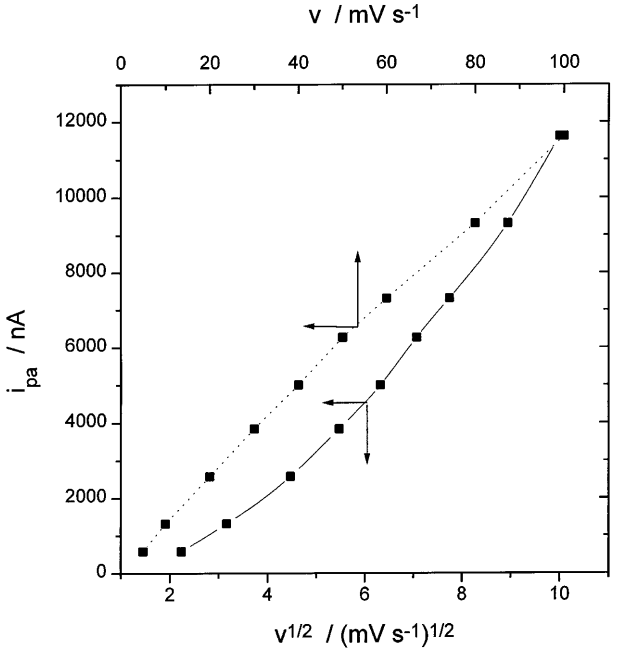

Fig. 6 Dependence of the anodic peak current (i_{pa}) on scan rate (v) and on the square root of the scan rate ($v^{1/2}$), obtained for a ZPRib modified carbon paste electrode in 0.1 M Hepes buffer, pH 7.0

rate, indicates that riboflavin is strongly adsorbed on ZP and that the electron transfer is very efficient [39].

Theory predicts that the peak width at half height, E_{fwhm}, should be $90.6/n$ for the ideal case [35, 42], where n is the number of electrons participating in the reaction. However, deviations from theory are often reported. Especially peak broadenings are very often observed for surface immobilised electroactive compounds owing to interactions between adsorbed/bound molecules [35, 42]. For low sweep rates (20 mV s^{-1}, Fig. 3) an E_{fwhm} of around 60 mV was noticed. For higher sweep rates (e.g., Fig. 5) a somewhat larger E_{fwhm} could be detected. However, the E_{fwhm} value for all investigated sweep rates (5–100 mV s^{-1}) remained less than 90.6 mV. This should possibly indicate that two electrons participate in the redox reaction, especially since it is known that two electrons participate in the redox reaction of flavins in an aqueous environment, both when dissolved [2, 3] and when adsorbed on graphite [4].

The peak current for a strongly adsorbed electroactive species is ideally given by [35, 40]:

$$I_p = [n^2F^2/4RT]\Gamma Av \quad (1)$$

where A is the surface area, v the scan rate, and Γ the coverage. The n value can be determined from Eq. (1) if the other parameters are known. Γ was determined by integrating the area under the cyclic voltammetric wave and gave a value of 6.8×10^{-10} mol cm^{-2} for an electrode with A equal to 0.12 cm^{-2} (stipulating the n value to be equal to 2). Applying these values in Eq. (1) for low scan rates (5–60 mV s^{-1}), a value of n was evaluated to be equal to 1.9 ± 0.1. This value further indicates that two electrons are transferred between the electrode and the adsorbed riboflavin during the redox process, meaning that the flavin is transferred between its fully reduced and oxidised states when running the CVs.

As argued above, a shift in the $E^{\circ\prime}$ of about 250 mV towards more positive potentials is observed, compared with that obtained for dissolved riboflavin in aqueous solutions at pH 7 [33, 41]. The shift in the $E^{\circ\prime}$ comparing the value in solution with that of the compound when immobilised or adsorbed on a surface gives evidence that the interaction forces differ between the oxidised and reduced forms with the surface [42]. It has been previously shown [23, 43] that when organic dyes such as phenoxazines are adsorbed on graphite, a shift in the $E^{\circ\prime}$ between 50 and 100 mV to more negative values is generally observed, indicating that the oxidised form more strongly interacts with graphite than the reduced form. However, for these compounds when adsorbed on ZP there has been registered a shift to more positive potentials in neutral solution [27]. In some cases the shift is about 300 mV towards the anodic direction [44]. This behaviour indicates that the reduced form more strongly interacts with ZP [43], as also observed in the case of riboflavin. A large shift towards more positive values suggests a strong interaction between riboflavin in its reduced form and ZP. In a previous paper [27], the strongly positive shift for the adsorbed phenoxazine (Nile blue) and phenothiazine (methylene blue) was explained by the acidity of the ZP and the observed $E^{\circ\prime}$ of these compounds when immobilised onto ZP was similar to that observed in acid media. However, it was previously shown [44] that adjusting the pH to more neutral values before adsorbing the dyes onto ZP had an effect

on the final $E^{\circ\prime}$ value of the adsorbed compound and somewhat less drastic shifts in the $E^{\circ\prime}$ were noticed. It is unclear whether the drying time and temperature will affect the final characteristics, but will be at focus in forthcoming investigations.

However, Shinkai and co-workers [45] assigned the shift of the $E^{\circ\prime}$ of some flavin towards more positive potentials to the high dielectric constant surrounding the flavin molecules when immobilised in highly charged polyelectrolyte complexes. Kajiki and co-workers [46] have shown that derivatisation of the N(5) of the flavin accelerates its ability for NADH oxidation, probably shifting the $E^{\circ\prime}$ towards more positive values, although no electrochemical studies were made. Another possibility is that the interaction of the riboflavin protons with the phosphate groups of the material results in drifting the electronic density on the flavin and stabilising the reduced form. However, the invariance of the $E^{\circ\prime}$ of riboflavin when adsorbed onto ZP with the solution pH (Fig. 7) remains unexplained. Thus, to conclude, the redox process of riboflavin adsorbed on ZP involves two electrons and its $E^{\circ\prime}$ is independent on the solution pH. Experiments carried out in different buffers showed different $E^{\circ\prime}$ values for the same pH (Table 1). The lowest value was observed in phosphate buffer and can be attributed to a competition between phosphate from the material and the buffer. When phosphate buffer was used the peak current intensity decreased with time, indicating that riboflavin leached out from the surface. In contrast when Hepes, Pipes or Tris were used, only a moderate shift to more negative values and no significant decrease in the current intensity was observed with time. Thus, the composition of the buffer solution can affect the $E^{\circ\prime}$ if the constituents of the buffer compete for the electrode material site and/or with riboflavin and consequently affect the $E^{\circ\prime}$ and the electrode stability.

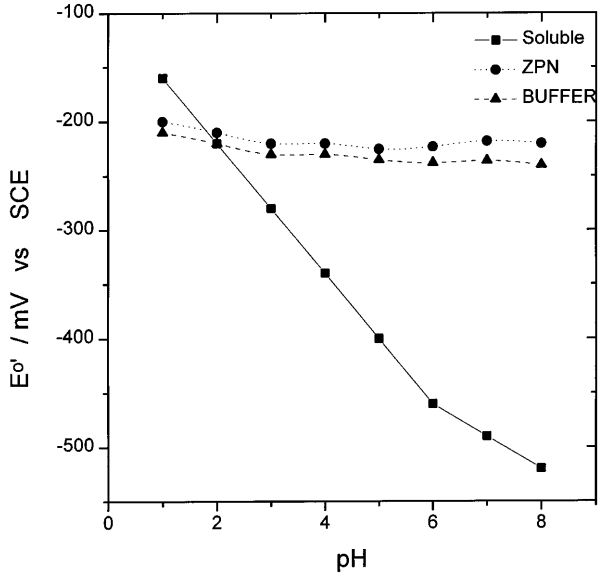

Fig. 7 Variation of $E^{\circ\prime}$ on solution pH for soluble and adsorbed riboflavin, obtained in 0.1 M KCl (*ZPN*) and in buffer (*BUFFER*) solution. The pH was adjusted with HCl or KOH. The scan rate was 20 mV s^{-1}

Table 1 Buffer effect on formal potential ($E^{\circ\prime}$) of riboflavin adsorbed on ZP obtained at pH 7.0 in 0.1 M buffer solution

Buffer	$E^{\circ\prime}$ (mV vs. SCE)
Phosphate	−370
Tris	−250
Pipes	−240
Hepes	−220

Electrocatalytic oxidation of NADH

Various flavoproteins are well known as catalysts, oxidising many compounds in the living cell, including NADH [2, 33, 47]. However, aqueous dissolved riboflavin very slowly electrocatalyses the oxidation of NADH in neutral media, because the difference between the $E^{\circ\prime}$ of the flavin (around −470 mV versus SCE [2, 33, 41]) and that of NAD^+/NADH (−560 mV [29, 48]) is too small to allow a rapid reaction rate. Thus, a shift of the $E^{\circ\prime}$ of the flavin to more positive values by means of derivatisation and immobilisation has been attempted by several researchers [49] to increase the reaction rate, although no good system has so far been described in the literature. The drastic shift in the $E^{\circ\prime}$ of riboflavin when adsorbed on ZP could be a good system to study the electrocatalysis of NADH oxidation brought about by a flavin. ZPRib modified electrodes were therefore investigated for their ability to electrocatalytically oxidise NADH. For all these investigations, 0.1 M Hepes buffer at pH 7.0 was used. Previous investigations [16, 23, 25, 50] have shown that the reaction rate between NADH and mediators is very strongly influenced by pH.

Figure 8 shows CVs obtained for the carbon paste electrode modified with ZPRib in the absence (dotted) and presence (solid line) of 10 mM NADH. In the presence of 10 mM NADH a small catalytic effect can be observed (Fig. 8, full line), revealed by the increase in the anodic wave and decrease in the cathodic wave. The catalytic effect is most clearly noticed for potentials more positive than the anodic peak potential. A CV obtained with unmodified ZP showed no electrocatalytic effect for NADH oxidation (results not shown).

The catalytic effect has been explained in a similar system [43, 50] by assuming that NADH diffuses to the electrode surface where it reduces the oxidised mediator ($ZPRib_{ox}$) to form NAD^+ and the reduced form the mediator ($ZPRib_{red}$), which in turn is electrochemically reoxidised:

$$ZPRib_{ox} + NADH \rightarrow ZPRib_{red} + NAD^+ + H^+ \quad (2)$$

$$ZPRib_{red} \rightarrow ZPRib_{ox} + 2e^- \quad (3)$$

Previously reported CVs of NADH oxidation obtained with electrodes modified with neutral mediators or with

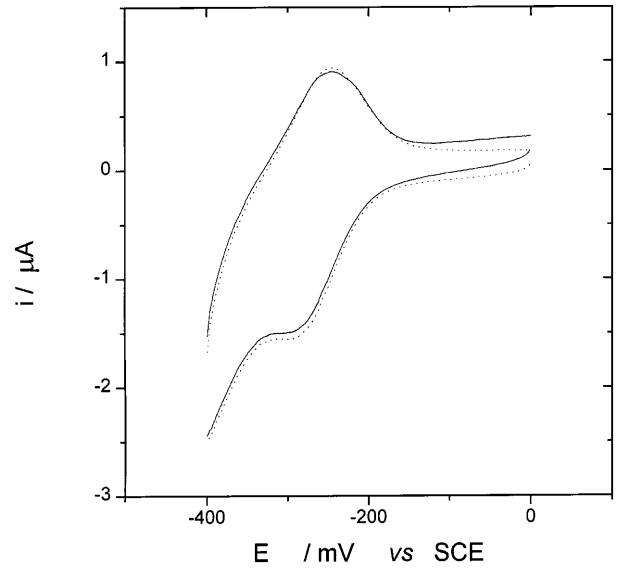

Fig. 8 Cyclic voltammograms obtained for a ZPRib modified carbon paste electrode in the absence (*dotted line*) and presence of 10 mM NADH (*solid line*), at a scan rate of 20 mV s^{-1}; the supporting electrolyte was 0.1 M Hepes buffer, pH 7.0

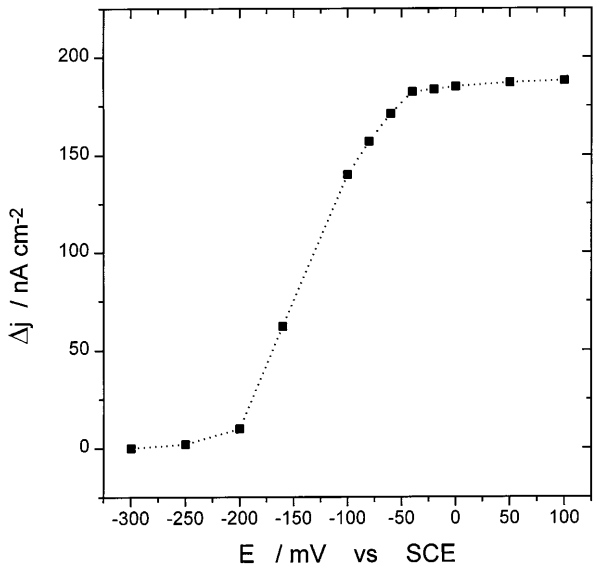

Fig. 9 Variation of the response current density (Δj) for 1 mM NADH with the applied potential for a ZPRib modified carbon paste rotating disk electrode (50 rpm) in 0.1 M Hepes buffer, pH 7.0

charged mediators with low $E^{\circ\prime}$ values reveal a similar behaviour, as reported here [9, 51]. Neutral mediators in general have much lower reaction rates than positively charged ones of similar $E^{\circ\prime}$ [52–54].

Cyclic voltammetry is, however, not the best method to use to extract precise quantitative information about the electrocatalytic process for a catalytic reaction, although this method has been used in many papers (e.g., in [55, 56]) to evaluate kinetic parameters.

To be able to study the electrocatalytic reaction in more detail to obtain kinetic and mechanistic information of the system, amperometric experiments using a rotating disk electrode can be used, evaluating the kinetic parameters through Koutecky-Levich coordinates [43, 50].

In Fig. 9 is presented a curve of the variation of the response of the catalytic current for 1 mM NADH with the applied potential and a rotation speed of 50 rpm. As can be seen, for potentials lower than −200 mV, virtually no catalytic current is observed, and for potentials higher than −50 mV the catalytic current reaches a maximum value. This suggests the potential use of such an electrode for electrocatalytic NADH oxidation, e.g. for biosensor purposes, as this potential falls within what has been stipulated as the optimal potential range (≈ −150 and 0 mV) to obtain least bias electrochemical contribution from possible interfering substances [15, 23, 57].

A series of experiments was therefore run using the ZPRib modified carbon paste electrode mounted in a rotating disk electrode device. All these experiments were run at −50 mV, where the maximum catalytic current is obtained (Fig. 9). Also at this potential the rate of reoxidation of $ZPRib_{red}$ [Eq. (3)] should not be limiting the current ($E^{\circ\prime}$ is −220 mV) and the total amount of ZPRib can be approximated to be equal to $ZPRib_{ox}$ so that first-order kinetics can be assumed.

A Levich plot (the dependence of the current with the square root of the rotation speed) is shown in Fig. 10 for 1 mM NADH. From this plot it can be concluded that the catalytic current is controlled by both the mass transport of NADH to the electrode surface as well as by the reaction between NADH and the adsorbed riboflavin [35, 43, 50], defined by the second-order rate

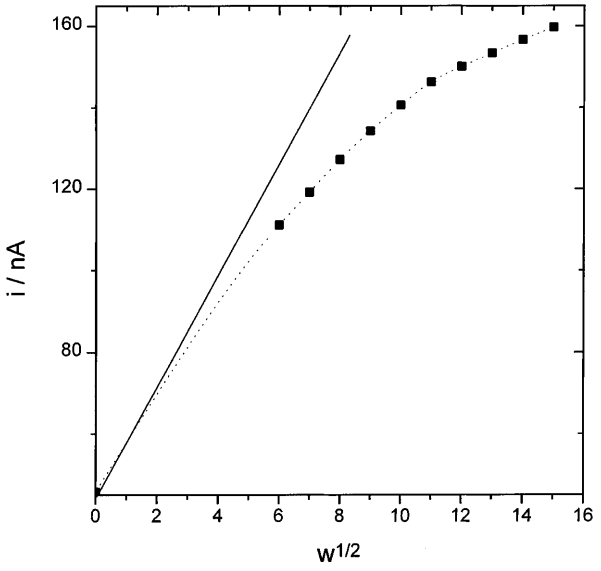

Fig. 10 Levich plot for NADH oxidation at a ZPRib modified carbon paste electrode, obtained for 1 mM NADH solution in 0.1 M Hepes buffer, pH 7.0, and an applied potential of −50 mV *versus* SCE. The *straight line* represents the theoretical curve with no kinetic restrictions

constant, k_{obs}. Figure 11 shows the Koutecky-Levich plot obtained for the same data as in Fig. 10. The intercept of the Koutecky-Levich plots can be used for calculating k_{obs} (see below). The response time for the electrode was about 15 s after the addition of the substrate, evaluated as the time to reach 95% of the maximum response. It is very short, considering the time for NADH to homogenise in the solution and to diffuse to the electrode after each addition.

The inset in Fig. 11 shows the variation of k_{obs} with the bulk concentration of NADH ([NADH]*) for the same electrode and, as seen, k_{obs} very much depends on the concentration of NADH. This behaviour is in accordance with previous observations between NADH and phenoxazine and phenothiazine type mediators adsorbed on graphite [23, 43, 50, 54], and can be interpreted in agreement with previous studies on the reaction mechanism between NADH and flavins [10, 46] that NADH and $ZPRib_{ox}$ first form a charge transfer complex intermediate (CT complex) in which the charge transfer occurs, which in turn decomposes into $ZPRib_{red}$ and NAD^+:

$$ZPRib_{ox} + NADH \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{CT complex} \xrightarrow{k_{obs}} ZPRib_{red} + NAD^+ + H^+ \quad (4)$$

This type of reaction mechanism is closely related to Michaelis-Menten kinetics and a Michaelis-Menten constant (K_M) can be defined as:

$$K_M = \frac{k_{-1} + k_{+2}}{k_{+1}} \quad (5)$$

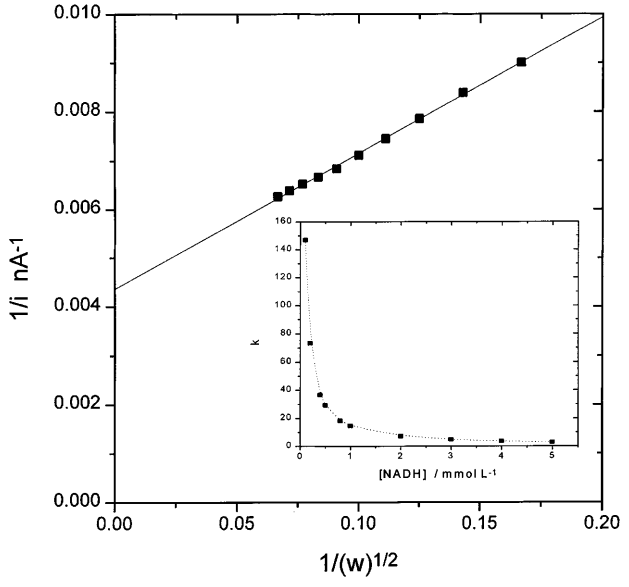

Fig. 11 Koutecky-Levich plot from data of Fig. 10. The *inset* shows the variation of k_{obs} as a function of the concentration of NADH

The heterogeneous second-order reaction rate constant, k_{obs}, for any concentration of NADH can be expressed as [43]:

$$k_{obs} = \frac{k_{+2}}{K_M + [NADH]} \quad (6)$$

We have previously derived the expression of the Koutecky-Levich equation for a system like this [43, 50]:

$$\frac{1}{i} = \frac{1}{nFA\left(\frac{k_{+2}}{K_M + [NADH]^*}\right)\Gamma[NADH]^*} + \frac{1}{0.620nFAv^{-1/6}D_{NADH}^{2/3}[NADH]^*\omega^{1/2}} \quad (7)$$

where v denotes the kinematic viscosity, ω the angular rotation of the electrode, D_{NADH} the diffusion coefficient of NADH, and [NADH]* the bulk concentration of NADH, respectively.

Replotting $1/k_{obs}$ versus [NADH]* gives a perfectly straight line (not shown), supporting Eq. (6) and the mechanism given in Eq. (4). From the intercept, k_{obs} at [NADH]* = 0 (k_{obs} for [NADH]* = 0 equals k_{+2}/K_M) can be evaluated, and from the slope k_{+2} can be evaluated [43, 50].

In Table 2 are summarised the parameters for NADH oxidation at the ZPRib-modified electrode. The second-order rate constant, $k_{obs([NADH]=0)}$, was in the present case found to be 816 M^{-1} s^{-1} and is comparable with that found for a gold electrode modified with a monolayer of pyrroloquinolinequinone (PQQ) on gold [58] and 1,2-benzophenoxazin-7-one (BPO) adsorbed on graphite [50]. The reason for comparing this system with those mentioned above is that they have $E^{\circ\prime}$ values at pH 7.0 similar to riboflavin adsorbed on ZP (PQQ: −125 mV versus SCE; BPO: −210 mV versus SCE) and that the catalytic functionality of the mediator is uncharged (even though PQQ as such is negatively charged). However, the evaluated apparent Michaelis-Menten constant, K_M (1.8 mM), for this system differs compared with those of both PQQ and BPO, reflecting a higher affinity for NADH in the present case compared with PQQ on Au on (109 mM) and a lower affinity compared with BPO adsorbed on graphite (1.1 mM). Although the affinity of BPO for NADH is higher, the complex dissociation step is slower in comparison with ZPRib and PQQ, which are similar. This behaviour indicates a reasonable catalytic activity for electrooxida-

Table 2 Electrochemical and kinetic parameters of adsorbed riboflavin on ZP for NADH electrooxidation

$E^{\circ\prime}$ (mV)	−220
Applied potential (mV)	−50
Coverage (mol cm^{-2})	6.8×10^{-10}[a]
K_M (mM)	1.8[a]
k_{obs} (M^{-1} s^{-1})	816[a]
k_{+2} (s^{-1})	1.47[a]
Sensitivity (nA M^{-1})	7200

[a] The values have 10% uncertainty

tion of NADH at the ZPRib-modified carbon paste electrode. The K_M of 1.8 mM is, however, comparable with the values found previously [23, 43, 50, 54] for phenoxazines and phenothiazines.

The results shown above on the variation of the catalytic current with the applied potential (Fig. 9) further supports that the catalytic reaction does not occur through a simple straightforward reaction mechanism. That the catalytic current starts to appear at around −250 mV is coherent with the $E^{\circ\prime}$ of ZPRib. However, that the catalytic current reaches a maximum at around −50 mV is puzzling as one would expect that maximum catalytic efficiency should be obtained already at potentials where the immobilised mediator is in its fully oxidised state (expected at potentials around +100 mV more positive than the $E^{\circ\prime}$ value, i.e., at around −150 mV). Previously, the same catalytic behaviour was shown for carbon electrodes modified with FAD [9], 1,2-benzophenoxazin-7-one [50] or Nile blue [51], i.e., that maximum currents for catalytic NADH were obtained far away from the $E^{\circ\prime}$ of the immobilised mediator. The reason for this is not clear but could possibly be due to the CT complex formed is itself electrochemically active but in a potential region more positive than that of the fully reduced mediator. This suggestion was supported by surface enhanced Raman scattering investigations of the catalytic performance at a Nile blue modified silver electrode [51].

Recently, there was reported a higher catalytic activity of PQQ for NADH oxidation in the presence of Ca^{2+} [58]; however, the presence of Ca^{2+} did not affect the $E^{\circ\prime}$ of PQQ. This property was explained by the interaction of Ca^{2+} with PQQ. When comparing the kinetic parameters (K_M and k_{+2}) for NADH oxidation by PQQ it was found that k_{+2} was virtually unchanged whereas K_M decreased from 109 mM to about 0.8 mM, explaining the effect of the positive charge with a drastically increased affinity for complex formation between the mediator and PQQ. More recent studies of the effect of Ca^{2+} on PQQ [59] and another *ortho*-quinone based mediator [60] confirm these studies. As already mentioned, in general it has been shown that positively charged mediators have much higher reaction rates with NADH than neutral mediators [52–54, 61]. The stability of positively charged mediators is also much improved compared with neutral analogues [54, 62]. The reason for this effect has not been elucidated yet but may reflect that a positive charge can stabilise the CT complex, preventing abortive side reactions assumed to occur in the charge transfer reaction as was proposed, for example, for neutral *ortho*-quinone type mediators [62].

Addition of 0.1 mM Ca^{2+} or Mg^{2+} to the contacting solution provoked a shift of the $E^{\circ\prime}$ of the adsorbed riboflavin of about 70 mV towards more positive values. Figure 12 shows the dependence of the $E^{\circ\prime}$ with the concentration of metal ion in the contacting solution, suggesting that both Ca^{2+} and Mg^{2+} interact strongly with riboflavin adsorbed on ZP. This phenomenon has,

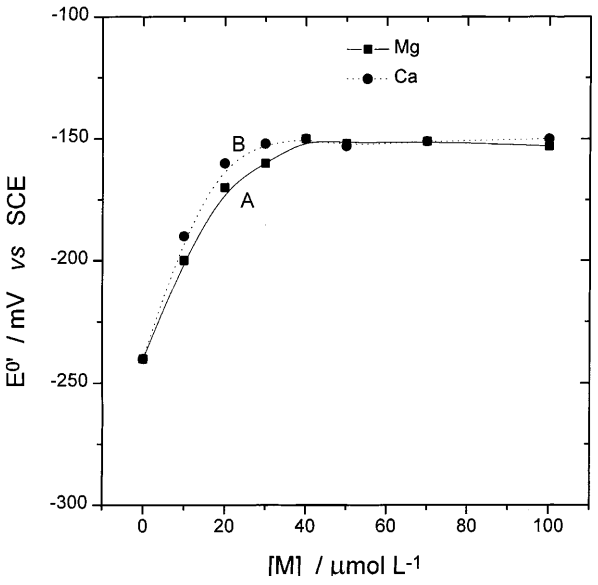

Fig. 12 Effect of Mg^{2+} (*A*) and Ca^{2+} (*B*) on the $E^{\circ\prime}$ of riboflavin adsorbed on ZP obtained at a scan rate of 20 mV s^{-1}; the supporting electrolyte was 0.1 M Hepes buffer, pH 7.0

at least to our knowledge, never been observed earlier electrochemically for flavins on any type of electrode material. Increases in the concentration for both Ca^{2+} and Mg^{2+} up to about 40 µM influence the $E^{\circ\prime}$ of the adsorbed riboflavin. However, Fukuzumi and co-workers [63–66] have in a series of papers shown that flavins form 1:1 complexes with Mg^{2+} and other divalent metal ions and that the catalytic activity of the flavin in its complexed form is drastically changed in comparison with its uncomplexed form. Electrochemical studies of flavins in protic and aprotic solutions containing various metals ions have also been carried out [67, 68], revealing strong interactions with various metal ions. For flavins, derivatisation of N(5) was shown to increase the catalytic activity for NADH oxidation [46]. These previous studies on the interaction and complex formation between flavins and metal ions, as well as derivatisation of the flavin at N(5), support our belief that riboflavin is bound to the ZP support through coordination to the zirconium, that phosphate ions in the contacting solution compete with riboflavin for coordination with zirconium, and that metal ions in the contacting solution may compete with zirconium for complex formation with riboflavin.

After addition of Ca^{2+} or Mg^{2+} the rate of the NADH electrooxidation also increased (Fig. 13), as was also observed for PQQ on Au in the presence of Ca^{2+} [58]. When comparing the CVs shown in Figs. 8 and 13 it is very obvious that in the presence of the divalent metal ion a much more efficient catalysis is noticeable. In Fig. 8, 10 mM NADH were used and the catalytic effect is most pronounced for potentials more positive than the $E^{\circ\prime}$ of the adsorbed riboflavin. In Fig. 13, only 1 mM NADH was used and the catalytic effect is seen well before the $E^{\circ\prime}$ of the mediator and a clear increase in the

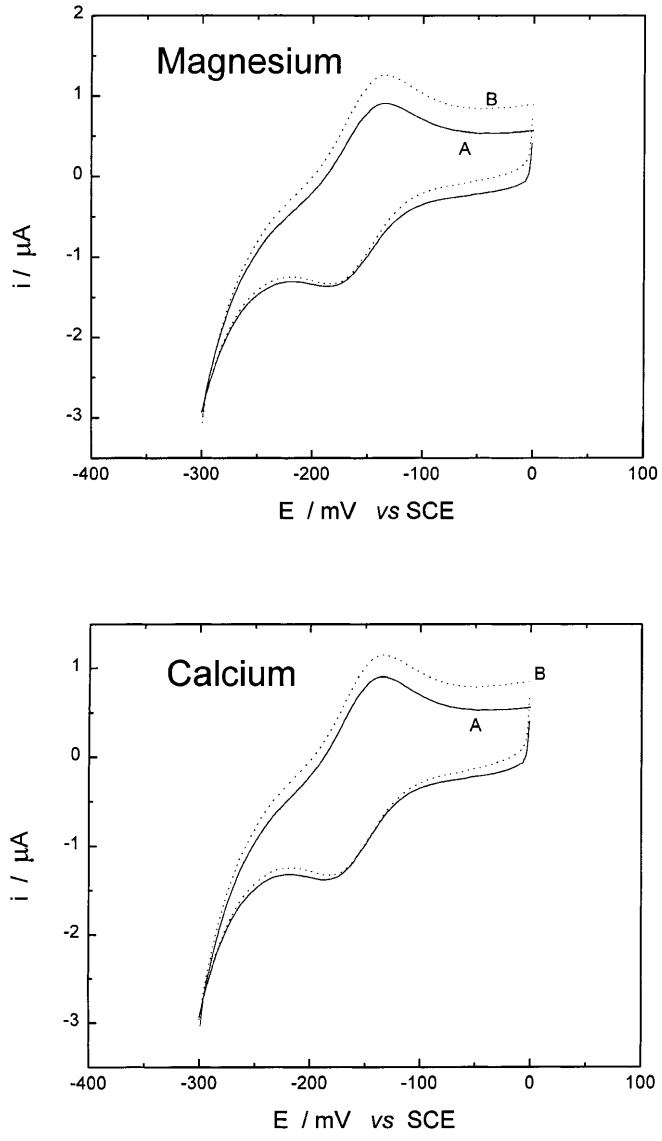

Fig. 13 Cyclic voltammograms obtained for a ZPRib modified carbon paste electrode in the absence (*A*) and presence of 1 mM NADH (*B*), at a scan rate of 20 mV s^{-1}; the supporting electrolyte was 0.1 M Hepes buffer, pH 7.0, plus 0.1 mM of Mg^{2+} (*above*) or 0.1 mM of Ca^{2+} (*below*)

peak current is observed. The reason for the more effective catalysis in the presence of the metal ion can be solely caused by the further shift of the $E^{\circ\prime}$ of the mediator to more anodic values, increasing the thermodynamic driving force for the reaction, but may also be caused by the interaction of the metal ion with the flavin, resulting in a mediator-metal complex with a net positive charge. Unfortunately, the stability of the electrode decreased significantly in the presence of the metal ion, shown by successively smaller waves of the CVs, probably due to the interaction of the metal ion both with the phosphate groups present on the ZP surface and with riboflavin, preventing any kinetic study of this system to be carried out with the rotating disk electrode approach. Investigations to clarify the mechanism involved in the catalytic reaction and explanations for the poor stability after addition of the metal ion will be the focus of future investigations.

Conclusions

Several important aspects are presented here, such as a new electrode material for immobilisation of riboflavin, increasing the activity for electrocatalytic oxidation of NADH, the invariance of the $E^{\circ\prime}$ of the immobilised riboflavin with the pH of the contacting solution, which would need a more extensive investigation to clarify the background mechanism, and the effect of Ca^{2+} and Mg^{2+} on the $E^{\circ\prime}$ and electrocatalytic activity for NADH oxidation. Future work in this direction will also be directed to further mechanistic investigations on the charge transfer reaction between NADH and the flavin, which may lead to further proof whether the charge is delivered as a single hydride or in a stepwise mode. The possibility to change the $E^{\circ\prime}$ of adsorbed riboflavin with the composition of the contacting solution [44] for a constant pH, and to change the pH of the solution while keeping the $E^{\circ\prime}$ of adsorbed riboflavin constant, gives the unique possibility to separately investigate the influence of pH and the influence of the difference in $E^{\circ\prime}$ values between NADH and the flavin on the reaction rate between these two compounds. Moreover, a basic understanding of the interaction between quinoic type mediators and ZP may lead to tailor-made modified electrodes that can be constructed for biosensor purposes, where the previous drawback with using electron-proton acceptor/donor type mediators having pH dependent $E^{\circ\prime}$ values will be circumvented. In parallel work, a first attempt to increase the understanding of the interaction between flavins and transition metal oxide modified electrodes used attenuated total reflectance infrared spectroscopy (AT-IR) [69], supporting the belief that N(5) in the isoalloxazine ring interacts with the metal. Future work, including studies of the interaction between riboflavin and Zr phosphate, are under way and will be reported separately.

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