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EFFICIENT INDIRECT ELECTROCHEMICAL IN-SITU REGENERATION OF NADH: ELECTROCHEMICALLY DRIVEN ENZYMATIC REDUCTION OF PYRUVATE CATALYZED BY D-LDH

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Summary: Using [Cp(Me)$_5$Rh(bipy)Cl]Cl (**1**) as redox catalyst for the continous NADH regeneration it was possible to perform an electrochemically driven enzymatic reduction of pyruvate to D-lactate catalyzed by D-LDH at a rate of 5 turnovers per hour. This is by a factor of 20 faster than the best results obtained until now. Current yields of 50 to 70 % may be obtained.

The regeneration of reduced pyridine nucleotides (NADH or NADPH) as cofactors for enzymatic reactions is of great technical interest. Enzyme coupled regenerations, although being already highly developed[1,2], are rather complicated systems. Therefore an electrochemical method would be principally of great importance. Electrochemical regenerations have to take place by an indirect pathway using redox catalysts which act as two electron or hydride atom transfer agents[3]. If one electron transfer agents like viologens are used as mediators, only electro-enzymic or electro-microbial reactions are possible which again are in need of an additional enzyme or a microorganism[4-6]. We have been able to form 1,4-NADH selectively by an indirect electrochemical method using tris(2,2'-bipyridine)rhodium(III)[3] or even more effectively by tris(2,2'-bipyridine-5-sulfonic-acid)rhodium(III) (Rh(bipy-5-SS)$_3$)[7] as two electron or hydride atom transfering mediators in the absence of an additional enzyme. These systems can be coupled internally to an enzymatic reduction of carbonyl compounds using for example horse liver alcohol dehydrogenase as catalyst[3,7]. In this way 46 turnovers for the mediator Rh(bipy-5-SS)$_3$ and 101 turnovers for the cofactor could be obtained[7]. The problem, however, is still the low reaction rate of about 3 to 5 turnovers per day. Now we are able to present a highly effective rhodium complex as mediator for the indirect electrochemical generation of NADH from NAD$^+$. This is pentamethylcyclopentadienyl-2,2'-bipyridine-chloro-rhodium(III) (**1**) which was first applied as catalyst for the photochemical hydrogen evolution at colloidal TiO_2[8].

Electroanalytical measurements by cyclic voltammetry in 0.1 M Tris/HCl buffer of pH 7.5 show a chemically irreversible reduction peak at - 0.76 V vs. Ag/AgCl for **1** (Fig. 1,broken lines). In the presence of equimolar amounts of NAD$^+$ the peak current for the complex is increasing by a factor of almost two (Fig. 1, solid lines) indicating a very fast reaction between the reduced rhodium complex and NAD$^+$ under regeneration of the oxidized form of the complex. The current increase is further intensified, if a complex to cofactor ratio of 1:2 is used. Higher concentrations of NAD$^+$ cause adsorption effects at the electrode surface. These results may be explained by the following second order electrocatalytic mechanism:

$$[Cp(Me)_5Rh^{III}(bipy)Cl]^+ + 2\ e^- \xrightarrow{\text{Cathode}} [Cp(Me)_5Rh^{I}(bipy)] + Cl^- \quad (1)$$
(1)

$$[Cp(Me)_5Rh^{I}(bipy)] + H^+ \longrightarrow [Cp(Me)_5Rh^{I}(bipy)H]^+ \quad (2)$$
(2)

$$[Cp(Me)_5Rh^{I}(bipy)H]^+ + NAD^+ + Cl^- \longrightarrow [Cp(Me)_5Rh^{III}(bipy)Cl]^+ + NADH \quad (3)$$
(3)

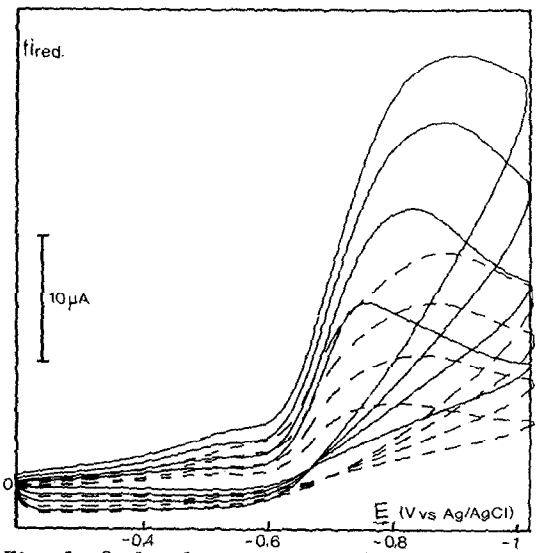


Fig. 1: Cyclovoltammograms of **1** in absence (broken lines) and presence of NAD^+(**1**:NAD^+=1:1, solid lines) at scan rates of 9, 25, 49, 81 mV/s(bottom to top)

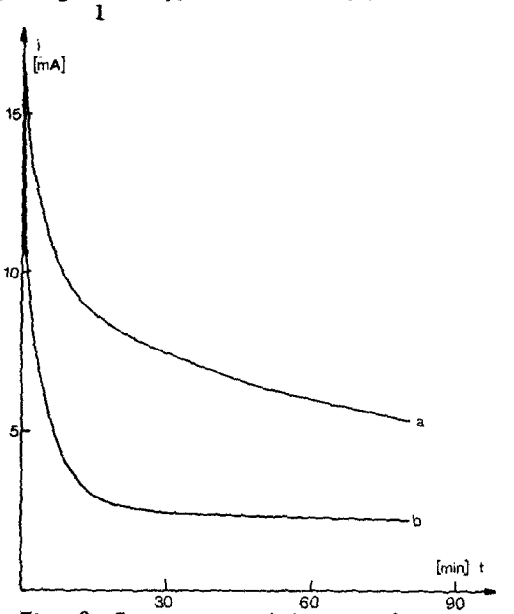


Fig. 2: Current-time behaviour for indirect electrolyses of NAD^+ (a) or cyclohexanone (b) under the conditions given in Table 1

This scheme not only explains the chemical irreversibility observed in the cyclovoltammogram because of the ligand exchange in steps (1) and (2) which is associated with the electron transfer at the cathode but also the cathodic peak current increase by addition of NAD^+, as the starting complex is regenerated by hydride transfer to NAD^+ in step (3). The mechanism is also consistent with the one proposed for the photoreduction of protons to hydrogen[8].

To be valuable as redox catalyst for the electrochemical in-situ regeneration of NADH for enzymatic reactions it should fulfill the following conditions: The reduced mediator should selectively reduce NAD^+ in presence of the enzyme substrate. Therefore the reaction rate towards the substrate should be considerably lower than towards NAD^+. The electrode potential for the reduction of the mediator must be so positive that direct cathodic reduction of NAD^+ leading to NAD dimers should not take place. Strong indications for a high selectivity of **2** towards NAD^+ reduction were obtained by cyclic voltammetry. In presence of cyclohexanone or pyruvate no increase of the cathodic peak current for the redox catalyst was observed. The results of indirect electrochemical reductions of NAD^+ and cyclohexanone using **1** as catalyst are given in Table 1.

Table 1: Comparison of the indirect electrochemical reduction of NAD^+ and cyclohexanone using **1** as redox catalyst[a]

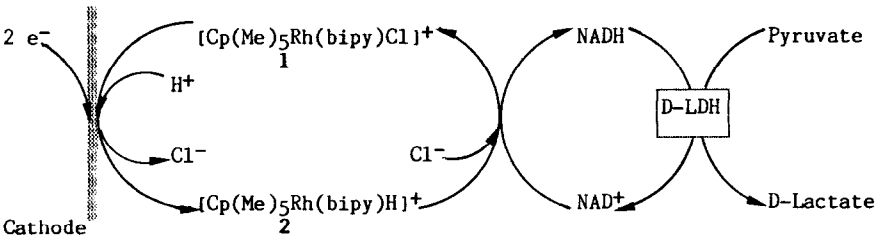
Substrate	t [min]	i [mA]	Charge [As]	Products NADH [M]	Products $(NAD)_2$ [M]	Products Cyclohexanol [M]	Turnovers 1	Turnovers 1/h	Current Yield [%]
NAD^+ 5.17×10^{-3}	40	6.9	24	1.9×10^{-3}	1.8×10^{-4}	---	4.2	6.3	78.6
	140	2.1	51	3.5×10^{-3}	3.3×10^{-4}	---	7.7	3.3	66.8
Cyclohexanone 5×10^{-3}	83	2.2	15	---	---	3.6×10^{-4}	0.72	0.52	23.2
	240	1.7	30	---	---	9.9×10^{-4}	1.98	0.49	31.8

[a] Using **1** (5×10^{-4} M) in 50 ml of 0.1 M Tris/HCl buffer of pH 7.5 in a divided cell at - 0.7 V vs. Ag/AgCl ; working electrode: Sigraflex carbon foil

The results show that NAD^+ is reduced at a considerably faster rate than cyclohexanone. This is nicely demonstrated by a comparison of the currents for NAD^+ and cyclohexanone reduction using **1** as mediator under the conditions given in Table 1 (Fig. 2). During indirect reduction of cyclohexanone the background current after 30 min is not significantly exceeded any more. Similar results are obtained with pyruvate as substrate.

NAD-dimer formation by direct cathodic reduction was studied using a high NAD^+/mediator ratio of 100:1 at potentials of -0.8, -0.7, and -0.6 V vs. Ag/AgCl. While at -0.8 V the NADH/$(NAD)_2$ ratio is 1:2, it increases to 5:1 at -0.7 V and to 100:1 at -0.6 V. Therefore it can be concluded that during indirect electrochemical in-situ regeneration of NADH NAD-dimer formation can be avoided, if NAD^+/mediator ratios of 10:1 or less and potentials of -0.6 V are applied. Therefore under these conditions the in-situ regeneration of NADH by indirect electrolysis using **1** as mediator according to scheme 1 was studied. As substrate pyruvate and as redox enzyme D-LDH were selected because by analysis of the enantiomeric

SCHEME 1



excess in D-lactate formed it can be established, if the reduction of the substrate by **2** under circumvention of the enzymatic reaction leading to racemic lactate could effectively be suppressed.

The results of two continuous electrolyses at -0.6 V using a concentration ratio of **1** : NAD^+ : pyruvate of 1:2:20 are given in Table 2.

Table 2: Results of the electrochemically driven D-LDH catalyzed enzymatic reduction of pyruvate to D-lactate[a]

Exp.No	t [min]	i [mA]	Charge [As]	Products D-lactate [M]	Products L-lactate [M]	Turnovers w.r.t **1**	Turnovers w.r.t NAD^+	Enantiomeric Excess [%] D-Lactate	Current Yield [%]
1	26	28	50	2.4×10^{-3}	5×10^{-4}	2.4	1.2		56
	50	26	100	6.0×10^{-3}	5×10^{-4}	6	3		61
	83	24	150	7.5×10^{-3}	5×10^{-4}	7.5	3.8		50
2	180	17	200	1.4×10^{-2}	4.5×10^{-4}	14	7	93.5	67

[a] Concentrations of **1**=1×10^{-3} M; NAD^+=2×10^{-3} M; pyruvate=2×10^{-2} M; D-LDH=1400 U (Exp. 1), 1300 U (Exp. 2) (Units with respect to pyruvate; from Staphylococcus epidermidis) in 50 ml 0.1 M Tris/HCl buffer of pH 7.5 in a divided cell at a Sigraflex carbon foil electrode and a potential of -0.6 V vs. Ag/AgCl; 25°C

It is shown that **1** is regenerated at a rate of about 5 turnovers per hour. The enantiomeric excess in D-lactate, evaluated by enzymatic determination of D-lactate and L-lactate using D- respectively L-LDH[9], was 93.5 %. This compares very well with the ee values obtained under enzymatic regeneration of NADH (ee values ranging from 92 to 97 %) indicating that practically no direct reaction between pyruvate and **2** had been taking place. The reaction rate is by a factor of more than 20 larger than that obtained with $Rh(bipy\text{-}5\text{-}SS)_3$ as mediator[7]. Also the current yields of 50 to 70 % are extremely good. Therefore it can be concluded that such an indirect electrochemical process could be an interesting alternative to the currently applied methods of enzymatic regeneration of NADH.

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