Efficient In-Situ Redox Catalytic NAD(P)$^+$ Regeneration in Enzymatic Synthesis Using Transition-Metal Complexes of 1,10-Phenanthroline-5,6-dione and Its *N*-Monomethylated Derivative as Catalysts☆

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In comparative studies, we have been able to demonstrate that redox catalysts based on transition-metal complexes using 1,10-phenanthroline-5,6-dione as a ligand or based on *N*-methylated 1,10-phenathroline-5,6-dione acting via hydride ion abstraction are superior to alternative methods for the redox catalytic aerobic or indirect electrochemical in situ NAD(P)$^+$ regeneration in enzymatic syntheses using alcohol dehydrogenases as production enzymes. Under preparative conditions in the gram scale we were able to obtain turnover frequencies of up to 130 turnovers per hour with respect to the redox catalyst. These are far larger than those of the presently most popular regeneration system.

Introduction

The most widely employed class of oxidizing enzymes are dehydrogenases such as horse liver alcohol dehydrogenase (HLADH),[1] alcohol dehydrogenase from *Thermoanaerobium brockii* (TBADH) or glycerol-dehydrogenase (GDH). These dehydrogenases depend upon NAD(P)$^+$ as the electron accepting cofactor. Thus, for the synthetic application of these enzymes an efficient and mild system for the cofactor regeneration is necessary.[1][2]

Whitesides[3] analyzed the present status of NAD(P)$^+$ regeneration and drew the following conclusions. The most widely used system was developed by Jones[1][4]. It consists of FMN as redox catalyst and catalase in the presence of oxygen and is especially ineffective. A very low bimolecular reaction rate between FMN and NADH of 0.2 $M^{-1}s^{-1}$ makes the use of high cofactor and high FMN concentrations necessary. Typical concentrations of the substrate are 14 mmol, with 1 mmol of NADH, and 20 mmol of FMN. Thus, in preparative runs, the turnover frequency often only reaches 0.06 cycles/h with regard to FMN. A maximum of 1.8 cycles/h can be obtained. If methylene blue is used as the redox catalyst in the presence of oxygen it is necessary to employ not only catalase to destroy the hydrogen peroxide formed, but also the enzyme diaphorase has to be present to speed up the reaction between methylene blue and the reduced cofactor. However, high concentrations of methylene blue and NADH are still necessary. These conditions are difficult to achieve because of the low solubility of methylene blue in aqueous buffer. Typical turnover frequencies of about 6 cycles/h can be reached. Diaphorase is unstable and quite expensive. Also, other chemical systems described thus far have limited use because they either react too slowly (4,7-phenanthrolin-5,6-dione[5]: 4-6 turnovers/h) or are chemically not stable enough under the basic conditions[6] which are usually favourable for the enzymatic oxidations. Alternatively, the cofactor regeneration can be performed using an additional regeneration enzyme. Thus, the enzyme-coupled regeration system consisting of glutamate dehydrogenase as the regeneration enzyme and 2-oxoglutarate as the cosubstrate have been applied successfully.[7] Total turnover numbers of 500 to 1000 for NAD can be reached. However, besides the need for a second enzyme, a valuable cosubstrate is necessary and the coproduct has to be separated from the desired product. In this case especially, the separation of the coproduct glutamate makes the system quite complicated. Drueckhammer and Wong[8] have applied FMN reductase as an additional enzyme to speed up the reaction between FMN and NADH. In this case a turnover frequency of 17 cycles/h for FMN and 170 cycles/h for NAD can be reached. However, the enzyme is quite expensive and not very stable.

Electrochemical regeneration of the oxidized cofactors is either possible by direct anodic oxidation[9] or via redox catalysts mainly applying *o*-quinonoid systems as mediators. While for the mediators the same disadvantages are observed as for the chemical systems, the direct electrochemical regeneration is problematic because electrode fouling has quite often been observed. Moreover, the anodic potential of about 800 mV vs. SCE is too positive for selective oxidations[9] because of the electrochemically highly irreversible anodic process.

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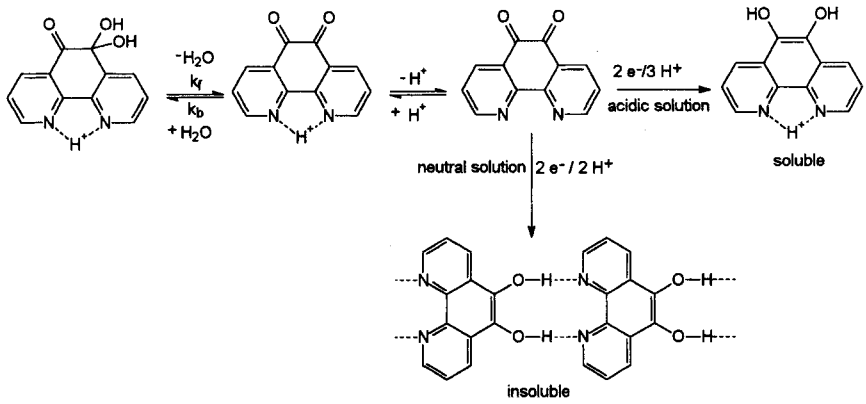
Scheme 1. Redox chemistry of PDon in aqueous buffer solution

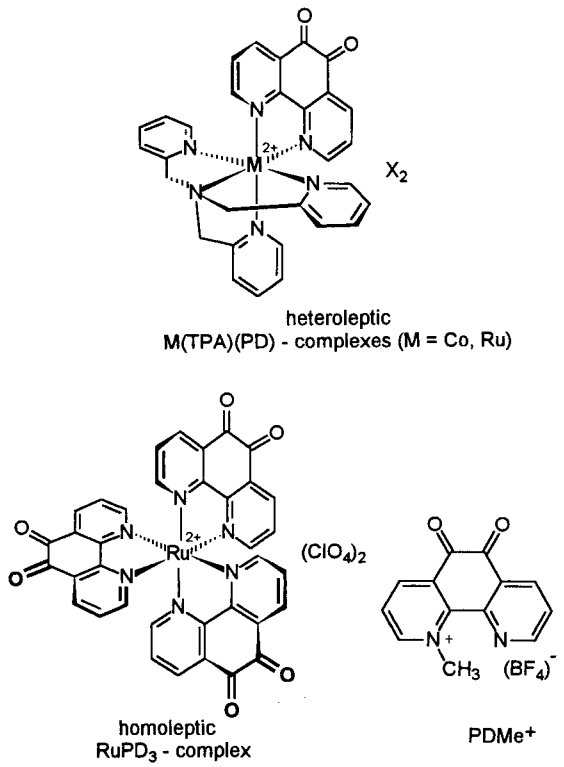


We have developed an efficient redox catalytic system for the chemical or electrochemical $NAD(P)^+$ regeneration based on 1,10-phenanthroline-5,6-dione (PDon) as a starting point. By itself this o-quinone is a very slow catalyst for the cofactor regeneration which takes place by hydride ion abstraction giving the hydroquinone form (PDol) which tends to precipitate by oligomerization via hydrogen bonding (Scheme 1).[10][11] Furthermore, it may also act as a chelating ligand for the zinc ion in the active centre of dehydrogenases.

To avoid these problems, we developed a new and very successful concept. To accellerate the hydride ion abstraction from NAD(P)H and to lower the oxidation potential to about 0 V vs. Ag/AgCl the electron density of the dione system was lowered by formation of transition-metal complexes using mainly ruthenium or cobalt as the central metal ion, or by mono *N*-methylation. At the same time, the positively charged species showed drastically increased water solubility and the precipitation of the reduced hydroquinone forms (PDol) was prevented by blocking of the nitrogen atoms of the PD ligand.[11] Thus, the following five systems have proved to be the most effective redox catalysts for the $NAD(P)^+$ regeneration. The first consists of tris(1,10-phenanthroline-5,6-dione)Ru^{II} perchlorate ($E_{p,ox} = -0.05$ V vs. Ag/AgCl at pH 7). In three cases, we prevented the precipitation of telomers or oligomers of the reduced Co^{II} or Ru^{II} complex by using a mixed ligand system consisting of one 1,10-phenanthrolin-5,6-dione (PDon) as the catalytically active unit and one *N,N,N*-tris(aminoethyl)amine (tren) or *N,N,N*-tris(2-pyridylmethyl)amin (TPA) ligand to block the other sites of the metal centre ($E_{p,ox} = -0.075$ to -0.090 V vs. Ag/AgCl at pH 7). In the last case, we blocked one nitrogen of the phenanthrolinedione by methylation to receive $PDMe^+$ ($E_{p,ox} =$ 0.0 V vs. Ag/AgCl at pH 7). The properties of the systems and the mechanism of the hydride ion abstraction from NAD(P)H have been studied in detail using electroanalytical and spectroscopic methods. The results have been reported previously[11].

The described compounds (Scheme 2) are well soluble in aqueous solutions, their stability is strongly enhanced in the oxidized as well as in the reduced form (the $[Ru(TPA)(PDon)]^{2+}$ complex is stable up to pH = 11.0), their redox potentials in neutral solution of pH = 7.0 have values around 0 V vs. Ag/AgCl and their hydride ion abstraction ability is also strongly enhanced. Thus, the most important requirements for mediators for a reasonable application in enzymatic syntheses seemed to be realized.

Scheme 2. Structures of the redox catalysts studied



Results and Discussion

Redox Catalytic Regeneration of the Oxidized Cofactor $NAD(P)^+$

As already previously communicated, the catalytic ability of the mediators can easily be investigated based on the redox catalytic aerobic NADH oxidation using the above mentioned mediators. Hydrogen peroxide is generated as

the reduction product because oxygen acts as the final electron acceptor. Thus, the mediators were dissolved in 0.1 M phosphate buffer of pH 6, pH 7, and pH 8 within a UV cuvette under an oxygen atmosphere. Then 60 equivalents of NADH were added and the reaction progress followed by UV spectroscopy at 340 nm, the absorption maximum of the reduced cofactor. The turnover frequencies were calculated based on the conversions during the first minute of the reaction (Table 1).

Table 1. Turnover frequencies (TN h^{-1}) for the redox catalytic aerobic NADH oxidation with PDon-based catalysts and comparison with literature data (4,7-phenanthroline-5,6-dione,[12] FMN[1][4])

Compound	TN h^{-1}[a] pH 6.0	TN h^{-1}[a] pH 7.0	TN h^{-1}[a] pH 8.0
4,7-Phenanthroline-5,6-dione		4.0–6.0	
FMN (pH 9.0)		1.8	
$[Ru(PDon)_3](ClO_4)_2$[b]	55	203	901
$[Ru(TPA)(PDon)](Cl_2)$	31	123	737
$[Co(TPA)(PDon)](BF_4)_2$	23	36	96
$PDMe(BF_4)$	33	73	96
$[Co(tren)(PDon)](BF_4)_2$[c]	–	–	117

[a] Turnover number for the catalyst per hour taken after the first minute of the reaction. – [b] in phosphate buffer pH = 8.5 up to 1209 turnovers/h could be found. – [c] pH = 8.2.

The data from Table 1 show that the reaction rate of the hydride ion transfer from NADH to the mediator is strongly increased through complexation of the phenanthroline system and by increasing the pH (up to 900 turnovers per hour). Even the methylation of the PDon system leads to reasonably better results.

As mentioned above, basic conditions for dehydrogenases are favoured for their application in enzymatic oxidation reactions. Fortunately the pH dependence of the turnover frequencies of the PDon-based mediators takes the same course so that best results could be expected. The new mediators are up to a factor of several hundred faster than the FMN system described by Jones and Taylor[1][4] for which up to 3 equivalents of the mediator with respect to the substrate had to be used.

For enzymatic applications of dehydrogenases in enzymatic syntheses the coproduction of hydrogen peroxide in the redox catalytic aerobic cofactor regeneration process is a disadvantage since both the enzymes and the cofactor NAD(P)H are oxidatively destroyed. The solution to this limitation is usually the addition of the enzyme catalase to destroy the hydrogen peroxide. A simple and easy to perform alternative would be the application of the anode as the end electron acceptor in an anaerobic regeneration system. Since the redox potentials of the mediators are around 0 V vs. Ag/AgCl, potentials which are more than 500 mV separated from the direct oxidation of the cofactor at the electrode, the indirect electrochemical cofactor regeneration using the PDon-based mediators should be very selective. Oxidatively labile substrates should also not be attacked at these potentials. The reaction path of this reaction is given in Scheme 3 and the results of the reactions are summarized in Table 2. For the measurements, the mediators were dissolved in 0.1 M phosphate buffer of pH 6, pH 7, and pH 7.9, 60 equivalents of NADH were added and the mixture electrolyzed as indicated in the experimental section. The reaction progress was followed by UV spectroscopy at 340 nm as described for the aerobic process with the difference that small samples were taken from the electrolysis cell at the appropriate times.

Scheme 3. Reaction path of the indirect electrochemical NADH oxidation (PDon = quinone form of the mediator; PDol = hydroquinone form of the mediator)

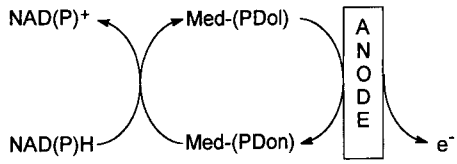


Table 2. pH dependence of the turnover frequency for the indirect electrochemical NADH oxidation

Compound	TN h^{-1}[a] pH 6.0	TN h^{-1}[a] pH 7.0	TN h^{-1}[a] pH 7.9
$[Ru(PDon)_3](ClO_4)_2$	30	35	39
$[Ru(TPA)(PDon)](Cl)_2$	23	21	21
$PDMe(BF_4)$	34	36	35
$[Co(TPA)(PDon)](BF_4)_2$	24	20	26

[a] Turnover number for the catalyst per hour taken after the first minute of the reaction.

In contrast to the redox catalytic aerobic NADH oxidation, much lower and practically pH-independent turnover frequencies were found. Although the turnover numbers are still strongly enhanced compared to the uncomplexed ligand and other quinoid systems there seems to be a limitation of the turnover frequency at about 40 TN/h. The reason is the heterogeneous charge-transfer process between the mediators and the anode so that the reaction rate is limited by the diffusional process to the electrode surface. A large effective electrode surface can overcome this limitation so that the enzymatic cofactor can effectively be reoxidized by the indirect electrochemical regeneration system (see preparative results).

Enzymatic Synthesis Using Horse Liver Alcohol Dehydrogenase (HLADH) and Redox Catalytic NAD$^+$ Regeneration

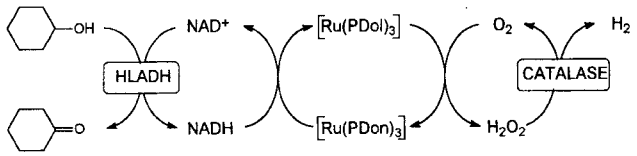
The next step in our investigations was to test the mediators in enzymatic systems so that we could get insights into the interactions of the mediator with the dehydrogenases. For this purpose we chose horse liver alcohol dehydrogenase (HLADH) as the production enzyme.

For most of the regeneration systems published up to now cyclohexanol was the test substrate. To be able to compare our results we therefore also used cyclohexanol. In most of the literature systems the reaction between the mediators and the reduced cofactor were the rate limiting step. However, as already pointed out, our redox catalytic aerobic regeneration using the $[Ru(PDon)_3]^{2+}$ complex as the catalyst was very fast so that we had to use very low mediator concentrations to keep the mediator/cofactor reaction as the rate limiting step instead of the enzymatic reac-

tion. On the other hand, we had to prove that the direct oxidation of the substrate through the mediator can be excluded. For these purposes, as unactivated secondary alcohol cyclohexanol and as activated alcohols cinnamic alcohol and *o*- and *m*-nitrobenzylic alcohol substrates were reacted with the oxidized mediators in aqueous buffer solution under aerobic conditions. Since no reaction occured at either room temperature or even at 60 °C, direct oxidation of the substrates through the mediators can be excluded.

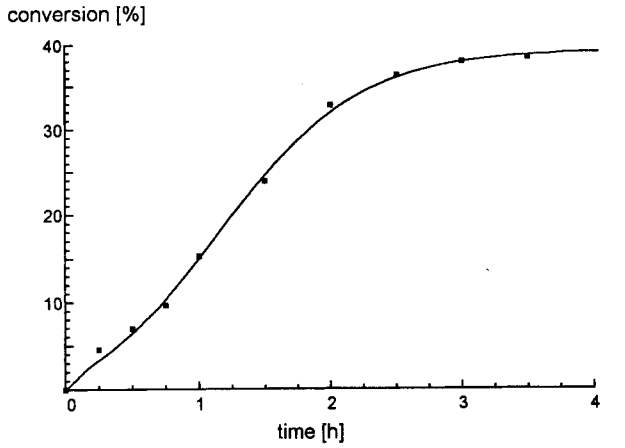
For the test reaction, the HLADH catalyzed oxidation of cyclohexanol under aerobic redox catalytic cofactor regeneration according to Scheme 4 we applied the following conditions: Cyclohexanol (468 mg; 4.7 mmol), NADH (27.6 mg, 39 μmol) and $[Ru(PDon)_3](ClO_4)_2$ (7.26 mg; 7.8 μmol) were applied in a ratio of 600:5:1 in 110 ml (0.1 M) aqueous phosphate buffer of pH 8 containing HLADH (12.5 mg; 20 U) and catalase (20 μl; 5200 U) under an oxygen atmosphere.

Scheme 4. Reaction scheme of the HLADH-catalyzed oxidation of cyclohexanol with redox catalytic aerobic regeneration of the cofactor (PDon = quinone form of the mediator; PDol = hydroquinone form of the mediator)



The reaction for which a time-yield diagram is given in Figure 1, shows that after an induction period for the transformation of NADH into NAD^+ the reaction rate reaches a maximum for reaction times between 1 and 2 hours. Product inhibition then starts to play a role so that the turnover stops after about 3 hours at a conversion of about 38%.

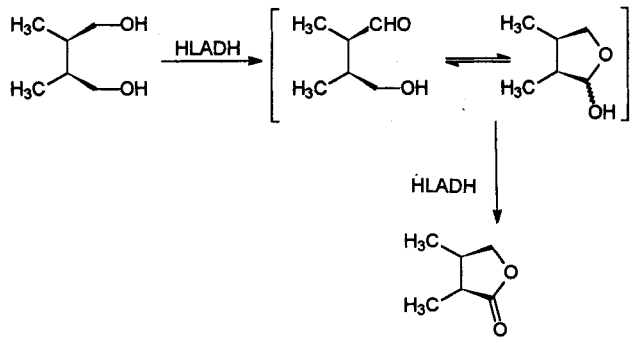
Figure 1. Reaction progress of the HLADH catalyzed oxidation of cyclohexanol using $[Ru(PDon)_3]^{2+}$ as mediator under aerobic conditions



For the conversion after 2 hours we calculated a turnover frequency of 98 cycles per hour for the mediator. Once again, these reaction rates have, thus far, not been reached by other published mediator systems.

For further enzymatic syntheses we chose the *meso*-diols as substrates to prevent product inhibition of the enzyme. This type of substrate can be oxidized in two steps, catalyzed by HLADH, to the corresponding hydroxyaldehyde which is in equilibrium with its lactol form and then irreversibly oxidized further to the lactone (Scheme 5).[1][4]

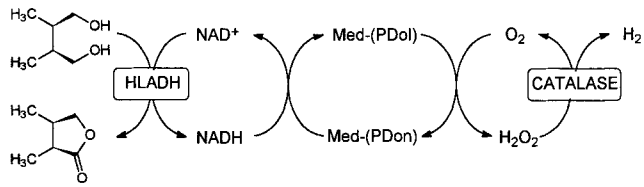
Scheme 5. HLADH-catalyzed oxidation of *meso*-2,3-dimethyl-1,4-butanediol



The lactones are themselves an interesting starting point for the synthesis of various natural products so that we focussed our investigation on the *meso*-diols *meso*-2,3-dimethyl-1,4-butanediol and *meso*-3,4-dihydroxymethylcyclohexene.

For the aerobic redox catalytic process given in Scheme 6 using different mediators the conditions were as follows: *meso*-2,3-dimethyl-1,4-butanediol (184 mg, 1.55 mmol), NADH (51.6 mg, 72.7 μmol) and the respective mediator (7.8 μmol) were applied in a ratio of 220:10.3:1 in 100 ml (0.1 M) aqueous phosphate buffer of pH 8 containing HLADH (12.5 mg, 20 U) and catalase (20 μl, 5200 U) under oxygen atmosphere. The progress of the reactions was followed by gaschromatography taking samples of 400 μl, extracting them with diethyl ether. The organic phases were dried over sodium sulphate and then injected on the HP-1 column.

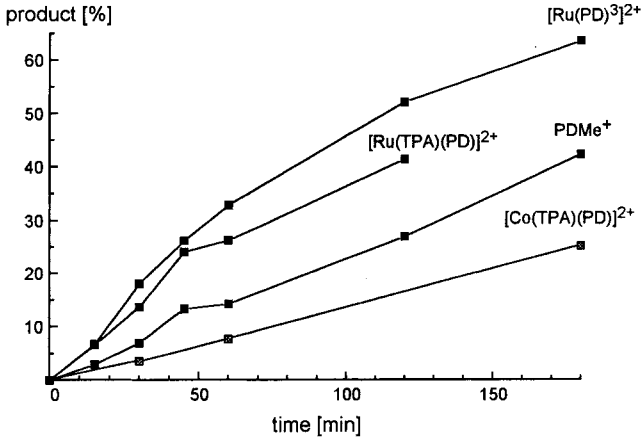
Scheme 6. Reaction scheme of the HLADH-catalyzed oxidation of 2,3-dimethyl-1,4-butanediol under aerobic regeneration of the cofactor (PDon = quinone form of the mediator; PDol = hydroquinone form of the mediator)



As can be seen from Figure 2 the highest reaction rates were obtained for the ruthenium complexes $[Ru(PDon)_3]^{2+}$ and $[Ru(TPA)(PDon)]^{2+}$ respectively. The turnover frequencies for the different mediators are based on the conversion after 60 minutes and are summarized in Table 3. However, for all mediators after 24 h reaction time no substrate could be detected any more and around 99% of the

desired product together with approximately 1% of the lactol form were detected.

Figure 2. Reaction progress for the HLADH catalyzed oxidation of *meso*-2,3-dimethyl-1,4-butandiol under redox catalytic aerobic regeneration of the cofactor



The calculated turnover frequencies for this process and especially for the ruthenium complexes do not reach the turnover numbers of the redox catalytic aerobic NADH oxidation (see Table 1) but they are again the best results published in this field up to now. One reason for the lower turnover frequency of the mediator is the lower ratio of cofactor to the mediator with a value of 10 to 1 while in the former case the ratio was about 60 to 1. For the application of the mediators in preparative enzymatic oxidations the ruthenium based mediators gave the best results and after 24 hours reaction time the mediators were still active and stable in solution without the formation of insoluble precipitates (Table 3). This demonstrates that the major requirements for a successful application of these mediators in aerobic enzymatic oxidations are fulfilled.

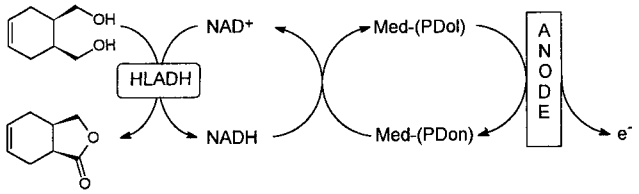
Table 3. Turnover frequencies of the mediators based on the conversion after 1 h reaction time for the redox catalytic aerobic cofactor regeneration in the HLADH catalyzed oxidation of *meso*-2,3-dimethyl-1,4-butandiol

Compound	TN h^{-1} [a]
$[Ru(PDon)_3](ClO_4)_2$	131
$[Ru(TPA)(PDon)](Cl)_2$	107
$PDMe(BF_4)_2$	65
$[Co(TPA)(PDon)](BF_4)_2$	30

[a] Turnover number for the catalyst per hour taken after 1 hour reaction time.

For the indirect electrochemical cofactor regeneration in HLADH catalyzed synthesis according to Scheme 7 we chose the cyclohexene derivative *meso*-3,4-dihydroxymethyl cyclohexene as the substrate. The conditions were as follows: *meso*-3,4-dihydroxymethyl-cyclohexene (204 mg, 1.44 mmol), NADH (60 mg, 85 μmol) and the respective mediator (16-23 μmol) were applied in ratios between 63:3:1 to 90:6:1 in 100 ml (0.1 M) aqueous phosphate buffer of pH 8 containing HLADH (12.5 mg; 20 U). The electrolysis was performed in an undivided cell at a graphite foil anode (55 cm^2) at a controlled potential of 50 mV vs. SCE. The progress of the reactions was followed by gas chromatography taking 400 μl samples, extracting them with diethyl ether. The organic phases were dried over sodium sulphate and then injected on the HP-1 column.

Scheme 7. Reaction scheme for the HLADH catalyzed oxidation of *meso*-3,4-dihydroxymethylcyclohexene under indirect electrochemical regeneration of the cofactor (PDon = quinone form of the mediator; PDol = hydroquinone form of the mediator)



The data presented in Figure 3 and Table 4 demonstrate that the reaction under indirect electrochemical conditions proceeds smoothly. Although the reaction rates are again very reasonable compared to other mediator systems the turnover frequencies are smaller in comparison with those of the aerobic processes described above. The reason for the smaller reaction rates under electrochemical conditions are based on the heterogeneity of the charge transfer process. This can be rationalized from the following observation. The oxidized form of the $PDMe^+$ mediator is light yellow in aqueous buffer solutions while its reduced hydroquinone form is red. Under aerobic conditions the solutions always stayed light yellow during the conversions with the $PDMe^+$ mediator indicating that the quinone form of the mediator is mainly present. However, under electrochemical conditions the colour of the solution turned red and stayed so during the whole reaction. This indicates that the major form of the mediator under the electrochemical conditions is the reduced hydroquinone form. The rate limiting step for the mediators under electrochemical condition thus is the charge transfer from the mediator to the anode. The reaction rates are further influenced by the diffusion coefficients so that under these conditions the low molecular weight $PDMe^+$ gave the best results. A larger electrode surface should thus have a positive effect on the reaction rates. This could be proved in larger scale preparative runs using a high surface area anode (see below). The poor results with the $[Co(TPA)(PDon)]^{2+}$ are partially due to the fact that a precipitate is formed from the reduced form of the mediator after a short time. As mentioned above, under indirect electrochemical conditions the reduced form of the mediator dominates while under aerobic conditions the more stable oxidized form is mainly present.

The investigation thus far showed that the best results for the aerobic regeneration pathway should be expected for the $[Ru(PDon)_3](ClO_4)_2$ complex or for the $[Ru(TPA)(PDon)](Cl)_2$ complex in enzymatic systems. On the other hand, the best results for the electrochemical regeneration system were obtained with $PDMe^+$ so that reactions of preparative scale were carried out with these mediators under the two alternative regeneration conditions.

Figure 3. Reaction progress for the HLADH catalyzed oxidation of the *meso*-diol under indirect electrochemical regeneration of the cofactor

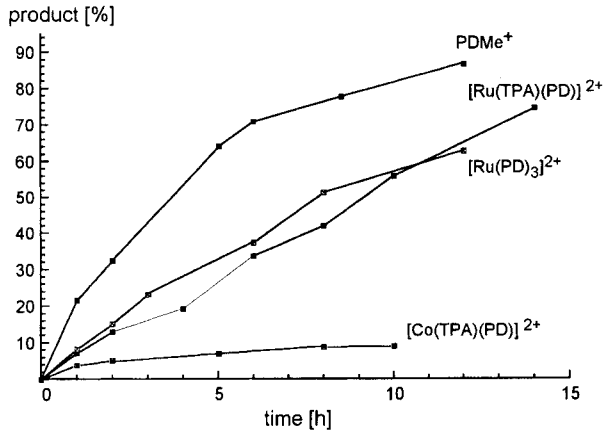


Table 4. Turnover frequencies for the mediators based on the conversion after 1 h reaction time during the indirect electrochemical regeneration of the cofactor in the HLADH catalyzed oxidation of *meso*-3,4-dihydroxymethyl cyclohexene

Compound	TN h^{-1}[a]
$PDMe(BF_4)_2$	31
$[Ru(PDon)_3](ClO_4)_2$	15
$[Ru(TPA)(PDon)](Cl)_2$	10
$[Co(TPA)(PDon](BF_4)_2$	5

[a] Turnover number for the catalyst per hour taken after 1 hour reaction time.

Larger Scale Enzymatic Synthesis Using Horse Liver Alcohol Dehydrogenase (HLADH) and Redox Catalytic NAD^+ Regeneration

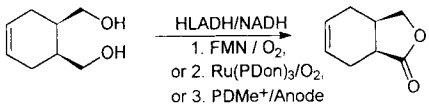
A larger scale enzymatic oxidation of 1g of the *meso*-3,4-dihydroxymethyl-cyclohexene with HLADH under aerobic regeneration of the cofactor according to Scheme 6 was carried out under an oxygen atmosphere at room temperature in aqueous buffer solution of pH = 7.8 using the mediator $[Ru(PDon)_3](ClO_4)_2$. After 28.5 hours the reaction was complete and the 900 equivalents of the substrate were oxidized so that the mediator underwent 1800 cycles resulting in an overall turnover frequency of 62 cycles/h. From the solution 90% of the desired compound could be isolated and the GC chromatographic investigation on a β-cyclodextrine phase gave >98% *ee* since no second enantiomer could be found. This indicates that the substrate is exclusively oxidized through the enzymatic pathway and not directly through the mediator because otherwise the product should be racemic.

An enzymatic oxidation of 1 g of the *meso*-3,4-dihydroxymethyl-cyclohexene with HLADH under indirect electrochemical regeneration of the cofactor according to Scheme 7 was carried out at room temperature in aqueous buffer solution of pH = 7.8 using the mediator $PDMe^+$. The electrolysis was performed in an undivided flow-through cell (250 ml volume of the whole system) equipped with a high surface area graphite felt anode at a controlled potential of 50 to 100 mV vs. SCE. After 20 hours the reaction was complete and the 350 equivalents of the substrate were oxidized so that the mediator underwent 700 cycles resulting in an overall turnover frequency of 35 cycles/h. Again, the product was enantiomerically pure. These experiments were compared with the published results by Jones[13] of the same substrate (Table 5).

Similarly, we performed the HLADH catalyzed oxidations of several other *meso* diols under redoxcatalytic aerobic (method 2) and indirect electrochemical (method 3) cofactor regeneration using $[Ru(PDon)_3]$ $(ClO_4)_2$ and $PDMe^+$ respectively as mediators at pH 7.8 (Scheme 8). In the case of *endo*-5,6-bis(hydroxymethyl)-2-norbornene, we observed at pH values of 9 to 8.8 that the lactol is the major product (lactol/lactone = 5:2).

We also performed the enzymatic oxidation of 2,4-pentanediol catalyzed by the alcohol dehydrogenase from Thermoanaerobium brockii (TBADH) under aerobic redox catalytic regeneration of $NADP^+$ in 100 ml of phosphate

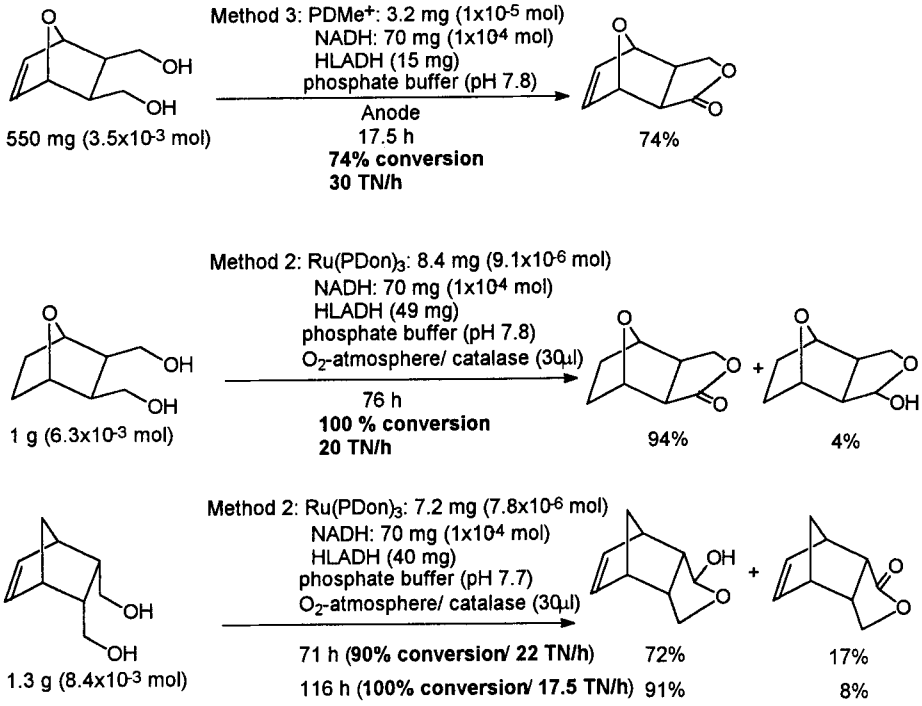
Table 5. Comparison of the enzymatic oxidation of a *meso*-diol 1. under indirect aerobic regeneration of NAD^+ using the system FMN/O_2 by Jones[13], 2. under indirect aerobic regeneration using $[Ru(PDon)_3](ClO_4)_2$ as the catalyst, and 3. under indirect electrochemical regeneration using $PDMe^+$ (*N*-methyl 1,10-phenthrolinium-5,6-dione tetrafluoroborate) as the catalyst



Conditions	1. FMN/O_2 (pH 9)	2. $Ru(PDon)_3/O_2$/catalase (pH 7.8)	3. $PDMe^+$/Anode (pH 7.8)
catalyst	9.72 g (20.3 mmol)	7.26 mg ($0.8 \cdot 10^{-5}$ mol)	3.2 mg ($1 \cdot 10^{-5}$ mol)
NAD	720 mg (1.1 mmol)	52 mg ($7.8 \cdot 10^{-5}$ mol)	70 mg ($1 \cdot 10^{-4}$ mol)
substrate	2 g (13.9 mmol)	1 g (7 mmol)	0.5 g (3.5 mmol)
HLADH	40 mg (50 U)	15 mg (19 U)	12.5 mg (16 U)
Catalase		30 µl (7800 U)	10 µl (2600 U)
time	48 h	28.5 h	20 h
conversion	83%	99.4%	99.5%
turnover frequency over the total reaction time	**0.025 TN/h**	**62 TN/h**	**35 TN/h**[a]
ratio substrate:NAD:catalyst	0.64:0.05:1	900:10:1	350:10:1

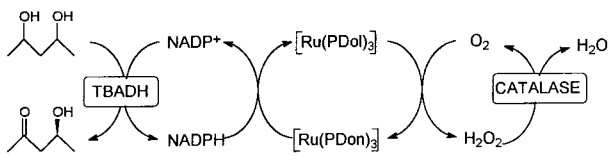
[a] After 4.5 h a turnover of 27% was reached corresponding to a turnover frequency of 82 TN/h.

Scheme 8. HLADH-catalyzed oxidation of *meso*-5,6-dihydroxymethyl-7-oxabicyclo[2.2.1]hept-2-ene under indirect electrochemical cofactor regeneration using $PDMe^+$ as the mediator



buffer of pH 7.3 according to Scheme 9. At a ratio of substrate to cofactor to mediator of 560:10:1 we obtained a conversion of 40% after 50 h to give (*4S*)-4-hydroxy-2-pentanone.[14] While for 2,3-butanediol at a ratio of substrate to cofactor to mediator of 200:10:1 at pH 7.4 we obtained 50% conversion after 6 h corresponding to 33 turnovers per hour.

Scheme 9. Enzymatic oxidation of 2,4-pentanediol-catalyzed by the alcohol dehydrogenase from *Thermoanaerobium brockii* (TBADH) under aerobic redox catalytic cofactor regeneration in phosphate buffer of pH 7.3 (PDon = quinone form of the mediator; PDol = hydroquinone form of the mediator)



Thus, it is obvious that the newly developed regeneration systems for NAD^+ are more efficient by several orders of magnitude than the FMN/O_2 system.[13] Presently, we are employing this system in combination with other $NAD(P)^+$ dependent enzymes.

Conclusion

In our studies we were able to demonstrate that redox catalysts on the basis of transition-metal complexes using 1,10-phenanthroline-5,6-dione as a ligand or on the basis of an *N*-methylated 1,10-phenanthroline-5,6-dione acting via hydride ion abstraction could solve most of the existing problems for an efficient and stable $NAD(P)^+$ regeneration system for alcohol dehydrogenase catalyzed oxidations. Under preparative conditions in the gram scale we were able to obtain turnover fequencies which are orders of magnitude larger than those of the presently most popular regeneration system. To render the electroenzymatic processes continuous, we are presently developing an electrochemical enzyme membrane reactor which is able to hold back the whole reaction system, enzyme, cofactor, and mediator, by using special membranes.

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Experimental Section

Instrumentation: UV-Vis spectra were recorded using a Cary 1 UV-Visible Spectrophotometer (Varian, Palo Alto, California, U.S.A.) and IR spectra were recorded at an FT-IR 1600 spectrometer (Perkin-Elmer, Überlingen, Germany). – The NMR spectra were obtained on a Bruker AC 200, AC 400, or WH 250 spectrometer. – Mass spectra by electron impact were obtained on an AEI MS-50 instrument at 70 eV. – Gas chromatographic analyses were performed on a HP 5980 GC (Hewlett Packard) equipped with a HP 1 column (12 m, 0.2 mm diameter, 0.33 µm film thick-

ness, nitrogen carrier gas). To determine the enantiomeric excess of the products gas chromatographic analysis was performed using a β-cyclodextrine phase (CS, Langerwehe) comparing the product with a racemic sample.

Chemicals: The buffer solutions were prepared by titration of 0.1 M KH_2PO_4 with 0.1 M K_2HPO_4, or 0.1 M H_3PO_4 solutions and the pH were adjusted using a pH-meter, pH 537 (WTW), with a glass electrode E56 (WTW). The synthesis of the redox catalysts has been described previously.[11b] The *meso*-diols were prepared according to literature procedures.[15][16] The enzymes and cofactors were commercially available (HLADH lyophylized, Fluka; TBADH, Sigma; NAD, Fluka).

General Procedures: The aerobic redox catalytic enzymatic syntheses were performed in a 250 ml round-bottom flask under an oxygen atmosphere while stirring carefully. 100 to 125 ml of phosphate buffer of pH 8.0 or 7.8, the substrate, mediator, cofactor, and enzymes were employed in the amounts as indicated in the results section.

The indirect electrochemical oxidations were performed under potential control using an AMEL potentiostat, Model 553 (AMEL, Milano, Italy). Two different electrolysis cells and electrode assemblies were employed. Cell 1 for $NAD(P)^+$ regeneration and preparative electro enzymatic oxidations in 200 mg scale consisted of an air-tight 110-ml beaker type glass cell with thermostating mantle and a top with five NS 14.5 junctions containing the connections to the working electrode, the reference electrode and the counter electrode, and an inert gas inlet. As the working electrode, a cylindrical carbon foil (Sigraflex®) of 55 cm^2 surface was placed at the wall of the electrolysis cell and contacted by a platinum wire. The platinum-wire counter electrode was placed concentrically in the middle of the cell and the tip of the luggin cappillary of the saturated calomel reference electrode (242 +/− 5 mV vs. NHE) was positioned at the surface of the working electrode. The electrolyses were performed at a potential of 50 mV vs. SCE. Cell 2, a flow-through cell equipped with a high surface area graphite felt anode that has been described previously[17], was used for the larger scale electrolyses in 500-mg or 1-g scale. A silver wire was employed as the pseudo-reference electrode and the electrolysis was performed at a controlled potential of 150 mV vs. the silver wire. The buffer solution containing the enzyme, the cofactor, the mediator and the substrate was circulated through the cell by a peristaltic pump. For isolation and characterization of the products, the whole solution was extracted several times with dichloromethane or diethyl ether, the organic phases dried over sodium sulphate, the solvent evaporated and the residue separated by liquid chromatography on flash silica gel (30−60 μm, Baker) using dichloromethane/methanol (20:1) as the eluent. The analytical data of the products compared well with the reported ones.[1][4][13] The enantiomeric excess determinations were performed by gaschromatography using a β-cyclodetrine phase after having proved the separation of the enantiomers by using a racemate of the respective products. The products of the TBADH catalyzed oxidation of diols were isolated by continous extraction of the aqueous phase with chloroform after saturation with sodium chloride followed by flash chromatography on silica gel using chloroform/acetone (2:1) as eluent.

☆ Dedicated to Professor *Hans J. Schäfer* on the occasion of his 60th birthday.

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