Efficient Photoelectrochemical *in-situ* Regeneration of NAD(P)$^+$ Coupled to Enzymatic Oxidation of Alcohols

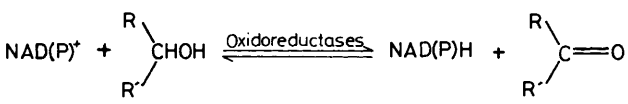
Romain Ruppert† and Eberhard Steckhan*
Institut für Organische Chemie und Biochemie der Universität Bonn, Gerhard-Domagk-Str. 1, D-5300 Bonn 1, FRG

The photoelectrochemical regeneration of NAD(P)$^+$ has been accomplished. With Ru(bipy)$^{2+}$ as a photosensitizer, methyl viologen, (MV^{2+}) as a primary electron acceptor (reoxidized at an anode), and NAD(P)H as an electron donor, NAD(P)$^+$ is formed. This photoelectrochemical NAD(P)$^+$ regeneration system has been coupled to enzyme-catalysed oxidations of alcohols.

The redox pairs NAD^+/NADH and $NADP^+$/NADPH are very important cofactors in biochemical redox processes. Currently, several hundred NAD^+- or $NADP^+$-dependent oxidoreductases are known. Application of these reactions in biotechnological processes for the synthesis of valuable chemicals requires effective means for the regeneration of the cofactor. For the reduction of carbonyl compounds (Scheme 1), this problem has been overcome by recycling catalytic amounts of coenzymes *via* coupled-enzyme,[1] coupled-substrate,[2] chemical,[3] and by indirect electrochemical[4] reduction. Efficient regeneration of catalytic quantities of coenzymes is even more important for oxidative processes since the equilibrium shown in Scheme 1 favours alcohol formation at all practical pH values.

Direct electrochemical oxidation of NAD(P)H is possible, and generates almost 100% enzymatically active NAD(P)$^+$. However, even at relatively high overpotentials, only low current densities are generally obtained.[5] Until now, a variety of organic mediators (for electrocatalytic recycling) have been studied,[6,7] but these systems are mainly investigated for their possible use as biosensors. Our group recently described an indirect electrochemical regeneration with trisbipyridyl iron(III) as an electron mediator for the enzymatic oxidation of alcohols.[8]

Maidan and Willner described a photochemical regeneration of coenzymes in multiphase systems,[9] however, the turnover numbers obtained for the expensive coenzymes remain very low. We now describe a photochemical regeneration, in which the primary electron acceptor can be reoxidized at an anode. The photoproduced enzyme-active NAD(P)$^+$ can be used in enzyme-catalysed oxidations.



Scheme 1.

Experimental

Materials.—Trisbipyridylruthenium dichloride [$Ru(bipy)_3$]Cl_2, trisphenanthrolineruthenium dichloride[10] [$Ru(phen)_3$]Cl_2, and [(1,3,6,8,10,13,16,19-octa-azabicyclo[6.6.6]eicosane)-cobalt(III)] chloride[11] [Co(sep)]Cl_3 were prepared according to published procedures. All other chemicals were purchased from Aldrich and used without further purification.

The cofactors [NAD(P)$^+$ and NAD(P)H] and the enzymes (horse liver alcohol dehydrogenase HLADH [EC 1.1.1.1] and alcohol dehydrogenase from *Thermoanaerobium brockii* TBADH [EC 1.1.1.2]) were obtained from Sigma. The anode materials (graphite electrodes) were purchased from Sigri.

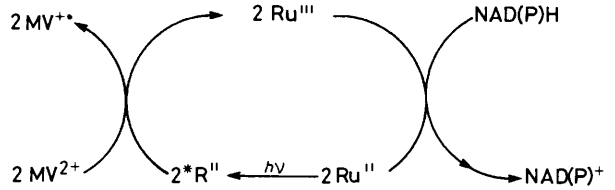
Apparatus.—The NAD(P)H concentration was determined by measuring the absorbance of the solutions at 340 nm (ε = 6 220 dm^3 mol^{-1} cm^{-1}) with a Cary 219 spectrophotometer. Illuminations were performed in a thermostatted Pyrex vessel with either a 250 W slide projector or a 450 W Xenon lamp from Müller Elektronik. Light was filtered through a 395 nm cut-off filter. The electrochemical equipment consisted of a FUG NTN 1400M-350 potentiostat and an integrator. Current *vs.* time curves were recorded on a BBC X–Y recorder. The products of the oxidations were determined on a Carlo Erba gas chromatograph equipped with a 50 m FFAP capillary column.

Reactor.—The photoelectrochemical reactor used was a water-jacketed beaker-type cell with a glass-frit cylinder as the cathode compartment. The working electrode was either a cylinder of carbon foil (area of 31 cm^2) or a cylinder of carbon felt (area of 27 cm^2 for the 50 cm^3 cell and 75 cm^2 for the 150 cm^3 cell; felt thickness: 5 mm). The reference electrode (Ag/AgCl) was connected to the anode compartment *via* a Luggin capillary.

The anolyte solution was degassed before irradiation and a small flow of argon was maintained over the solution during the experiments. The cathode compartment was filled with the same buffer solution and a platinum wire was used as the cathode.

Results and Discussion

Photosensitized NAD$^+$ Formation.—The oxidized form $Ru(bipy)^{3+}$ of the photosensitizer, obtained after oxidative quenching of the excited state $*Ru^{II}$, is able to oxidize NADH to NAD^+. This can be deduced from the electroanalytical data, which are well known: $E_0[Ru^{III}/Ru^{II}]$ = 1.26 V[12] and $E_0[NADH/NADH^{+\bullet}]$ = 1.05 V *vs.* NHE for the first mono-electronic oxidation step which is immediately followed by deprotonation and further electron transfer[13] (Scheme 2). This



Scheme 2. Photochemical regeneration of NAD(P)$^+$.

† *Present address:* ENSCL, Lab. de Chimie Organique Appliquée, U.A. C.N.R.S. 402, Cité Scientifique, 59652 Villeneuve d'Ascq, France.

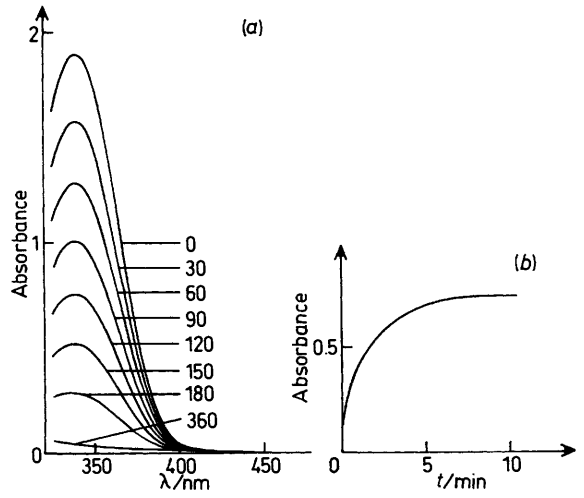
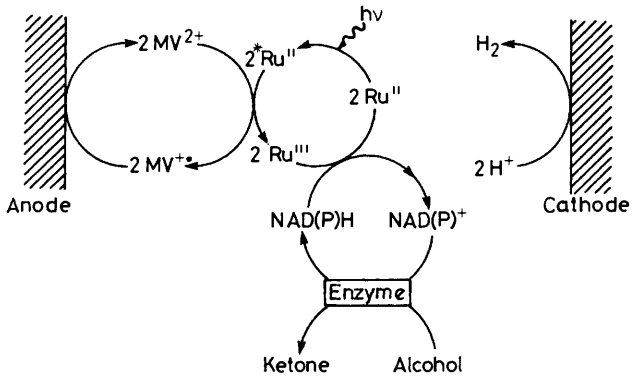


Figure 1. (*a*) NADH disappearance at time intervals of 30 s of illumination. 0.1 mol dm^{-3} Tris–HCl buffer (pH 9) containing $[Ru(bipy)_3]Cl_2$ at concentration 2.7×10^{-5} mol dm^{-3} and $[MV^{2+}]$ 2.4×10^{-4} mol dm^{-3}. The difference spectrum is recorded with an initial NADH concentration of 3.5×10^{-4} mol dm^{-3} in aerated solution; (*b*) after irradiation, NADH formation due to the addition of EtOH (30 mm^3) and HLADH (0.8 units) as a function of time (absorbance at 338 nm).



Scheme 3. Photoelectrochemical cofactor regeneration system with coupled enzymatic oxidation of alcohol.

Table 1. Photoelectrochemical enzymatic oxidation of cyclohexanol with various quenchers.[a]

Quencher	Current under irradiation/mA	Turnover number after 8 h[b] Ru^{II}	NAD^+	Quencher
MV^{2+}	2.5	128	6.4	6.4
$Co(sep)^{3+}$	2	86	4.3	4.3
A-Q-Sulph.[c]	1.35	57	2.9	2.9

[a] Tris–HCl buffer (50 cm^3) (0.1 mol dm^{-3}; pH 9): $[Ru(phen)_3]Cl_2$: 5×10^{-5} mol dm^{-3}, quencher: 0.001 mol dm^{-3}, NAD^+: 0.001 mol dm^{-3}, cyclohexanol: 0.05 mol dm^{-3} and 16 units HLADH. Irradiation with a 250 W lamp at 25 °C and an applied potential of 0.4 V (carbon-foil anode). [b] The turnover number is defined as the ratio of the number of moles of cyclohexanone produced in 8 h to the number of moles of photosensitizer, quencher, or cofactor initially introduced. [c] A-Q-Sulph. = Anthraquinone-2-sulphonate sodium salt.

can also be demonstrated by monitoring during irradiation the disappearance of the absorption due to NADH (at 340 nm) in a solution containing the photosensitizer, a quencher, and NADH [see Figure 1(*a*)].

Table 2. Photoelectrochemical enzymatic oxidation of cyclohexanol with various cofactor concentrations.[a]

$[NAD^+]$/ mol dm^{-3}	Cyclohexanone produced/mmol	Turnover number after 8 h Ru^{II}	NAD^+	MV^{2+}
10^{-3}	0.56	255	11.3	11.3
5×10^{-4}	0.51	205	20.5	10.3
2×10^{-4}	0.51	204	51	10.2

[a] Experimental conditions as in Table 1, except that a carbon-felt anode (27 cm^2) was used.

Addition of an enzyme (ADH) and an alcohol (EtOH) after complete photochemical oxidation of NADH clearly showed that enzyme-active NAD^+ was formed. The absorption at 340 nm was restored [see Figure 1(*b*)], showing typical enzymatic kinetics. Due to the equilibrium described in Scheme 1, the oxidation of ethanol (and reduction of NAD^+) could not proceed to completion and the concentration of NADH reached a plateau.

In the spectroscopic measurements, oxygen served as the ultimate electron acceptor. In preparative-scale experiments, oxygen must be removed since its reduction products ($O_2^{-\bullet}$ or H_2O_2) deactivate the enzyme and the cofactor. It was decided, therefore, to regenerate the quencher electrochemically.

Photosensitized Enzymatic Oxidation of Alcohols.—Many enzymatic redox processes are dependent on the NAD(P)H cofactors. Thus coupling of the photosensitized $NAD(P)^+$ regeneration cycle with enzyme-catalysed processes can allow various synthetic applications. The $NAD(P)^+$ regeneration system that includes the sensitizers $[Ru(bipy)_3]Cl_2$ or $[Ru(phen)_3]Cl_2$, an electron acceptor, and NAD(P)H as electron donor was coupled with $NAD(P)^+$-dependent enzymes. The oxidation of cyclohexanol to cyclohexanone and of butan-2-ol to butan-2-one as test systems was catalysed by alcohol dehydrogenases [HLADH: EC 1.1.1.1 (NAD^+ dependent) and TBADH: EC 1.1.1.2 ($NADP^+$ dependent), respectively].

The electron acceptor is reduced during the photochemical cycle, and its reduced form has been used to mediate the reduction of dibromo compounds in a multiphase system.[9] To ensure that the oxidative part of the complete system works optimally, we decided to regenerate the electron acceptor electrochemically, so that the reductive step could not be rate-determining. In this way, high turnover numbers were expected for the expensive cofactors NAD^+ and $NADP^+$. The complete photoelectrochemical system is described in Scheme 3. The reduction reaction in the electrochemical cell caused the evolution of hydrogen at the platinum cathode. In the homogeneous multiphase system described, the choice of the electron acceptor of the photochemical cycle was dictated by the reduction potential of the ultimate organic electron acceptor. The application of an anode as the ultimate electron acceptor allows the potential to be varied and also allows the use of various quenchers [or even the use of other photosensitizers which are able to oxidize NAD(P)H], therefore allowing ready optimization of the enzymatic part of the system.

We have tested some quenchers that have been used previously in the photochemistry of the sensitizer $[Ru(bipy)_3]Cl_2$.[14–16] The results obtained in the enzymatic oxidations are given in Table 1. The best results were obtained with the viologen quencher MV^{2+}. However, during these runs, we used a carbon-foil anode and the anolyte remained deep blue (the reduced form of the viologen $MV^{+\bullet}$ has an absorption maximum at *ca.* 610 nm) during irradiation, indicating that the area of the anode was not sufficient to reoxidize totally the

Table 3. Photoelectrochemical enzymatic oxidation of butan-2-ol as a function of time.[a]

			Turnover number			
t/h	Current/mA	Butan-2-one produced/mmol	Ru^{II}	$NADP^+$	MV^{2+}	Enzyme
3	12.7	0.9 (6 mmol dm^{-3})	120	30.1	6	32 000
6	10.5	1.5 (10 mmol dm^{-3})	199	50	9.9	53 000
9	9.2	2 (13.4 mmol dm^{-3})	267	67	13.3	71 500

[a] Tris–HCl buffer (150 cm^3; 0.1 mol dm^{-3}; pH 9): $[Ru(phen)_3]Cl_2$: 5 × 10^{-5} mol dm^{-3}, $NADP^+$: 2 × 10^{-4} mol dm^{-3}, MV^{2+}: 0.001 mol dm^{-3}, butan-2-ol: 0.05 mol dm^{-3} and 42 units TBADH. Irradiation with a 450 W Xenon lamp at 37 °C and an applied potential of 0.4 V (carbon felt anode; 75 cm^2).

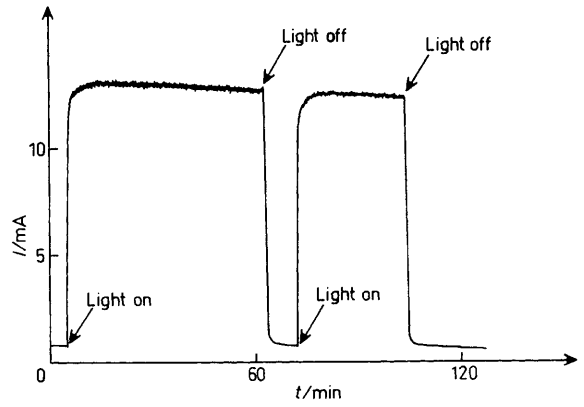


Figure 2. Current *vs.* time curve in the dark and under irradiation recorded in preparative-scale enzymatic oxidations. Experimental conditions as described in Table 3.

photochemically formed radical cation. The problem can be overcome by using an anode with a larger active surface, for example a carbon-felt electrode. On exclusion of the cofactor or the enzyme, almost no oxidation of the alcohol occurs. This indicates clearly that cyclohexanol and butan-2-ol are oxidized by the $NAD(P)^+$-dependent alcohol dehydrogenases and not by the photochemically produced Ru^{III} nor at the anode.

For the alcohol dehydrogenases (HLADH and TBADH) used in this work it was not necessary to use a 0.001 mol dm^{-3} $NAD(P)^+$ concentration to reach the maximal velocity of the enzymatic oxidation reaction.[17] As described in Table 2, it was shown that the overall reaction rate was almost invariant in the $NAD(P)^+$ concentration range 10^{-4}–10^{-3} mol dm^{-3}. With the lowest concentration of NAD^+, the electron donor to photosensitizer ratio was equal to four. This represents an extremely small amount of donor compared with the amounts usually used in classical photochemical systems.

Finally, we could also demonstrate that such a photoelectrochemical system works very well with $NADP^+$ dependent enzymes. We chose TBADH as the enzyme because it can withstand higher temperatures without loss of activity. The reaction could then be performed at 37 °C or higher. The amount of butan-2-one *vs.* time is shown in Table 3. After 9 h irradiation, butan-2-one (2 mmol) was produced using the 30 μmol $NADP^+$ initially introduced. The initial turnover frequency obtained of 10 h^{-1} for $NADP^+$ compares very well with other cofactor-regeneration systems. The high activity observed is also outlined by the almost quantitative current yield obtained. Figure 2 shows a typical current *vs.* time curve. The current measured in the dark remained very low (almost equal to the background current). As the light was switched on, the current immediately increased and reached a value of *ca.* 13 mA in the 150 cm^3 cell. When the light was switched off, the current decreased rapidly and reverted to the initial value, equal to that of the current in the dark. This clearly demonstrates that the regeneration of $NADP^+$ is a homogeneous photochemically driven reaction.

The diminution of the reaction rate observed in this system is not due to enzyme or cofactor loss, but can totally be accounted for by product inhibition. We found that the rate of butan-2-ol (50 mmol dm^{-3}) oxidation catalysed by TBADH (3 units) is dimished to 50% in presence of a 5 mmol dm^{-3} concentration of butan-2-one and to 25% in the presence of a 25 mmol dm^{-3} concentration of butan-2-one at 30 °C and pH 8.0.[18] After a given alcohol conversion, the reaction stops, if the oxidation product is not removed from the solution. We are currently working on similar systems with continuous extraction of the oxidation products.

Conclusions

We have developed a photoelectrochemical cofactor-regeneration system with coupled enzyme-catalysed oxidation of alcohol. In this process, the cofactor and the enzyme remain stable during the course of the reaction.

The oxidized photosensitizer formed during the photochemical cycle is reduced by NAD(P)H and the cofactor used in enzymatic oxidation. Such an 'electron-donor system' [$NAD(P)^+$ + enzyme + substrate] may be of special interest in the photochemically driven reduction of water. Generally, the sacrificial electron donors are introduced in great excess and are lost during the course of the reaction. Here, it is possible to envisage the photoreduction of water with an electron donor which is enzymatically regenerated. The large number of $NAD(P)^+$ dependent enzymes allows a desired oxidation processes to be chosen.

Acknowledgements

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