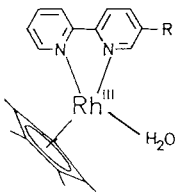
Continuous Generation of NADH from NAD$^{\oplus}$ and Formate Using a Homogeneous Catalyst with Enhanced Molecular Weight in a Membrane Reactor**

By *Eberhard Steckhan,** *Sabine Herrmann, Romain Ruppert, Jörg Thömmes*, and *Christian Wandrey*

Homogeneous catalysts, which are often highly efficient and selective even at low reaction temperatures, have usually been restricted to use in batch processes. On the other hand, heterogeneous catalysts, though well suited for continuous processes, often exhibit low selectivity. Only moderate success has been achieved so far in attempts to exploit the potential advantages of homogeneous catalysts by binding them to polymers in order to make them heterogeneous. Many problems remain to be solved; these include the nonuniform and partly unknown structures of the heterogeneous catalysts thereby obtained, hindered diffusion due to extensive crosslinking of the polymer matrix, low catalytic activity, loss of metal, and self-poisoning of the active centers.[1] Recently, we reported that the Rh^{III} complex **1** is an effective homogeneous catalyst for regeneration of the enzyme cosubstrates nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) either with formate as hydride donor[2] or electrochemically. The rhodium catalyst reacts with NAD(P)$^{\oplus}$ much faster than with carbonyl compounds, thereby allowing selective reduction of the cofactors, provided that suitable concentration ratios are chosen.[3] After 100 catalytic cycles involving **1**, no formation of 1,6-NADH could be detected enzymatically.



1, R = H
2, R = CH_2OPEG
3, R = CH_2OEt

An effective system for in situ regeneration of these expensive cosubstrates in enzymatic reactions is a prerequisite for the use of oxidoreductases as extraordinarily active catalysts in stereospecific organic synthesis. The usual enzymatic regeneration is not unproblematic.[4] A system employing formate dehydrogenase (FDH) as regeneration enzyme and formate as hydride donor has been brought to technical perfection.[5] Continuous production of enantiomerically pure amino acids is achieved by retaining the amino acid dehydrogenases, FDH, and polymer-bound high-molecular-weight NADH (PEG-NADH)[6] in an enzyme membrane reactor (EMR) behind an ultrafiltration membrane.

In principle, **1** could serve the same function as FDH.[2] Compared with the FDH system, this nonenzymatic generation of NADH has the following important advantages: (1) The reaction is zero order in NAD$^{\oplus}$ and, in contrast to FDH, **1** exhibits no product inhibition. (2) NADPH can be generated under the same conditions. (3) Complex **1** is more stable than an enzyme and not oxygen-sensitive.

By binding **1** to polyethyleneglycol (PEG, MW 20000), we have obtained, for the first time, a homogeneous catalyst (**2**) that can be retained by an ultrafiltration membrane, but which does not give rise to the problems associated with a heterogeneous catalyst, since it remains water soluble. The properties of **1** are therefore largely maintained. We were thus able to replace the FDH in the ultrafiltration flow reactor and generate NADH continuously in a homogeneous catalytic reaction. Because of the improved solubility of the PEG-bound complex, the stationary concentrations of catalyst can be larger than those possible with FDH. In addition, the reaction parameters can be widely varied.

In order to demonstrate that the catalytic properties of the rhodium complex **2**,[7] with enhanced molecular weight, are maintained in the ultrafiltration flow reactor, the kinetic parameters for the reduction of NAD$^{\oplus}$, NADP$^{\oplus}$, and PEG-NAD$^{\oplus}$[6] with formate catalyzed by **2** must be determined and compared with the values obtained for the analogous low-molecular-weight complex **3**.[8] The turnover frequencies[9] for the reduction of (PEG-)NAD(P)$^{\oplus}$ are only lowered by a factor of 0.56–0.66 by the increased molecular (MW 20000) weight of the rhodium complex (Table 1). The

Table 1. Turnover frequencies [h^{-1}] for complexes **2** and **3** in the catalysis of the reduction of NAD$^{\oplus}$, NADP$^{\oplus}$, and PEG-NAD$^{\oplus}$ (0.5 M HCOONa, 3.8×10^{-4} M cosubstrate, 2.5×10^{-5} M **2** or **3** in degassed, thermostated 0.1 M sodium phosphate buffer at pH 7.0; UV absorption at 340 nm monitored as a function of time).

	NAD$^{\oplus}$		NADP$^{\oplus}$		PEG–NAD$^{\oplus}$	
T [°C]	**3**	**2**	**3**	**2**	**3**	**2**
25	20.3	13.3	20.7	12.3	16.6	9.9
38	67.5	43.3	67.5	39.5	56.4	31.8

increase in molecular weight has no effect, however, on the activation energies of the reactions determined from the Arrhenius equation.[10] The reaction catalyzed by **2** is also zero order in NAD$^{\oplus}$. This can be determined by simple variation of the initial concentration of NAD$^{\oplus}$.[11] The reaction mechanism may be formulated as follows:

$$[Cp^*Rh(bpy\text{-}5\text{-}CH_2OPEG)(H_2O)]^{2\oplus} + HCOO^{\ominus} \rightleftharpoons [Cp^*Rh(bpy\text{-}5\text{-}CH_2OPEG)(HCOO)]^{\oplus} + H_2O$$

$$[Cp^*Rh(bpy\text{-}5\text{-}CH_2OPEG)(HCOO)]^{\oplus} \rightleftharpoons [Cp^*Rh(bpy\text{-}5\text{-}CH_2OPEG)H]^{\oplus} + CO_2$$

$$[Cp^*Rh(bpy\text{-}5\text{-}CH_2OPEG)H]^{\oplus} + NAD^{\oplus} + H_2O \rightleftharpoons [Cp^*Rh(bpy\text{-}5\text{-}CH_2OPEG)(H_2O)]^{2\oplus} + NADH$$

The retention of the catalytic properties of **2**, even in the reduction of PEG-NAD$^{\oplus}$, is of great importance, because this allows **2** to be used in a membrane reactor as shown in Figure 1. NADH generation was used to check the suitability of the polymer-bound high-molecular-weight complex **2** for use in a flow reactor. For this purpose, a novel, pressure-stable (to 3 bar) glass flow reactor equipped with an overhead stirrer was employed (Fig. 2). Unlike the reaction in the EMR, the reaction chamber is situated above the ultrafiltration membrane. This reactor has several basic advantages: Since it is made of glass, the reaction can be followed visual-

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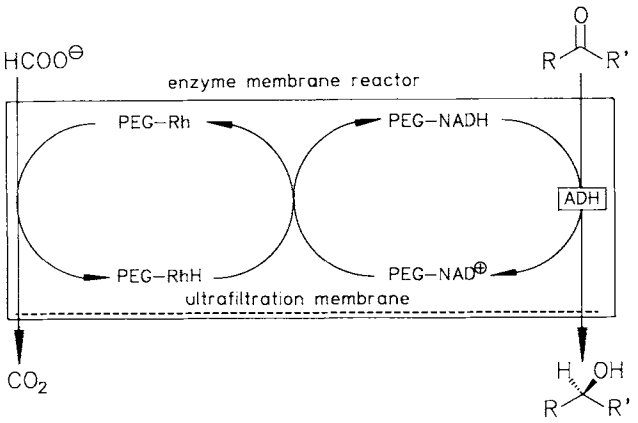

Fig. 1. Concept for the use of **2** in an enzyme membrane reactor (ADH: alcohol dehydrogenase).

ly. In addition, the reactor material should have no effect on the reaction. Moreover, the reactor can be employed for a wide variety of purposes; for example, a continuous electrochemical generation of NADH could be easily achieved by

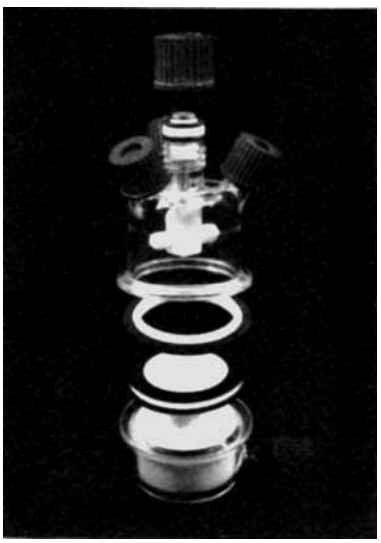

Fig. 2. Flow-through membrane reactor for continuous generation of NADH.

introducing electrodes. Reactions involving gaseous species also pose no problems. A schematic drawing of the experimental setup for continuous generation of NADH is shown in Figure 3.

With the aim of easily isolating the NADH formed, we used an unbuffered ammonium formate/$NAD^{\oplus}$ substrate solution (pH 5.6) in the initial continuous run. After addition of **2**, the conversion of $NAD^{\oplus}$ rapidly climbs to 70%, whereby **2** affords a turnover frequency of 26.3 h^{-1} (Fig. 4A). Although the subsequent decrease in conversion can be slowed by a longer residence time (Fig. 4B), only after replacement of ammonium formate by sodium formate after 30 h of continuous operation (Fig. 4C) does the conversion increase from 30 to 45%. A stationary state is then reached. The large decrease in conversion in the presence of ammonium formate could be due to the incorporation of NH_3 into the ligand sphere of the Rh atom. This ligand exchanges more slowly than H_2O and $Cl^{\ominus}$ with formate. Accordingly, the use of strongly coordinating ligands in the reaction medium should be avoided. In a second experiment with an $NAD^{\oplus}$/sodium formate substrate solution (4.1 mM/0.5 M) buffered to pH 8 with phosphate (0.1 M), the conversion increases at 25 °C in the presence of a doubled concentration of

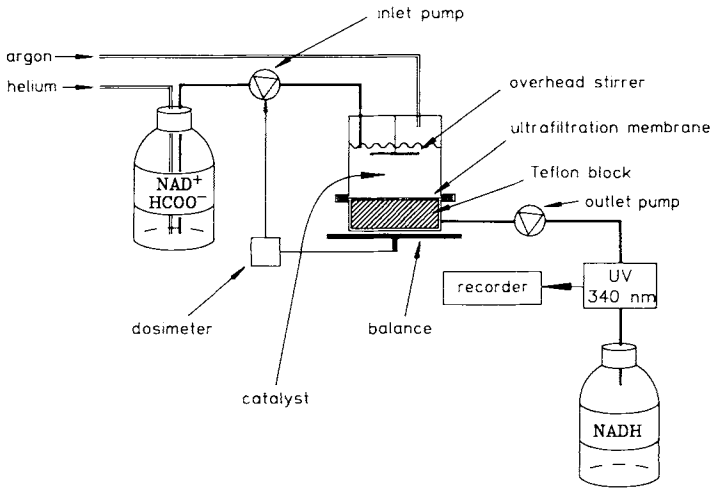

Fig. 3. Experimental setup for continuous generation of NADH.

2 to 70% and then decreases, though more slowly than in the first experiment, over twenty residence times to a stationary conversion of 50%. Before the stationary state was reached, the residence time was doubled by doubling the reaction volume. Conversions of up to 80% were sometimes

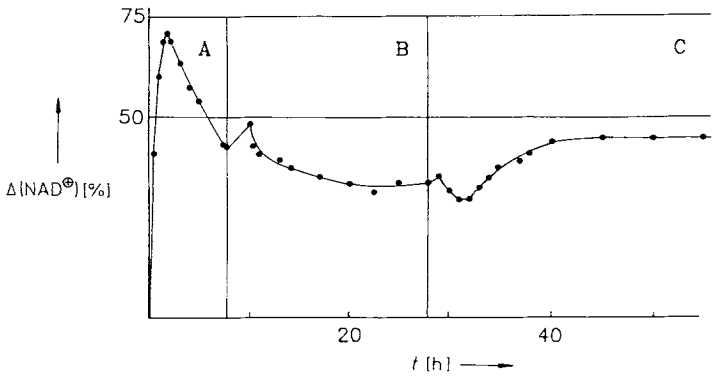

Fig. 4. Dependence of $NAD^{\oplus}$ conversion $\Delta(NAD^{\oplus})$ on time t for continuous generation of NADH (5 mM $NAD^{\oplus}$, 0.13 mM **2**; A,B, 0.5 M $HCOONH_4$; C, 0.5 M HCOONa; residence time A,C, 1 h; B, 2 h; membrane precoated with 300 mg PEG 35000).

achieved. The quantification of $NAD^{\oplus}$ in the substrate and product solution shows that material balance is maintained over the entire period of the experiment. The cause of the decrease in conversion has not yet been determined. However, loss of complex end groups due to hydrolysis, as well as washing out of **2**, can be excluded.[12] The increase in the conversion caused by temporarily doubling the reaction volume in the second experiment shows that an irreversible deactivation of **2** cannot be a reason for the decrease in conversion.

The experiments show that the increased-molecular-weight redox catalyst **2** exhibits the same properties as the low-molecular-weight model. In the flow reactor, a continu-

ous conversion of 45–50% can be maintained after the stationary state has been reached. The redox catalyst thereby displays a turnover frequency of 16.9 h^{-1}. The entire period of the experiment involved more than 1000 reaction cycles. The space–time yield is 44.4 mmol L^{-1} d^{-1}. Taking into consideration the kinetic reaction data, complete conversion can be expected for analogous runs at the stationary state when a 2.2-fold concentration of **2** is employed. This would correspond to a space–time yield of 98.4 mmol L^{-1} d^{-1}. Thus, the rhodium catalyst **2**, with enhanced molecular weight, is very well suited for the continuous regeneration of NADH and NADPH, as well as of their polymer-bound high-molecular-weight forms in coupled enzymatic reactions. The procedure described here offers a widely variable alternative for the enzymatic regeneration of NAD(P)H.

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1, 125568-56-1; **2**, 125591-72-2; **3**, 125591-71-1; PEG, 25322-68-3; $NAD^{\oplus}$, 53-84-9; $NADP^{\oplus}$, 53-59-8; PEG-$NAD^{\oplus}$, 53-84-9; NADH, 58-68-4; NADPH, 53-57-6; NaHCOO, 141-53-7; PEG-2,2'-bipyridyl-5-methyl deriv., 125591-70-0; 5-ethoxymethyl-2,2'-bipyridine, 125568-55-0; 5-brommethyl-2,2'-bipyridine, 98007-15-9.

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[6] Polyethyleneglycol-(20000)-N^6-(2-aminoethyl)-NADH and -$NAD^{\oplus}$: A. F. Bückmann, M. R. Kula, R. Wichmann, C. Wandrey, *J. Appl. Biochem. 3* (1981) 301.
[7] Experimental procedure for **2**: Freshly sublimed potassium *tert*-butyl alkoxide (115 mg, 1 mmol) was added under argon atmosphere to a solution of PEG (MW 20000; 5 g, 0.25 mmol) in 1 L of dry THF. After refluxing for 20 h, the reaction solution was treated with 5-bromomethyl-2,2'-bipyridine (500 mg, 2 mmol) and then refluxed for an additional 28 h. It was then allowed to cool, 200 mL of water was added, and the THF was removed in vacuo. After dialysis and freeze-drying, 4.7 g of colorless, cotton-wool-like product was obtained. UV: λ_{max} [nm] = 242, 303. Part of the product (4.3 g) was dissolved in 1 L of dry methanol and the resulting solution was treated with di-μ-chloro(dichloro)bis(pentamethylcyclopentadienyl)dirhodium(III) (400 mg, 0.65 mmol) (synthesis: B. L. Booth, R. N. Hazeldine, M. Hill, *J. Chem. Soc. A. 1969*, 1299) and stirred for 1 h. Water (1 L) was then added and the organic solvent was removed in vacuo. After dialysis and freeze-drying, 4.1 g of a pale orange product was obtained. UV (H_2O, pH 7.5, 25 °C): λ_{max} [nm] (ε[L mol^{-1} cm^{-1}]) = 233 (12700), 308 (6150), 318 (6300).
[8] Physical data for 5-ethoxymethyl-2,2'-bipyridine and for **3**: Synthesis of 5-ethoxymethyl-2,2'-bipyridine from 5-bromomethyl-2,2'-bipyridine (synthesis: J. G. Eaves, H. S. Munro, D. Parker, *Inorg. Chem. 26* (1987) 644) by reaction with $EtO^{\ominus}$. ^1H NMR (60 MHz, $CDCl_3$, TMS int.): δ = 1.26 (t, 3H, ^3J = 7 Hz), 3.57 (q, 2H, ^3J = 7 Hz), 4.53 (s, 2H), 7.07–8.73 (m, 7H). MS (70 eV, 300 μA): *m/z* 214 ($M^{\oplus}$, 42%), 185 ($M^{\oplus}$ – Et, 28), 169 ($M^{\oplus}$ – OEt, 100), 157 (bpy + H, 55), 141(22), 115(5), 78 (py, 13), 51(8). UV (H_2O; pH 3.0; 25 °C): λ_{max} [nm] (ε[L mol^{-1} cm^{-1}]) = 242 (8900), 304 (1680). Preparation of the rhodium complex was carried out in analogy to U. Kölle, M. Grätzel, *Angew. Chem. Int. Ed. Engl. 26* (1987) 567; ^1H NMR (60 MHz, D_2O): δ = 1.23 (t, 3H, ^3J = 7 Hz), 1.58 (s, 15H), 3.63 (q, 2H, ^3J = 7 Hz), 7.57–8.97 (m, 7H). UV (H_2O; pH 7.5; 25 °C): λ_{max} [nm] (ε[L mol^{-1} cm^{-1}]): 233 (31600), 308 (15300), 318 (15700). Correct elemental analysis (C, H, N).
[9] Turnover frequency = (PEG-)NAD(P)H concentration/(catalyst concentration × time).
[10] The activation energies for the reaction of $NAD^{\oplus}$ and (PEG-)$NAD^{\oplus}$ (3.8×10^{-4} M each) in sodium formate (0.5 M) in the presence of **2** and **3**, respectively (2.6×10^{-5} M each) were determined at 17.3, 25.7, 32.1, and 40.0 °C in sodium phosphate buffer at pH 7.0. For the reduction of $NAD^{\oplus}$, E_a is 64.0 and 68.2 kJ mol^{-1} for **2** and **3**, respectively; for the reduction of (PEG-)$NAD^{\oplus}$, E_a is 70.3 and 68.6 kJ mol^{-1} for **2** and **3**, respectively.
[11] The reaction order of zero in $NAD^{\oplus}$ was confirmed by applying a differential method of determining the reaction order to two NADH concentration–time curves.
[12] Complex **2** is stable upon dialysis (six times against 9 L of bidistilled H_2O) and under flow-through conditions in H_2O (six residence times) in an Amicon ultrafiltration unit.

$Se_{10}^{2\ominus}$, a Bicyclic Polyselenide

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With the exception of the undecaselenide **1**, whose anion has a spirocyclic structure,[1] and the recently described hexadecaselenide 2, whose anion consists of Se_6 rings and two $Se_5^{2\ominus}$ chains,[2] all other structurally investigated polyselenides are acyclic. Known compounds are the diselenide **3**,[3] the tetraselenides **4**–**8**,[4–7] the pentaselenides **9**–**12**,[7–10] and the hexaselenides **13** and **14**.[11,12]

$(PPh_4)_2Se_{11}$ **1** Cs_4Se_{16} **2** Na_2Se_2 **3** [Ba(222-Crypt)]Se_4 **4** $[Ba(en)_4]Se_4$ **5**

$(NC_4H_8O)_2Se_4$ **6** $(NC_5H_{10})_2Se_4$ **7** $[Ph_3PNPPh_3]_2Se_4 \cdot 4CH_3CN$ **8**

Rb_2Se_5 **9** Cs_2Se_5 **10** [Cs([18]crown-6)]$_2Se_5 \cdot CH_3CN$ **11** $(PPh_4)_2Se_5$ **12**

$(NBu_4)_2Se_6$ **13** $[(CH_3)_3N(CH_2)_{13}CH_3]_2Se_6$ **14**

Although the conditions leading to formation of polyselenides are not understood in detail, important factors include reaction conditions such as solvent and temperature in addition to the size, charge, and shape of the counterion. For instance, the tetraselenide **8** is formed from Cs_3TaSe_4 in acetonitrile in the presence of $[Ph_3PNPPh_3]Cl$ at room temperature,[7] whereas, as we report here, heating of a polyselenide solution in dimethylformamide (DMF) at 100 °C in the presence of $[Ph_3PNPPh_3]Cl$, followed by cooling of the solution, affords the previously unknown bicyclic decaselenide $[Ph_3PNPPh_3]_2Se_{10} \cdot DMF$ as black crystals in very good yield.

The crystal structure analysis[13] showed that, per formula unit, the compound contains one molecule of DMF disordered over two mutually orthogonal positions. The $Se_{10}^{2\ominus}$ ion is located on a crystallographic twofold axis; its symmetry corresponds to point group C_2. The two selenium six-membered rings have chair conformation (Fig. 1). Compared to the decalin molecule, the decaselenide ion contains four additional valence electrons if a selenium atom is regarded as isolobal to a CH_2 group. This has several consequences for the central Se–Se unit: The selenium atoms are coordinated in a distorted pseudo-trigonal-bipyramidal fashion, whereby the selenium atoms Se3 und Se3' are linked equatorially. The two lone pairs on these selenium atoms can

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