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Chemical and Electrochemical Oxidation of Aqueous Solutions of NADH and Model Compounds *

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Summary

Aqueous solutions of NADH and of model compounds such as 1,4-dihydro-N1-propyl- and 1,4-dihydro-N1-benzyl-nicotinamide are oxidized on rotating Pt disk electrode at +0.66 ± 0.02 V (N.H.E.). This two-electron wave is pH independent in the range of 7–13. Both macroelectrolysis on Pt at +0.85 V (N.H.E.) and chemical oxidation by [Fe(CN)6]3− lead to NAD+ or model compounds. Differences between biochemical, chemical and electrochemical oxidation of NADH are discussed, especially in terms of energetics and mechanisms of reactions.

Introduction

It is now well known that the nicotinamide adenine dinucleotide (NAD+)—dihydronicotinamide adenine dinucleotide (NADH) system plays a prominent part in the biological oxidation-reduction reactions. It participates in a large number of metabolic pathways as well as in the respiratory chain. However, the mechanism of these reactions has not yet been elucidated, even in the absence of pyridine nucleotide dependent enzymes.

In order to simplify the study of their redox properties NAD+ and NADH have been commonly replaced by model compounds in which a simple alkyl group takes the place of the ribose–pyrophosphate–adenine moiety: their redox and spectrophotometric characteristics are very similar to the NAD+–NADH ones.

Extensive works have been published on the reduction of pyridine nucleotides (mainly NAD+) and of model compounds (mainly N1-methyl nicotinamide salts) 1–7 but the oxidation of NADH and its analogs has given rise to still few and partial studies. 8 In unbuffered acetonitrile

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HAAS\textsuperscript{9-10} reported a one electron oxidation leading to NAD\textsuperscript{+} or model compound by macroscale electrolysis. In pH 8.6 aqueous solutions of NADH he obtained\textsuperscript{10} an oxidation wave on rotating Pt electrode \([U_{1/2} = 0.90 \pm 0.02 \text{ V (N.H.E.)}]\) and on rotating carbon electrode \([U_{1/2} = +0.67 \pm 0.03 \text{ V (N.H.E.)}]\) leading to NAD\textsuperscript{+} by a two electron macroscale electrolysis. The pH range of these studies was not large enough to bring useful information on the mechanism energetics of the electron and proton transfer.

Furthermore attention should be paid for the "primary acid modification" of aqueous NADH or models solutions: at pH below 7 the 5,6 double bond of 1,4 dihydronicotinamides is saturated, the UV absorption band is shifted from 355 to 290–300 nm and the chemical oxidation of the new compound is more difficult.\textsuperscript{11-22}

Being aware of the possible spontaneous reactions of NADH and models in aqueous buffered solutions, we have determined the principal electrochemical features of these compounds and followed their complete oxidation both by macroscale electrolysis and by chemical reaction.

**Experimental**

**Reagents**

NADH was obtained by "MANN" (from NAD\textsuperscript{+} 90% reduced). All other products were prepared in our laboratory:

- NMeN\textsuperscript{+}I\textsuperscript{−} according to Holman and Wiegand\textsuperscript{23} (M.P.: 211°C, reported 204°C, "SIGMA" and "K and K" reagents: 210–211°C),
- NBzN\textsuperscript{+}Cl\textsuperscript{−} according to Kim and Chaykin\textsuperscript{20} (M.P.: 245°C, reported 241°C),
- NPrN\textsuperscript{+}Br\textsuperscript{−} according to Anderson and Berkelhammer\textsuperscript{12} (M.P.: 170–175°C reported 168–172°C),
- NMeNH was prepared by the procedure given by Karrer\textsuperscript{11} as modified by Haas\textsuperscript{10} (M.P.: 84°C, reported 84–85°C). Stored in a freezer at −20°C with P\textsubscript{2}O\textsubscript{5}, it nevertheless decomposed to a red, viscous residue and could not be recovered by any recrystallization,
- NPrNH was prepared according to Suelter and Metzler\textsuperscript{24} (M.P.: 92°C, reported 86–96°C),
- NBzNH according to Anderson and Berkelhammer\textsuperscript{12} (M.P.: 123°C dec., reported 120°C).

The last three dithionite reductions were performed in a glove bag with permanent N\textsubscript{2} circulation; all reaction, extraction or recrystallization solvents were previously deoxygenated by a N\textsubscript{2} stream.

Buffer solutions were prepared from chemicals of analytical reagent grade, at a concentration 100 or 200 time greater than those of examined compound (usually 0.1 \textit{M} acidic and basic form).
Control of purity. Determination of concentration

We checked all our aqueous solutions with a Cary 14 recording spectrophotometer and a pair of five millimeters silica cells.

With freshly prepared solutions of our products we found the following $\varepsilon_{\text{max}}$ and $\lambda_{\text{max}}$: $\varepsilon_{\text{max}} = 7250 \pm 50$ for NBzNH; $\varepsilon_{\text{max}} = 7100 \pm 50$ for NPrNH at $\lambda_{\text{max}} = 355 \text{ nm}$, $\varepsilon_{\text{max}} = 3950 \pm 50$ for NBzN$^{+}$Cl$^{-}$ and NPrN$^{+}$Br$^{-}$ at $\lambda_{\text{max}} = 265 \text{ nm}$. These values are in good agreement with those previously reported. Therefore, all concentrations of freshly prepared solutions were given with an error range of 2%.

Apparatus

For voltammetry and polarography a conventional three electrodes cell of 20 cm$^3$, thermostated at 25.0 $\pm$ 0.1 °C was employed.

Although the potential are given vs. N.H.E. all measurements were made with Ag|AgCl|KCl sat. reference electrodes, which potentials were frequently controlled vs. a saturated calomel electrode.

For drop–time controlled polarography we used a 30 cm capillary with a mercury height of 50 cm; the free drop–time was 8.2 s and the mercury flow rate 0.85 mg/s; the drop–time was controlled by a Tacussel electrical hammer (M.P.O. animated by a G.C.M.R.) and regulated at 0.5 s.

The working electrodes used in voltammetry were disks of 1.0 mm diameter of Pt, Au and glassy carbon.

Polarography and voltammetry were carried out with different Tacussel apparatuses (PRT 500 LC, Servovit 9 B, S6 RZ millivoltmeter) and a Sefram XY Luxytrace bigalvanometric recorder.

For coulometry, a Tacussel IG 4 – 1100 electronic integrator was used, checked by a current recorder Sefram–Graphispot. During the beginning of macroelectrolysis a Fontaine A–60 V power supply was placed between the counter electrode and the generator.

Procedure

Test solutions of NBzN$^{+}$Cl$^{-}$ and NPrN$^{+}$Br$^{-}$ were prepared by adding small quantities of highly concentrated solutions to known volume of deoxygenated buffer; because of the weaker solubility of NBzNH and NPrNH the solutions were directly prepared in the buffer and then deoxygenated.

The disk electrodes for voltammetry were mechanically treated by successive polishings using emery papers. The glassy carbon electrode was, according to Haas 10 dipped during 30 s in concentrated H$_2$SO$_4$. The rotation speed was 1000 r.p.m. for all measurements.

The sweep rate was 0.120 V/min for voltammetry and 0.250 V/min for d.c. polarography.
Electrochemical properties of NADH and model compounds in aqueous solution

The size of NADH molecule and the nature of the ribose–pyrophosphate–adenine side chain on the pyridine ring have two consequences: first, a rather small mobility and consequently weak diffusion currents; second, some adsorption reactions which are likely to make difficult the determination of the electrochemical properties of the compound under investigation.

Therefore in this study we have mostly used model compounds of NADH, namely the 1-4, dihydro-N1-benzylnicotinamide (NBzNH) and the 1-4, dihydro-N1-propyl nicotinamide (NPrNH).

Electrochemical properties of the NBzNH

The NBzNH molecule is oxidizable at a rotating Pt disk electrode; the current–potential curve corresponding to buffered aqueous solutions, containing $5 \times 10^{-4} M$ of NBzNH, is reproducible within $\pm 20$ mV fluctuation, whatever is the mechanical or chemical treatment of the electrode surface.

We registered different curves by anodic and cathodic potential scan in the range from $+0.2$ up to $+1.2$ V (N.H.E.); the two corresponding $U_{1/2}$ values differ by about 30 mV. Thus all given $U_{1/2}$ values refer to anodic potential sweep.

Influences of pH variations

Checking the solutions by UV spectrophotometry we observed that NBzNH is reasonably stable when the pH ranges from 7.0 to 13.0 in aqueous buffered solutions.

Within these limits, we registered a single pH independent oxidation wave, corresponding to $U_{1/2} = +0.66 \pm 0.02$ V (N.H.E.) and $I_d = 5.8 \pm 0.2 \mu A \, m M^{-1}$.

The slope of this wave is rather weak, $U_{3/4} - U_{1/4} = 90 \pm 7$ mV, and corresponds to an irreversible process.

Influence of the NBzNH concentration

The observed anodic wave shows an amplitude proportional to the NBzNH concentration between $3 \times 10^{-5}$ and $5 \times 10^{-4} M$; the half–wave potential does not significantly depend upon the concentration.

Determination of the number of exchanged electrons

We recorded, within the same experimental conditions, the current–potential curves for solutions of known compounds, namely ferricyanide, hydroquinone and 1-4 dihydroxyanthraquinone (Table 1). All these
molecules are roughly of the same size as NBzNH; so if one compares their limiting diffusion current with that measured for NBzNH, one can deduce that two electrons are involved in the voltammetric oxidation process of NBzNH.

Table 1. Comparison of diffusion currents obtained by voltammetry on the same rotating Pt disk electrode.

<table>
<thead>
<tr>
<th>Products</th>
<th>Molecular weight</th>
<th>Number of electron exchanged</th>
<th>$I_d/c$ $\mu A$/mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Fe(CN)$_6$]$^{3-}$</td>
<td>329</td>
<td>1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>$\beta$-hydroquinone</td>
<td>110</td>
<td>2</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td>1,4-dihydroxyanthraquinone</td>
<td>240</td>
<td>4</td>
<td>12.2 ± 0.6</td>
</tr>
<tr>
<td>NPrNH</td>
<td>166</td>
<td></td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>NBzNH</td>
<td>214</td>
<td></td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td>NADH</td>
<td>664</td>
<td></td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

Influence of the rotation speed and of the material of the electrode

The anodic wave observed with a rotating Pt disk shows an amplitude proportional to the square root of the electrode rotation speed (between 200 and 2000 r.p.m.); the potential does not depend on this parameter. In order to check the possible influence of electrode material, we compared the current-potential curves registered for the same NBzNH solution ($5 \times 10^{-4} M$), at pH 8.2, with different disk electrodes, made of Pt, Au, and glassy carbon. We obtained:

with Pt: $U_{1/2} = 0.66 \pm 0.02$ V (N.H.E.); $U_{3/4} - U_{1/4} = 90 \pm 7$ mV

with Au: $U_{1/2} = 0.60 \pm 0.025$ V (N.H.E.); $U_{3/4} - U_{1/4} = 100 \pm 10$ mV

with C: $U_{1/2} = 0.48 \pm 0.04$ V (N.H.E.); $U_{3/4} - U_{1/4} = 120 \pm 10$ mV

These results indicate that the nature of the electrode material does not alter the shape of the irreversible oxidation wave but influences significantly its half-wave potential.

Electrochemical properties at pH lower than 7

At pH below 7 the NBzNH molecule is spontaneously altered by saturation of the 5,6 bond in the pyridine ring.
This chemical reaction results on the one hand in a modification of the UV absorption spectrum: the absorption peak at 365 nm is replaced by another, located between 290 and 300 nm; on the other hand, the redox properties of the solution are changed (Fig. 1): the reaction product shows at the rotating Pt disk electrode a single pH-independent anodic wave with $U_{1/2} = 1.075 \pm 0.01$ V (N.H.E.) in the 4.6–7.0 pH range.

Comparison between the electrochemical properties of NADH and of its model compounds in aqueous solutions

With a rotating Pt disk electrode we registered an anodic wave in aqueous solutions of NADH and NPrNH.

NPrNH presents as model compound of NADH an important disadvantage; it evolves in solution still more rapidly than NBzNH:

so it is still more necessary to use freshly prepared solutions and to check their UV spectrum during the voltammetric recordings. The current potential curves registered for $5 \times 10^{-4} M$ NPrNH solutions, at pH 8.2 and 9.2, show half-wave potentials, slopes and limiting diffusion currents which are equal to the above mentioned results for similar NBzNH solutions (Fig. 1 and Table 1).
For the purpose of comparison, we performed a measurement with a $10^{-3}$ M NADH solution at pH 9.5 on a rotating Pt disk electrode; the corresponding anodic wave shows an equal potential to that observed for NBzNH and NPrNH (Fig. 1). However, the diffusion current reaches a limiting value about ten times weaker (Table I). Furthermore no distinct wave can be observed for a return potential sweep or later anodic–cathodic sweeps, unless the electrode surface has been mechanically repolished. The electrochemical oxidation reaction of NADH is thus partially perturbed or hidden by some surface reaction, probably an adsorption one.

**Total oxidation of 1,4-dihydro N$_1$-benzyldihydronicotinamide**

In order to elucidate the mechanism of the reaction $\text{NADH} \rightarrow \text{NAD}^+$, we made several total oxidations of NBzNH, which is stable enough in the 7–13 pH range. For unambiguously identifying the formation of N$_1$-benzylnicotinamide (NBzN$^+$), we measured the electrochemical characteristics of NBzN$^+$Cl$^-$ and registered its UV spectrophotometric data.

**Polarographic reduction of aqueous buffered solutions of NBzN$^+$Cl$^-$**

We registered current potential curves obtained by drop–time controlled polarography of $10^{-4}$ M NBzN$^+$Cl$^-$ aqueous solutions. The first reduction wave is pH-independent over the investigated pH range (4.6 to 10.9); it corresponds to the potential $U_{1/2} = -0.635 \pm 0.01$ V (N.H.E.) and so it appears at significantly higher potential values than the first polarographic reduction wave of the N$_1$-methyl and N$_1$-propyl-nicotinamide salts at the same concentration (Fig. 2 and ref. [4] and [6]), $U_{1/2} = -0.83 \pm 0.03$ V (N.H.E.). We obtained limiting diffusion current values with $10^{-4}$ M solutions which are about 30% smaller in the case of NBzN$^+$Cl$^-$ and NPrN$^+$Br$^-$ than for solutions of NMeN$^+$I$^-$ with the same concentration.

A second wave emerges at pH higher than 8 from the background discharge; the half–wave potential seems to be independent of the alkyl group of the pyridine ring; except for a value obtained with NBzN$^+$ in a butylamine buffer at pH 10.9 (Fig. 2).

As for the reduction of NMeN$^+$I$^-$, limiting currents of both waves are proportional to N$_1$-alkyl–nicotinamide salt concentration and an increase of this concentration leads to potential shifts: $U_{1/2}$ of the first reduction wave shifts toward less negative potentials, whereas $U_{1/2}$ of the second wave shifts toward more negative potentials.

In addition to this general scheme NBzN$^+$Cl$^-$ first reduction wave splits progressively when its concentration rises above approximately a $3 \times 10^{-4}$ M level. As for the second pyrimidine reduction wave in the
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3.0–5.2 pH range, when such a phenomenon was observed and studied in details\(^{25,26}\) the height of the first split wave is concentration-independent whereas the height of the sum of the two split waves is proportional to the concentration.

![Diagram](image)

Fig. 2. Diagram $U_{1/2}$ vs. pH for the reduction of $10^{-4} M$ aqueous solutions of $N_1$-methyl-nicotinamide iodide ($R = CH_3$), $N_1$-propynicotinamide bromide ($R = C_3H_7$), and $N_1$-benzynicotinamide chloride ($R = CH_2C_6H_5$) by drop-time controlled polarography.

Macroelectrolysis of aqueous buffered solutions of NBzNH

Seven different macroelectrolyses on Pt gauze were conducted in $5 \times 10^{-4} M$ NBzNH aqueous solutions, buffered at pH 8.2. The potential was controlled at $+0.850 \pm 0.05 \text{ V (N.H.E.)}$. Analysis of the product of these electrolyses was performed through UV spectrophotometry and drop-time controlled polarography (Fig. 3 and 4). Results indicate clearly that the electrolysis product is NBzN$^+$. The quantity of removed NBzNH is equal to the quantity of formed NBzN$^+$ and both quantities are proportional to the number of integrated coulombs. In spite of numerous attempts to have reproducible results and to clean thoroughly the Pt gauze, $n$ values were scattered between 1.2 and 1.9 $F/mol$, the mean and more frequent value being 1.55 $F/mol$.

As shown on curve 1 of Fig. 4 the initial NBzNH solution presents a small polarographic reduction wave. It was observed in borate, Tris and glycinate buffers (pH 8.2 and 9.8) at $U_{1/2} = -1.09 \pm 0.03 \text{ V (N.H.E.)}$. Its height in $5 \times 10^{-4} M$ NBzNH solution is in the ratio 1 to 5 with $5 \times 10^{-4} M$ NBzN$^+$ wave height. As NBzNH contains less than 2% impurity (checked by its UV spectrum), this polarographic wave cannot be related to a foulness resulting from the synthesis of this compound.
Fig. 3.
U.V. spectra of a $5 \times 10^{-4} M$ 1-4, dihydro-N$_1$-benzylnicotinamide aqueous solution (buffered at pH 8.3) during a macroscale electrolysis at $+0.85$ V (N.H.E.). Initial solution: curve (1), solution after 0.25 (2), 0.50 (3), 1.0 (4) and 1.2 F/mole (5) electrolysis. $5 \times 10^{-4} M$ NBzN$^+$Cl$^-$ aqueous solution in the same buffer (---).

Fig. 4.
Current vs. potential curves of a $5 \times 10^{-4} M$ 1-4, dihydro-N$_1$-benzyl-nicotinamide aqueous solution (buffered at pH 8.3) by voltammetry on a rotating Pt disk electrode (anodic part) and controlled drop-time polarography (cathodic part) during a macroscale electrolysis at $+0.85$ V (N.H.E.). Initial solution: curve (1), solution after 0.25 (2), 0.50 (3), 1.0 (4), and 1.2 F/mole (5) electrolysis.
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Chemical oxidation of NBzNH

Total chemical oxidations of $5 \times 10^{-4} \, M$ NBzNH solutions were performed by $[\text{Fe(CN)}_6]^{3-}$ in borate and Tris buffers (pH 8.2): the reaction is rapid and complete as checked by UV spectrophotometry and electrochemistry. The reaction product was identified as NBzN$^+$ through its polarographic patterns. As $[\text{Fe(CN)}_6]^{3-}$ presents an important UV absorption in the 265 nm region, it is not possible to follow NBzN$^+$ concentration by this method: this was obtained by polarography.

The decrease of NBzNH concentration (followed by UV spectrophotometry at 355 nm (Fig. 5) and not by voltammetry, because of the interference with the $[\text{Fe(CN)}_6]^{3-}$ oxidation) equals the increase of NBzN$^+$ concentration, both variations being proportional to the added quantity of $[\text{Fe(CN)}_6]^{3-}$. In spite of numerous attempts to have reproducible results, the number of $[\text{Fe(CN)}_6]^{3-}$ molecules necessary to oxidize one molecule of NBzNH, varied from one experiment to another in the 1.2–2.0 range.

Fig. 5.

UV spectra of a $5 \times 10^{-4} \, M$ 1–4, dihydro-N$_1$-benzyl-nicotinamide aqueous solution (buffered at pH 8.3) during a chemical oxidation by a $[\text{Fe(CN)}_6]^{3-}$ solution of concentration 0 (1), (1* after 24 h), $2 \times 10^{-4}$ (2), $6 \times 10^{-4}$ (3), $8 \times 10^{-4}$ (4), $10 \times 10^{-4} \, M$ (5). $5 \times 10^{-4} \, M$ NBzN$^+$Cl$^-$ solution in the same buffer (…) and $5 \times 10^{-4} \, M$ NBzN$^+$Cl$^-$ + $10^{-3} \, M$ Fe(CN)$_6^{4-}$ solution in the same buffer (— — —).

Discussion

Aqueous buffered solutions of NADH and of model compounds such as 1–4, dihydro-N$_1$-propyl- and N$_1$-benzyl-nicotinamide present a single 2 electron voltammetric wave on rotating Pt, Au and glassy carbon disk electrodes. The registered half–wave potential is about 200 mV smaller than the value obtained by Haas and does not depend on pH in the 7.0–13.0 range.
Both macroelectrolysis at +0.85 V (N.H.E.) and \([\text{Fe(CN)}_6]^{3-}\) oxidation gives a total reaction from NBzNH to NBzN+. Häas obtained the same 100% yield with NMeNH and NCiBzNH in basic acetonitrile and with NADH in a buffered aqueous solution at pH 8.6. Whereas, in all these experiments he found a \(F/\text{mol} \) ratio very close to 2, we observed erratic values around 1.6 both by electrochemical and chemical oxidation. Obviously a side reaction occurs during these controlled oxidations, giving about 20% of the amount of NBzN+. It cannot be related to a reaction with dissolved \(O_2\), for all experiments were conducted under \(N_2\) atmosphere. It cannot either be related to an oxidation by the buffer components (borate and Tris). Finally, this side reaction is not the saturation of the 5,6 bond of pyridine ring, happening at pH 7.0, for the product of such a reaction is oxidizable on Pt only at 1.07 V (N.H.E.) and can be detected spectrophotometrically at 290 nm. Discussion is still open on the interpretation of these discrepancies which, to our knowledge, are not linked to experimental errors.

The results obtained during voltammetric oxidation of NADH and model compounds can be interpreted in terms of mechanism of reactions. The pH-independence of the limiting current and of the half-wave potential shows that the energetics of possible acid–base reactions are not integrated in the redox ones. As cyclic voltammetry and ring–disk voltammetry experiments have clearly proved that within about 30 ms NBzN\(^+\) is formed during NBzNH oxidation, a deprotonation should occur after a slow loss of electron and the overall mechanism should be controlled by the activation energy of the first electron departure. Thus, two mechanisms can be proposed for the electrochemical oxidation of NADH model compounds:

\[
\begin{align*}
\text{NADH} & \rightarrow \text{NADH}^+ & \quad \text{slow} & \quad \text{NADH}^2+ & \quad \text{rapid} & \quad \text{NAD}^+ \\
\text{NADH} & \rightarrow \text{NADH}^+ & \quad \text{slow} & \quad \text{NAD}^+ & \quad \text{rapid} & \quad \text{NAD}^+ \\
\end{align*}
\]

\((a)\) reversible at \(-0.7\) V (N.H.E.)

These oxidation mechanisms differ from the two–step reduction mechanisms of NAD\(^+\) and model compounds:

\[
\begin{align*}
\text{NAD}^+ & \quad \overset{+e^-}{\xrightarrow{a}} & \quad \text{NAD}^* & \rightarrow \text{NAD--NAD} & \quad \text{NAD}^* & \quad \overset{+e^-+H^+}{\xrightarrow{b}} & \quad \text{NADH} \\
\end{align*}
\]

\((a)\) reversible at \(-0.7\) V (N.H.E.); \((b)\) at \((-0.075-0.06\) pH) V (N.H.E.)

This means the nonsense of zero current potential measurements with NAD\(^+\)--NADH system, if no mediator and even enzyme is added. It seems that most important energetical and mechanical informations could be gathered on this biological system by the study of the influence of these mediators and of the enzymatic functions on the electrochemical properties of both NAD\(^+\) and NADH.
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