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The Mechanism of Addition-Reactions to the C 5-Atom of the Nicotinamide Moiety of *NADH***

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The C 5-atom of the nicotinamide moiety of *NADH* undergoes addition-reactions with protons and positive halogen atoms from N-halogen-succinimides; the final products of these reactions were the corresponding 6-hydroxy-(5-halogeno-)-1,4,5,6-tetrahydronicotinamide adenine dinucleotides. A common quantitative mechanism for all these reactions has been worked out, and all kinetic parameters have been reported.

(Keywords: NADH; Nucleophilic addition-reactions)

Der Mechanismus von Additions-Reaktionen am C 5-Atom des Nikotinamidanteils von NADH

Das C 5-Atom des Nikotinamidteils von *NADH* addiert die Protonen und die positiven Halogenatome aus Halogen-Succinimiden; Endprodukt jeder Reaktion war das entsprechende 6-Hydroxy-(5-halogen)-1,4,5,6-tetrahydronikotinamid-Adenin-Dinukleotid. Ein gemeinsamer Mechanismus für alle diese Reaktionen wurde ausgearbeitet und alle kinetischen Parameter ermittelt.

Introduction

The first systematic investigation of addition-reactions to the nicotinamide moiety of N-substituted 1,4-dihydronicotinic acid derivatives has been started by *Wallenfels* [1–3], clearly indicating the nucleophilic character of the C 5-atom of the dihydronicotinamide ring. The C 5-atom of *NADH* undergoes addition-reactions with protons, metal ions, halogen atoms, carbonyl group, and other electron defficient reaction centers [4, 5]. The mechanism of these important addition-reactions has not been investigated, except with protons [6–9], the proposed mechanism for the

** Dedicated to Prof. *Gerhard Pfleiderer* on the occasion of his 65th birthday.

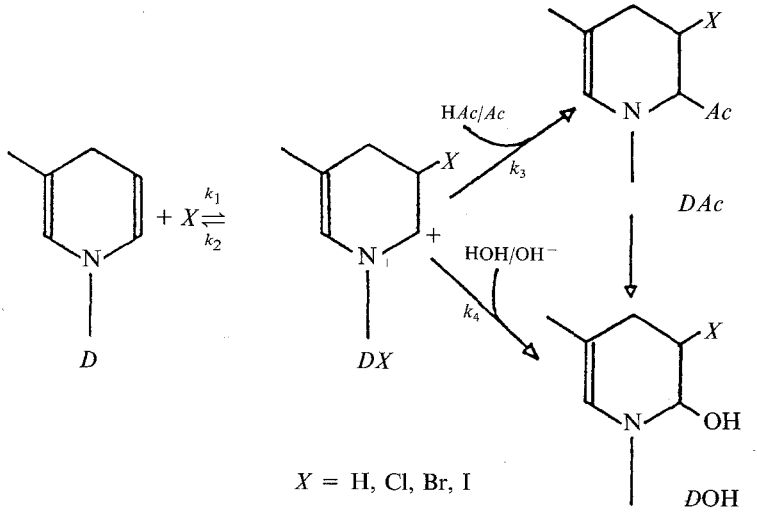
proton-addition reaction has been worked out only in a qualitative fashion. Addition-reactions to the nucleophilic C 5-atom of *NADH* in aqueous media are of biological and technological importance, especially in the biotechnological reactors working with immobilized *NAD(P)H*. For this reason, we have worked out a quantitative mechanism for the addition-reactions of *NADH* with protons and positive halogen atoms in aqueous media.

Results

Reaction Mechanism

Spectroscopic, chemical and kinetic evidence indicated [6, 8] that the addition of protons and positive halogen atoms to the C 5-atom of the nicotinamide moiety of *NADH* proceeds, in aqueous media, by the following mechanism:

Scheme 1



The mechanism depicted on Scheme 1 shows the reversible addition of the electron defficient reactant (*X*) to the C 5-position of the 1,4-dihydronicotinamide ring (*D*), forming a cation intermediate (*DX*). The cation intermediate decomposes in aqueous media irreversibly by four pathways: reacting with undissociated acid (H*Ac*) and its anion (*Ac*) to form a stable product (*DAc*), and with water and hydroxyl anion to form 6-hydroxy-(5-halogeno)-1,4,5,6-tetrahydronicotinamide adenine dinuc-

leotide (*D*OH). The anion adduct (*DAc*) was unstable and hydrolyzed easily into *D*OH.

For the mechanism shown in Scheme 1 the following approximations were valid (capital letters denote concentrations):

$$-\frac{\mathrm{d}\,D}{\mathrm{d}\,t} = k_1 \cdot D \cdot X - k_2 \cdot DX \tag{1}$$

$$-\frac{\mathrm{d}\,DX}{\mathrm{d}\,t} = k_2 \cdot DX + k_4 \cdot \mathrm{HOH} \cdot DX + k_3 \cdot Ac \cdot DX - k_1 \cdot \mathrm{D} \cdot \mathrm{X} \tag{2}$$

$$-\frac{\mathrm{d}\,D}{\mathrm{d}\,t} = k_4 \cdot \mathrm{HOH} \cdot DX + k_3 \cdot Ac \cdot DX + \frac{\mathrm{d}\,DX}{\mathrm{d}\,t} \tag{3}$$

The reaction rates of the cation intermediate (*DX*) with OH^- and H*Ac* were too small and therefore omitted from Eq. (2).

In order to calculate the rate constants (k_1, k_2, k_3 and k_4) from the experimental measurements, a development of a simple mathematical model was required, connecting reaction rates (d *D*/d *t*) with reactant concentrations (*D*, *X*, HOH, *Ac*) and the rate constants ($k_1 - k_4$) in a simple, straight-forward fashion. Presteady-state measurements required rapid mixing techniques and a development of complicated models. On the other hand, an introduction of a steady-state approximation presented a simple mathematical model, connecting reaction rates with reactant concentrations in a linear relationship. Such a model made possible an estimation of the rate constants with sufficient precision.

In the steady-state (d *DX*/d $t \sim 0$), the concentrations of *D*, *X* and *DX* were in equilibrium:

$$K = \frac{DX}{D \cdot X} \tag{4}$$

$$DX = K \cdot D \cdot X \tag{5}$$

$$-\frac{\mathrm{d}\,D}{\mathrm{d}\,t} = K \cdot X \cdot D(k_4 \cdot \mathrm{HOH} + k_3 \cdot Ac) \tag{6}$$

The coenzyme (D_0) was divided between the 1,4-dihydroform (*D*), the cation intermediate (*DX*), and the products (*P*):

$$D_0 = D + DX + P \tag{7}$$

$$D = \frac{D_0 - P}{1 + K \cdot X} \tag{8}$$

The combination of Eq. (6) and Eq. (8) provided a simple linear

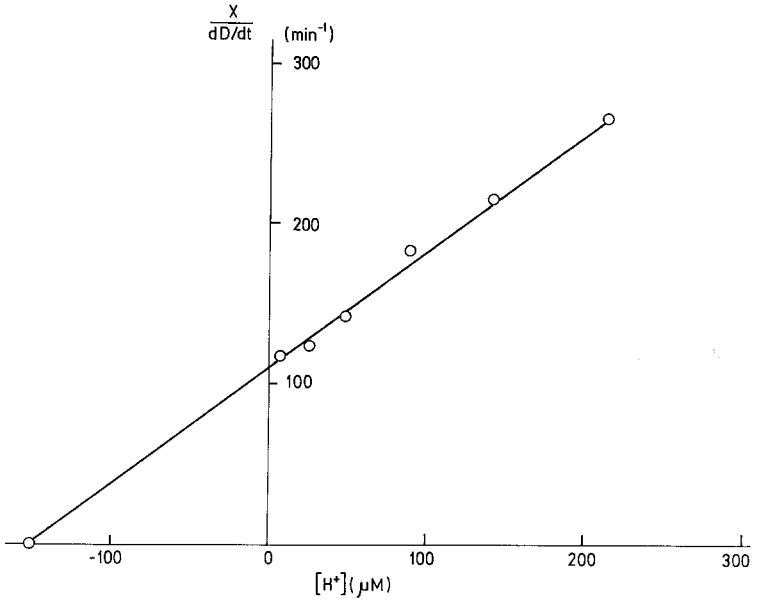


Fig. 1. Estimation of the equilibrium constant (K) for the proton-addition reaction, according to Eq. (10). Initial concentration of *NADH* was 27.56 μ*M* in 0.05 *M* sodium acetate buffers of indicated *pH*, at 25 °C

relationship between the reaction rates, reactant concentrations and the reaction constants:

$$-\frac{\mathrm{d}D}{\mathrm{d}t} = \frac{K \cdot X(D_0 - P)}{1 + K \cdot X} \cdot (k_4 \cdot \mathrm{HOH} + k_3 \cdot Ac) \tag{9}$$

$$-\frac{X}{\mathrm{d}D/\mathrm{d}t} = \frac{1}{K(D_0 - P)} \cdot \left(\frac{1}{k_4 \cdot \mathrm{HOH} + k_3 \cdot Ac}\right) + \frac{X}{D_0 - P} \cdot \left(\frac{1}{k_4 \cdot \mathrm{HOH} + k_3 \cdot Ac}\right) \tag{10}$$

Reaction with Protons

With the aid of Eqs. (9) and (10), the mechanism of the Scheme 1 has been put to a quantitative test. Figure 1 shows the dependence of the reaction rate $\left(\frac{X}{\mathrm{d}D/\mathrm{d}t}\right)$ on the concentration of protons (Eq. 10).

Equation (10) is valid only under the steady-state assumption, which

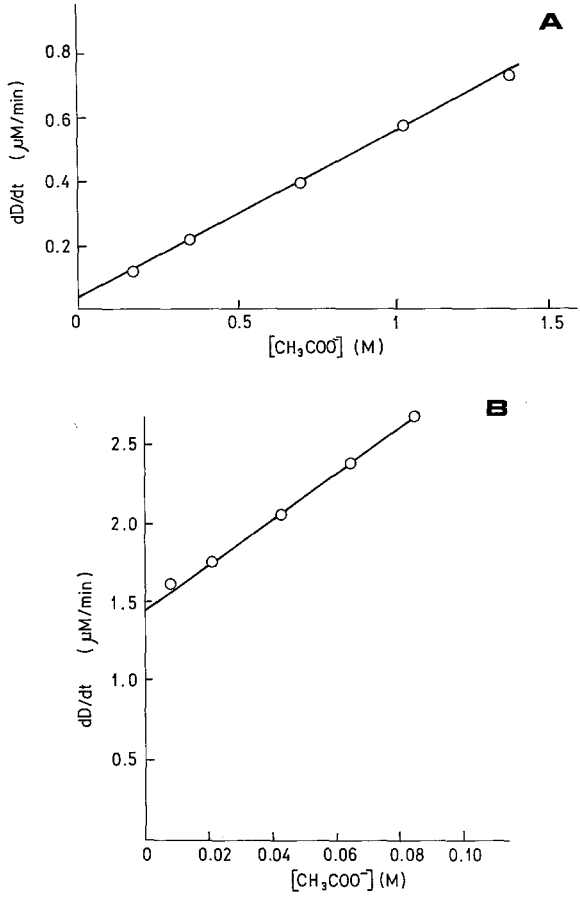


Fig. 2. Estimation of k_3 (*A*) and k_4 (*B*). Concentration of *NADH* was 80 μ*M* in (*A*) at *pH* 5.5, and 30 μm in (*B*) at *pH* 3.67; rate constants were measured in sodium acetate buffers of increasing concentrations

was corroborated by the two lines of evidence: first, Eq. (3) requires a constant reaction rate (d *D*/d *t*) in the steady-state, which was true for the reaction rates between *pH* 4 and 7 (data not shown), and, second, a presteady-state kinetics would provide a linear relationship between the reaction rate and the concentration of protons (Eq. 1), contrary to the observed saturation kinetic (Fig. 1). Thus, the experimental data of Fig. 1 have been drawn according to Eq. (10) with two restrictions: first, the experimental data below *pH* 4 were not sufficiently reliable due to the presteady-state interference, and, second, the concentration term for the products (*P*) was omitted from the Eq. (10), due to the negligible con-

centration of products at the beginning of reactions, especially above *pH* 4. The replotting of the data from Fig. 1 according to *Hill* indicated that a single proton reacted with the dihydropyridine nucleus (data not shown). Thus, Fig. 1 allowed the estimation of the dissociation constant (*K*) with sufficient precision.

Figure 2 A shows the estimation of the k_3-constant for the acetate anion at *pH* 5.5, according to Eq. (9); the rate constant for any other anion may be estimated in the same way. Again, the steady-state assumption was corroborated by the constancy of reaction rates (data not shown) and by the linear relationship between the reaction rate and the anion concentration; in a presteady-state, the reaction rate would be almost insensitive to the concentrations of anions.

Figure 2 B shows the estimation of the k_3-constant and the k_4-constant at *pH* 3.67 from the slope and an intercept on the ordinate, according to Eq. (9). The estimation of the k_4-constant from the experiment presented in Fig. 2 B was the least accurate, since the reaction rates at different buffer concentrations were not linear (data not shown), indicating the interference of the presteady-state conditions.

It is interesting to note that both constants k_3 and k_4 may be estimated from the data on Fig. 2 A or 2 B from the slopes and the intercepts on the ordinate, respectively. The k_3-value calculated from Fig. 2 A was very similar to the same value calculated from Fig. 2 B; on the other hand, the estimation of the k_4-value from the intercept on Fig. 2 A was very inaccurate, due to the low intercept.

The rate constants for the formation (k_1) and the decomposition (k_2) of the cation intermediate cannot be determined directly. Therefore, the mechanism shown in Scheme 1 was programmed into a computer algorithm using the values for k_3, k_4 and *K*. The experimental results, obtained at *pH* 3.67 and *pH* 3.92 in a low salt buffer, have been fitted in the calculation until the best fit for k_1 has been obtained (Table 1).

In this way, all four constants ($k_1 - k_4$) and the equilibrium constant (*K*) have been determined. The second-order rate constant for the reaction of *DX* with the undissociated acid (H*Ac*) was too low for an accurate estimation, and the reaction rate of *DX* with OH^- was too low due to the very high ratio HOH/OH^-.

Reactions with the Positive Halogen Atoms

Titration of *NADH* in a neutral buffer with N-chloro-, N-bromo-, or N-iodosuccinimide indicated a binding of a single halogen atom to a molecule of *NADH*, and formation of a single product. A selective formation of a single reaction product was deduced from the stochiometry of the spectrophotometric titration and by TLC (see Exp. part); it was

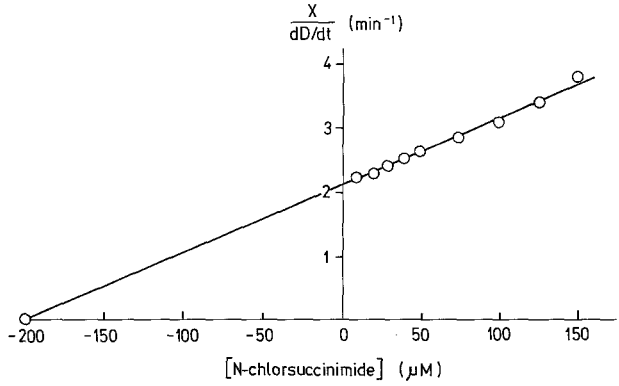


Fig. 3. Estimation of the equilibrium constant (K) for the chlorine-addition reaction, according to Eq. (10). Initial concentration of *NADH* was 27.6 μ*M* in 0.05 *M* sodium phosphate buffer, *pH* 7.0, at 25 °C

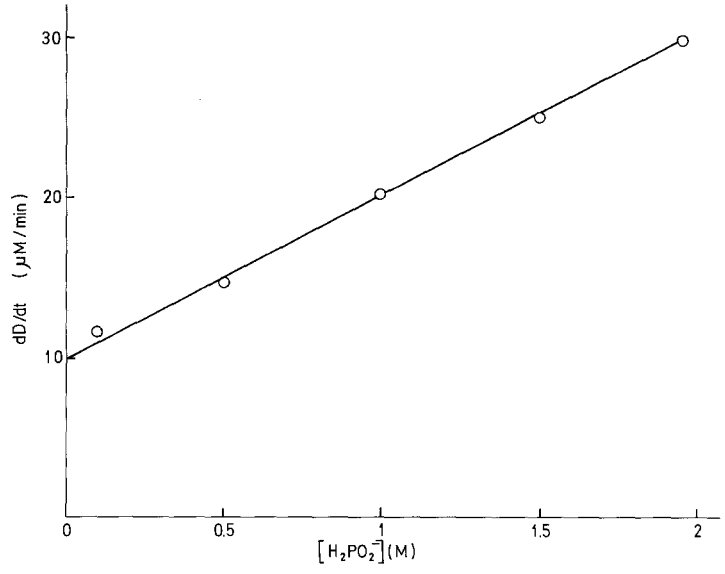


Fig. 4. Estimation of k_3 and k_4 for the chlorine-addition reaction. Concentration of *NADH* was 23.2 μ*M* and N-chloro-succinimide 35 μ*M*, in sodium phosphate buffers of increasing concentrations, at *pH* 7.0

assumed that the reaction product in each case was the corresponding 6-hydroxy-5-halogeno-1,4,5,6-tetrahydronicotinamide adenine dinucleotide [1–4].

Reactions of *NADH* with iodo- and bromosuccinimide were too fast to be measured, and with chlorosuccinimide sufficiently slow to be monitored spectrophotometrically. The addition of N-chlorosuccinimide to *NADH*-solutions caused a rapid decrease in the absorbance of the coenzyme at 340 nm. This evidence and chemical considerations [1–4] indicated a formation of a *NADH*-halogen adduct by a mechanism according to Scheme 1 (Eq. 1–10).

Figure 3 shows an estimation of the equilibrium constant (K) for the chlorine-addition reaction at *pH* 7.0 (Eq. 10); the validity of the steady-state assumption has been confirmed as described above. Figure 4 shows the estimation of the k_3-constant and the k_4-constant from the slope and an intercept on the ordinate in a phosphate buffer, *pH* 7.0 (Eq. 9). The rate constants for the formation (k_1) and the decomposition (k_2) of the cation intermediate have been calculated using a computer programme in a manner similar to the proton-addition reaction (Table 1).

Discussion

There is some evidence in the literature supporting the mechanism of Scheme 1. The most important evidence was the isolation and the identification of 1-(2,6-dichlorobenzyl)-6-hydroxy-1,4,5,6-tetrahydronicotinamide [8], the chemical evidence for the reversibility of reaction $D + X \rightleftharpoons DX$ [8], and the assumption that the cation intermediate (DX) was devoid of an absorption band at 340 nm [8]. We have used these facts to postulate the mechanism shown in Scheme 1; further we used the corresponding mathematical model (Eq. 1–10) to check its validity.

There was no direct physical evidence for the existance of the cation intermediate (DX). However, a satisfactory agreement of the proposed mechanism (Scheme 1) with our experimental data proved the validity of the mechanism and, by inference, the existence of the cation intermediate. The computer simulation of the proton-addition reactions indicated the following: the steady-state concentration of DX increased with decreasing *pH*, and the steady-state concentration of DX was achieved rapidly above *pH* 4, but rather slowly at *pH* 3.67 (Fig. 5). The assumption of the irreversibility of reactions $DX + OH^- \rightleftharpoons DOH$ and $DX + Ac^- \rightleftharpoons DAc$, vs. the reversibility had little influence on the calculation of rate constants.

Table 1 summarizes the rate constants calculated for the proton- and the chlorine-addition reactions. The equilibrium constant (K) was very

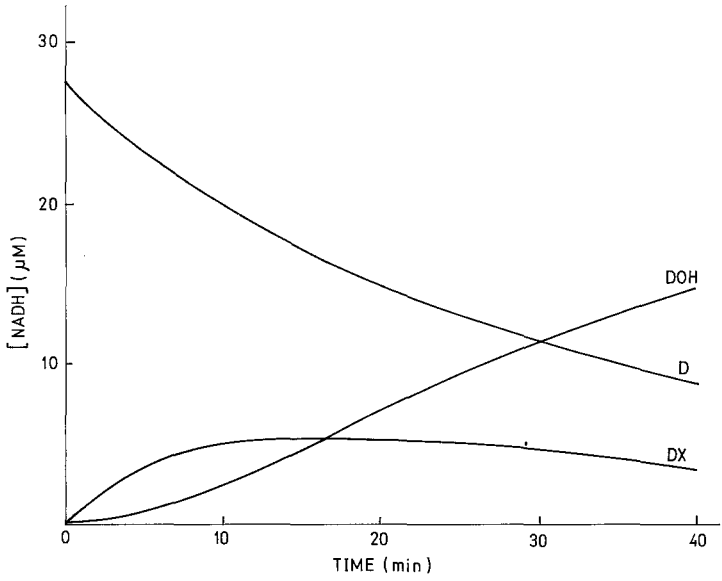


Fig. 5. Computer simulation of the progress of the proton-addition reaction at *pH* 3.67. Initial concentration of *NADH* was 27.6 μ*M* sodium acetate buffer at 25 °C; rate constants were taken from Table 1

Table 1. *Rate constants for the proton- and the chlorine-addition reactions at 25 °C*

Type of reaction	k_1 $M^{-1}\,min^{-1}$	k_2 min^{-1}	k_3 $M^{-1}\,min^{-1}$	k_4 $M^{-1}\,min^{-1}$	K M^{-1}
Proton-addition reaction	170	0.026	0.30[a]	0.0013	6 500
Chlorine-addition reaction	13 300	2.660	2.46[b]	0.0443	5 000

[a] Reaction rate with an acetate anion CH_3COO^-
[b] Reaction rate with the orthophosphate anion $H_2PO_4^-$

similar for both reactions. The reaction rate for the forward reaction (k_1) was two orders of magnitude higher for the chlorine-, compared to the proton-addition reaction; reaction rates with bromo- and iodo-succinimides were much higher, making these reactions practically irreversible. The reactivity of *DX* (k_3, k_4) depended on the substituent at position-5; the least reactive was with hydrogen, progressively increasing from the chloro- to the bromo- and iodo-substituents.

Experimental

NADH, N-chloro-, N-bromo-, and N-iodo-succinimide were the products of Sigma Chemie GmbH, Taufkirchen (Federal Republic of Germany); all other chemicals were of analytical grade purity. Twice distilled water from an all glass assembly was applied throughout.

Kinetic measurements were performed in a spectrophotometer Specord UV-VIS (Carl Zeiss, Jena, German Democratic Republic), with thermostated küvette-holders. The disappearance of the 1,4-dihydropyridine structure was monitored at 340 nm; the cation intermediate was devoid of an absorption band at 340 nm [8]. On all figures d D/d t represents the initial reactions rates. The formation of 6-hydroxy-[5-halogen]-1,4,5,6-tetrahydronicotinamide adenine dinucleotides from *NADH* was accompanied by a shift of a 260 nm absorption band to 265 nm, and an increase in molar absorbances by 50–75%. *NADH* and all 6-hydroxy-[5-halogeno]-1,4,5,6-tetrahydronicotinamide adenine dinucleotides were difficult to distinguish by TLC (Silicagel G), due to the very similar R_f-values.

The mechanism shown in Scheme 1 has been mathematically described by Eqs. (1) to (10); the model based thereupon has been programmed into a computer, and the k_1-constant in the model has been varied until the best fit with the experimental data has been obtained. All data have been processed on a computer Delta-Iskra 341/10, Ljubljana (Yugoslavia) (PDP 11/34).

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