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Oxidation of NADH Involving Rate-Limiting One-Electron Transfer

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Abstract: The kinetics and mechanism of the oxidation of NADH by ferrocenium cation (**1a**) and several substituted ferrocenium cations in aqueous 1-propanol are reported. All reactions were found to be first order in both NADH and the ferrocenium cation. The rate of oxidation of NADH by **1a** was found to be independent of pH and showed no change in rate when [4-^{1}H,4-^{2}H]NADH or [4-^{2}H,4-^{2}H]NADH was used. All of these results are consistent with rate-limiting one-electron transfer. A plot of log k vs. $E°$ for the ferrocenium/ferrocene couples gave a straight-line segment with slope = 16.0 V^{-1}. By extrapolation of this line to the diffusion-controlled rate limit the $E°$ for the NADH/NADH$^{+\cdot}$ couple was found to be 1.05 V vs. NHE in PrOH/H$_2$O. When the Rehm–Weller relationship was used, the $\Delta G^\ddagger$ value for NADH/NADH$^{+\cdot}$ self-exchange was calculated to be 5.6 kcal mol^{-1}. The best estimates in aqueous solution are $E°$ = 0.93 V and $\Delta G^\ddagger$ = 5.6 kcal mol^{-1}. The mechanistic implications of these results are briefly discussed.

The redox couples NADH/NAD$^+$ and NADPH/NADP$^+$ represent extremely important coenzymes in biological systems. As a result there has been a long-standing interest in the chemical reactions of NADH and NADH analogues in enzyme-free systems.[1] Most reactions of NADH and its analogues proceed with *net* transfer of H$^-$ from the 4-position of the dihydropyridine ring to the oxidizing agent. For example, NADH models reduce *N*-methylacridinium ion to *N*-methylacridan[2] and trifluoroacetophenone to the corresponding alcohol.[3] In such reactions either a single step hydride transfer or a multistep mechanism is in principle possible. We felt it was important to directly investigate the multistep e$^-$, H$^+$, e$^-$ mechanism of the oxidation of NADH to NAD$^+$.[4] The mechanism by which such an oxidation could occur is given in Scheme I.

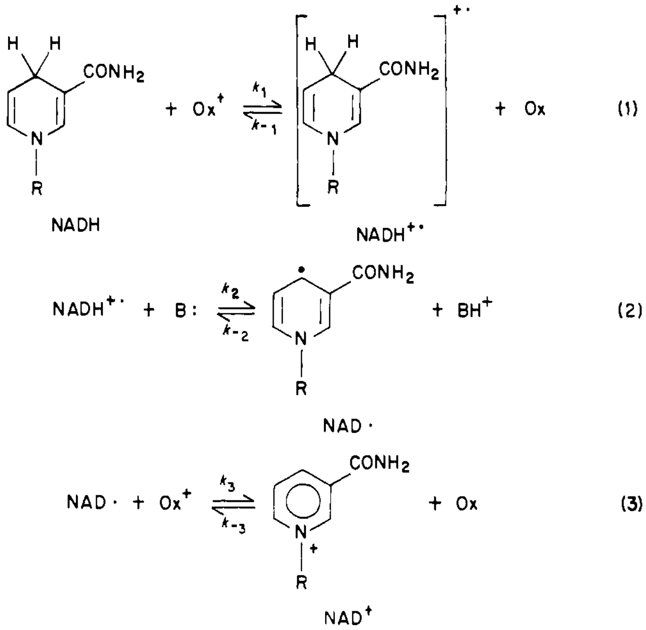
Such a mechanism is abnormal in comparison to many biological reactions, since in the above reaction the 4-hydrogen from NADH is transferred to solvent, not to the oxidant. On the other hand, if the first step—one-electron transfer—is rate limiting, the kinetic data have significance in a wider context. The rates of electron-transfer reactions can be understood theoretically, and some quantitative predictions are possible.

In the present study we provide evidence for a NADH oxidation which appears to proceed by initial rate-limiting one-electron transfer from NADH to the oxidizing agent. In addition we have been able to provide an estimate of the $E°$ for the one-electron, no proton NADH/NADH$^{+\cdot}$ couple as well as an estimate of the NADH/NADH$^{+\cdot}$ self-exchange rate constant for electron transfer. This has led to a clarification of the energetics of NADH/NADH$^{+\cdot}$/NAD$\cdot$ interconversions, and we have been able to draw conclusions about the possibility of one-electron transfer in other NADH redox reactions.

Results and Discussion

Kinetics and Mechanism. The general requirements for one-electron NADH oxidants are as follows: (1) the oxidant must have an appropriate one-electron $E°$ (good thermodynamic oxidant); (2) it should have a sufficiently rapid self-exchange rate constant for one-electron transfer (good kinetic oxidant); (3) it must be unlikely to be reduced by hydride transfer; (4) it must be stable in aqueous or a mixed aqueous/organic solvent; and (5) it is desirable to have available a series of closely related oxidants. A series of compounds which meet these requirements are the substituted ferrocenium cations **1** which are the one-electron oxidation products of the corresponding ferrocenes **2**. We were able to prepare all of the reported ferrocenium compounds as stable PF$_6^-$ salts (Table I). Reactions of NADH with **1** were conducted in 46/54 (w/w) 1-PrOH/H$_2$O. This mixture ensures solubility and was previously used in a study of electron exchange between **1** and **2**.[5]

Scheme I



net reaction: NADH + 2Ox$^+$ $\longrightarrow$ NAD$^+$ + 2Ox + H$^+$
(R = adenosine diphosphoribosyl)

The stoichiometry of the reaction was investigated by reacting various molar ratios of **1a**/NADH. In a reaction of 2 equiv of

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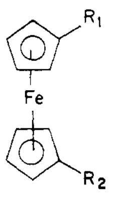

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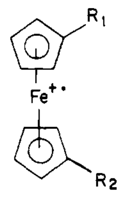

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1a with 1 equiv of NADH, both reactants were completely consumed. Product **2a** and enzymically active NAD$^+$ were isolated in >75% yield. For 1/1 and 1/2 ratios the stoichiometry was determined by spectrophotometrically assaying the amount of NADH left at the end of the reaction. Values of 2.1 and 1.9, respectively, were found for the moles of **1a** consumed per mole of NADH oxidized. This stoichiometry is in agreement with results previously reported for ferricyanide oxidation of NADH.[6]

In kinetic studies the solvent contained NaClO$_4$ and 0.01 M pH 7.0 phosphate buffer. The NaClO$_4$ was sufficiently soluble to allow variation in the salt concentration from 0.1 to 0.5 M. The solution was buffered in order to minimize the acid-catalyzed hydration of NADH. Reactions were run with NADH in at least 15-fold excess over the ferrocenium salt so that pseudo-first-order kinetics could be observed. If it is assumed (see below) that the initial electron transfer step (Scheme I, step 1, Ox$^+$ = **1**) is rate limiting then:

$$-\frac{d[\mathbf{1}]}{dt} = 2k_1[\mathrm{NADH}][\mathbf{1}] \quad (1)$$

and the observed pseudo-first-order rate constant (k_{obsd}) is related to the observed second-order rate constant ($k_{1,\mathrm{obsd}}$) by,

$$k_{1,\mathrm{obsd}} = k_{\mathrm{obsd}}/2[\mathrm{NADH}] \quad (2)$$

In practice, the reactions were clearly first order in **1** as plots of ln ($A_t - A_\infty$) vs. time were linear over 3 or more half-lives with $r > 0.999$. The reported rate constants are the average of 6–16 individual kinetic runs using at least two different concentrations of NADH and have a standard deviation of ±5% or less. The range of half-lives measured was 5–250 s. For **1a** the concentration of NADH was varied from 3.3 to 9.5 mM. A plot of the observed pseudo-first-order rate constants vs. NADH concentration is shown in Figure 1. The good linear correlation and the intercept near zero show that the reaction is also first order in NADH. It is, therefore, second order overall.

With other oxidants, oxidations of NADH and its analogues generally show large primary kinetic isotope effects when the compound is deuterated in the 4-position.[7] This is to be expected when the reaction proceeds by hydride transfer or if the second step, proton loss, is rate limiting in the multistep mechanism. On the other hand, if the initial one-electron transfer is rate limiting there should only be a secondary isotope effect on the rate of electron transfer. The rate of reaction of **1a** with NADH-d_1 and NADH-d_2 was investigated. No isotope effect, $k_H/k_D = 1.0 \pm 0.05$, was found for these reactions (see Table II).[8] It therefore appears that H$^-$, H$^+$, or H· transfer are not involved in the rate-limiting step.

In agreement with this conclusion are the results in Tables III and IV. In a multistep e$^-$, H$^+$, e$^-$ process such as that outlined in Scheme I, it is possible for the deprotonation step to be rate limiting. The kinetics of the oxidation of NADH by **1a** were conducted at varying pHs and buffer concentrations. It can be seen that these changes in the basicity of the medium did not have a measurable effect on the rate of reaction.

In Scheme I, the back electron transfer reaction k_{-1} should have a large rate constant. To investigate the possibility that the back electron transfer step is important, the effect of added **2a** on the rate of oxidation of NADH by **1a** was studied. For seven runs with 0.3 M NaClO$_4$ and 0.26–0.65 mM **2a** added, the average $k_{1,\mathrm{obsd}}$ was 2.48 ± 0.15 M^{-1} s^{-1}, well within experimental error of $k_{1,\mathrm{obsd}}$ = 2.40 M^{-1} s^{-1} found without added **2a**. In 1-PrOH/H$_2$O NADH with high concentrations of NAD$^+$ is unstable, therefore inhibition by NAD$^+$ was tested with 1.0–1.5 mM NAD$^+$. At these concentrations the decomposition of NADH in a stock solution was found to be negligible during the time scale of the kinetic experiments. The average of seven runs in the presence of 0.3 M NaClO$_4$ was $k_{1,\mathrm{obsd}}$ = 2.34 ± 0.11 M^{-1} s^{-1}, again within experimental error of the value found with no added NAD$^+$.

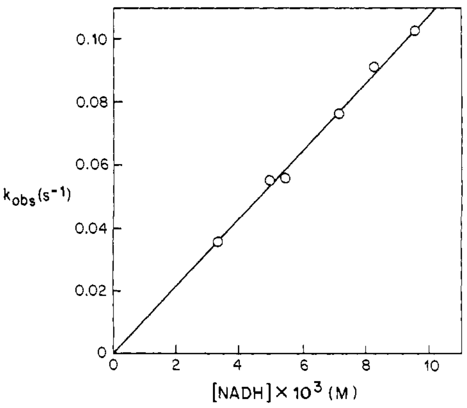

Figure 1. Plot of the observed pseudo-first-order rate constants vs. NADH concentration. [NaClO$_4$] = 0.1 M.

In conclusion, the stoichiometry, reaction order, independence of the rate on pH, or added **2a** and NAD$^+$, and lack of a primary kinetic isotope effect are all consistent with rate-limiting one-electron transfer from NADH to **1a**. This is Scheme I where Ox$^+$ is **1a** and k_1 is rate limiting.

An interesting aspect of this mechanism is that in order for the initial one-electron transfer to be rate limiting k_2[B] must be greater than k_{-1} [**2a**]. From a recent pulse radiolysis study[9a] it is known that NADH$^{+\cdot}$ is completely deprotonated after 1 μs. Therefore k_2[B] must be greater than 10^6 s^{-1} in aqueous solution at pH 7. A laser flash photolysis study put this value at 1.2 × 10^7 s^{-1}.[9b] Assuming that k_{-1} is the diffusion-controlled rate constant and **2a** ~0.3 mM (a representative value at the end of the reaction) then k_{-1}[**2a**] ~ 10^6 s^{-1}. Therefore, even at the end of the reaction $k_2[\mathrm{B}] \geq k_{-1}[\mathbf{2a}]$ and the electron-transfer step should be rate limiting. A previous study had reported rate-limiting one-electron transfer in the oxidation of *n*-propyldihydronicotinamide by ferricyanide.[10] However, in that instance there was a pH effect, ferrocyanide inhibition, and a small deuterium isotope effect, all of which are consistent with the deprotonation step being at least partially rate limiting in that system. A study of the oxidation of NADH by ferric ion was complicated by the low pH employed.[11]

Kinetic–Thermodynamic Correlations. By studying the relationship between $E°$ and log k for a series of ferroceniums, we can achieve a deeper understanding of the one-electron chemistry of NADH and such measurements have been made. A complicating, practical factor was the effect of NaClO$_4$ on the rate. An increase in the concentration of NaClO$_4$ resulted in a decrease in the observed second-order rate constant. This could be due to a kinetic salt effect (more polar solvent) or to ion pairing of the reactants with the salt. Because we wished to compare the rates for various compounds it was decided to estimate the rate in the absence of salt by extrapolation. Assuming that ion pairing is the cause of the salt effect provides a satisfactory means to treat

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Table I. Preparation of Ferrocenium Hexafluorophosphates

				elemental anal.			
				calcd		found	
1	R_1	R_2	% yield (method)[a]	C	H	C	H
a	H	H	82 (A)	36.29	3.05	36.12	3.17
b	HgCl	H	56 (B)	21.22	1.60	21.50	1.89[b]
c	*n*-Bu	H	77 (A)	43.44	4.69	43.23	4.69
d	Et	H	74 (A)	40.14	3.93	40.06	4.11
e	CH_3	CH_3	75 (A)	40.14	3.93	40.05	4.05
f	*o*-$CH_3C_6H_4$	H	84 (B)	48.48	3.83	48.44	3.80
g	C_6H_5	H	39 (B)	47.20	3.47	47.05	3.53
h	*p*-ClC_6H_4	H	64 (B)	43.52	2.97	43.59	3.04

[a] A = oxidation using concentrated H_2SO_4; B = oxidation using *p*-benzoquinone. See Experimental Section for details. [b] Hg calcd 35.4, found 34.2 ± 2.0 by atomic absorption.

Table II. Oxidation of NADH, NADH-*d*$_1$, and NADH-*d*$_2$ by **1a**[a]

	k, M^{-1} s^{-1}		
[$NaClO_4$]	NADH	NADH-*d*$_1$	NADH-*d*$_2$
0.1	5.41	5.44	5.18
0.2	3.29	3.36	3.30

[a] Conditions cited in text. Rate constants are ±5%.

Table III. Rate Constants (k, M^{-1} s^{-1}) for the Oxidation of NADH by **1a** at Varying pH[a]

	pH			
[$NaClO_4$]	6.0	7.0	8.0	9.0
0.1	5.30	5.41	5.52	5.27
0.2	3.36	3.29	3.37	3.19
0.3	2.38	2.40	2.46	2.43
0.4	1.99	1.90	1.92	2.04

[a] See Experimental Section for explanation of pH. Rate constants are ±5%.

Table IV. Rate Constants (k, M^{-1} s^{-1}) for the Oxidation of NADH by **1a** at pH 7.0 at Varying Buffer Concentrations[a]

	[phosphate]		
[$NaClO_4$]	0.005	0.01	0.02
0.1	5.48	5.41	5.20
0.2	3.28	3.29	3.22
0.3	2.53	2.40	2.50

[a] Rate constants are ±5%.

the data, regardless of the actual origin of the effect. If the ion pair was not an active oxidant, the $k_{1,\mathrm{obsd}}$ value measured at a particular [ClO_4^-] would have to be corrected to account for the proportion of **1** which was ion paired. By doing this, k_0, the rate constant with no salt present, and the apparent equilibrium constant, K, for ion pairing could be obtained. Therefore a plot of $1/k_{1,\mathrm{obsd}}$ vs. $[ClO_4]^-$ yields K/k_0 as the slope and $1/k_0$ as the

$$1/k_{1,\mathrm{obsd}} = K[ClO_4^-]/k_0 + 1/k_0 \qquad (3)$$

intercept (eq 3). For each of the ferrocenium derivatives studied $k_{1,\mathrm{obsd}}$ values were measured at four or more concentrations of $NaClO_4$ in the range 0.1–0.5 M. The k_0 and K values are given in Table V.

Conductivity measurements were made by using ferrocenium hexafluorophosphate in 1-PrOH/H_2O (ferrocenium perchlorate has been reported to be a detonator and therefore the synthesis of large quantities of this compound was not attempted). The conductivity data were interpreted by using the Fuoss equation[12] with the constants in the equation determined in the usual manner

$$\Lambda = \Lambda_0 - S(C\gamma)^{1/2} + EC\gamma \log C\gamma + (J - B\Lambda_0)C\gamma - KC\gamma\Lambda f^2 \qquad (4)$$

with K set equal to zero. The limiting conductance (Λ_0) was found to be 54.0 and K was calculated to be 20 ± 10^{-1} M^{-1}. It should be noted that small errors in the observed conductances can result in large errors in the calculated K. Furthermore, the concentrations are so high that this is not an unequivocal test for ion pairing.

Table V. Oxidation of NADH by Ferrocenium Ions

1	$E°$, V	k_0,[a] M^{-1} s^{-1}	K,[b] M^{-1}
a	0.529	13.7	15.6
b	0.533	16.3	15.9
c	0.504	4.55	12.5
d	0.490	2.99	9.0
e	0.445	0.629	7.6
f	0.580	39.8	11.2
g	0.580	41.3	8.3
h	0.613	90.9	10.9

[a] Rate constants extrapolated to zero salt concentration. Correlation coefficient for plots of [$NaClO_4$] vs. $k_{1,\mathrm{obsd}}^{-1}$, r = 0.999. [b] Apparent ion-pairing equilibrium constant from plots of [$NaClO_4$] vs. $k_{1,\mathrm{obsd}}^{-1}$.

The validity of the method for evaluating k_0 was tested by using a different anion in the electrolyte. The kinetics of the oxidation by **1a** were studied in the presence of NaCl instead of $NaClO_4$. It therefore would be expected that a plot of $1/k_{1,\mathrm{obsd}}$ vs. [Cl^-] would yield a different slope,[13] but the same value of k_0, when compared with ClO_4^-. Experimentally a k_0 of 13.4 M^{-1} s^{-1} and a K of 10.7 M^{-1} were found which indicates that the k_0 is valid.

The one-electron $E°$ values were determined in 1-PrOH/H_2O with $NaClO_4$ as the electrolyte for the **1**/**2** couples by using cyclic voltammetry. The compounds generally showed good reversibility at a sweep rate of 50 mV/s with the ratio of anodic to cathodic peak currents of ~1.0 and peak separations (ΔE_p) of 60–65 mV. **2b** gave ΔE_p > 70 mV at 50 mV/s but ΔE_p decreased to 60–65 mV at 20 mV/s. A salt effect was observed on the $E°$ which could be due to ion pairing, solvation, changes in liquid junction potential, or a combination of these effects. The change in $E°$ was observed to be approximately linear when plotted against $[NaClO_4]^{1/2}$. The reported $E°$ for each **1**/**2** couple was therefore calculated by extrapolating this plot to zero $NaClO_4$ concentration. The extrapolated $E°$ values are given in Table V.

In subsequent paragraphs the rate of a diffusion-controlled reaction between **1** and NADH becomes important. The Smoluchowski equation (eq 5) provides a method for estimating k_{diff}

$$k_{\mathrm{diff}} = 4\pi D R_c \qquad (5)$$

where D is the sum of the diffusion coefficients and R_c is the sum of the two reaction radii. When it is assumed that R is approximately constant as the solvent is changed, D and R_c can be calculated as follows.

$$D_a = kT/6\pi\eta R_a \qquad (6)$$

$$D_a/D_b = \eta_b/\eta_a \qquad (7)$$

The viscosity (η) of the 46/54 (w/w) 1-PrOH/H_2O solution at 25 and 30 °C was obtained by interpolation from the data of

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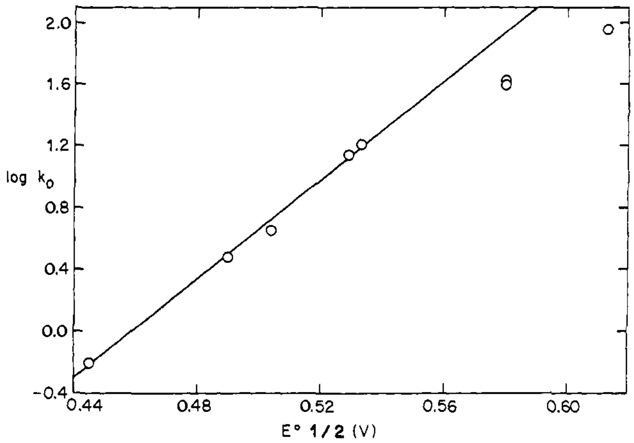

Figure 2. Plot of log k_0 vs. **1**/**2** redox potential.

Kikuchi and Oikawa.[14] The values obtained were $\eta = 0.0253$ and 0.0220 P, respectively. With use of $D_{\mathrm{NADH}} = 3.3 \times 10^{-6}$ cm^2 s^{-1} in H_2O at 25 °C[15] the values for D_{NADH} in 1-PrOH/H_2O are found to be 1.2×10^{-6} cm^2 s^{-1} at 25 °C and 1.4×10^{-6} cm^2 s^{-1} at 30 °C. The values for **1a** were estimated from data for **2a**. With use of eq 6 and $D_{1a} = 2.4 \times 10^{-5}$ cm^2 s^{-1}[16] in acetonitrile, R_{2a} is calculated to be 2.6 Å. By the same method as that used for NADH, D_{2a} is calculated to be 3.9×10^{-6} cm^2 s^{-1}. NADH has a structure in which the hydrodynamic radius may be much greater than the reaction radius for electron transfer from the dihydronicotinamide moiety. The R_{NADH} was estimated to be 3.0 Å on the basis of molecular models. Therefore at 30 °C $D = 5.3 \times 10^{-6}$ cm^2 s^{-1} and $R_c \sim 5.6$ Å which gives a value of log $k_{\mathrm{diff}} = 9.57$. This value is very close to log $k_{\mathrm{diff}} = 9.49$ which is obtained by using a modified Debye equation in which the radii of the two molecules are assumed to be approximately equal.

$$k_{\mathrm{diff}} = 8RT/3\eta \qquad (8)$$

A value of log $k_{\mathrm{diff}} = 9.5$ will be used in subsequent calculations.

Within the initial one-electron transfer (Scheme I, step 1) there are actually three processes which must occur. The two reactants must diffuse together to form an encounter complex, an electron can then hop within the complex, and finally there must be diffusional separation of the products. In principle any of these three processes can limit the rate of the overall electron transfer.[17] As the rate-limiting process changes, curvature will be observed in plots of thermodynamic vs. kinetic data. At the one extreme if diffusion together of the reactants is the process which limits the rate of the overall electron transfer log k_0 = log k_{diff}. In this situation increasing the oxidizing power of the oxidant does not affect the rate as it is already at its maximal rate. When the oxidant becomes weaker the electron hop within the encounter complex becomes rate limiting. This will be true when $E°$ for **1**/**2** is approximately the same as $E°$ for the NADH/NADH$^{+\cdot}$ couple. Eventually the one-electron transfer becomes so endothermic that the other extreme is reached and diffusional separation limits the overall electron transfer. In this case, the back reaction of the products (**2** + NADH$^{+\cdot}$) would be diffusion controlled and structure independent. In this latter case a plot of log k_0 vs. $E°_{1/2}$ should give a line with slope 16.6 V^{-1}.

In Figure 2 is shown a plot of the experimental log k_0 vs. $E°_{1/2}$. The points are experimental and the line is drawn with a slope of 16.6 V^{-1}. The experimental points from the five weakest oxidants form a line with a slope of 16.0 V^{-1} which is in good agreement with the prediction. The agreement substantiates the mechanistic proposal that all the ferroceniums react by rate-limiting one-electron transfer. Considering this electron transfer in more detail, Figure 2 shows that these five ferroceniums react in a very endothermic one-electron transfer where separation of the **2**/NADH$^{+\cdot}$ pair is rate limiting. The other three ferroceniums—the more powerful oxidants—do not fall on the 16.6-V^{-1} segment and react with the electron hop as the actual rate-determining process.

The one-electron NADH/NADH$^{+\cdot}$ $E°$ can be estimated by extrapolating the segment with slope 16.0 V^{-1} up to the diffusion-controlled rate limit.[18] At this point log k_0 = log k_{diff} and therefore $E°_{\mathrm{NADH/NADH^{+\cdot}}} = E°_{1/2}$. The $E°$ for the one-electron NADH/NADH$^{+\cdot}$ couple determined by this extrapolation is 1.05 V vs. NHE. The accuracy of this value naturally depends upon the slope of the line used to do the extrapolation and the value used for k_{diff}. A 10% change in the slope of the line results in a 0.05-V shift in $E°_{\mathrm{NADH/NADH^{+\cdot}}}$ while each 0.1 log unit change in log k_{diff} results in a 0.006-V shift. Thus, this kinetic estimation of a thermodynamic quantity could be inaccurate by as much as 0.1 V. Even considering this inaccuracy, *the very positive potential found is indicative of the instability of NADH$^{+\cdot}$. Only very powerful one-electron oxidants will be able to oxidize NADH by a mechanistic pathway involving one-electron transfer.* For comparison, the one-electron $E°$ for *N*-benzyldihydronicotinamide in CH_3CN solvent has been determined by two groups.[19] The values obtained were 0.80 and 1.00 V vs. NHE. Considering that the kinetics of fluorescence quenching of the excited dihydronicotinamide was used by both groups to estimate $E°$, the lack of agreement is substantial. It must also be realized that the solvent does have an effect on $E°$ (see below). Estimates for an aqueous solution made by using a nonaqueous solvent can only be fortuitously accurate.

Since the rate-limiting process for the reactions involving the aryl-substituted ferrocenium cations (**1f**–**h**) is the electron hop, the Marcus equation[20] (eq 9) can be used to calculate the self-exchange rate constant for electron transfer between NADH and NADH$^{+\cdot}$ ($k_{\mathrm{NADH/NADH^{+\cdot}}}$). λ_{11} is equal to four times the activation energy ($\Delta G^{\ddagger}_{11}$) for self-exchange between NADH and NADH$^{+\cdot}$

$$\Delta G^{\ddagger} = ((\lambda_{11} + \lambda_{22})/8)(1 + 2\Delta G°/(\lambda_{11} + \lambda_{22}))^2 \qquad (9)$$

while λ_{22} is the corresponding value for the **1**/**2** couple. The only unknown is λ_{11} since λ_{22} can be evaluated from the literature,[21] $\Delta G°$ can be calculated from the redox potentials, and $\Delta G^{\ddagger}$ can be evaluated from the rate constant by using eq 10. The value

$$k = Ze^{-\Delta G^{\ddagger}/RT} \qquad (10)$$

for λ_{11} was calculated to be 31.4 kcal mol^{-1}[22] which yields $k_{\mathrm{NADH/NADH^{+\cdot}}} \sim 2 \times 10^5$ M^{-1} s^{-1}.

An alternative method for calculating $\Delta G^{\ddagger}_{11}$ from these data is to use the Rehm–Weller relationship[23] (eq 11). Klinger and

$$\Delta G^{\ddagger} = \Delta G°/2 + [(\Delta G°/2)^2 + ((\Delta G^{\ddagger}_{11} + \Delta G^{\ddagger}_{22})/2)^2]^{1/2} \qquad (11)$$

Kochi have noted[24] that this relationship calculates a more con-

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[22] In this treatment we have assumed that NADH behaves as if it were uncharged, and, therefore, assumed that the work required to bring the reactants together (w^R) and to separate the products (w^P) is negligible since in both cases one of the components is uncharged. The neutral dihydronicotinamide moiety in NADH undergoes reaction, but the phosphate backbone does have a 2– charge. If this charge were important an effect of the ionic strength on the rate constant might be expected. Unfortunately the magnitude of this effect cannot be evaluated due to the possible ion pairing of **1**.

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Table VI. Calculated and Experimental Rate Constants (k, M^{-1} s^{-1}) for the Oxidation of NADH

compound	$E°/V^a$	$\Delta G^\ddagger_{22}$, kcal/mol	log $k_{(calcd)}$	log $k_{(exptl)}{}^a$	Δ log k^b
(a) promazine radication cation	0.71^c	3.3^c	5.3	5.7^d	0.4
(b) chlorpromazine radical cation	0.78^c	3.3^c	6.1	6.9^d	0.8
(c) promethazine radical cation	0.86^c	3.3^c	7.0	7.4^d	0.4
(d) *m*-benzosemiquinone	0.81^e	3.2^e	6.6	6.9^d	0.3
(e) $Br_2^{-}\cdot$	1.69^f	g	10.0^g	9.0^d	−1.0
(f) $Fe(CN)_6^{3-}$	0.45^h	8.5^i	0.5	0.5^j	0.0
(g) benzoquinone	0.099^k	4.4^l	−3.7	0.7^j	4.4
(h) 2-methylbenzoquinone	0.023^k	4.4^l	−4.9	-0.1^j	4.8
(i) 2,5-dimethylbenzoquinone	-0.065^k	4.4^l	−6.3	-1.1^j	5.2
(j) 2,3-dimethylbenzoquinone	-0.074^k	4.4^l	−6.4	-0.6^j	5.8
(k) *N*-methylacridinium	0.0^m	2.7^n	−5.3	2.0^o	7.3
(l) *N*-propylnicotinamide	-0.845^p	2.7^n	−19.3	-2.5^q	16.8

[a] In aqueous solution, generally pH 7.0, various temperatures (21–30 °C). [b] log k(exptl) − log k(calcd). [c] Pelizzetti, E.; Mentasti, E. *Inorg. Chem.* **1979**, *18*, 583. [d] Reference 9a. [e] Steenken, S.; Neta, P. *J. Phys. Chem.* **1982**, *86*, 3661. $\Delta G^\ddagger_{22}$ value was calculated by simultaneously solving three questions involving three known rate constants and three known $E°$ values. [f] Endicott, J. F. In "Concept of Inorganic Photochemistry"; Adamson, A. W., Fleishauer, P. D., Ed.; Wiley-Interscience: New York, 1975. [g] Unknown, for such an exergonic electron transfer log k is estimated to be 10, the diffusion-controlled limit. [h] Extrapolated to 0.5 M KCl from: Hanania, G. I. H.; Irvine, D. H.; Eaton, W. A.; George, P. *J. Phys. Chem.* **1967**, *71*, 2022. [i] Pelizzetti, E.; Mentasti, E.; Pramauro, E. *Inorg. Chem.* **1978**, *17*, 1688. [j] Carlson, B. W.; Miller, L. L., unpublished results. [k] Ilan, Y. A.; Czapski, G.; Meisel, D. *Biochim. Biophys. Acta* **1976**, *430*, 209. [l] Meisel, D.; Fessenden, R. W. *J. Am. Chem. Soc.* **1976**, *98*, 7505. $\Delta G^\ddagger_{22}$ for all of the methyl-substituted benzoquinones was assumed to be the same as that measured for benzoquinone and 2,5-dimethylbenzoquinone. [m] Reference 19a, in CH_3CN. [n] Estimated, based upon $\Delta G^\ddagger_{22} \sim 2.7$ kcal mol^{-1} for protonated 2,2′-bipyridine/2,2′-bipyridine radical ($bpyH^+/bpyH\cdot$). Krishnan, C. V.; Creutz, C.; Schwarz, H. A.; Sutin, N. *J. Am. Chem. Soc.* **1983**, *105*, 5617. [o] Creighton, D. J.; Hajdu, J.; Mooser, G.; Sigman, D. S. *J. Am. Chem. Soc.* **1973**, *95*, 6855. [p] Assumed to be the same as that for the *N*-methyl derivative. Blankenhorn, G. *Eur. J. Biochem.* **1976**, *67*, 67. [q] Calculated from the reverse rate constant and the K vale. Jones, J. B.; Taylor, K. E. *Can J. Chem.* **1976**, *54* 2974.

sistent value for $\Delta G^\ddagger_{11}$ in the endergonic region than the Marcus relationship. Their results indicate that Marcus theory may underestimate k_{11}. The Rehm–Weller relationship yields a $\Delta G^\ddagger_{11}$ value of 5.6 kcal mol^{-1} (λ_{11} = 22.4 kcal/mol) which yields $k_{NADH/NADH^{+}\cdot} \sim 9 \times 10^6$ M^{-1} s^{-1}. The Rehm–Weller relationship will be used in subsequent calculations. These λ_{11} values indicate how large a barrier exists for electron transfer between NADH and $NADH^{+}\cdot$, i.e., how much NADH has to reorganize in order to lose an electron to any one-electron oxidant. In comparison to ferrocene ($\Delta G^\ddagger_{22}$ = 5.6 kcal mol^{-1}),[25] NADH requires about the same amount of reorganization. This indicates that there is a kinetic barrier to mechanisms involving electron transfer from NADH in addition to a large thermodynamically imposed limitation.

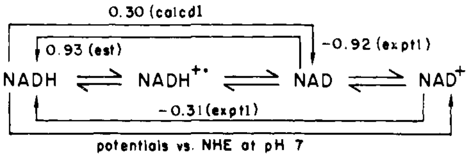
It is of particular interest to know $E°$ and $\Delta G^\ddagger_{NADH/NADH^{+}\cdot}$ for aqueous solution rather than $PrOH/H_2O$. Because of solubility problems it was not possible to measure **1**/NADH rates in aqueous solution. Therefore, some estimate of $E°$ and log k changes will be made as a less-attractive alternative. Consider first changes in $E°$ for **1**/**2** couples as the solvent changes.

Because hydroxymethyl ferrocene (**2i**) is slightly water soluble the one-electron $E°$ for **1i**/**2i** was determined by using cyclic voltammetry in H_2O as well as 1-$PrOH/H_2O$. The values obtained, extrapolated to zero salt concentration, were 0.536 V in $PrOH/H_2O$ and 0.430 V in H_2O. As a second example the $E°$ for **1a** was determined in H_2O containing 0.1 M $NaClO_4$ to be 0.395 V. By comparison with the salt effect data for **1i** the extrapolated $E°$ was estimated to be 0.400 V. This is 0.129 V cathodic of the $E°$ of 0.529 V found in 1-$PrOH/H_2O$. These two results indicate that $E°$ values for **1**/**2** are about 0.12 V smaller in water. The quantitative conclusions from a recent report[26] which assumes no difference in ferrocenium $E°$ values in going from water to propanol/water should be revised.

For the effect of solvent on the rate to be investigated, the oxidation of NADH by promethazine radical cation was studied in 50% 2-$PrOH/H_2O$ by using pulse radiolysis. It has previously been postulated that this reaction involves one-electron transfer. The rate constant found was 3.2×10^7 M^{-1} s^{-1} which is within experimental error of the $2.8 \pm 0.8 \times 10^7$ M^{-1} s^{-1} found in H_2O.[9a] Thus, there appears to be a substantial solvent effect on $E°$, but not on the rate of one-electron oxidations of NADH. Therefore, the best estimates for aqueous solution are $E°_{NADH/NADH^{+}\cdot}$ 0.93 V (NHE) and $\Delta G^\ddagger_{11}$ = 5.6 kcal mol^{-1}.

It is worth emphasizing that there is a good deal of confusion in the literature about "one-electron" NADH potentials. It should now be clear that there is a value for one-electron oxidation (yielding $NADH^{+}\cdot$) and a value for one-electron, one-proton oxidation (yielding $NAD\cdot$).

With use of this value for the $E°_{NADH/NADH^{+}\cdot}$ the thermodynamic cycle for stepwise $NADH/NAD^+$ interconversion may be completed as follows, where experimental, calculated, and estimated reduction potentials are shown in V vs. NHE at pH 7.[27] In addition the pK_a for $NADH^{+}\cdot$ can be estimated to be −3.5 from the thermodynamic data. This compares well with the value of −4 estimated previously from photochemical data in CH_3CN.[28]



It is of mechanistic interest to predict the rates of various one-electron oxidants with NADH, and since the appropriate $E°$ and $\Delta G^\ddagger_{22}$ values are known for several oxidants this can be done by using the Rehm–Weller relationship. The results for such calculations for NADH reactions in aqueous systems are shown in Table VI. Compounds a–f have been proposed to be one-electron oxidizing agents while g–l could react by various routes including hydride transfer as well as mechanisms involving an initial electron transfer. A general trend can be seen from these results. The calculated rate constants for the supposed one-electron oxidants are within 1.0 order of magnitude of the experimental rate constants.[29] The agreement is very good as the range of measured rate constants is more than eight orders of magnitude.

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The other oxidants, however, have calculated rate constants which are 4–16 orders of magnitude less than the experimental rate constants.

This comparison then suggests that one-electron transfer mechanisms hold for all the ferroceniums and oxidants a–f. Since for any oxidation involving initial electron transfer the overall rate cannot be greater than the rate of the electron-transfer step, the discrepancy between calculated and measured rates suggests that compounds g–l in Table VI react by a mechanism which avoids the higher barrier imposed by electron transfer. One such mechanism is a hydride transfer.[19a,30]

The above calculations allow comparison of a wide variety of compounds with widely varying $E°$. There is, however, considerable uncertainty in the calculated rate. It is, therefore, equally interesting to directly compare a pair of oxidants. For two weak oxidants like **1a** and benzoquinone both of which should fall on the 16.6-V^{-1} line it can be shown that the rates of reaction can be compared without knowing $E°_{NADH/NADH^{+\cdot}}$ or $\Delta G^{\ddagger}_{NADH,NADH^{+\cdot}}$. For such compounds there is then less uncertainty in the comparison. With use of the $E°$ values for **1a** and benzoquinone the calculated difference in log k is 5.0. Experimentally the difference of log k is 0.45. Benzoquinone reacts 4×10^4 times faster than predicted, and this again suggests that the benzoquinone reaction does not involve $NADH^{+\cdot}$ as an intermediate.

Conclusions and Summary

The oxidation of NADH by ferrocenium cations in aqueous propanol has been shown to proceed by a multistep mechanism involving initial rate-limiting one-electron transfer. Analysis of the data for a series of ferrocenium cations has yielded as estimate of the one-electron $E°$ and self-exchange rate constant for the $NADH/NADH^{+\cdot}$ couple. Calculations using the Rehm–Weller relationship allow mechanistic differentiation for several NADH oxidations in the literature.

Experimental Section

Materials. Ferrocene (Aldrich) was vacuum sublimed twice before use; 1,1′-dimethylferrocene (Alfa) was recrystallized from ethanol/H_2O; 1-(chloromercuri)ferrocene (Aldrich) was recrystallized from acetone; *n*-butylferrocene (Pfaltz & Bauer) was used without further purification. Ethylferrocene[31] and the arylferrocenes[32] were prepared by literature procedures. All compounds gave satisfactory melting points, IR spectra, NMR spectra, and/or mass spectra. β-NADH and NAD^+ were obtained from Sigma Chemical Co. Other materials were readily available from commercial sources and were either used without further purification or recrystallized according to standard procedures.

Solvent and pH. 1-Propanol (Fisher) was distilled before use. H_2O was triply distilled. The solvent used was prepared by mixing equal volumes (46/54 w/w) of 1-PrOH and an aqueous solution buffered to the appropriate pH by 0.02 M buffer. The pH values cited refer to the pH of the aqueous solution before mixing. The pH 6.0, 7.0, and 8.0 solutions were made from phosphate buffers and the pH 9.0 solution from boric acid/NaOH buffer. $NaClO_4$ was then dissolved in the appropriate volume of solvent to give the desired concentration. The range of buffer concentrations used was limited by the solubility of the buffer salt. Unless otherwise indicated reactions were run in 46/54 (w/w) 1-PrOH/H_2O containing 0.01 M pH 7.0 phosphate buffer. The pH values observed in the solvent changed ca. 0.5 pH unit with a change of 1.0 pH unit in the aqueous solution.

Preparation of Ferrocenium Hexafluorophosphates (1a–h). Two general methods[33,34] were used to prepare the ferrocenium hexafluorophosphates as blue or green solids.

Method A. Approximately 2 mmol of the ferrocene derivative were dissolved in 4 mL of concentrated H_2SO_4. After 1 h the blue or green solution was diluted to 40 mL with H_2O and filtered through a fine sintered glass funnel. 30 mmol of NH_4PF_6 in 10 mL of H_2O were added and the solution cooled in an ice bath. The salt was collected by filtration, washed with 2 × 10 mL of cold H_2O, and then copiously washed with absolute ether. In some instances the salt was reprecipitated from dilute H_2SO_4 with NH_4PF_6.

Method B. Approximately 1 mmol of the ferrocene derivative and an equal molar amount of benzoquinone were dissolved in 50–100 mL of absolute ether. 2–4 mL of 60% HPF_6 were added and the solution immediately turned blue or green. After the mixture was cooled in an ice bath the salt could be isolated by filtering, washing with 2 × 10 mL of cold H_2O, and washing copiously to remove any remaining ferrocene derivative, benzoquinone, or hydroquinone. The phenylferrocenium hexafluorophosphate was somewhat soluble in ether so less extensive washing with ether was used.

Pertinent data for the ferrocenium hexafluorophosphates is collected in Table I.

Preparation of [4-^{1}H,4-^{2}*H*]- and [4-^{2}*H*,4-^{2}*H*]NADH. [4-^{1}H,4-^{2}H]-NADH (NADH-d_1) stereospecifically labeled with deuterium in the *pro-R* position was prepared by the method of Fisher and Brown.[35] [4-^{2}H,4-^{2}H]NADH (NADH-d_2) was prepared from NADH-d_1 as follows: NADH-d_1 and 3,5-*di-tert*-butyl-*o*-benzoquinone were reacted in 2/1 (v/v) H_2O/CH_3CN to produce NAD^+ containing a mixture of NAD^+-d_0 and NAD^+-d_1. The solution was extracted with ether, and the aqueous layer was made 0.5 M in Tris base and adjusted to pH 9.6 with 1 M NaOH. The NAD^+ was then reduced in the standard manner with alcohol dehydrogenase and ethanol-d_6. The NADH product was isolated as in the case of NADH-d_1. This procedure was repeated to yield NADH-d_2 which had >90% total deuterium at the 4-position (NMR).

During the isolation of these compounds a quantity (≤10%) of α-NADH was produced by epimerization. The control experiment was done in which β-NADH was epimerized with use of the isolation procedure used for NaDH-d_1 and NADH-d_2. This material yielded, within experimental error, the same rate constants for oxidation of **1a** as had been obtained with β-NADH.

Product Identification. NADH (125 μmol) and 250 μmol of **1a** were reacted in 20 mL of 46/54 (w/w) 1-PrOH/H_2O which had been bubbled with argon. When the blue color of the ferrocenium cation was discharged (~75 min) the solution was extracted with ether. The ether was removed and the resulting orange compound was sublimed to give 35 mg of a compound which was found to be ferrocene based upon its melting point and IR (75% isolation yield). The aqueous layer was treated with five times its volume of cold acetone and left in a refrigerator. The fine white powder was collected and assayed for NAD^+ by observing the change in absorbance at 340 nm when a solution of the product was enzymically reduced with alcohol dehydrogenase and ethanol. On the basis of this method 96 mmol of NAD^+ were present (77% yield).

Kinetics. The kinetics were investigated under pseudo-first-order conditions with NADH in at least 15-fold excess. The progress of the reaction was monitored by following the decrease in the ferrocenium absorption of the λ_{max} in the visible region. These wavelengths were in the region 615–750 nm. Typical concentrations of NADH were 4.0–8.0 mM while typical ferrocenium concentrations were 0.2–0.4 mM. The concentration of NADH was determined by syringing 40 μL of the NADH solution to be used in the kinetic experiment into 2.0 mL of aqueous pH 7.0 phosphate buffer. The absorbance at 340 nm was read and converted to concentration by using Beer's law ($\epsilon = 6.22 \times 10^3$ M^{-1} s^{-1}) and the known dilution factor (2.04/.04). The solvent was deoxygenated before use by bubbling it with prepurified argon and mixing of solutions was done in a nitrogen purged glove bag. Aliquots (2.0 mL) of NADH solution were syringed into cuvettes and septum capped. The cuvettes were thermostated to 30.0 ± 0.2 °C in the cell holder of a Cary 219 spectrophotometer. The absorbance due to NADH was stable over times longer than the temperature equilibration time. To aid in data collection the spectrophotometer was interfaced with an Apple II Plus computer used with a Texas Instruments TI99/4 Color Monitor. To initiate the reaction 20–80 μL of **1**, which had been dissolved in the same solvent as the NADH, kept under an inert gas, and thermostated, were syringed into the cuvette. Time vs. absorbance readings were then recorded and evaluated through the use of integrated rate equations. The reactions were all followed for three or more half-lives and plots of ln $(A_0 - A_\infty)/(A_t - A_\infty)$ vs. time gave r >0.999.

Effect of Added 2a and NAD$^+$. **2a** (0.26–0.65 mM) was added to the solvent before the kinetics were observed. The [NADH] was approximately 7 mM with **1a** being present in 0.2–0.35 mM concentrations. There was some difficulty encountered keeping **2a** in solution during the reaction as **2a** is not very soluble in the solvent.

NAD^+ (0.98–1.52 mM) was added to the solvent before the kinetics were observed. The [NADH] was approximately 7 mM with [**1a**] approximately 0.25 mM. At these concentrations there was no difficulty encountered with NADH decomposition.

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J. Am. Chem. Soc. 1984, 106, 7239–7244

Pulse Radiolysis. The experimental procedures for these measurements have been reported previously.[9a]

Electrochemical Measurements. Cyclic voltammetry was performed with a PAR Model 173 potentiostat in conjunction with a PAR 175 universal programmer. Cyclic voltammograms were recorded on a Houston Instruments Model 2000 *x–y* recorder. The working electrode was a glassy carbon electrode of area 0.08 cm^2 which had been sealed in heat-shrinkable Teflon. The electrode was cleaned by successive abrasion with Buehler Micropolish A (0.3 micron) and Buehler Micropolish B (0.05 micron) on a polishing cloth, wiping with a Kimwipe, rinsing with water, and air drying. The counter electrode was a carbon rod. The reference electrode was a SCE, and the observed potentials were converted to the NHE by addition of 0.242 V. All measurements were made at 21 ± 1 °C under a nitrogen atmosphere.

Conductivity Measurements. It was found that allowing 1-PrOH to sit in glass resulted in a gradual increase in conductivity. The 1-PrOH was therefore distilled immediately before mixing with triply distilled H_2O (46/54 (w/w)). A standard conductivity cell employing two platinum disks 1 cm in diameter separated by 3 cm in a cell of ~15 mL volume was used. The cell constant was determined by using aqueous KCl solutions. Temperature was controlled at 30.0 ± 0.02 °C with use of a Fisher Water Bath Model 80. Resistance was measured by using a Marconi Instruments Universal Bridge TF2700. The solvent was bubbled with argon before use, and solutions were mixed in a nitrogen-purged glove bag.

Acknowledgment. This work was supported by the National Science Foundation. Stimulating discussions with Jean-Michel Dumas-Bouchiat are acknowledged.

Registry No. 1a, 11077-24-0; **1b**, 92314-56-2; **1c**, 34778-21-7; **1d**, 92314-57-3; **1e**, 51512-99-3; **1f**, 92314-59-5; **1g**, 52633-42-8; **1h**, 92314-61-9; NADH, 58-68-4; [4-^{2}H]NADH, 10012-96-1; [4,4-$^2H_2$]NADH, 60764-22-9.

Supplementary Material Available: Tables of the rate constants for the oxidation of NADH by ferrocenium ions **1** at varying $NaClO_4$ concentrations and $E°$ values for **1**/**2** couples at varying $NaClO_4$ concentrations (2 pages). Ordering information is given on any current masthead page.

Binding of the Antitumor Drug *cis*-$[PtCl_2(NH_3)_2]$ to Crystalline $tRNA^{Phe}$ at 6-Å Resolution

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Abstract: Monoclinic crystals of yeast $tRNA^{Phe}$ soaked in reactive hydrolysis products of *cis*-$[PtCl_2(NH_3)_2]$ and in a low chloride ion buffer show drug binding in the vicinity of residues G3-G4, C25-m^2_2G26, G42-G43-A44-G45, and A64-G65. Drug binding invariably results in crystal deterioration with loss of the diffraction pattern beyond a resolution of ~6 Å so that detailed geometric data concerning the binding of the drug cannot be obtained from the present study. Considered together with previous work performed in this area, however, the most probable interpretation of these results is that the *cis*-$[Pt(NH_3)_2]^{2+}$ moiety forms an intrastrand cross-link at certain GG and AG sites within $tRNA^{Phe}$ thereby causing a local distortion of the macromolecule. Features common to each of these binding sites are that at least one G residue is involved, that the adjacent bases in the native undistorted macromolecule are stacked on top of one another, and that the N7 donor atoms of these bases are ~4 Å apart and are not, themselves, involved in hydrogen-bonding interactions elsewhere in the macromolecule. Binding at one CG site is observed and a base-flip by the C residue is invoked to rationalize the drug binding at this site. A preference for the drug to bind at AG rather than GA sites is noted and an explanation offered.

cis-$[PtCl_2(NH_3)_2]$ (*cis*-DDP) is a potent antitumor drug while *trans*-$[PtCl_2(NH_3)_2]$ is inactive.[2–4] There now exists a strong body of evidence[5–14] to suggest that it is the ability of the *cis*-$[Pt(NH_3)_2]^{2+}$ moiety to cross-link between the N7 atoms of two adjacent G residues on the same strand of DNA, and the inability of the trans complex to behave similarly, that is the reason for the diverse pharmacological properties of these two relatively simple inorganic complexes. Since it is the *cis*-$[Pt(NH_3)_2]^{2+}$ moiety that actually forms the cross-link, binding to bipolymers depends very much upon the extent of hydrolysis of the drug and reaction of *cis*-DDP itself is slow compared with the more reactive aquo species such as *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ and *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$.[15] It is believed that DNA is the biological target of the drug rather than RNA or protein.[16]

Evidence that the *cis*-$[Pt(NH_3)_2]^{2+}$ moiety forms an intrastrand cross-link with DNA via the N7 atoms of two adjacent G residues stems from several lines of experimentation. First, a greater buoyant density change was seen for poly(dG)·poly(dC) reacted with *cis*-DDP than was observed for platinated poly(dG-dC) when

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