

## 226 - Effect of Lewis Acids on the Electrochemical Reduction of Nicotinamide Adenine Dinucleotide \*

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### Summary

Electrochemically, nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ;  $\text{DPN}^-$ ; coenzyme I) is reduced in two steps, *i.e.*, a pH-independent one-electron reduction to the free radical, which, at more negative potential, is reduced in a one-electron, one-proton process to  $\text{NADH}$ . The variation in the electrochemical pattern resulting from interaction with two LEWIS acids known to be involved biologically with  $\text{NAD}^+$ , *i.e.*,  $\text{Ca(II)}$  and  $\text{Zn(II)}$ , has been examined. Potentiometric titration indicates that  $\text{Ca(II)}$  forms a weak 1 : 1 complex with  $\text{NAD}^+$  at pH 7.2 (formation constant equals 38); *d.c.* polarography supports such complexation. The nature and effects of these complexes have been explored, *e.g.*,  $\text{Ca(II)}$  increases the rate of charge transfer to that expected for a reversible reaction for the first reduction step only at relatively high concentration but has a marked effect on the second step even at low concentration.  $\text{Zn(II)}$  apparently forms a complex with a decomposition product of  $\text{NAD}^-$ , which catalyzes the  $\text{Zn(II)}$  reduction.

### Introduction

In recent years, the electrochemical reduction of nicotinamide adenine dinucleotide ( $\text{NAD}^-$ ; diphosphopyridine nucleotide;  $\text{DPN}^+$ ; coenzyme I), which is one of the most common respiratory coenzymes, has been extensively studied.<sup>1-6</sup> The overall electrochemical redox behavior of the  $\text{NAD}^+/\text{NADH}$  redox system is summarized in Fig. 1.<sup>7</sup>

At a potential of  $-0.9$  to  $-1.1$  V, depending on the background electrolyte,  $\text{NAD}^+$  is reduced in a one-electron ( $1e^-$ ) pH-independent process to a free radical,  $\text{NAD}^\cdot$ , which rapidly dimerizes. In the range

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of pH 7 to 9, the 1 e<sup>-</sup> reduction of the free radical to NADH can be observed. Dimer and NADH can be oxidized back to NAD<sup>+</sup> at considerably more positive potential.

At present, the authors and their collaborators are attempting to obtain a more detailed picture of the electrochemical reaction pathways for the reduction of NAD<sup>+</sup> and the oxidation of NADH. One aim of such studies is clarification of the differences and similarities between the

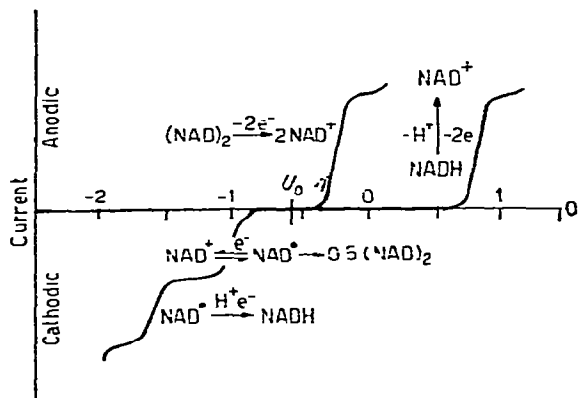


Fig 1

Composite representation of the polarographic and voltammetric waves for the NAD<sup>+</sup>–NADH system in relation to the calculated formal potential,  $U_c^\circ$  of  $-0.557$  V for the half-reaction,  $\text{NAD}^+ + 2e^- + \text{H}^+ \rightleftharpoons \text{NADH}$ , at pH 7 and 25 °C Potentials are vs S.C.E [Reproduced with permission from Ref 7]

electrochemically and biologically observed behaviors of the NAD<sup>+</sup>/NADH redox system.<sup>7</sup> The present paper reports the results of added Ca(II) and Zn(II) on the observed electrochemical behavior of NAD<sup>+</sup>. These two LEWIS acids were examined because of their importance in biological systems, *e.g.*, the implication of zinc as a LEWIS catalyst in liver alcohol dehydrogenase for which NAD<sup>+</sup> is the coenzyme.<sup>8-11</sup>

## Experimental

### Chemicals

Compounds were obtained from the following sources: nicotinamide adenine dinucleotide (NAD<sup>+</sup>), adenosine diphosphoribose sodium salt (ADPR), and yeast alcohol dehydrogenase from P-L BIOCHEMICALS and 1-methylnicotinamide chloride from SIGMA. Reagent grade chemicals were obtained from the following sources: tetraethylammonium chloride (TeaCl), and tetraethylammonium hydroxide (TeaOH) from ALDRICH; tetraethylammonium perchlorate (Teap), zinc perchlorate, calcium perchlorate, and perchloric acid from G. FREDERICK SMITH; 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) from SIGMA, and other chemicals from J. T. BAKER.

Purity of NAD<sup>+</sup> was determined from enzymatic assays.<sup>12</sup>

### Apparatus

Polarographic and other voltammetric studies were conducted in a water jacketed one-compartment cell with a LUGGIN capillary.<sup>13</sup> For *d.c.* and *a.c.* polarography, a PAR Model 174 polarographic unit was used; for *a.c.* polarography (modulation voltage = 5 mV peak; 40 and 100 Hz), a PAR Model 121 lock-in amplifier/phase detector was interfaced with the Model 174. For fast-scan cyclic voltammetry, a potentiostat was used which was designed to have a minimal response time.<sup>13</sup> Data were recorded on a HOUSTON INSTRUMENTS Series 2000 x-y recorder and on a TEKTRONIC 5103N cathode ray oscilloscope equipped with suitable modules and a camera.

### Procedures

Standard procedures were used. All measurements were made at 25 °C. All potentials are referred to the aqueous saturated calomel electrode (S.C.E.). The care taken with the *a.c.* polarographic measurements has been described.<sup>14</sup>

### Initial one-electron reduction

#### Effect of Ca(II)

1. *d.c.* and *a.c.* polarography. The *d.c.* and *a.c.* polarographic behavior of mixtures of 0.42 mM NAD<sup>-</sup> and Ca(II) (0.25 to 100 mM) were studied at pH 7.2 and 9.3 in 0.1 M Teap and 0.01 M Tris buffer (pH was adjusted by addition of HClO<sub>4</sub>). The addition of Ca(II) had little effect on the *d.c.* polarographic wave until sufficient Ca(II) had been added to raise significantly the ionic strength of the solution. At that point, the slope of the *d.c.* wave became more steep,  $U_{1/2}$  shifted 15 mV positive and the diffusion current constant,  $I_d$ , decreased 10–15 % (Table 1).

Essentially the same behavior was observed for the *a.c.* polarographic response of NAD<sup>+</sup> on Ca(II) addition (Table 2). At low Ca(II) concentrations, changes in the *a.c.* peak were slight. At 50 and 100 mM Ca(II) levels, the *a.c.* summit potential,  $U_s$ , was shifted 10–25 mV more positive, the half-peak width decreased, and the peak became broader on the negative side of  $U_s$ ; in the absence of Ca(II), the peak was broader on the positive side of  $U_s$ .

Since the presence of Ca(II) at the 50 or 100 mM level produces an *a.c.* polarographic response for the reduction of NAD<sup>+</sup>, which more closely approximates that predicted for a reversible charge transfer followed by an irreversible dimerization,<sup>15</sup> it appears that the addition of Ca(II) must do at least one of the following:

- (1) increase the rate of charge transfer, and/or
- (2) decrease an intermolecular interaction between NAD<sup>+</sup> and its reduction products. These two alternatives can not be distinguished solely on the basis of changes in the *a.c.* and *d.c.* polarographic behavior.

Table 1. *d.c.* Polarographic data for the one-electron reduction of NAD<sup>+</sup> in the presence of Ca(ClO<sub>4</sub>)<sub>2</sub><sup>a</sup>

Ca(II) (mM)	$U_{1/2}$ (V)	$U_{1/4} - U_{3/4}$ (mV)	$I_d^b$
pH 7.2			
0	-1.077	64	1.17
0.50	-1.073	58	1.14
5.0	-1.073	56	1.18
50	-1.058	61	1.05
pH 9.3			
0	-1.074	63	1.17
0.50	-1.073	58	1.04
10.0	-1.070	62	1.10
50	-1.063	57	1.05
100	-1.056	53	0.99

<sup>a</sup> All solutions are 0.42 mM NAD<sup>+</sup> in 0.10 Teap and 0.01 M Tris

<sup>b</sup> Polarographic diffusion current constant =  $I_d/cm_i^{2/3} t^{1/6}$ , calculated from average diffusion current

2. *Cyclic voltammetry.* The results of rapid-scan cyclic voltammetry (scan rate  $v > 100$  V/s) of NAD<sup>+</sup> in the presence and absence of Ca(II) suggest that large Ca(II) concentrations increase the rate of charge transfer.

In 0.1 M Teap with 0.01 M Tris buffer adjusted to pH 9.3, 0.30 mM NAD<sup>-</sup> shows, in the presence of 50 mM Ca(II), a cathodic  $U_p$  of  $-1.18 \pm 0.005$  V, a slope of  $60 \pm 5$  mV for  $U_{p/2} - U_p$  of the cathodic peak, and a separation of 60 mV between the cathodic and anodic peaks for  $v = 200$  V/s. The slope and peak separation indicate that, for the solution conditions specified, the rate of charge transfer is sufficiently rapid to appear reversible, *i.e.*,  $k_s$  exceeds 1 cm/s, and any homogeneous reactions involving redox participants are being outrun.<sup>16</sup> In the absence of Ca(II), the separation between cathodic and anodic peaks is 116 mV, which corresponds to a charge transfer rate of 0.2 cm/s.<sup>17</sup>

Since the dimerization involving the free radical produced on the initial 1 e<sup>-</sup> transfer and any interaction involving NAD<sup>+</sup> are being outrun at a scan rate of 200 V/s, the changes in voltammetric behavior caused by the action of Ca(II) must be due to an increase in the rate of charge transfer. This does not prove, however, that Ca(II) would not also affect an intermolecular interaction between NAD<sup>+</sup> and the dimer.

Table 2. *a.c.* Polarographic data for the one-electron reduction of  $\text{NAD}^-$  in the presence of  $\text{Ca}(\text{ClO}_4)_2$  <sup>a</sup>

Ca(II) (mM)	$-U_s$ (V)	$U_{+s/2}^b - U_s$ (mV)	$U_s - U_{-s/2}^c$ (mV)	$\Phi_s^d$	$I(\omega t)^e$ (nA)
pH 9.6; 40 Hz					
0	1.128	60	57	16°	160
0.50	1.125	56	58	13°	170
50	1.108	52	56	12°	170
100	1.103	51	55	12°	170
pH 9.6; 100 Hz					
0	1.133	64	57	18°	180
0.50	1.133	62	57	17°	200
50	1.116	55	59	16°	200
100	1.105	53	55	14°	180
pH 7.2; 40 Hz					
0	1.122	65	55	15°	180
0.50	1.122	61	58	14°	200
50	1.119	59	54	15°	180
50	1.112	55	52	14°	170
pH 7.2; 100 Hz					
0	1.129	68	60	17°	220
0.50	1.130	65	59	17°	200
50	1.130	59	59	18°	210
50	1.117	56	57	14°	190
pH 7.2; 400 Hz					
0.0	1.134	72	61	27°	290
0.50	1.135	67	60	25°	310
5.00	1.128	67	64	27°	300

<sup>a</sup> All solutions are 0.42 mM  $\text{NAD}^-$  in 0.10 M Teap and 0.01 M Tris

<sup>b</sup> Potential on the positive side of the *a.c.* polarographic peak, where the in-phase current is one-half of  $I_s$

<sup>c</sup> Potential on the negative side of the *a.c.* polarographic peak, where the in-phase current is one-half of  $I_s$

<sup>d</sup> Phase angle at the *a.c.* polarographic summit potential

<sup>e</sup> Total faradaic current.

### Effect of Zn(II)

**I. d.c. and a.c. polarography.** Addition of NAD<sup>+</sup> (final concentration = 0.42 to 1 mM Zn(II) in 0.10 M Teap and 0.01 M Tris buffer at pH 7.2 produced quite striking changes in the electrochemical behavior of the Zn(II)/Zn redox couple. In the absence of NAD<sup>+</sup>, Zn(II) showed a kinetically controlled wave with  $U_{1/2} = -1.41$  V and a slope of 92 mV. The corresponding a.c. polarographic peak had  $U_p = -1.510$  V and a half-peak width of 2.4 mV.

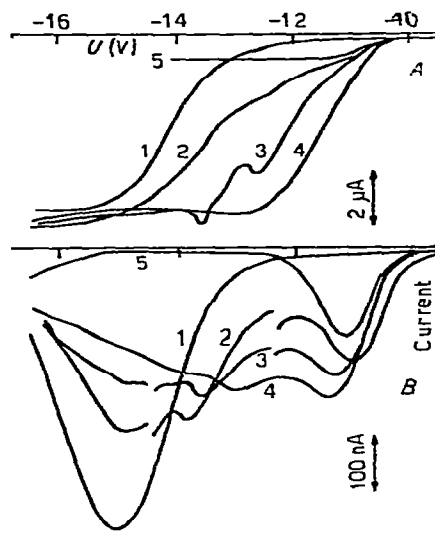


Fig 2

(A) Variation of the d.c. polarographic behavior of mixtures of Zn(II) and NAD<sup>+</sup>. Solution composition and nature: 1, 1 mM Zn(II) in 0.10 M Teap and 0.01 M Tris at pH 7.2; 2, solution 1, 80 min after addition of 0.42 mM NAD<sup>+</sup>; 3, solution 2, 100 min after NAD<sup>+</sup> addition; 4, solution 2, 245 min after NAD<sup>+</sup> addition; 5, solution 4 after Zn(II) was precipitated with Na<sub>2</sub>HPO<sub>4</sub>. (B) Variation of the a.c. polarographic behavior (in-phase current) of mixtures of Zn(II) and NAD<sup>+</sup> (controlled drop-time = 2.0 s; a.c. modulation voltage = 5 mV peak; frequency = 100 Hz). Solution composition and nature: 1, 1 mM Zn(II) in 0.10 M Teap and 0.01 M Tris at pH 7.2; 2, solution 1, 85 min after addition of 0.42 mM NAD<sup>+</sup>; 3, solution 2, 145 min after NAD<sup>+</sup> addition; 4, solution 2, 235 min after NAD<sup>+</sup> addition; 5, solution 4 after Zn(II) was precipitated with Na<sub>2</sub>HPO<sub>4</sub>.

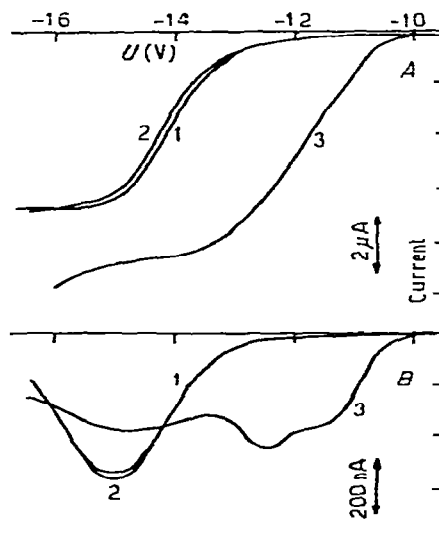
When 0.42 mM NAD<sup>+</sup> was added to the Zn(II) solution, the foot of the d.c. Zn(II) wave shifted positive and overlapped the NAD<sup>+</sup> reduction wave, whose  $U_{1/2} = -1.07$  V; the shift was time-dependent with an overall shift in  $U_{1/2}$  of 260 mV in four hours (Fig. 2). When NAD<sup>+</sup> was first added, three waves appeared. The first came in the potential region of the NAD<sup>+</sup> reduction wave. Before this wave reached a plateau, a second wave of roughly equal height began, which also did not reach a plateau but was overlapped by a third wave which apparently was due to reduction of Zn(II). With time, the third wave shifted positive, beginning to merge with the first two waves. The second wave began to develop into a sharp polarographic maximum about two-thirds of the way up the third wave. A second sharp maximum appeared at the top of the third wave. As the third wave continued to shift positive, the first maximum disappeared leaving behind an inflection in the third wave slope in the potential region where this maximum had previously been observed. The second maximum remained at the top of the third wave and became quite broad. When Zn(II) was then precipitated by adding Na<sub>2</sub>HPO<sub>4</sub>, the NAD<sup>+</sup> wave reappeared and was the same as

in the absence of Zn(II) except for a 6% decrease in the diffusion current constant which may have been due to some hydrolytic decomposition of  $\text{NAD}^+$ .

The *a.c.* polarographic response of the  $\text{NAD}^+$ -Zn(II) mixture was compatible with the *d.c.* polarographic behavior (Fig. 2). When  $\text{NAD}^+$  was added to Zn(II), three peaks were formed. The first peak, corresponding to reduction of  $\text{NAD}^+$  to  $\text{NAD}$ , increased in amplitude and shifted to more negative potential with time. The Zn(II) peak split into two peaks, both of which shifted positive and decreased in amplitude.

Fig. 3

(A) Variation of the *d.c.* polarographic behavior of mixtures of Zn(II), ADPR and 1-methylnicotinamide. Solution composition and nature 1, 1 mM Zn(II) in 0.10 M Teap and 0.01 M Tris at pH 7.2 at 5 and 180 min after the addition of Zn(II); 2 solution 1, 150 and 195 min after the addition of 0.5 mM ADPR, 3, solution 2, 45 and 120 min after the addition of 0.5 mM 1-methylnicotinamide (B) Variation of the *a.c.* polarographic behavior (in-phase current) of mixtures of Zn(II), ADPR and 1-methylnicotinamide (controlled drop-time = 2.0 s; *a.c.* modulation voltage = 5 mV peak; frequency = 100 Hz). Solution composition and nature 1, 1 mM Zn(II) in 0.10 M Teap and 0.01 M Tris at pH 7.2 at 10 and 170 min after the addition of Zn(II); 2, solution 1, 160 and 200 min after the addition of 0.5 mM ADPR; 3, solution 2, 60 and 100 min after the addition of 0.5 mM 1-methylnicotinamide.



In order to determine whether  $\text{NAD}^+$  was responsible for the shift in the Zn(II) wave, *d.c.* and *a.c.* polarograms were run for a solution of 1 mM Zn(II) in 0.10 M Teap and 0.01 M Tris buffer at pH 7.2 over a 3-hr period; no shift in  $U_{1/2}$  or  $U_s$  was observed (Fig. 3). Sufficient adenosine diphosphoribose (ADPR) was added to the Zn(II) solution to give a 0.5 mM concentration (ADPR is similar to  $\text{NAD}^+$  except that it does not contain the nicotinamide moiety). After 2.5 hr,  $U_{1/2}$  for Zn(II) had shifted only 5 mV negative; after over three hours, the total shift was 10 mV. Comparable shifts were observed in  $U_s$ .

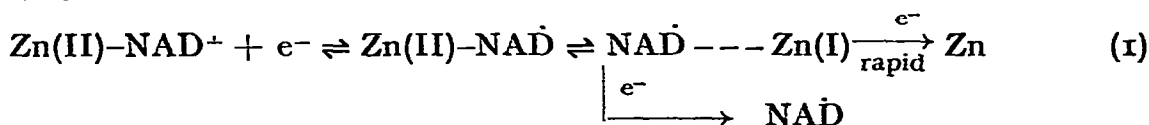
At this point, sufficient 1-methylnicotinamide was added to give a 0.5 mM concentration. After 45 min, the Zn(II) wave had shifted 210 mV positive (Fig. 3). Because of this shift, the 1-methylnicotinamide wave could not be resolved. The composite wave had an  $U_{1/2}$  of  $-1.193$  V and a slope of 135 mV. *a.c.* polarograms of the mixture run an hour after the addition of 1-methylnicotinamide revealed that the Zn(II) peak had split into two peaks with  $U_s$  of  $-1.250$  and  $-1.490$  V;  $U_s$  for 1-methylnicotinamide could not be resolved because it was overlapped by

the more positive Zn(II) peak. During an additional 40-min period, there was no change in the *a.c.* polarogram.

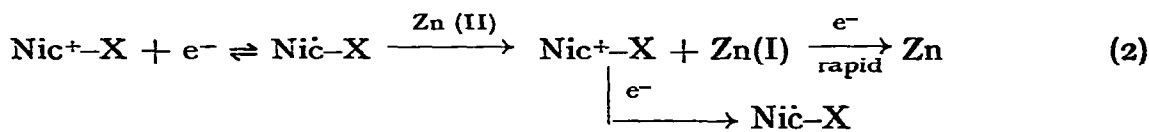
The behavior of the Zn(II)/Zn redox couple in the presence of ADPR and 1-methylnicotinamide suggests that the nicotinamide moiety is in some fashion responsible for the Zn(II) wave pattern observed.

2. *Mechanism of Zn(II)-nicotinamide interaction.* The fact that the shifts in  $U_{1/2}$  occur quite slowly in the presence of NAD<sup>+</sup> and in less than 45 min in the presence of 1-methylnicotinamide suggests two possibilities:

(a) Zn(II) complexes slowly with NAD<sup>+</sup> and more rapidly with 1-methylnicotinamide. The resulting complexed Zn(II) either is more easily reduced or is reduced in a manner akin to the following mechanism:



(b) The reduction of Zn(II) is catalyzed by a decomposition product of NAD<sup>+</sup>, which contains the nicotinamide moiety:



In the case of 1-methylnicotinamide, X is the methyl group.

Since the ratio of Zn(II) : NAD<sup>+</sup> was 5 : 2, it seems unlikely that a slow complexation with NAD<sup>+</sup>, which could at most complex only 40 % of the Zn(II), would cause the entire *d.c.* polarographic Zn(II) wave to shift. Therefore, the behavior of Zn(II)/Zn redox couple appears to be due to a catalytic reduction (equation 2) involving a decomposition product of NAD<sup>+</sup>. Since the changes in the Zn(II) redox couple were not brought about by intact NAD<sup>+</sup>, this particular phenomenon was not examined further.

### NAD free radical reduction

As previously indicated, the second step in the electrochemical reduction of NAD<sup>+</sup> to NADH involves a charge transfer to the uncharged free radical nicotinamide moiety, which produces the second polarographic wave or peak:





*Effect of Ca(II)*

The reduction of NAD to NADH was examined, using *d.c.* and *a.c.* polarography on a 0.42 mM NAD<sup>+</sup> solution (0.1 M Teap and 0.01 M Tris at 9.3), to which 0.25 to 100 mM Ca(II) was added (Tab. 3). Cyclic voltammetry could not be used since Ca(II) shows an adsorption peak in the same potential region as the NAD reduction peak.

Table 3. *d.c.* Polarographic behavior of the NAD/NADH redox couple in the presence of Ca(II) <sup>a</sup>

Ca(II) (mM)	$U_{1/2}$ (V)	$U_{1/4} - U_{3/4}$ (mV)	$I_d^b$
0.00	1.670	90	1.30
0.25	1.670	85	
0.50	1.665	75	1.25
1.00	1.665	85	
10.0	1.665	60	1.15
50.0	1.665	65	1.15
100	1.660	55	1.10

<sup>a</sup> All solutions are 0.42 mM NAD<sup>+</sup> in 0.1 M Teap and 0.01 M Tris at pH 9.3

<sup>b</sup> Polarographic diffusion current constant, calculated from average diffusion current.

Although  $U_{1/2}$  was relatively insensitive to Ca(II) addition (Tab. 3), the slope of the polarographic wave became markedly steeper with increasing Ca(II) concentration, *e.g.*, 90 mV in the absence of Ca(II) and 55 mV at the 100 mM Ca(II) level. Addition of 100 mM Ca(II) decreased the diffusion current constant by 20 %.

The *a.c.* polarographic response of the NAD/NADH redox couple was relatively sensitive to Ca(II) addition (Tab. 4). At a Ca(II) : NAD<sup>+</sup> ratio of 1.2 : 1, the in-phase summit current,  $I_s$ , for the reduction of NAD had increased 35 %. In the presence of 100 mM Ca(II), the increase in  $I_s$  was slightly over 100 %,  $U_s$  shifted slightly positive, and the peak width,  $\Delta U_{s/2}$ , substantially decreased.

The most viable explanation for the changes brought about by the addition of Ca(II), is that the presence of Ca(II) increases the rate of charge transfer for the reduction of NAD. The following behavior is predicted<sup>18</sup> for an *a.c.* polarographic peak for an increase in  $k_{s,h}$  when the charge transfer is preceded by a reversible chemical reaction, *e.g.*,

Table 4 *a c* polarographic behavior of the  $\text{NAD}^+/\text{NADH}$  redox couple in presence of  $\text{Ca(II)}$  <sup>a</sup>

$\text{Ca(II)}$ (mM)	$U_s$ (V)	$U_{+s/2}^b - U_s$ (mV)	$U_s - U_{-s/2}^b$ (mV)	$\Delta U_{s/2}$ (mV)	$I_s$ (nA)
40 Hz					
0.00	1.690	68	60	128	56
0.25	1.685	65	67	132	59
0.49	1.682	57	58	115	75
1.00	1.690	62	57	119	78
50	1.680	57	43	100	122
100	1.674	50	38	88	110
100	1.671	51	40	91	132
100 Hz					
0.00	1.696	76	72	148	57
0.25	1.690	67	66	133	63
0.49	1.692	67	62	126	78
1.00	1.694	67	61	128	80
10.0	1.696	61	55	116	103
50	1.687	57	48	105	118
100	1.680				117
100	1.677				122

<sup>a</sup> All solutions are 0.42 mM  $\text{NAD}^+$  in 0.10 M Teap and 0.010 M Tris at pH 9.3

<sup>b</sup> Defined in footnotes *b* and *c* of Table 2

formation of  $\text{NAD}^+-\text{HX}$ , where HX denotes a proton donor, or of  $\text{NADH}^+$  as a preprotonation step:

- (a) The in-phase *a.c.* current should increase.
- (b) The summit potential should shift slightly positive.
- (c) The negative half-summit potential,  $U_{-s/2}$ , should shift substantially positive.
- (d) The positive half-summit potential,  $U_{+s/2}$ , should shift very little.

Examination of Table 4 shows that the addition of  $\text{Ca(II)}$  brought about the type of behavior just described. The increase in steepness of the *d.c.* polarographic wave (Tab. 3) on  $\text{Ca(II)}$  addition is also indicative of an increase in  $k_{s,h}$ .

Titration of Ca(II) with  $\text{NAD}^-$ 

Since the presence of Ca(II) modifies the electrochemical behavior of  $\text{NAD}^+$ , Ca(II) in a pH 7.2 0.01  $M$  Tris buffer was titrated with  $\text{NAD}^-$  to determine to what degree  $\text{NAD}^+$  would complex with Ca(II). The titration was monitored potentiometrically with a liquid-membrane ion-selective calcium electrode. Teap had to be excluded from the titration since the tetraethylammonium ion showed a significant interference with the Ca(II) electrode.

Table 5. Formation constant of  $\text{NAD}^-$ : Ca(II) complex in 0.01  $M$  Tris at pH 7.2

Total [Ca(II)] (mM)	Total [ $\text{NAD}^-$ ] (mM)	Uncomplexed [Ca(II)] <sup>a</sup> (mM)	[ $\text{NAD}^+$ Ca(II)] (mM)	$K_f^{b,c}$ ( $M^{-1}$ )
1.028	0.580	0.994	0.034	$63 \pm 19$
0.941	1.194	0.900	0.041	$40 \pm 10$
0.881	1.615	0.823	0.058	$45 \pm 8$
0.804	2.155	0.744	0.060	$38 \pm 6$
0.740	2.609	0.667	0.073	$43 \pm 5$
0.669	3.112	0.598	0.071	$39 \pm 4$
0.624	3.430	0.552	0.072	$39 \pm 4$
0.561	3.874	0.504	0.057	$30 \pm 3$
0.514	4.203	0.447	0.067	$36 \pm 3$
0.474	4.482	0.405	0.069	$39 \pm 3$
0.437	4.746	0.370	0.067	$39 \pm 3$

<sup>a</sup> The free Ca(II) concentration was determined potentiometrically

<sup>b</sup> The uncertainty in  $K_f$  reflects an uncertainty of  $\pm 0.2$  mV in the potentiometric determination of the free Ca(II) concentration

<sup>c</sup> Weighted mean based on the uncertainty in each  $K_f$  determination =  $38 M^{-1}$ ; standard deviation =  $\pm 4$

Since the formation constant of the Ca(II)- $\text{NAD}^+$  complex was not sufficiently large for the titration to show a clear cut end-point, it was necessary to calculate the formation constant,  $K_f$ , from the potentiometrically determined free Ca(II) concentration (Tab. 5). A value for  $K_f$  of  $38 \pm 4 M^{-1}$  (mean and standard deviation for 11 measurements) was determined for a 1:1 ratio Ca(II): $\text{NAD}^+$  complex.

Inasmuch as the formation constant is quite small, it seems unlikely that the complexation of  $\text{NAD}^+$  with Ca(II) is entirely responsible for

changes in the electrochemical behavior of the NAD<sup>•</sup>/NADH redox couple, unless the free radical NAD<sup>•</sup> reacts with Ca(II) exceedingly rapidly to form a Ca(II) : NAD<sup>•</sup> complex, which has a very large  $K_f$ .

#### Effect of Ca(II) adsorption on $k_{s,h}$

Since the changes in the wave II polarographic behavior are substantial even at low Ca(II) concentration, *e.g.*, 0.5 mM, it is unlikely that these changes are due in any large measure to an increase in ionic strength. However, adsorption of at least some Ca(II) at the electrode-solution interphase in the potential region where reduction of NAD<sup>•</sup> occurs, could be responsible for the increase in the heterogeneous rate constant for the NAD<sup>•</sup> reduction.

Ca(II) shows an *a.c.* polarographic peak at a potential only slightly negative of the reduction potential of the NAD<sup>•</sup>/NADH couple. The size of the peak is dependent on the Ca(II) concentration with the peak being sufficiently large at 50 and 100 mM Ca(II) that it begins to overlap the *a.c.* peak for the free radical reduction. Moreover, on cyclic voltammetry of 0.42 mM NAD<sup>+</sup> in 0.1 M Teap with a 0.01 Tris buffer at pH 9.3 and 50 mM Ca(II), the adsorption peak due to Ca(II) completely obscures the NAD<sup>•</sup> reduction peak.

The adsorption of a positively charged species such as Ca(II) at an electrode-solution interphase, which is at a potential negative to the potential of zero charge, will have the effect of reducing the apparent negative charge on the electrode. As a result, there will be less electrostatic repulsion between the electrode surface and ions of the same charge, *e.g.*, NAD<sup>•</sup>-HX which has a net charge of -2 due to the presence of two singly ionized phosphate groups, and the population of negative ions at the outer HELMHOLTZ plane will increase. With a decrease in electrostatic repulsion between NAD<sup>•</sup>-HX and the electrode surface, an increase in the rate of charge transfer is quite reasonable.

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