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An Electrochemical Study of the Kinetics of NADH Being Oxidized By Diimines Derived from Diaminobenzenes and Diaminopyrimidines

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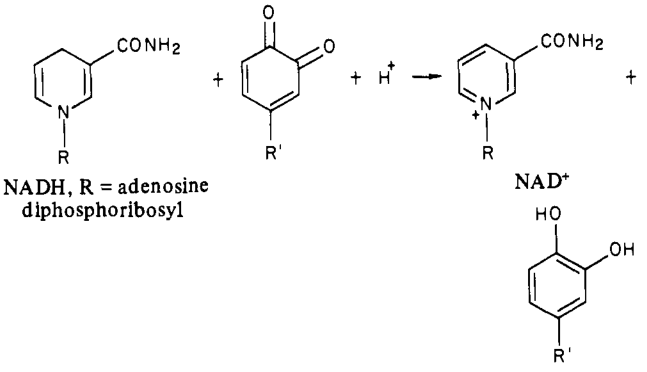
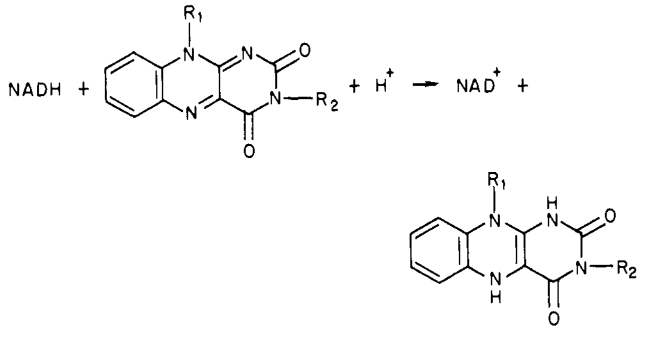
Abstract: On a vitreous carbon electrode, in aqueous, pH 7 solution, NADH is oxidized to NAD^+ at about 0.6 V vs. SCE. Using cyclic voltammetry we can show that electroactive aromatic diamines, like 1,4-diaminobenzene, catalyze this reaction, allowing it to be performed at potentials as low as 0.1 V. The catalysis mechanism involves a two-step process. The diamine is oxidized to a diimine at the electrode. The diimine or another related oxidant then, in turn, attacks NADH in the solution. Controlled potential electrolysis showed that NAD^+ is the product and $n = 2$. The effectiveness of fourteen diaminobenzenes and seven diaminopyrimidines as catalysts was evaluated. Rate constants for the homogeneous NADH oxidation step were measured for 11 compounds and k for the aromatic diamines is about 10^4 M^{-1} s^{-1}. There is no correlation between the two-electron E_0' values for diamines and k. 1,2-Diimines are somewhat faster NADH oxidants than 1,4-diimines, which are faster than 1,4-quinones. In contrast to the rapid rates for two-electron diamine/diimine catalysts, one-electron catalysts like *N*,*N*,*N'*,*N'*-tetramethyl-1,4-diaminobenzene react very slowly. It is proposed that the mechanism involves transfer of the 4-hydrogen of NADH to a protonated diimine. Unusual examples of mediation of the chemically irreversible NADH oxidation by mediators with chemically irreversible couples are also reported.

The importance of the coenzyme redox couple $NADH/NAD^+$ has engendered several studies of NADH oxidations in the absence of any enzyme. Although NAD^+ can be produced by many oxidants, flavins, certain quinones, and *N*-methylacridinium ion have been shown to be especially reactive with NADH or its analogues.[1-3] The pertinent reactions for two of these reagents are shown.





The present work explores a series of oxidants for NADH. Most of these can be considered to evolve from a flavin or quinone model. We were interested in identifying kinetically superior oxidants for NADH and in exploring structure/reactivity and thermodynamic/kinetic relationships. An electrochemical method was used here to measure rate constants.[4] Therefore, the information available from the literature on the electrochemical oxidation of NADH is of interest.[5,6] Consider, in particular, reaction on a glassy carbon electrode in pH 7. It has been found that NADH is converted to NAD^+ and $E_{1/2}$ is ca. 0.6 V vs. SCE. The formal potential for the $NADH/NAD^+$ couple at 25 °C and pH 7 is −0.56 V. The approximately 1.2 V difference between these values is due to the activation energy for the electrochemical process. The kinetics for NADH oxidation on vitreous carbon have recently been studied by Moiroux and Elving.[5] They proposed a mechanism in which NADH was converted to its cation radical, which lost a proton to solvent giving a radical. This was in turn oxidized to NAD^+. Interestingly, the initial electron transfer was not fast, but irreversible, even though the cation radical deprotonated only on a time scale of 0.01 s. It was, thus, in effect proposed that the large activation energy arose because of the slowness of the initial electron transfer. This mechanism is quite different from those proposed for most chemical oxidations of NADH.[1-3] In these reactions it is proposed and usually demonstrated that the 4-pyridinyl hydrogen is transferred to the oxidant, not to the solvent. The exact pathway for this overall hydride transfer is a matter of debate.[1-3]

Scheme I

$$NADH \rightarrow NADH^{+\cdot} + e^-$$
$$NADH^{+\cdot} \rightarrow NAD\cdot + H^+$$
$$NAD\cdot \rightarrow NAD^+ + e^-$$

For various reasons it would be desirable to lower the electrochemical activation energy and oxidize NADH at a lower potential. This problem has been approached using "mediators".[7,8] Thus, for example, Kuwana and co-workers[7] have oxidized dop-

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[1] Creighton, D. J.; Hadjic, J.; Sigman, D. S. "Bioorganic Chemistry"; Academic Press: New York, 1978; Vol. IV, Chapter 14.

[2] Kill, R. J.; Widdowson, D. A. "Bioorganic Chemistry"; Academic Press: New York, 1978; Vol. IV, Chapter 8.

[3] Recent references are cited in: (a) Ohno, A.; Shio, T.; Yamamoto, H.; Oka, S. *J. Am. Chem. Soc.* **1981**, *103*, 2045. (b) Colter, A. K.; Saito, G.; Sharon, F. J. *Can. J. Chem.* **1977**, *55*, 2741. (c) van Eikeren, P.; Grier, D. L.; Eliason, J. *J. Am. Chem. Soc.* **1979**, *101*, 7406.

[4] Preliminary communication: Kitani, A.; Miller, L. L. *J. Am. Chem. Soc.* **1981**, *103*, 3595.

[5] Moiroux, J.; Elving, P. J. *J. Am. Chem. Soc.* **1980**, *102*, 6533 and references therein to earlier studies of Elving and co-workers.

[6] Dryhurst, G. "Electrochemistry of Biological Molecules"; Academic Press: New York, 1977. Blaedel, W. J.; Jenkins, R. A. *Anal. Chem.* **1975**, *47*, 1337. Leduc, P.; Thevenot, D. *Bioelectrochem. Bioenerg.* **1974**, *1*, 96; *J. Electroanal. Chem.* **1973**, *47*, 543. Jaegfeldt, J.; *Ibid.* **1980**, *110*, 295. Hermolin, J.; Kirowa-Eisner, E.; Kosower, E. *J. Am. Chem. Soc.* **1981**, *103*, 1591.

[7] Tse, D. C.-S.; Kuwana, T. *Anal. Chem.* **1978**, *50*, 1315.

[8] Electroanalytical schemes involving NADH, NAD^+, enzymes, and redox reagents have also been discussed, see references in: Cheng, F. S.; Christian, G. D. *Anal. Chem.* **1977**, *49*, 1785.

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amine **1** to dopaquinone **2** at 300 mV and used the electrogenerated dopaquinone to oxidize the NADH, which is also present. Because the oxidation of dopamine at the carbon surface and the oxidation of NADH by quinone are both quite fast, the quinone catalyzes NADH electrooxidation. In electrochemical terms, the mediator allows one to obtain an equal or greater rate (current) for NADH oxidation at a less positive electrode potential.

The concept of mediation has been recently used to devise chemically modified electrodes for NADH oxidation. In particular, it proved possible to prepare an electrode with dopamine units firmly bound to the surface.[9] This electrode in an aqueous, pH 7 solution could be cycled between the hydroquinone and quinone forms at $E_0' = 180$ mV. When NADH was added to the solution it could be oxidized at this potential. We believe that more effective and more stable modified electrodes can be developed, and the data reported here will be used for such work.

The task of finding a good mediator is not trivial. In addition to finding water soluble and chemically reversible redox couples, one requires fast mediator redox kinetics at the electrode and a kinetically rapid, but thermodynamically not too exothermic, reaction with NADH. We perceive NADH oxidation as an example which belongs to a large class of redox reactions. These reactions have strong structural requirements for rapid rates (see below) and, therefore, often have slow electrochemical kinetics. Mediated reactions are in these cases of special importance. From a biochemical viewpoint the mediators described here interconnect "one-electron" (the electrode) and "two-electron" (NADH) redox systems. Quinone diimines are shown to be effective because they can engage in both "two-electron" or "one-plus-one-electron" redox processes. The importance of this problem is exemplified by recent studies of quinoid anticancer drugs which are proposed to act in vivo as catalysts for NADPH reduction of oxygen.[10]

Experimental Section

Reagents. β-NADH and NAD^+ were obtained from Sigma Chemical Co. The mediators were obtained from Sigma Chemical Co., Aldrich Chemical Co., or Eastman Kodak Co. All chemicals were stored according to directions and were used without further purification. Buffer solutions were pH 7.0 phosphate buffer (KH_2PO_4 + NaOH, 0.05 M) obtained from Fisher Scientific Co. All test solutions contained 0.1 M sodium perchlorate as supporting electrolyte and were prepared just prior to each experiment.

Compound **7c** was prepared. 4,5-Dimethyl-1,2-diaminobenzene (2.00 g, 14.6 mM), iodomethane (2.07 g, 14.6 mM), and sodium carbonate (4 g) were stirred and refluxed in 70 mL of acetonitrile for 3 h. The solid was filtered off and the products in solution were applied to a silica gel column eluted with a mixture of cyclohexane and ethyl acetate. 4,5 *N*-Trimethyl-1,2-diaminobenzene (1g, 43%) was isolated as a brown solid: mp 72–74 °C; NMR ($CDCl_3$) δ 6.45 (s, 1 H), 6.40 (s, 1 H), 3.10 (s, 3 H), 2.80 (s, 3 H), 2.20 (s, 6 H); mass spectrum 150 ($M^{+\cdot}$, 100%), 135, 108.

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. At higher sweep rates, voltammograms were recorded on the X–Y recorder in conjunction with a Physical Data Model 512A transient recorder. The working and counterelectrodes were vitreous carbon, 3.0-mm diameter disks sealed in a glass tube. The carbon electrodes were cleaned by abrasion with Magomet polishing compound (No. 40-6440 AB) on a polishing cloth, wiping with a Kimwipe, rinsing with water, and air drying. The reference electrode was a SCE (Coleman H 4750-1) and all potentials refer to this electrode. All measurements were made at 21 ± 1 °C, under a nitrogen atmosphere.

The rate constants for reaction between various diimines and NADH were determined by cyclic voltammetry. The concentration of mediator was generally 0.1 mM and the NADH concentration was 1.0 mM. The potential sweep rates were in the range from 10 to 500 $mV{\cdot}s^{-1}$. Rate constants were estimated with use of the working curve of Figure 3 from Andrieux et al.[11] This curve was derived from an assumed mechanism in which a one-electron mediator catalyzes a one-electron reduction with a rate-limiting electron transfer. Since a ratio of peak currents is measured, we assume that the two-electron oxidation of NADH catalyzed by a two-electron mediator can be treated identically to the one-electron case. The theory also assumes that all diffusion coefficients are equal. *D* for a diaminobenzene is about two times larger than *D* for NADH.[5]

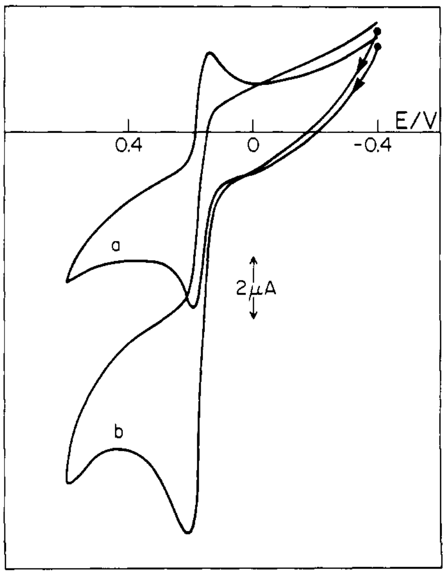

Figure 1. Cyclic voltammograms in pH 7.0 phosphate buffer, ν = 10 $mV{\cdot}s^{-1}$: (a) 0.19 mM 1,4-diaminobenzene, (b) 0.82 mM NADH added.

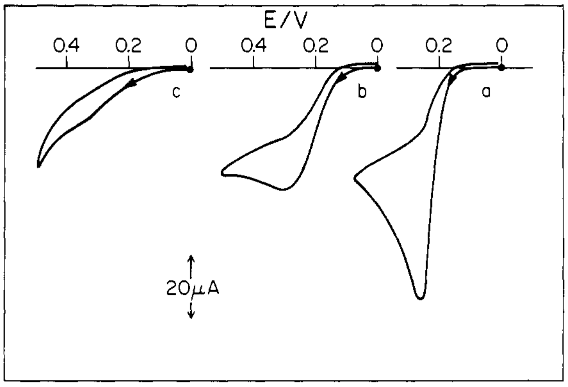

Figure 2. Cyclic voltammograms (first sweep) in the presence of NADH. Sweep rate: 0.1 $V{\cdot}s^{-1}$. Mediator concentration: 0.1 mM. NADH concentrations: 3 mM. (a) 4,5-Dimethyl-1,2-diaminobenzene; (b) *N,N*-dimethyl-1,4-diaminobenzene; (c) *N*-acetyldopamine.

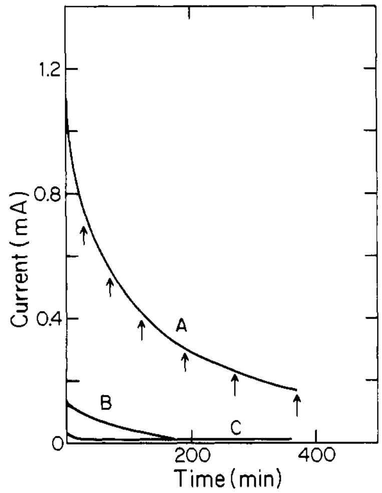

Figure 3. Current transients during controlled potential electrolysis at 0.26 V: (A) 0.15 mM 4,5-dimethyl-*o*-phenylenediamine plus 2.34 mM NADH, (B) 0.15 mM 4,5-dimethyl-*o*-phenylenediamine, (C) 1.62 mM NADH.

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[9] Degrand, C.; Miller, L. L. *J. Am. Chem. Soc.* **1980**, *102*, 5728.

[10] Bachur, N. R.; Gordon, S. L.; Gee, M. V. *Cancer Res.* **1978**, *38*, 1745.

[11] Andrieux, C. P.; Blocman, C.; Dumas-Bouchiat, J. M.; M'Halla, F.; Saveant, J. M. *J. Electroanal. Chem.* **1980**, *113*, 19. We thank Professor Saveant for supplying a preprint of this paper.

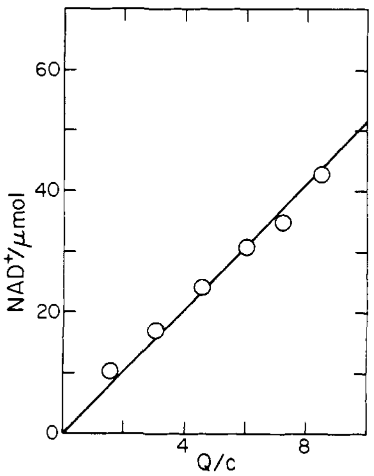


Figure 4. Plots of NAD$^+$ produced vs. quantity of electricity passed during catalytic oxidation of NADH. Straight line calculated assuming 100% current efficiency.

Because the rates are high the catalytic current in the presence of NADH will be "artifically" small due to its slower diffusion and the calculated rate constant will be smaller than the true value. This will, however, amount to less than a factor of 2 and we have not attempted a more accurate analysis.

It is further assumed that the presence of NADH does not affect the heterogeneous kinetics of the mediator couple. Because most diimine and quinone couples are quasireversible (ΔE_p typically 40–50 mV at 20 mV·s^{-1}, but unless otherwise noted $\Delta E_p > 60$ mV at 200 mV·s^{-1}) the kinetics of these couples may be especially sensitive to the presence of NADH. This could cause errors, but we see no evidence that this is the case. In particular, the voltammograms at sufficiently high ν or low NADH concentration are unperturbed by the presence of NADH. As expected from Figure 4 of Andrieux et al., all the E_p^a values for mediator plus NADH appeared very close to E_p^a for the mediator alone. This was even true when the mediator alone showed a rather large ΔE_p value.

Finally, it will be realized that the pH was not varied and the proton concentration is incorporated into k.

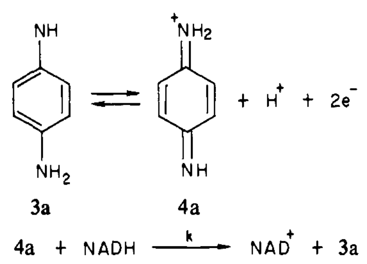
In controlled potential electrolysis, graphite rods (diameter = 6 mm, length = 30 mm) were used as working and counter electrodes. The anode and cathode chambers were separated by a sintered glass disk. The amount of electricity passed was measured by a PAR Model 179 digital coulometer. The amount of NAD$^+$, produced by mediated oxidation of NADH, was monitored by cyclic voltammetry, using a separate analytical electrode. The reduction peak of NAD$^+$, observed at −1.1 V vs. SCE, was recorded at various times during the electrolysis and the NAD$^+$ concentration was calculated from a calibration curve.

Results

In this study 25 mediators for NADH electrooxidation were surveyed, and rate constants were measured for the more active of these. Special attention was given to reactions of NADH with quinone diimines. The technique employed, cyclic voltammetry, has been used extensively to study mediated processes.[8,12] It allowed us to rapidly survey a number of compounds and to measure rate constants. An electrochemical approach was particularly useful because the oxidants of interest are rather unstable. It would be impractical, for example, to isolate *o*-quinone diimines and measure their NADH reaction rate in an independent experiment. The voltammetric approach has the disadvantage, however, that one does not use the pure oxidant to measure the rate.

With the use of cyclic voltammetry, it is a simple matter to detect mediation. Consider a specific, but representative, experiment. First, the voltammogram for a 0.78 mM NADH solution at pH 7.0 was recorded. A clean, vitreous carbon disk electrode was used. At a sweep rate (ν) of 10 mV·s^{-1} an anodic peak was observed, E_p^a = 600 mV vs. SCE, with peak current i_p^a = 4.8 μA. This is in accord with previous reports.[5,6] Separately, the cyclic voltammogram of the mediator, 1,4-diaminobenzene (**3a**) (0.19 mM) was recorded under the same conditions. The voltammogram (Figure 1) showed a quasireversible couple E_p^a = 190 mV, i_p^a = 3.6 μA (background corrected), E_p^c = 140 mV. This indicated that the diimine oxidation product was, as expected,[13] stable. Since ΔE_p is theoretically 30 mV for a 2 e$^-$, 2 H$^+$ oxidation, this reaction is considered quasireversible, i.e., the electron and proton transfers are rather rapid on the time scale of this experiment. This time scale is, of course, set by the sweep rate; at faster, ν, ΔE_p increased and at slower ν it was closer to the theoretical 30 mV. Finally, the voltammogram of a mixture of 0.19 mM **3a** and 0.81 mM NADH was recorded. As shown in Figure 1, there was a considerable enhancement of the anodic peak, i_p^a = 10.9 μA at E_p 200 mV, and the cathodic peak is virtually absent. The enhanced anodic current comes from the mediated oxidation of NADH to NAD$^+$, which regenerates **3a**. Under the conditions used here, this approach will detect mediation if the reaction rate constant between oxidized mediator and NADH is greater than 10^2 M^{-1}·s^{-1}.

Scheme II



Qualitative Mediation Results. The survey undertaken here extended over mediators with the general structures: hydroquinones/quinones, diaminobenzenes/quinone diimines, and corresponding diaminopyrimidine/diimine derivatives. Several one-electron couples were also tested. The initial experiments used 0.1 mM reduced mediator and 1.0 mM NADH at pH 7.0 and sweep rates between 10 and 500 mV·s^{-1}. The E_0' values are actually the average between the anodic peak potential, E_p^a, and the cathodic peak potential, E_p^c. The voltammograms which showed mediation always had E_p^a values within 20 mV of E_p^a for the mediator alone.

Initial studies with hydroquinone/quinone mediators confirmed the observations of Kuwana and Tse[7] and more fully demonstrated that *o*-quinones were more effective for NADH electrooxidation than were *p*-hydroquinones. Thus *p*-benzoquinone (E_0' = 150 mV) gave no observable mediation while 4-methyl-1,2-dihydroxybenzene (E_0' = 150), dopamine (E_0' = 180), and *N*-acetyldopamine (**5**) (E_0' = 200) and observable catalytic currents. Figure 2c shows the curve for *N*-acetyldopamine. This compound was of special interest because intramolecular cyclization of the quinone form[14] is blocked. This cyclization complicates the use of dopamine. The use of Dopa as mediator failed, perhaps because of this rapid cyclization.

A comparison of the results for *N*-acetyldopamine and 1,4-diaminobenzene suggested that other compounds like the latter should be studied. 2-Chloro-1,4-diaminobenzene (**3b**) and 2-methoxy-1,4-diaminobenzene (**3c**) showed electrochemistry similar to the parent, i.e., 2 e$^-$ quasireversible oxidations, ΔE_p = 40 mV at ν = 20 mV·s^{-1}, and provided effective NADH mediator systems. *N*,*N*-Dimethyl-1,4-diaminobenzene (**3d**) has a somewhat slower electrochemical rate (ΔE_p = 120 mV at 20 mV·s^{-1}), but it proved to be an extremely effective mediator for NADH (Figure 2b). *N*-Acetyl-1,4-diaminobenzene (**3e**) was briefly investigated. It showed E_0' = 360 mV and was a successful NADH mediator. ΔE_p was, however, 280 mV at 20 mV·s^{-1}, and so there was considerable doubt as to the nature of the oxidation product. Finally, the more complex 1,4-diamino-9,10-anthraquinone (E_0' = 490 mV, ΔE_p = 40 mV at 20 mV·s^{-1}) was shown to mediate NADH oxidation. All of these diamine compounds have a propensity

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(12) See papers cited in ref 11.

(13) Adams, R. N. "Electrochemistry at Solid Electrodes"; Marcel Dekker: New York, 1969.

(14) Brun, A.; Rosset, R. *J. Electroanal. Chem.* **1974**, *49*, 287.

Chart I

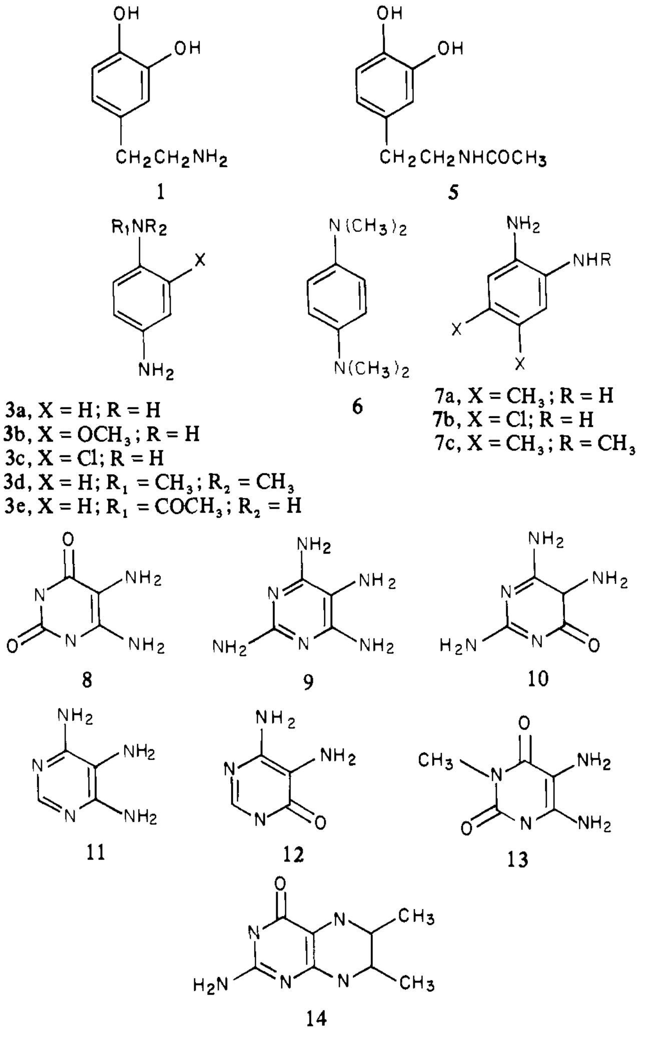

Table I. Peak Potentials and NADH Mediation for Pyrimidine Derivatives[a]

reduced mediator	$E_p{}^a$, mV	$E_p{}^c$, mV	NADH catalysis[b]
9	120	−20	+
10	90	−140	+
11	340	230[c]	+
12	270	100[c]	+
13	140	−180	−[d]
14	10	−160	−[d]

[a] Measured at pH 7.0 at 20 mV·s^{-1}. [Mediator] = 0.1 mM. [NADH] = 1 mM. Reference electrode SCE. [b] (+) and (−) indicate mediation or lack of it. [c] No cathodic peak at 0.02 V·s^{-1}. Measured at 500 mV·s^{-1}. [d] No catalysis was evident at 10 mV·s^{-1}.

to lose protons as well as electrons upon oxidation, producing imine functions. This is important for NADH catalysis as demonstrated by the lack of catalysis observed at the first peak for *N,N,N′,N′*-tetramethyl-1,4-diaminobenzene (**6**). This compound undergoes 1 e$^-$ oxidation (ΔE_p = 60 mV) and its cation radical is not effective in oxidizing NADH. Even though there is considerable thermodynamic driving force, the reaction is kinetically slow.

Based upon the differing results for *o*- and *p*-quinones, it was of considerable interest to survey mediation by *o*-diaminobenzenes. As expected from literature reports,[15] 1,2-diaminobenzene ($E_p{}^a$ = 400), 4-chloro-1,2-diaminobenzene ($E_p{}^a$ = 330), 3,4-diaminobenzoic acid ($E_p{}^a$ = 400), and *o*-aminophenol ($E_p{}^a$ = 220) all gave voltammograms at ν = 50 mV·s^{-1} with no cathodic peak on the return half-cycle. This results because the oxidation products, orthodiimines or quinone imines, are quite unstable. Since the oxidized mediators did not survive on the time scale of cyclic voltammetry it seemed unlikely that they could catalyze NADH oxidation. In contrast to this expectation, all of these compounds mediated NADH oxidation! Since the reactions of the oxidized mediators are complex and produce polymers, the mediation mechanism was not explored. It may, however, by speculated that the orthodiimines are trapped by NADH before they decompose.

It was reasoned that if the instability of *o*-quinone diimines resulted from attack on ring positions, this reaction could be blocked by substitution. Therefore, 4,5-dimethyl-1,2-diaminobenzene (**7a**) and 4,5-dichloro-1,2-diaminobenzene (**7b**) and *N*,4,5-trimethyl-1,2-diaminobenzene (**7c**) were tried as mediators. These compounds showed cyclic voltammograms with quasireversible couples suggestive of 2 e$^-$, 2 H$^+$ oxidation and in the presence of NADH they proved to be excellent catalysts (see Figure 2a), better than their para analogues.

The diaminobenzenes discussed above do not occur in nature so that these results have no direct significance for biology. Closer analogues to nature are aminopyrimidines. Pyrimidines are, of course, naturally occurring and flavins are important biological redox reagents with a diaminopyrimidine partial structure. Dryhurst and co-workers have elucidated some of the electrochemistry of pyrimidines and, in particular, have shown[16] that 5,6-diaminouracil (**8**) is oxidized to a diimine in a quasireversible 2 e$^-$, 2 H$^+$ reaction.[16] This diimine is hydrolyzed on a time scale of minutes at pH 6. We, therefore, expected to observe catalytic behavior analogous to that of the diaminobenzenes.

The cyclic voltammograms for tetrasubstituted pyrimidines **8**–**10** (Table I) showed quasireversible couples. The peak separations, ΔE_p, were quite large even at ν = 50 mV·s^{-1}, but since they increased as ν increased and there was no evidence for a different couple at fast sweep rates, we assume that these compounds are oxidized to diimines which are stable on this time scale and that the redox kinetics are slow. This has not, however, been unequivocally demonstrated. Trisubstituted pyrimidines gave no cathodic peak on the return half-cycle at 50 mV·s^{-1}. Small cathodic peaks were seen, however, at 500 mV·s^{-1}.

As shown in Table I, the polyaminopyrimidines **8**–**12** gave enhanced anodic peaks when NADH was added. In contrast, pterin (**14**) and 1,3-dimethyl-5,6-diaminouracil (**13**) were not successful mediators when tested at ν = 10 mV·s^{-1}. The trisubstituted compounds **11** and **12** were quite active, giving oxidation rates apparently comparable to that for *N,N*-dimethyl-1,4-diaminobenzene.

Controlled Potential Electrolysis. The formation of NAD$^+$ from NADH in a mediated reaction could be detected by cyclic voltammetry. The potential was cycled from 0.0 to +0.30 V and then back to −1.4 V. If both mediator, e.g., 4,5-dimethyl-1,2-diaminobenzene (**7a**), and NADH were present a cathodic peak was produced at −1.1 V. This potential was identical with the peak potential for NAD$^+$ reduction and the peak was absent if only the mediator was present.

In order to demonstrate that **7a** provided a stable mediator system and to also measure the coulometric yield for mediated NAD$^+$ production, controlled potential electrolyses were performed at 0.26 V. The current–time curve is shown in Figure 3 as curve A. Separate experiments using an equivalent concentration of **7a** gave curve B. The quantity of electricity necessary to completely oxidize **7a** (integral of curve B) was 2.1 F/mol. Another experiment (curve C) showed that in the absence of **7a** NADH was essentially electroinactive at this potential. Consider now curve A. The arrows attached to this curve indicate sampling points where the concentration of NAD$^+$ was measured by means of cyclic voltammetry. A plot of the amount of NAD$^+$ vs. the quantity of electricity passed is shown in Figure 4. The straight line was calculated assuming 100% current efficiency and the

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(15) Heineman, W. R.; Wieck, H. J.; Yacynych, A. M. *Anal. Chem.* **1980**, *52*, 345.

(16) Dryhurst, G.; Owens, J. L. *J. Electroanal. Chem.* **1977**, *80*, 171.

Table II. Kinetic Data for NADH Oxidation by 1,4-Diaminobenzene[a]

ν, $mV \cdot s^{-1}$	$i_{p,o}$[a,b], μA	i_p[a,c], μA	$k_N \times 10^{-3}$,[d] $M^{-1} \cdot s^{-1}$	$k_A \times 10^{-3}$,[e] $M^{-1} \cdot s^{-1}$
200	4.93	6.85	5.5	2.5
100	3.55	5.94	4.0	2.1
50	2.57	5.76	3.6	2.2
20	1.72	4.92	2.3	1.6

[a] Data obtained at pH 7.0, [NADH] = 1 mM, [3a] = 0.1 mM. Currents are the average of two measurements on one solution at each sweep rate. [b] Peak current from mediator alone, E_p = 190 mV. [c] Peak current from NADH plus mediator. E_p = 200 ± 20 mV in all cases. [d] Calculated according to Nicholson. [e] Calcu-according to Andrieux.

points are experimental. The current efficiency for NAD^+ production was 100% within the experimental error.

In this experiment the electrolysis was stopped at 7 h after the preparation of the sample solution in order to avoid the possible decomposition of NADH. At this stage, the conversion of NADH to NAD^+ was 70% and the turnover number for **7a** was 11.4, i.e., each molecule of **7a** had catalyzed 11 NADH oxidations. Since the number of electrons involved in the electrochemical oxidation of 4,5-dimethyl-1,4-diaminobenzene is two, the data are consistent with a process like Scheme II. A precisely similar controlled potential electrolysis and analysis for NAD^+ was performed using 2,4,5,6-tetraaminopyrimidine (**9**) as mediator for NADH oxidation. At 0.20 V it was shown that NAD^+ was produced in slightly greater than 100% yield assuming $n = 2$.

Kinetic Studies. Measurement of i_p^a for the mediator alone and in the presence of NADH, and assumption of a simple catalytic scheme, like Scheme II, allows calculation of a rate constant, k, for reaction of oxidized mediator with NADH in solution.[11] The validity of the rate constant can be checked by measuring these two i_p^a values at various sweep rates and concentrations. Raw data for one example, 1,4-diaminobenzene, are provided in Table II. Conversion of such data into an *accurate* rate constant is problematic. The rate constant for reaction of dopaquinone with NADH has previously been estimated ($k = 3.6 \pm 0.4 \times 10^4\ M^{-1} \cdot s^{-1}$ at 23 °C), using double potential step chonocoulometry and a kinetics model which assumes pseudo-first-order kinetics,[17] i.e., that the NADH concentration in the diffusion layer is not substantially depleted by reaction. Because the rates measured here were often fast, this assumption was not valid. The invalidity was evident because the calculated[18] k values varied with sweep rate. As shown in Table II for the **3a** with NADH reaction, slow sweep rates gave low values of k. Therefore, a more sophisticated kinetic treatment due to Andrieux et al.[11] was used. Properly applied this treatment can account for depletion of the NADH and more satisfactorily constant k values were, indeed, obtained. The comparison for one example is shown in Table II. Rate constants for 11 compounds are shown in Table III. As enucleated in the Experimental Section there are several assumptions made in calculating these values, so that there is still some doubt about their accuracy. There is, however, good reason to believe that the *relative* rate constants determined by this method are sufficiently accurate to merit detailed comparison. A few k values were also measured by chronopotentiometry. The only kinetic theory available assumes pseudo-first-order kinetics[19] and we were not surprised to find k values which increased with the value of the current at which the potential–time transient was measured. It is of at least historical interest, however, to compare a few values obtained by chronopotentiometry with those from cyclic voltammetry. See the last column of Table III.

The data demonstrate that diimines provide very rapid NADH oxidation rates, $k \approx 10^4\ M^{-1} \cdot s^{-1}$. 1,2-Diimines react faster than 1,4-diimines, which in turn react more rapidly than 1,4-quinones. It will be noted in Table III that k for several compounds, particularly the more reactive ones, drifts to higher values with increasing sweep rate. We were especially concerned with the rapid oxidant **7a**. Further experiments showed that when the ratio NADH/**7a** was varied from 2.0 to 40, $k = (4.6 \pm 1) \times 10^4\ M^{-1} \cdot s^{-1}$ with no apparent drift ($\nu = 200\ mV \cdot s^{-1}$). This showed that the reaction was first order in NADH, but it did not explain the change in k as ν varies. This will be investigated more thoroughly, Until proven otherwise, it should be assumed that **7a** reacts by a mechanism which is not compatible with the assumptions inherent in the model of Andrieux et al.

Discussion

In this work a large number of diaminobenzenes and other potential mediators of NADH oxidation have been surveyed. It has been shown that diimines are formed from diamines in quasireversible electrode reactions and that these redox couples can effectively catalyze the electrooxidation of NADH. The catalysis mechanism involves formation of an oxidized species from the diamine at the electrode followed by a homogeneous oxidation of NADH, regenerating the diamine, e.g., Scheme II. The homogeneous rate constants of $\sim 10^4\ M^{-1} \cdot s^{-1}$ can be compared with the $k = 10^2\ M^{-1} \cdot s^{-1}$ for N-methylacridinium oxidation,[1] which has been considered to be quite fast.

In addition to the many examples in the present work which appear as "classical" electrochemical mediation processes, it is important to note that several cases involving apparent mediation by electrochemically irreversible (slow) or chemically irreversible (unstable product) mediators have been revealed.[20] The latter are especially unexpected and deserve careful study.

A more careful consideration of the diamine mediation mechanism can be made by comparing the qualitative and quantitative relative rates and the corresponding values of E_0'. It is expected that thermodynamically powerful oxidants (more positive E_0') will also be kinetically superior. The data demonstrate that there is no correlation of k and the two-electron E_0' values of mediator couples. Instead, there is an important dependence of k on the structure of the mediator. Consider the following comparisons.

1. Although 1,4-diaminobenzene (E_0' = 160 mV) provides an excellent mediator, p-hydroquinone (E_0' = 150 mV) and p-aminophenol (E_0' = 110 mV) are ineffective on this time scale.
2. The simply substituted 1,4-diaminobenzenes **3a**, **3b**, and **3c** have E_0' values which differ by 110 mV (2.4 kcal mol^{-1}) and yet k varies by less than 15%.
3. 1,2-Diaminobenzenes are faster than 1,4-diaminobenzenes. Compare specifically N,4,5-trimethyl-1,2-diaminobenzene (**7c**) (E_0' = 120 mV, $k = 59 \times 10^3$) or tetraaminopyrimidine (**9**) (E_0' = 50 mV, $k = 6.1 \times 10^3$) with **3a** (E_0' = 160 mV, $k = 2.1 \times 10^3$) in which thermodynamically weaker, two-electron oxidants react more, not less, rapidly.

Because of the strong structural requirements for fast rates it was of interest to attempt mediation using one-electron couples which were unlikely to be involved in any proton, hydrogen atom, or hydride transfers. Both N,N,N',N'-tetramethyl-1,4-diaminobenzene (**6**) (E_0' = 60 mV) and hydroxymethylferrocene (E_0' = 230 mV) gave well-behaved, reversible, one-electron couples (ΔE_p = 60 mV). Neither compound showed evidence for mediation of NADH oxidation, even at $\nu = 10\ mV \cdot s^{-1}$.[22]

The results suggest that NADH is only slowly oxidized by one-electron-transfer, proton-loss-to-solvent mechanisms, e.g., Scheme I. It is proposed that diimines rapidly react with NADH by transfer of the 4-hydrogen to the oxidant. Although this transfer may occur in stepwise fashion the net result is a hydride

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(20) The catalysis of NADH oxidation at the chemically irreversible second wave for N,N,N',N'-tetramethyl-1,4-diaminobenzene has been described.[4]

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(22) The one-electron E_0' values for $3 \rightleftarrows 15$ are of obvious interest, but to our knowledge these have not been measured. These values would not necessarily correlate with the two-electron E_0' values and could correlate with k.

Table III. Rates of NADH Oxidation

reduced mediator	E_0', mV	$k_A \times 10^{-3}$,[a] $M^{-1} \cdot s^{-1}$ 500 $mV \cdot s^{-1}$	200 $mV \cdot s^{-1}$	100 $mV \cdot s^{-1}$	50 $mV \cdot s^{-1}$	20 $mV \cdot s^{-1}$	$k_{av} \times 10^{-3}$ [b]
1	180	16	18	13			16
3a	160		2.5	2.1	2.2	1.6	2.1 (1.6)
3b	110		1.8	1.9	1.6	1.5	1.7
3c	220		1.9	1.9	1.6	1.4	1.7
3d	190		47	36	35	27	36 (17)
5	200			8.8	6.9	7.0	7.6
7a	210	76	42	30			49 (49)
7b	330	65	51	68			61
7c	120		63	58	47	67	59 (27)
9	50		7.3	6.2	4.7		6.1 (6.2)
10	−25		1.0	0.9	1.1		1.0

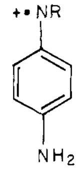
[a] Determined by cyclic voltammetry, using the Andrieux, et al. method. [b] Average *k*. Values in parentheses determined by chronopotentiometry.

transfer. If this is correct then the kinetic analysis used is a meaningful one. In agreement with this hypothesis is the high overpotential of the direct electrochemical oxidation. Indeed, because there is an alternative mechanism available to these diamine mediators, very large rate enhancements can be observed. Peak catalytic currents nearly equal to the i_p^a for direct NADH oxidation can be obtained at potentials 500 mV less positive. It is emphasized that the catalytic efficiency of two-electron oxidants is not governed by the Marcus–Hush considerations which can control catalysis by one-electron, outer-sphere mediators.[21]

Catalysis of a two-electron oxidation by a two-electron mediator can in principle occur by a variety of "one electron at a time" pathways, and the available data do not rule all of these out. The data do, however, strongly suggest that the mediator plays a chemical role greater than just transferring electrons and show that a simple one-electron mediator, e.g., a ferrocene, will have to have its $E_0' > 250$ mV to be effective.[22] Following this line of reasoning it is suggested that protonated diimines, e.g., **4a**, not the corresponding neutrals are the species which attack NADH. Although the pK_a's of **4a** and its analogues are not known,[23] they should be better chemical oxidants than the neutral diimines. In this regard we draw attention to the fact that metal hydride reductions of imines require very mild reducing agents, but acidic solutions. This remote analogy might allow one to understand why diimines are faster NADH oxidants than quinones. In further agreement with the hypothesis that protonated diimines are attacking NADH is the relatively rapid rate for mediation using *N*,*N*-dimethyl-1,4-diaminobenzene (**3d**). This compound cannot form a neutral diimine.

It is, of course, commonly established that most chemical NADH oxidations proceed by hydride transfer or its two-step (e^-, H) or three-step (e^-, H^+, e^-) equivalents. All three mechanisms involve transfer of the 4-hydrogen to the oxidant, as proposed here for diimines. Specific analogues are the oxidations of NADH model compounds by quinones and flavins.[1,2]

The diamine cation radical, e.g., **15**, must be considered as a reasonable alternative as the oxidant which actually attacks NADH. This species should be a good one-electron oxidant and



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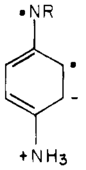

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if its one-electron E_0' is sufficiently large it might rapidly attack NADH. This attack on NADH would, however, have to be more rapid than deprotonation and reoxidation of **15** to produce the diimine. Interestingly, NADH could not react by transfer of its 4-hydrogen to the nitrogen of **15**, because the resulting neutral species **16** would be very unstable.

The structural differences between protonated 1,2-diimines and protonated 1,4-diimines could be used in several ways to account for the difference in rates. In particular, there are some special hydrogen-bonding possibilities available to protonated 1,2-diimines. These cations should be weak acids because of internal hydrogen bonding. They could also form transition states with NADH which have special stability due to interspecies hydrogen bonding. Even these speculations must, however, be cautiously considered in light of the inconstancy of *k* for mediation by **7a**.

We believe these rapid catalytic reactions may have utility for synthesis[24] or analysis. An analytical application has been suggested[4] and we have shown that NADH can be differentiated from ascorbate, using **3d**.

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