POLAROGRAPHIC, POTENTIOMETRIC AND KINETIC STUDIES OF NAD MODEL COMPOUNDS

by

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We accept this thesis as conforming to the required standard

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ABSTRACT

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The effects of varying the 1- and 3-substituents of NAD$^+$ (I) model compounds on reactions (1) through (4) have
been investigated. The one-electron reduction in reaction (1) was observed polarographically. A reaction constant $p^*$ of +3.7 was found for the effect of 1-substituents and a reaction constant $p$ of +11 to +12 was found for the effect of 3-substituents. The potential of the two-electron reduction of pyridinium ions in reaction (2) was measured potentiometrically. Reaction constants of +2.8 and +9 to +11 were found for the effects of 1- and 3-substituents, respectively. Reaction constants of -1.9 and -6 were found for the effects of 1- and 3-substituents on the rates of oxidation of
1,4-dihydropyridines by flavins, reaction (3). A similar reaction constant of -2.0 to -2.6 was found for the effect of 1-substituents on the acid decomposition of 1,4-dihydropyridines, reaction (4). These results suggest that the mechanism of oxidation of 1,4-dihydropyridines by flavins may be more complex than the commonly accepted mechanism of hydride transfer.

Reactions (1) through (4) were also investigated using NAD\(^+\), nicotinamide mononucleotide (NMN) and two 1,1'-alkylenebis(3-carbamoylpyridinium) compounds (II). The oxidation-reduction properties of these four compounds can be explained by considering the inductive effects of the 1-substituents.
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Dedicated to Vikki
1. INTRODUCTION

Nicotinamide-adenine dinucleotide (Ia, NAD) and nicotinamide-adenine dinucleotide phosphate (Ib, NADP) are coenzymes involved in oxidation-reduction reactions within living systems.

\[
(a): \ R=H \quad (\text{NAD}^+) \\
(b): \ R=\text{PO}_3^- \quad (\text{NADP}^+)
\]

The coenzymes are also referred to by several other names. Throughout this work, the nomenclature and abbreviations proposed by the Commission on Enzymes of the International Union of Biochemistry will be used. The pyridine ring was identified by Warburg, et al. as the reactive portion of the molecule and has led to the general term pyridine nucleotides being used.
for these compounds and for the mononucleotide (II, NMN). In the oxidized state, abbreviated NAD\(^+\), the pyridine ring exists as the pyridinium salt (III). Reduction of the coenzymes involves the addition of two electrons and one proton to form a dihydropyridine. It was originally thought that the reduced form of NAD, abbreviated NADH, was a 1,2- or a 1,6-dihydropyridine. It has since been shown that enzymatic reduction of NAD\(^+\) yields only the 1,4-dihydropyridine (IV).\(^3,4\) The chemical and biological properties of NAD\(^+\), NADH and model compounds have been reviewed.\(^1,4,5,6,10\) Only material which is pertinent to this study will be reviewed here.
1.1 Acid Decomposition of 1,4-Dihydropyridines:

The characteristic ultra-violet absorption of 1,4-dihydropyridine centered near 340 nanometers is rapidly lost in the presence of acid and replaced by a peak at 290 nanometers. If left in acid, this compound which has been called "primary acid modification compound" is further altered with a corresponding decrease in the absorption at 290 nanometers. The suggested mechanism involves protonation of V at C-5 to give VI, followed by a nucleophilic attack at C-6 of the pyridine ring. In aqueous solutions, the nucleophile would generally be a water molecule, with subsequent deprotonation yielding the 6-hydroxy-1,4,5,6-tetrahydropyridine (VII), which is the "primary acid modification compound". The reaction is subject to general acid catalysis. In aqueous media, the protonation step is rate-determining. It is this step which causes the shift in the ultra-violet absorption maximum to 290 nanometers. The further reaction of the "primary acid modification compound" (VII), which is much slower, is suspected
to involve protonation at C-3 with subsequent nucleophilic attack at C-2.
1.2 Nucleophilic Addition to Pyridinium Salts:

The electron deficiency of the pyridinium ring makes it susceptible to attack by nucleophiles. Sund\textsuperscript{1} lists several anions which have formed addition compounds with pyridinium salts (VIII). Most of these form 1,4-dihydropyridines (IXa), but hydroxide and sulfoxylate ions apparently form 1,2- (IXb) or 1,6-dihydropyridines.\textsuperscript{1}

\textbf{[Diagram of structures]}

The addition of cyanide has received considerable attention. The addition to NAD\textsuperscript{+}, which is rapid and quantitative in 1 M. cyanide,\textsuperscript{13} gives a product with an absorption band near 325 nanometers. This reaction has been used as an assay for NAD\textsuperscript{+}\textsuperscript{13,14} since it allows a simple spectrophotometric determination to be made. The direct spectrophotometric determination of NAD\textsuperscript{+} is impossible if other aromatic compounds are present. Both rate and equilibrium constants have been determined for the addition of cyanide ion to a number of pyridinium salts\textsuperscript{15,16,58} (see also section 1.6, Substituent Effects). It has been suggested that the equilibrium constants for cyanide addition measures the ease of reduction, and therefore the redox potentials, of pyridinium salts.\textsuperscript{1,17,58}
1.3 Oxidation of 1,4-Dihydropyridines by Flavins:

A variety of compounds are capable of oxidizing NADH and dihydropyridine models to the corresponding pyridinium salts. One biologically important group with this capability are the flavins (X), riboflavin (Xa) and riboflavin adenosine pyrophosphate(Xc). The coenzymes are also referred to as flavin mononucleotide (FMN) for (Xb) and flavin adenine dinucleotide (FAD) for (Xc).

Within mitochondria, much of the NADH is oxidized by a flavoenzyme, NADH dehydrogenase. The electrons are transferred from the flavin through the various enzymes of the respiratory chain (also referred to as the electron transfer chain) to ultimately reduce oxygen. During this process, three molecules of high energy compound adenosine triphosphate are formed.

The importance of flavoenzyme oxidations of NADH has spawned considerable interest in the mechanism of the reaction between riboflavin and 1,4-dihydropyridines. Singer and Kearney found that riboflavin readily catalyzed the oxidation of NADH by oxygen or cytochromes (heme molecules which are part of the respiratory chain). The reaction has since been studied both aerobically and anaerobically. The mechanism which has been suggested is shown in scheme 1 using FMN as the oxidant. A direct hydrogen transfer occurs from the 4-carbon of the NADH pyridine ring to the 5-position of a 5-deaza-flavin. The hydrogen transfer step is preceded by the formation of a flavin-NADH complex.
for which, by analogy with some other flavin reactions, a covalent intermediate has been proposed.\textsuperscript{62,63} Other evidence

\[
\begin{align*}
\text{(a)} & \quad \text{(b)} & \quad \text{(c)} \\
R = & \quad \text{CHOH} & \quad \text{CHOH} & \quad \text{CHOH} \\
& \quad \text{CHOH} & \quad \text{CHOH} & \quad \text{CHOH} \\
& \quad \text{CHOH} & \quad \text{CHOH} & \quad \text{CHOH} \\
& \quad \text{CHOH} & \quad \text{CHOH} & \quad \text{CHOH} \\
\end{align*}
\]

has been presented in favour of a charge transfer complex\textsuperscript{25,26,64} as has also been proposed in the oxidation of NADH models by trifluoroacetophenone.\textsuperscript{65} A possible covalent intermediate is XI, which could decompose to products by electron and proton migrations as shown in scheme 2a.

\[
\begin{align*}
\text{H}^+ + \text{NADH} + \text{FMN} & \rightleftharpoons \text{NAD}^+ + \text{FMNH}_2 \\
\text{FMNH}_2 + \text{FMN} & \rightleftharpoons (\text{FMNH}_2 - \text{FMN}) \rightleftharpoons 2\text{FMNH} \\
\end{align*}
\]

scheme 1.
In a charge transfer complex the hydrogen could be transferred as either a hydride (one step mechanism) or a hydrogen atom (two step mechanism) with intermediate free-radical formation (scheme 2). Flavin radicals are formed during the reaction but these are believed to be formed in secondary reactions rather than in the initial oxidation-reduction step. No evidence has been found for the formation of NAD radicals during the reaction. The protonated pyridine radical (XIV) has been observed in electrochemical oxidations of NADH models. It is known to undergo rapid deprotonation to the radical (XIII) or rapid disproportionation to the pyridinium (III) and dihydropyridine (IV) compounds. The rate of oxidation of the protonated radical (XIV) by riboflavin is unknown, but it is unlikely that the hydrogen-atom transfer step would be rate determining in pathway (d). Since deuterium isotope effects indicate the involvement of hydrogen bond breaking and formation in the transition state, pathway (d) is unlikely to be operative. The rate constant for the oxidation of the neutral NAD radical (XIII) by riboflavin has been estimated as between $4 \times 10^9$ and $5 \times 10^9$ M$^{-1}$·sec$^{-1}$. It is unlikely that the oxidation of (XIII) by the flavin radical would be very different from this rate. The overall oxidation-reduction reaction is ten orders of magnitude slower than this radical oxidation. Such an enormous difference in these two rates would seem to leave no practical difference between pathways (b) and (c) in scheme 2. McCormick et al., have measured the rates of
Scheme 2
oxidation of NADH by a series of riboflavin analogues. They concluded that, in the transition state, a negatively charged species, probably a hydride ion, is approaching the flavin, but their results may also be explicable by the development of a negative charge at N-5 of the flavin molecule as in pathway (a).
1.4 Oxidation-Reduction Potentials of Pyridinium-Dihydropyridine Systems:

The redox potential of the NAD$^+$/NADH system, represented by equation 1, has been determined potentiometrically and by equilibration with systems of known redox potential. The measured cell potential is related to the concentration of oxidized and reduced species by the Nernst equation (equation 2) where $E$ is the measured cell potential, $E^\circ$ is the standard cell potential, $R$ is the gas constant, $T$ is the absolute temperature, $n$ is the number of electrons transferred, $F$ is the Faraday, and $a_{\text{red}}$ and $a_{\text{ox}}$ are the activities of the reduced and oxidized species respectively. For convenience, in dilute solutions, the activities can be replaced by concentrations. For the NAD$^+$/NADH system the cell potential is given by equation 3. At any given pH, the midpoint potential,

$$E = E^\circ - \frac{RT}{nF} \ln \frac{[\text{NADH}]}{[\text{NAD}]} - \frac{2.303RT}{2F} \text{ (pH)}$$

the potential at which the concentration of NADH equals the concentration of NAD$^+$, is given by equation 4. The reduction
potentials of biologically important compounds are often quoted at pH 7, a pH which is much more meaningful physiologically than is pH 0. This potential can be calculated from the midpoint potential at any other pH by equation 5.

$$E^\circ_{7} = E^\circ - \frac{2.303RT}{4F}(pH-7)$$  \((5)\)

The cell potential of the NAD\(^+\)/NADH system is not directly measurable. Rodkey\(^14,32\) has utilized a mediator system to measure these potentials. The NAD\(^+\)/NADH system is equilibrated with an electromotively active indicator such as benzyl viologen. After equilibrium has been reached, the potential of the indicator can be measured with a platinum electrode. Since equilibrium has been attained, the measured potential corresponds to the potential of the NAD\(^+\)/NADH system. Rodkey has determined the value of -318 millivolts for \(E^\circ_7\) at 30\(^\circ\).\(^14\) After determining a temperature coefficient of 1.31 millivolts per degree, he calculated a value of -311 millivolts for \(E^\circ_7\) at 25\(^\circ\).\(^32\) The reduction potential (\(E^\circ_7\)) of the NADP\(^+\)/NADPH system has been determined in a similar manner as -316 millivolts at 30\(^\circ\).\(^31\)

By equilibrating the unknown redox system with a known one, the unknown standard reduction potential can be
found from the overall equilibrium constant. For the reaction:

\[ \text{OX}_A + \text{RED}_B \xrightarrow{\text{RED}_A + \text{OX}_B} \]

the equilibrium constant in dilute solutions is given by equation 6.

\[ K = \frac{[\text{RED}_A][\text{OX}_B]}{[\text{OX}_A][\text{RED}_B]} \]  

(6)

\( K \) is related to \( E^\circ \) through the Nernst equation with \( E^\circ_{\text{cell}} = 0 \).

\[ E^\circ_{\text{cell}} = \frac{RT}{nF} \ln K \]  

(7)

\( E^\circ_{\text{cell}} \) is related to the standard reduction potentials of \( A \) and \( B \) by equation 8.

\[ E^\circ_{\text{cell}} = E^\circ_A - E^\circ_B \]  

(8)

Combining (7) and (8) allows one to determine \( E^\circ_A \) from knowledge of the equilibrium constant and \( E^\circ_B \).

\[ E^\circ_A = E^\circ_B + \frac{RT}{nF} \ln K \]  

(9)

The main difficulty with this approach for the determination of \( E^\circ \) for the \( \text{NAD}^+ / \text{NADH} \) system is the knowledge of
$E^\circ$ for reference systems which can be equilibrated with NAD. Clark\textsuperscript{30} discusses the problems related to this and calculates values for $E^\circ_7$ for the NAD$^+/\text{NADH}$ system spanning a potential range from -314 to -322 millivolts. Of these, Clark suggests the value of -315 millivolts as the most reliable. The NADP$^+/\text{NADPH}$ system has been equilibrated with the NAD$^+/\text{NADH}$ system.\textsuperscript{30} The reduction potential was estimated to be about 5 millivolts more negative than the potential for the NAD$^+/\text{NADH}$ system. Potentiometric measurements on the NADP system gave the same value for $E^\circ_7$ as was found for the NAD system.\textsuperscript{31}

Little attention has been given to the redox potentials of model systems. Karrer and coworkers\textsuperscript{33,34} have measured the reduction potential of 1-methyl-, 1-ethyl-, 1-propyl-, and 1-butylnicotinamides potentiometrically without the use of a potential mediator. Clark\textsuperscript{30} has used their data to calculate the values of $E^\circ_7$ given in Table 1. These values are suspect since no mediators were used and the potentials were reportedly unstable. Leach, Baxendale, and Evans\textsuperscript{35} determined the reduction potential of 1-methylnicotinamide using benzyl viologen as a mediator. They prepared the dihydropyridine electrochemically, a method which produces pyridine diners as well as dihydropyridine.\textsuperscript{35,5} (See also section 1.5, Electrochemistry.) The dimer is known to react rapidly with benzyl viologen,\textsuperscript{1} whereas NADH, a 1,4-dihydropyridine, reacts very slowly.\textsuperscript{1,14} It is uncertain whether they were actually measuring the reduction potential of the pyridinium/dihydropyridine system or the pyridinium/dimer
TABLE I

Redox Potentials of NAD Models

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₃</th>
<th>E°' (mv.)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃</td>
<td>CONH₂</td>
<td>-419, -417</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-290</td>
<td>35</td>
</tr>
<tr>
<td>C₂H₅</td>
<td>CONH₂</td>
<td>-427</td>
<td>30</td>
</tr>
<tr>
<td>C₃H₇</td>
<td>CONH₂</td>
<td>-430</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-387</td>
<td>58</td>
</tr>
<tr>
<td>C₄H₉</td>
<td>CONH₂</td>
<td>-412</td>
<td>30</td>
</tr>
<tr>
<td>α-TAG</td>
<td>CONH₂</td>
<td>-267</td>
<td>59</td>
</tr>
<tr>
<td>β-TAG</td>
<td>CONH₂</td>
<td>-267</td>
<td>58</td>
</tr>
<tr>
<td>CH₂OCH₂C₆H₅</td>
<td>CONH₂</td>
<td>-300</td>
<td>58</td>
</tr>
<tr>
<td>CH₂CH₂OC₆H₅</td>
<td>CONH₂</td>
<td>-361</td>
<td>58</td>
</tr>
<tr>
<td>CH₂C₆H₅</td>
<td>CONH₂</td>
<td>-361</td>
<td>58</td>
</tr>
<tr>
<td>CH₂C₆H₃Cl₂</td>
<td>CONH₂</td>
<td>-349</td>
<td>58</td>
</tr>
<tr>
<td>CH₂C₆H₃Cl₂</td>
<td>COCH₃</td>
<td>-287</td>
<td>58</td>
</tr>
<tr>
<td>CH₂C₆H₃Cl₂</td>
<td>COOC₂H₅</td>
<td>-354</td>
<td>58</td>
</tr>
<tr>
<td>CH₂C₆H₃Cl₂</td>
<td>CON(CH₃)₂</td>
<td>-391</td>
<td>58</td>
</tr>
<tr>
<td>CH₂C₆H₃Cl₂</td>
<td>COO⁻</td>
<td>-404</td>
<td>58</td>
</tr>
</tbody>
</table>

TAG = \[
\begin{array}{c}
\text{AcO} \\
\text{OAc} \\
\text{AcO} \\
\text{OAc}
\end{array}
\]

AcO
Table I (cont.)

<table>
<thead>
<tr>
<th>$R_1$</th>
<th>$R_3$</th>
<th>$E^0'$ (mv.)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPPRA</td>
<td>COCH$_3$</td>
<td>-257</td>
<td>58</td>
</tr>
<tr>
<td>RPPRA</td>
<td>CONHOH</td>
<td>-315</td>
<td>17</td>
</tr>
<tr>
<td>RPPRA</td>
<td>CONNH$_2$</td>
<td>-339</td>
<td>17</td>
</tr>
<tr>
<td>RPPRA</td>
<td>CHNOH</td>
<td>-342</td>
<td>17</td>
</tr>
<tr>
<td>RPPRA</td>
<td>COC$_6$H$_5$</td>
<td>-242</td>
<td>17</td>
</tr>
<tr>
<td>RPPRA</td>
<td>COCH(CH$_3$)$_2$</td>
<td>-243</td>
<td>17</td>
</tr>
<tr>
<td>RPPRA</td>
<td>CSNH$_2$</td>
<td>-280</td>
<td>17</td>
</tr>
<tr>
<td>RPPRA</td>
<td>CHCHCONH$_2$</td>
<td>-342</td>
<td>17</td>
</tr>
</tbody>
</table>

RPPRA = \[ \text{Diagram of RPPRA molecule} \]
system. The potentials of a group of NAD analogues, containing various 3-substitutents, have been determined by Anderson and Kaplan\textsuperscript{17} by enzymatic equilibration with the NAD system. These authors used as their reference the value of -320 millivolts determined by Burton and Wilson\textsuperscript{36} for $E^\circ$ of NAD. The values listed in Table 1 were calculated using the value of -315 millivolts suggested by Clark\textsuperscript{30} for the NAD$^+$/NADH system.

Wallenfels and co-workers\textsuperscript{58} have calculated the reduction potentials of several NAD model compounds from the equilibrium constants for cyanide addition by assuming that the ratio of these equilibrium constants for two pyridinium salts will be the same as the ratio of the reduction equilibrium constants. They used a reduction potential of -315 millivolts for NAD as an anchor for the series.
1.5 Electrochemical Reduction of Pyridinium Salts:

An example of a current-potential curve obtained from polarographic reduction at a dropping mercury electrode is shown in Figure 1. Along the portion of the curve from A to B, the applied potential is too positive for reduction of the electroactive species to occur and only the baseline current is observed. As the potential becomes more negative, the electroactive species is reduced causing the increase in current between B and C in Figure 1. At potentials more negative than point C, the electroactive species is being reduced as rapidly as it diffuses to the electrode surface. Thus, the plateau current is known as the diffusion current, \( i_d \).

For most electrode processes, whether oxidations or reductions, the current-potential curve can be described by equation 10, where \( E \), \( E_{1/2} \), \( i \), and \( i_d \) are described in Figure 1, \( R \) is the universal gas constant, \( T \) is the temperature, \( F \) is the Faraday, \( n \) is the number of electrons transferred in the electrode process, and \( \alpha \) is a number between 0 and 1 called the transfer coefficient. \( E^o \) is simply the potential at which the right hand term of equation (10), the log-current function, equals zero and is defined in different ways for different reduction mechanisms. If the electrode process is reversible, i.e. if both the forward and backward
Figure 1. A sample polarogram.
electron transfers are sufficiently rapid that the equilibrium concentrations of oxidized and reduced species are attained at the electrode surface, $\alpha$ has a value of 1. At 25°C, a plot of $E$ versus log $(i_d - i)/(i)$ will be linear with a slope of 59/n millivolts. The half-wave potential is related to the standard reduction potential, $E^\circ$, of the electroactive species by equation 12 where $D_{ox}$ and $D_{red}$ are the diffusion coefficients of the oxidized and reduced species respectively.

For an irreversible process, the electron transfer process is slow and the equilibrium concentrations are not attained at the electrode surface. The half-wave potential is a function of the rate constant of the electron transfer reaction.

$$E_{1/2} = E^\circ + \frac{RT}{\alpha nF} \ln \left( \frac{0.8861^{1/2}}{k_o} \right)$$  \hspace{1cm} (13)$$

where $\tau$ is the drop time, $D$ is the diffusion coefficient, and $k_o$ is the rate of the electrode reaction at the potential $E^\circ$. The transfer coefficient, $\alpha$, is less than 1. In the absence of any complicating factors, such as adsorption, chemical reaction, etc., a plot of potential, $E$, versus log $(i_d - i)/(i)$ should be linear with a slope greater than 59/n millivolts. Vlcek has summarized a list of criteria for determining the reversibility of an electrode process. 37
The case of a one-electron reduction with subsequent dimerization of the reduction product has been considered theoretically. Bonnaterre and Cauquis predict that this case will, in general, result in a non-linear plot of potential versus log \((i_d - i)/(i)\). Their treatment includes five particular situations and, for each, the logarithmic functions of the current which should give rise to a straight line when plotted against the applied potential.

(1) If the electrode process is itself irreversible, the dimerization reaction will have no effect. In the four remaining individual cases the electrode process is assumed reversible.

(2) If the dimerization rate is much slower than the electrode process it will not affect the concentration of reduction product at the electrode surface and, thus, will have no effect on the polarographic wave.

(3) If the dimerization rate is faster than the electrode reaction, the electrode reaction will appear to be irreversible. The current and potential are related by equation 10, with the half-wave potential being given by equation 12.

(4) If the dimerization equilibrium is displaced towards the dimer and is almost as rapid as the electrode reaction, the current and potential are related by equation 14. \(K\) is

\[ E = E^\circ + \frac{RT}{F} \ln \left( \frac{i_d - i}{i} \right)^{1/2} \]  

(14)
the equilibrium constant of the dimerization reaction, and \( \delta \) is the thickness of the diffusion layer which, at the dropping mercury electrode, is given by equation 16. All other symbols have the same meaning as in previous equations. The half-wave potential is given by equation 17, where \( C \) is the concentration of the electroactive species.

\[
E_{1/2} = E^* + \frac{RT}{2F} \ln (KC)
\]  

(17)

(5) If the dimerization reaction is irreversible, the current and potential are related by equation 18.

\[
E = E^* + \frac{RT}{F} \ln \left( \frac{i_d - i}{i} \right)^{2/3}
\]  

\[
E^* = E^* + \frac{RT}{3F} \ln \frac{\lambda_f^2}{2 F D} + \frac{\delta^2 k_d}{D}
\]  

(18)

\( \lambda_f^2 \) is the rate constant of the dimerization reaction and all other symbols have their previous meanings. The half-wave potential is given by equation 21. These equations agree
with those developed by Mairanovskii for an irreversible dimer-
ization.\textsuperscript{39}

The electrochemical behavior of NAD\textsuperscript{+} and other pyri-
dinium salts has recently been reviewed.\textsuperscript{5,40} The mechanism
suggested for the electrochemical reduction of these salts is
shown in scheme 3. At a potential of approximately -670
millivolts (versus the normal hydrogen electrode, N.H.E.),
NAD\textsuperscript{+} (V) is reversibly reduced (reaction 1) to the radical
(XV), NAD\textsuperscript{*} giving rise to wave I in Figure 2.\textsuperscript{40,45,51-53}
The radical rapidly dimerizes (reaction 2) converting this
first reduction step into an overall irreversible process.
The half-wave potentials of some NAD\textsuperscript{+} analogues are listed
in Table II. The presence of the radical intermediate has
been shown using cyclic voltammetry with a rapid-return scan
rate.\textsuperscript{40-42}

The half-wave potentials of NAD and 1-methyl-3-carb-
amoylpseudopyridinium salts for the reduction process in steps 1
and 2 in scheme 3, are independent of pH between pH 1 and
10.\textsuperscript{40,43,44,45} The pyridine mononucleotide, NMN\textsuperscript{+}, half-wave
potential shifts to a more negative value by about 80 milli-
vols as the pH increases from 5 to 7.5.\textsuperscript{40} The potential for
this compound is otherwise independent of pH.\textsuperscript{40} Ionic strength
variations have no effect on the half-wave potentials of
N-alkylpyridinium salts. The half-wave potentials of NAD\textsuperscript{+}

\[ E_{1/2} + E^0 + \frac{RT}{3F} \ln \frac{\delta^2}{3D} + \frac{RT}{3F} \ln k_d C \] (21).
and NMN$^+$ are shifted to more positive potentials by 30 millivolts and 100 millivolts, respectively, as the ionic strength is increased from 0.1 to 2.0 M. The effects of ionic strength and pH on the half-wave potential of NMN$^+$ have been explained as being due to complex formation between the negatively charged phosphate group (II) and the pyridinium ring.\textsuperscript{40} The complex would reduce the positive charge in the pyridinium ring, thus making reduction more difficult. Above pH 7.5, the phosphate group exists as the dianion; below pH 5, it exists in the monoprotonated form, the monoanion. Protonation of the phosphate or high ionic strengths would destroy
Figure 2. A typical polarogram of a pyridinium salt in a weakly basic solution (1-(2'-hydroxyethyl)-3-carbamoylpyridinium chloride at pH 9.9).
<table>
<thead>
<tr>
<th>R</th>
<th>Medium</th>
<th>$E_{1/2}$(mv.)</th>
<th>wave I</th>
<th>wave II</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$</td>
<td>aqueous pH 1-13</td>
<td>-780 to -880</td>
<td>-1.4 to -1.5</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>C$_2$H$_5$</td>
<td>aqueous pH 9.65</td>
<td>-830</td>
<td>-1410</td>
<td>40,60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aqueous</td>
<td>-790</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32% ethanol</td>
<td>-790</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>C$_3$H$_7$</td>
<td>aqueous pH 9.65</td>
<td>-820</td>
<td>-1410</td>
<td>40,60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aqueous</td>
<td>-790</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32% ethanol</td>
<td>-780</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>C$_4$H$_9$</td>
<td>aqueous pH 9.65</td>
<td>-810</td>
<td>-1410</td>
<td>40,60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aqueous</td>
<td>-800</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32% ethanol</td>
<td>-760</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>C$<em>5$H$</em>{11}$</td>
<td>aqueous</td>
<td>-790</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32% ethanol</td>
<td>-750</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>C$<em>6$H$</em>{13}$</td>
<td>aqueous</td>
<td>-790</td>
<td>-</td>
<td>5</td>
<td></td>
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<tr>
<td></td>
<td>32% ethanol</td>
<td>-740</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>C$<em>7$H$</em>{15}$</td>
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<td>-750</td>
<td>-</td>
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<tr>
<td></td>
<td>32% ethanol</td>
<td>-730</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
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<td>C$<em>8$H$</em>{17}$</td>
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<td>-710</td>
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<td>-</td>
<td>5</td>
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<td>C$<em>{10}$H$</em>{19}$</td>
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<td>-</td>
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<td></td>
<td>32% ethanol</td>
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<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>Medium</td>
<td>$E_{1/2}$ (mV)</td>
<td>wave I</td>
<td>wave II</td>
<td>Ref.</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------</td>
<td>----------------</td>
<td>--------</td>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>$\text{CH}_2\text{C}_6\text{H}_5$</td>
<td>aqueous pH 7-9</td>
<td>-760</td>
<td>-1410</td>
<td></td>
<td>40, 52, 60</td>
</tr>
<tr>
<td>$\text{CH}_2\text{C}_6\text{H}_4\text{SO}_3$</td>
<td>aqueous pH 7-9</td>
<td>-720</td>
<td>-</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>$\text{CH}_2\text{CH}_2\text{SO}_3$</td>
<td>aqueous pH 7-9</td>
<td>-780</td>
<td>-</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>50% dioxane</td>
<td>-630</td>
<td>-</td>
<td></td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>50% dioxane</td>
<td>-770</td>
<td>-</td>
<td></td>
<td>61</td>
</tr>
</tbody>
</table>
the complex with a resultant positive shift in the half-wave potential. Below pH 5, the half-wave potentials of NMM + and NAD + are identical.

Electrolysis at a potential on the plateau of wave I leads to a product having a molecular weight consistent with an NAD dimer.5,45 The dimers have generally been assumed to have the 6,6'-structure (XVI) on the basis of the ultraviolet spectra of the electrolyzed solutions5,46 but the use of this procedure has been questioned.5 Underwood and Burnett have isolated three dimeric products from the electrolysis of 1-benzyl-3-acetylpyridinium chloride. They assigned to these the 4,4', 6,6' and 4,6'-structures on the basis of NMR spectra.5 They have also tentatively assigned the 4,4'-structure, on the same basis, to an apparently homogeneous dimeric product recovered after electrolysis of an NAD + solution.5 After electrolysis of solutions of NAD + or 1-methyl-3-carbamolpyridinium salts, a polarographic oxidation wave corresponding to reoxidation of the dimers is observed at approximately -10 millivolts and -110 millivolts, respectively.5,44

During the polarography of dilute solutions (less than 10^-5 M.) of NAD +, a prewave has been observed at a potential 30 millivolts more positive than wave I. A similar prewave has been observed in a cyclic voltammetry study of 1-ethyl-, 1-propyl-, and 1-benzyl-3-carbamolpyridinium chlorides,41 which was tentatively attributed to reduction of pyridinium ions adsorbed on the mercury surface.41,47 The prewave
observed during the reduction of 3-acetylpyridine has been studied in greater detail. In this case the prewave is due to normal reduction of the pyridine moiety. After a monolayer of dimer has adsorbed, reduction of the remaining pyridine molecules requires a more negative potential in order to overcome the inhibition.

Wave II in Figure 2 is due to further reduction of the radical (step 4 in scheme 3) and is only observed in alkaline solutions. High ionic strength or the presence of tetraalkylammonium salts is required to separate Wave II of NAD$^+$ from the background wave. The half-wave potentials are difficult to determine for this wave but appear to fall in the vicinity of -1.4 to -1.5 volts at pH 9. Plots of $E$ vs. log ($i_d - i$)/($i$) indicate that the reduction process is irreversible. A pH dependence of -30 millivolts per pH unit has been reported for Wave II of 1-methyl-3-carbamoylpyridinium iodide, although one study reported only a slight pH dependence. Wave II of NAD$^+$ is reportedly independent of pH. The product of the Wave II reduction process is a dihydropyridine, but its structure is subject to controversy. Good evidence has been presented favouring a near quantitative yield of 1-methyl-3-carbamoyl-1,4-dihydropyridine. It has also been proposed that the isomeric 1,6-dihydropyridine is formed, based on the ultraviolet spectrum of the electrolyzed solution. During the electrolysis of NAD$^+$ at a potential on the plateau of Wave II some dimer is formed in addition to dihydropyridines.
NADH measured by enzymatic assay plus dimer, measured by the height of the reoxidation wave, does not account for the total NAD⁺ electrolyzed. The formation of the 1,6-dihydropyridine isomer of NADH is postulated to account for the balance. Since the dimerization rates are similar for the NAD radical and the 1-methyl-3-carbamoylpyridine radical, the presence of dimer in the electrolysis product of NAD⁺ and not in the case of 1-methyl-3-carbamoylpyridinium ion is unexplained. In aprotic solvents, the second wave does not appear unless a proton donor is present.
1.6 Substituent Effects in Pyridinium/Dihydropyridine Systems:

Substituent effects are normally analyzed through Hammett's sigma-rho linear free-energy relationships. In their standard form

$$\log \frac{k_x}{k_H} = \rho \sigma_x$$

where $\sigma_x$ is the empirically derived substituent constant for the substituent $x$, $k_x$ and $k_H$ are the rate or equilibrium constants of a reaction when the molecule contains the substituent $x$ or hydrogen, respectively, and $\rho$ is the resulting slope. The original substituent constants were derived from the ionization of benzoic acids with an assigned slope, $\rho$, of 1.0. A variety of sets of substituent constants have been developed covering a variety of structural relationships between substituent and reaction centre.54 Among the most commonly used substituent constants are $\sigma_m$, $\sigma_p$, $\sigma^*$, and $\sigma_I$. The standard reaction for each is shown in Figure 3.

Half-wave potentials have generally been used in place of $\log k$ in polarographic linear free-energy relations.55 This procedure, although simpler than calculating $\log K$ or $\log k_o$ for the electrode reaction, prevents $\rho$ values determined from polarography being compared with $\rho$ values determined in any other way. The value of $\rho$ for a reaction measures the change in charge at the reaction centre in going from reactants to transition state or products. If $\rho$ is positive, the reactants possess a greater degree of positive charge or a lesser degree
\[ \sigma_m: \quad \text{XCH}_2\text{COOH} \rightleftharpoons \text{XCH}_2\text{COO}^- + H^+ \]

\[ \sigma(x) = pK(H) - pK(x) \]

\[ \sigma_p: \quad \text{XCH}_2\text{COOH} \rightleftharpoons \text{XCH}_2\text{COO}^- + H^+ \]

\[ \sigma(x) = pK(H) - pK(x) \]

\[ \sigma^*: \quad \text{XCH}_2\text{COOR} \xrightleftharpoons{\text{H}^+} \text{XCH}_2\text{COOH} + \text{ROH} \]

\[ \sigma(x) = \left[ \log\left(\frac{k_x}{k_{\text{HOH}}}\right) + \log\left(\frac{k_x}{k_{\text{H}^+}}\right) \right] / 2.48 \]

\[ \sigma^*: \quad \text{XCH}_2\text{COOH} \xrightleftharpoons{\text{H}^+} \text{XCH}_2\text{COO}^- + H^+ \]

\[ \sigma(x) = 0.251[pK(H) - pK(x)] \]

**Figure 3.**
of negative charge as in the ionization of carboxylic acids. Larger absolute values of $\rho$ indicate a greater degree of charge alteration at the reaction centre.

In the choices of pyridine nucleotide models, little attention has been paid to finding a model which closely parallels NAD in its behaviour. The models which have been used were generally nicotinamides substituted at the 1-position with simple alkyl groups (e.g. methyl, ethyl, etc.) or benzyl groups. Direct comparisons of rate constants (or reduction potentials) have been made between NAD and model compounds, apparently on the assumption that such quantities are independent of substituent effects.\(^{26,35,41}\)

Elving\(^{40}\) and Sund\(^1\) allude to the possible effects of substituents on the properties of pyridinium salts and 1,4-dihydropyridines. Lamberg, et al.\(^{56}\) have conducted a qualitative study of 3-substituted-1-methylpyridinium iodides and their NAD\(^+\) analogues. They investigated the cyanide addition reaction, the sodium dithionite reduction, and the aldehyde oxidase oxidation of each compound. The higher cyanide affinity of NAD\(^+\) over the 1-methyl analogue was attributed to an increase in the electron-withdrawing ability of the carbamoyl group induced by hydrogen bonding with the ribose side chain. Consideration was also given to the possibility of an inductive effect operating through the 1-position. In his review, Sund\(^1\) discusses the work of Wallenfels and Diekmann\(^{16}\) in which the cyanide affinities of various 1- and 3-substituted pyridinium salts were found to be linearly related to the
absorption maxima of the cyanide adducts. No attempt was made to correlate either set of data with substituent constants. Kosower\textsuperscript{57} has determined a $\rho$ of 13.4 for the variation of $\lambda_{\text{max}}$ of the charge transfer band of 3- and 4-substituted-1-ethylpyridinium iodides.

Anderson and Kaplan\textsuperscript{17} have determined the redox potentials of several 3-substituted analogues of NAD. They observed potentials ranging from 73 millivolts more positive than NAD to 22 millivolts more negative than NAD. They suggested that a qualitative correlation exists between the redox potential and the equilibrium constant for the addition of cyanide. Unfortunately, substituent constants are unavailable for most of the substituents which they used.

The most thorough investigation in this area is that of Lindquist and Cordes,\textsuperscript{15} who studied the cyanide addition reaction of a series of 1-alkyl-3-carbamoylpseuduridinium halides. Plots of the addition rates and equilibrium constants versus $\sigma^*$ yielded values of 2.2 and 3.7, respectively, for $\rho^*$. No effect on the rate or equilibrium constants from internal charge transfer complex formation was observed. They were unable to explain the abnormally high affinity of $\text{MMN}^+$, $\alpha$-NAD$^+$ (the NAD isomer containing an $\alpha$-riboside linkage to the pyridine ring), and $\beta$-NAD$^+$ (the natural coenzyme) toward cyanide ion, but their results point to a direct interaction between pyridine ring and the ribose side chain, in addition to an inductive effect.
2. SCOPE OF THE INVESTIGATION.

This investigation was originally undertaken to determine the free energy levels of a series of NAD model compounds relative to NAD, and ultimately to find NAD models whose equilibrium with riboflavin and FMN could be directly measured. It was found that little was known about the effect of substituents on the properties of pyridinium salts and 1,4-dihydropyridines (often in the work, the NAD$^+$ models (VIII) will be referred to by the general term pyridinium ions and the NADH models (V) by the general term of dihydropyridines). In an effort to fill this gap, several properties of 1- and 3-substituted pyridines have been investigated. Series I consists of 1-substituted-3-carbamoylmethylpyridines and Series II consists of 1-carbamoylmethyl-3-substituted pyridines. For comparison, NAD, NMN, and two 1,1'-alkylenebis(3-carbamoylpyridinium) salts (XIX)a and XIXb) have also been investigated.

In view of the importance of NAD-flavin reactions, the rates of oxidation of the dihydropyridines by riboflavin or FMN have been investigated. Dihydropyridines tend to decompose
TABLE III

Substituents on the Compounds Studied

<table>
<thead>
<tr>
<th>Series I:</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>a: CH&lt;sub&gt;2&lt;/sub&gt;COO&lt;sup&gt;-&lt;/sup&gt;</td>
<td></td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>b: CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>c: CH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td></td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>d: CH&lt;sub&gt;2&lt;/sub&gt;COCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>e: CH&lt;sub&gt;2&lt;/sub&gt;OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>f: CH&lt;sub&gt;2&lt;/sub&gt;COOCH&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>g: CH&lt;sub&gt;2&lt;/sub&gt;COOCH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>h: CH&lt;sub&gt;2&lt;/sub&gt;CN</td>
<td></td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
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<td></td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Series II:</th>
<th></th>
<th>COCH&lt;sub&gt;3&lt;/sub&gt;</th>
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<tr>
<td>j: CH&lt;sub&gt;2&lt;/sub&gt;CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
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</tr>
<tr>
<td>k: CH&lt;sub&gt;2&lt;/sub&gt;CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>CN</td>
</tr>
<tr>
<td>l: CH&lt;sub&gt;2&lt;/sub&gt;CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>F</td>
</tr>
<tr>
<td>m: CH&lt;sub&gt;2&lt;/sub&gt;CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>H</td>
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<tr>
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<td></td>
<td>OH</td>
</tr>
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</table>
in acid and an investigation of this reaction was necessary in order to determine the pH ranges in which these compounds could effectively be studied.

Polarography was used to examine the effect of substituents on the reduction of pyridinium salts to the corresponding pyridine radicals. Potentiometry was used to examine the effect of substituents on the reduction of the salts to the corresponding 1,4-dihydropyridines. The choice of suitable models for NAD in various reactions should be aided by a better understanding of substituent effects in these areas. Furthermore, light may be shed on any special effects conferred by the presence of the adenosine moiety in NAD.
3. RESULTS

3.1 Acid-catalyzed Decomposition of Dihydropyridines

The rates of the acid decomposition reaction (equation 22) of the dihydropyridines were followed spectrophotometrically near the absorbance maximum of the dihydropyridine (V). The kinetic analysis follows from the equation for the absorbance of the solution (equation 23) and the equation for a first-order reaction (equation 27). Rearranging the equation for the absorbance at any given wavelength (equation 23) to calculate for the concentration of reactant leads to equation (25)

\[
\frac{A}{\lambda} = \varepsilon_{\text{reac}} C_{\text{reac}} + \varepsilon_{\text{prod}} C_{\text{prod}} \tag{23}
\]

\[
\frac{A}{\lambda} = (\varepsilon_{\text{reac}} - \varepsilon_{\text{prod}}) C_{\text{reac}} + \varepsilon_{\text{prod}} C_{\text{initial}} \tag{24}
\]

\[
C_{\text{reac}} = \frac{(A/\lambda) - \varepsilon_{\text{prod}} C_{\text{initial}}}{\varepsilon_{\text{reac}} - \varepsilon_{\text{prod}}} \tag{25}
\]
wherein, if the reaction is irreversible, then \( \varepsilon_{\text{prod}} C_{\text{initial}} \) is equivalent to the absorbance at infinite time divided by the cell path length (equation 26). Substitution of equation (26) into the equation for a first-order reaction (equation 27)

\[
\frac{-dC_{\text{reac}}}{dt} = kC_{\text{reac}}
\]

(27)

gives equation (28) which integrates to equation (29). Thus

\[
\frac{-d(A - A_\infty)}{dt} = k(A - A_\infty)
\]

(28)

\[
\ln(A - A_\infty) = kt + \text{const.}
\]

(29)

for a first-order reaction going to completion, the rate constant can be determined from a plot of \( \ln(A - A_\infty) \) against time.

Such a plot for the decomposition of 1-methyl-3-carbamoyl-1,4-dihydropyridine (Vb) is shown in Figure 4. It
Figure 4. Pseudo-first order decomposition of 1-methyl-3-carbamoyl-1,4-dihydropyridine

1.0 M. Tris buffer

$pH = 7.05$

$A_\infty$ calculated from this intercept
is obvious that the initial decomposition reaction is accompanied by a much slower secondary reaction. The absorbance at infinite time for the initial reaction, $A_\infty$, cannot be determined by allowing the absorbance to become constant with time because of the occurrence of the secondary reaction. The absorbance at infinite time for the initial reaction can, however, be estimated by extrapolating the line of $\ln(A - A_\infty)$ versus $t$ for the secondary reaction backwards as shown in Figure 4. $A_\infty$ estimated in this way was generally five to ten percent of the initial absorbance higher than the absorbance recorded at the end of the secondary reaction, an amount sufficient to cause a three to five percent change in the slope of the log $(A - A_\infty)$ versus $t$ plot. The extinction coefficient, $\varepsilon$, of the initial product from the decomposition of 1-carbamoylmethyl-3-fluoro-1,4-dihydropyridine (IV$^c$) was so large that application of this method caused a five-fold change in the calculated

![IVc](image)

rate constant. A similar value of $A_\infty$ could be estimated by varying $A_\infty$ until the first five half-lives of data give the best correlation coefficient. This method was subsequently used to determine the rate constants for the initial
decomposition reactions. Pseudo-first-order rate constants were determined primarily in the presence of acetate buffer at pH's ranging from 3.4 to 5.6 and at buffer concentrations of 1.0 and 0.1 M. At least four decomposition reactions were carried out on most dihydropyridines and these data were used to calculate the second-order rate constants for catalysis by $\text{H}_2\text{O}$, $\text{H}_3\text{O}^+$, and acetic acid by equation (30). The $\text{H}_3\text{O}^+$ and acetic acid rate constants are listed in Table IV. The water catalyzed rate represented only a few percent of the rate due to $\text{H}_3\text{O}^+$ and acetic acid and, as a result, there were very large variations in the calculated values.

The more rapidly decomposed dihydropyridines, 1-methyl-3-carbamoyl-, 1-carboxymethyl-3-carbamoyl-, 1-(2'-hydroxyethyl)-3-carbamoyl-, and 1-carbamoylmethyl-3-fluoro-1,4-dihydropyridine were also decomposed in tris(hydroxymethyl)aminomethane (hereafter called Tris) buffer. The Tris catalyzed rate constants are also listed in Table IV. The Tris catalyzed rate constants could not be determined accurately for the remaining dihydropyridines as these reactions had half-lives of days or weeks with the hydrogen-ion catalysis generally being the major reaction. The decomposition of 1-carbamoylmethyl-3-fluoro-1,4-dihydropyridine in acetate buffer was too rapid to be followed by normal spectrophotometric techniques. In fact, this compound decomposed in 1.0 M. sodium perchlorate solution.

$$k_{\text{obs}} = k_{\text{H}_2\text{O}}[\text{H}_2\text{O}] + k_{\text{H}_3\text{O}^+}[\text{H}_3\text{O}^+] + k_{\text{AcOH}}[\text{AcOH}] \quad (30)$$
<table>
<thead>
<tr>
<th>R$_1$</th>
<th>R$_3$</th>
<th>$k_{H_3O^+}$ ($M^{-1}$-sec$^{-1}$)</th>
<th>$k_{AcoH} \times 10^3$ ($M^{-1}$-sec$^{-1}$)</th>
<th>$k_{Tris} \times 10^4$ ($M^{-1}$-sec$^{-1}$)</th>
<th>Minimum pH for use of dihydropyridine$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_2$COO$^-$</td>
<td>CONH$_2$</td>
<td>77</td>
<td>33</td>
<td>1.2</td>
<td>9.1 (Tris)</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>CONH$_2$</td>
<td>1050</td>
<td>157</td>
<td>3.6$\pm$0.7</td>
<td>9.7 (Tris)</td>
</tr>
<tr>
<td>CH$_2$CH$_2$OH</td>
<td>CONH$_2$</td>
<td>400$\pm$280</td>
<td>62$\pm$22</td>
<td>1.19$\pm$0.11</td>
<td>9.3 (Tris)</td>
</tr>
<tr>
<td>CH$_2$OCH$_3$</td>
<td>CONH$_2$</td>
<td>18$\pm$5</td>
<td>7.7$\pm$2.3</td>
<td>-</td>
<td>8.1 (Tris)</td>
</tr>
<tr>
<td>CH$_2$COCH$_3$</td>
<td>CONH$_2$</td>
<td>12</td>
<td>7.7</td>
<td>-</td>
<td>8.1 (Tris)</td>
</tr>
<tr>
<td>CH$_2$COOCH$_3$</td>
<td>CONH$_2$</td>
<td>7.8$\pm$1.3</td>
<td>4.8$\pm$0.7</td>
<td>-</td>
<td>7.5 (Tris)</td>
</tr>
<tr>
<td>CH$_2$CN</td>
<td>CONH$_2$</td>
<td>0.45$\pm$0.07</td>
<td>0.32$\pm$0.02</td>
<td>-</td>
<td>7.0 (Acetate)</td>
</tr>
<tr>
<td>CH$_2$CONH$_2$</td>
<td>CONH$_2$</td>
<td>10.5$\pm$3.0</td>
<td>5.3$\pm$0.7</td>
<td>-</td>
<td>7.3 (Tris)</td>
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<tr>
<td>CH$_2$CONH$_2$</td>
<td>COCH$_3$</td>
<td>0.30$\pm$0.05</td>
<td>0.22$\pm$0.03</td>
<td>-</td>
<td>6.5 (Acetate)</td>
</tr>
<tr>
<td>CH$_2$CONH$_2$</td>
<td>CN</td>
<td>0.39$\pm$0.06</td>
<td>0.23$\pm$0.03</td>
<td>-</td>
<td>6.5 (Acetate)</td>
</tr>
<tr>
<td>CH$_2$CONH$_2$</td>
<td>F</td>
<td>14500</td>
<td>-</td>
<td>86</td>
<td>11.5 (Tris)</td>
</tr>
</tbody>
</table>

Special Compounds:

- NADH | 10.2$\pm$3.6 | 0.95$\pm$0.10 | - | 7.1 (Tris) |
- NH$_2$NH | 7.7$\pm$0.8 | 0.90$\pm$0.43 | - | 7.5 (Tris) |

$^\dagger$ Calculated for a maximum decomposition of 10% over a period of 24 hours.
within minutes and had a half-life (calculated from the rate constants in Table IV) at pH 10 of only about four hours.

The primary purpose of studying the acid decomposition of the dihydropyridines [reaction (22)] was to determine the minimum pH at which each dihydropyridine shown in Table IV could be studied. This pH limit represents a 10% decomposition over 24 hours in 0.1 M. buffer. Some compounds decompose too rapidly to be used in acetate buffer but had very slow Tris catalyzed decomposition. The rates of the very slow Tris catalyzed decomposition were estimated from an empirical equation [equation (31)] based on the acetate and Tris catalyzed

\[ k_{\text{Tris}} = \frac{k_{\text{AcOH}}}{400} \]  

rates for 1-carboxymethyl-, 1-methyl-, and 1-(2'-hydroxyethyl)-3-carbamoyl-1,4-dihydropyridines (IV a,b,c).

a: \( R_1 = \text{CH}_2\text{COO}^- \)
b: \( R_1 = \text{CH}_3 \)
c: \( R_1 = \text{CH}_2\text{CH}_2\text{OH} \)
A secondary result which came out of the study of the dihydropyridine decomposition was the determination of the Hammett reaction constant $\rho$. This quantity measures the influence of substituents on the reaction and can be determined from equation (32), where $\sigma$ represents the electronic effect of the substituent.†

$$\log \chi/\chi_0 = \rho \sigma$$ (32)

$\chi = \text{rate constant, } k, \text{ or equilibrium constant, } K$

$\chi_0 = \chi \text{ for the unsubstituted compound}$

The logarithms of the acetic acid and hydrogen ion catalyzed rate constants of the Series I compounds (IV) are plotted against $\sigma^*$ in Figures 5 and 6. The $\sigma^*$ substituent scale is used because of the similarity in the structural

†The most commonly met substituent constants are $\sigma_m$ and $\sigma_p$ for meta- and para- substituents in aromatic rings and $\sigma^*$ for substituents acting on an adjacent reaction centre through only an inductive effect.
Figure 5. Plot of second order rate constants for the acetic acid catalyzed decomposition of dihydropyridines (Table IV) against $\sigma^*$ (Table V)

$\log k_{acoll} (M^{-1}\text{-sec}^{-1})$

$\sigma^*$

$-0.5$

$-1.0$

$-1.5$

$-2.0$

$-2.5$

$-3.0$

$-3.5$

$r = 0.992$

$\rho^* = -2.0$

$-\text{CH}_3$

$-\text{CH}_2\text{CH}_2\text{OH}$

$-\text{CH}_2\text{COO}^-$

$\text{CH}_2\text{OCH}_3$

$\text{CH}_2\text{CONH}_2$

$-\text{CH}_2\text{COCH}_3$

$-\text{CH}_2\text{COOCH}_3$

$-\text{CH}_2\text{CN}$

$R_1$
Figure 6. Plot of second order rate constants for the hydrogen ion catalyzed decomposition of dihydropyridines (Table IV) against $\sigma^*$ (Table V).

$\log k_{H^+} (\text{m}^{-1} \text{sec}^{-1})$

- CH$_3$
- CH$_2$COO$^-$
- CH$_2$CH$_2$OH
- CH$_3$OCH$_2$
- CH$_2$COCH$_3$
- H$_2$NOCCH$_2$
- CH$_2$COOCH$_3$
- CH$_2$CN

$r = 0.990$
$\rho^* = -2.6$
relationship between substituent and reaction centre in reaction (22) and the reaction used to define $\sigma^*$, reaction (33). The

$$R-COOR' \xrightarrow{H^+ \text{ or } OH^-} R-COOH + R'OH$$

(33)

values of $\sigma^*$ used are listed in Table V and were calculated from Charton's values of $\sigma_I$ using equation (34).† Since

$$\sigma^* = \sigma_I / 0.45$$

(34)\textsuperscript{70}

†The $\sigma^*$ scale, designed to be a measure of polar substituent effects only, is defined from the rates of equation (33) by the equation:

$$\sigma^* = \frac{1}{2.48} \left[ \log \left( \frac{k_R}{k_{CH_3}} \right)_{OH^-} - \log \left( \frac{k_R}{k_{CH_3}} \right)_{H^+} \right]$$

The factor of 2.48 is included to make $\rho^*$ values comparable to normal Hammett $\rho$ values (which are based on the ionization of benzoic acids.) Polar substituent effects have also been described by $\sigma'$,\textsuperscript{74} obtained from the ionization of $X-COOH$ by the equation:

$$\sigma' = \frac{1}{1.464} \log \left( \frac{K_X}{K_H} \right)$$

The factor of 1.464 is again designed to make $\rho'$ values from this scale comparable to normal Hammett $\rho$ values. Taft\textsuperscript{73} defined an inductive substituent constant, $\sigma_I$, based on his $\sigma^*$ values but corrected by a factor of 0.45 to make the $\sigma_I$ scale equivalent to the $\sigma'$ scale of Roberts and Moreland, thus $\sigma_I(X) = 0.45 \times \sigma^*(CH_2X) = \sigma'(X)$\textsuperscript{73}. $\sigma_I$ has since been redefined from the ionization of $X-CH_2COOH$ by the equation $\sigma_I = 0.251 \log \left( \frac{K_X}{K_H} \right)$\textsuperscript{72} where the factor of 0.251 is used to maintain the original scale of $\sigma_I$\textsuperscript{37} i.e., to make $\sigma_I$ comparable to $\sigma'$. This latter definition of $\sigma_I$ has further expanded the list of inductive substituent constants, and, by application of equation (34), the list of $\sigma^*$ values.

$$\sigma^* = \sigma_I / 0.45$$

(34)\textsuperscript{70}
TABLE V

A list of $\sigma^*$ values used in this work

$$\begin{array}{cccc}
R_1(CH_2X) & \sigma_I(X) & \sigma^*(R_1)^a & \sigma^*(R_1)^b \\
\text{CH}_2\text{COO}^- & -.17 & -.38 & - \\
\text{CH}_3 & .00 & 0.00 & 0.00 \\
\text{CH}_2\text{CH}_2\text{OH} & .05 & 0.11 & - \\
\text{CH}_2\text{OCH}_3 & .25 & 0.55 & 0.66 \\
\text{CH}_2\text{COCH}_3 & .29 & 0.64 & 0.62 \\
\text{CH}_2\text{COOCH}_3 & .34 & 0.76 & 0.66 \\
\text{CH}_2\text{COOCH}(\text{CH}_3)_2 & .34^c & 0.76 & - \\
\text{CH}_2\text{CONH}_2 & .27 & 0.60 & - \\
\text{CH}_2\text{CN} & .55 & 1.30 & 1.25 \\
\end{array}$$

$^a$Calculated from $\sigma_I$ by $\sigma^*(\text{CH}_2X) = \sigma_I(X)/0.45$.

$^b$Values from Wells.

$^c$Not given by Charton but was estimated from the identical values for the carboxethoxy and carboxethoxy groups.
Charton's data gives a more extensive list of polar substituent constants than are available from the original $\sigma^*$ values.\(^{69}\) The error limits for each compound are $\pm 1$ standard deviation, as given in Table IV. Where error limits are not given, these have been approximated by the worst error among the other compounds and are shown in Figures 5 and 6 as broken lines.

Reaction constants $\rho^*$ of -2.0 and -2.6 are found from the slopes, $\Delta \log k/\Delta \sigma^*$, (equation 35) of Figures 5 and 6 respectively. The two values of $\rho^*$ are close enough together that,

$$\rho = \frac{\Delta \log k}{\Delta \sigma} \quad (35)$$

considering the error in many of the rate constants, there may be no real difference between the two, particularly since the larger reaction constant is for the reaction catalyzed by the stronger acid, $H_3O^+$. Usually the more reactive reagent is less selective and therefore should have a smaller reaction constant. However, a similar reversal of this generalization has been reported by Bruice and Benkovic\(^{71}\) for this reaction in which the more rapidly decomposed 1-propyl-3-carbamoyl-1,4-dihydropyridine (XX) is also more sensitive to changes in the $pK_a$ of the acid catalyst than is NADH (XXI). Possibly the mechanism of this reaction is not as simple as has been proposed.\(^{10}\)
(XX)

(XXI)
3.2 Base-catalyzed Decomposition Reactions

Some pyridinium salts (VIII) and dihydropyridines (V) were found to be susceptible to a variety of base catalyzed decomposition reactions. Among the simpler reactions were hydrolysis of ester and amide functions. Amide hydrolysis, which has been observed previously in NAD and model compounds\textsuperscript{71,75} reached significant levels near pH 10.5, which therefore represented the upper pH limit for any experiment since all compounds studied have an amide group at either the 1- or 3-positions. Amide groups in the 1-position seemed to be slightly more susceptible to hydrolysis than those in the 3-position but no quantitative measurements were made in this regard.

Two esters used in this work, 1-carbomethoxymethyl- and 1-carbo-i-propoxymethyl-3-carbamoylpyridinium chloride (VIII f and g) underwent rapid hydrolysis in alkaline solutions. The methyl ester hydrolysis had a half-life of approximately 3 minutes (determined polarographically) at pH 9.2 and the i-propyl ester hydrolyzed at about 15% of this rate. Therefore these two compounds could not be used above pH 7.5 for
the i-propyl ester and 6.5 for the methyl ester. The methyl ester of the 1-carbomethoxymethyl-3-carbamoyl-1,4-dihydropyridine (IVf) was hydrolyzed several hundred fold more slowly than was the corresponding pyridinium salt. Acid catalyzed decomposition of the corresponding dihydropyridines prevented their use below pH 7.5 meaning potentiometric measurements, which require the presence of both the pyridinium salts and the dihydropyridines, could not be made on the methyl ester at all and could only be made near pH 7.5 with the i-propyl ester. The polarography and the study of the oxidation of the dihydropyridines by flavins were not greatly affected by the hydrolyses.

Several pyridinium salts underwent base-catalyzed reactions at the pyridinium ring.\textsuperscript{71} Pseudo-base formation\textsuperscript{1,71} either represented only a small fraction of the total decomposition rate or the pseudo base underwent further reactions. At least two products were formed from 1-cyanomethyl-3-carbamoylpyridinium chloride (IIIh) above pH 7, the major one being a new pyridinium salt with a more negative reduction potential.

\begin{align*}
\text{(VIII)} & \\
\text{(IV)} & \\
f: R_1 &= \text{CH}_2\text{COOCH}_3 \\
g: R_1 &= \text{CH}_2\text{COOCH(CH}_3)_2
\end{align*}
1,1'-methylenebis(3-carbamoylpyridinium chloride) (XIXa),  
1,1'-ethylenebis(3-carbamoylpyridinium chloride) (XIXb),  
1-carbamoylmethyl-3-cyanopyridinium chloride (XXIIk) and  
1-carbamoylmethyl-3-acetylpyridinium chloride (XXIIj) above  
\( \text{pH} \)'s 5, 7, and 9.5 respectively. No attempt was made to  

\[
\begin{align*}
  \text{XXIIj} & : \quad j: R_3 = \text{COCH}_3 \\
  \text{XXIIk} & : \quad k: R_3 = \text{CN} \\
\end{align*}
\]

isolate the decomposition products as this preliminary investiga
tion was designed only to find the highest pH at which the  
compounds could be used.
During potentiometric measurements, it was found that NAD$^+$ (Ia) and/or NADH decomposed at pH 8.8 to give a material with very similar reduction potentials (both potentiometric and polarographic) to NMN$^+$. On the basis of potentiometric and polarographic reduction potentials, (see section 3.6) the decomposition product was tentatively identified as the nononucleotide (II) arising from hydrolysis of the pyrophosphate bond of NAD. The only decomposition of NAD previously reported in weakly alkaline solution is the hydrolysis of the ribose-pyridine bond, a reaction which could not possibly be causing the results observed here (see section 3.6).
3.3 Limitations on pH in the Study of NAD Model Compounds

Table VI summarizes the results of the base-catalyzed decompositions given in Section 3.2 and the acid catalyzed decompositions given in Section 3.1. The lower pH limit is the pH at which the acid-decomposition of the dihydropyridines (reaction 22) occurs to an extent of no more than 10% over a period of 24 hours. The upper pH limit is the maximum pH at which ring reactions of the pyridinium salt such as reaction (36) are not observed over a period of 24 hours, or the pH at which the hydrolysis reactions (37) through (39) occur to an extent of no more than 10% over 24 hours. The minimum pH for 1-carbamoylmethyl-3-fluoro-1,4-dihydropyridine (Vf) was so high that potentiometric measurements and the determination of the oxidation rate by flavins could not be made. To ensure that
### TABLE VI

**Stability Limits for Pyridinium Salts and Dihydropyridines**

<table>
<thead>
<tr>
<th>( R_1 )</th>
<th>( R_3 )</th>
<th>( \text{minimum pH of dihydropyridine} )</th>
<th>( \text{maximum pH} )</th>
<th>Base reaction</th>
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<tr>
<td>( \text{CH}_2\text{COO}^- )</td>
<td>( \text{CONH}_2 )</td>
<td>9.1</td>
<td>10.5</td>
<td>3-carbonoyl hydrolysis</td>
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<tr>
<td>( \text{CH}_3 )</td>
<td>( \text{CONH}_2 )</td>
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<td>10.5</td>
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</tr>
<tr>
<td>( \text{CH}_2\text{CH}_2\text{OH} )</td>
<td>( \text{CONH}_2 )</td>
<td>9.3</td>
<td>10.5</td>
<td>&quot;</td>
</tr>
<tr>
<td>( \text{CH}_2\text{OCH}_3 )</td>
<td>( \text{CONH}_2 )</td>
<td>8.1</td>
<td>10.5</td>
<td>&quot;</td>
</tr>
<tr>
<td>( \text{CH}_2\text{COCH}_3 )</td>
<td>( \text{CONH}_2 )</td>
<td></td>
<td>10.5</td>
<td>&quot;</td>
</tr>
<tr>
<td>( \text{CH}_2\text{COOCH}_3 )</td>
<td>( \text{CONH}_2 )</td>
<td>7.5</td>
<td>5.7</td>
<td>1-carbomethoxymethyl hydrolysis</td>
</tr>
<tr>
<td>( \text{CH}_2\text{COOCH}((\text{CH}_3) )</td>
<td>( \text{CONH}_2 )</td>
<td>7.5(^a)</td>
<td>6.6</td>
<td>1-carbo-i-propoxymethyl hydrolysis</td>
</tr>
<tr>
<td>( \text{CH}_2\text{CN} )</td>
<td>( \text{CONH}_2 )</td>
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<td>7.0</td>
<td>ring reactions</td>
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<tr>
<td>( \text{CH}_2\text{CONH}_2 )</td>
<td>( \text{CONH}_2 )</td>
<td>7.8</td>
<td>10.5</td>
<td>3-carbamoyl hydrolysis</td>
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<td>( \text{CH}_2\text{CONH}_2 )</td>
<td>( \text{COCH}_3 )</td>
<td>6.5</td>
<td>9.5</td>
<td>ring reactions</td>
</tr>
<tr>
<td>( \text{CH}_2\text{CONH}_2 )</td>
<td>( \text{CN} )</td>
<td>6.5</td>
<td>7.0</td>
<td>&quot;</td>
</tr>
<tr>
<td>( \text{CH}_2\text{CONH}_2 )</td>
<td>( \text{F} )</td>
<td>11.5</td>
<td>10.5</td>
<td>1-carbamoylmethyl hydrolysis</td>
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Table VI (cont.)

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<th>Special Compounds</th>
<th>minimum pH of dihydropyridine</th>
<th>maximum pH</th>
<th>Base reaction</th>
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<tr>
<td>NAD</td>
<td>7.1</td>
<td>9.9</td>
<td>unknown</td>
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<tr>
<td>NMN</td>
<td>7.5</td>
<td>10.5</td>
<td>3-carbamoyl hydrolysis</td>
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</tbody>
</table>

This value was not measured but was assumed to be the same as that of 1-carbomethoxymethyl-3-carbamoyl-1,4-dihydropyridine.
no base-catalyzed reactions of 1-cyanomethyl-3-carbamoyl-
pyridinium chloride (VIIIh) interfered, potentiometric
measurements on this compound were made at pH 5.6, despite the higher decomposition rate of the dihydropyridine (Vh) at this pH. Potentiometric measurements were not attempted on 1-carboxymethoxymethyl-3-carbamoylpyridinium chloride (IIIf). Potentiometric measurements on 1-carbo-i-propoxymethyl-3-carbamoylpyridinium chloride (IIIg) were made at pH 7 which was a compromise between the acid and base catalyzed decompositions. Although both the ester hydrolysis of (IIIg) and the acid-decomposition of (IVg) were faster than desired, potentiometric measurements were still possible. If a pH much above or below 7 was used, either the ester hydrolysis of (IIIg) or the acid-decomposition of (IVg) reached an intolerable rate.

\[ (III) \quad (IV) \]

f: \( R_1 = \text{CH}_2\text{COOCH}_3 \)

\( g: R_1 = \text{CH}_2\text{COOCH(CH}_3\text{)}_2 \)
3.4 Substituent Constants of the 3-Carbamoylpyridinium Group

In order to properly analyze the behaviour of 1,1'-methylenebis(3-carbamoylpyridinium chloride) (XIXa) and 1,1'-ethylenebis(3-carbamoylpyridinium chloride) (XIXb) the inductive effect of the pyridinium group must first be known.

Intuitively, one would expect the inductive substituent constant for a pyridinium group (XXIII) to closely resemble that of

the trimethylammonio group or ammonio group. To test this hypothesis, the pKa of the substituted acetic acid (IIIa) was
determined spectrophotometrically (Figure 7) and found to be 
1.7 ± 0.1, from which a $\sigma_I$ of 0.76 can be calculated using 
Charton's correlations [equation (40)]. This value is in 

$$\sigma_I = 0.251 \log \frac{K_x}{K_H} \tag{40}$$

fact in good agreement with $\sigma_I$ for the trimethylammonio group 
(0.73)$^7$ and approximately 20% larger than the value of $\sigma_I$ 
for the ammino group ($\sigma_I = 0.60)^7$ so the value for $\sigma_I$ of (XXIV) 

![Chemical Structures](XXIV)(XXII)

was assumed to be close to 20% larger than $\sigma_I$ for the $\text{CH}_2\text{NH}_3^+$ 
group. ($\sigma_I = 0.36)^7$ giving it a value of 0.44. The corres-
ponding $\sigma^*$ substituent constants for the substituents (XXV 
and XXVI) have been calculated from equation (34)$^7$ giving 

![Chemical Structures](XXVI)(XXV)
Figure 7. Spectrophotometric determination of the pKa of the 1-carboxymethyl-3-carbamoylpyridinium ion.

\[
p_{\text{Ka}} = 1.8
\]

\[
p_{\text{Ka}} = 1.7
\]

Absorbance at 240 nm.

pH
values of 1.7 and 1.0 respectively. Having determined the

\[ \sigma^{*}(\text{XCH}_2) = \sigma_{I}(X)/0.45 \]  \hspace{1cm} (34) \dagger\n
inductive effect of a second pyridinium ring, the properties of (XIXa) and (XIXb) can be investigated with the purpose of discovering if the proximity of the two pyridine rings has any effect in addition to the inductive effect.

\[
\begin{align*}
\text{H}_2\text{NOC} & \quad \text{CONH}_2 \\
\text{N} & \quad \text{CH}_2 \\
\text{N} & \quad \text{N}
\end{align*}
\]

(XIX)

\[ \dagger \text{See footnote on page 48 (Section 3.1)} \]
3.5 Polarography of Pyridinium Salts

The polarographic results were in good agreement with previous studies. At pH 5.6, NAD$^+$ (Ia), NMN$^+$ (II), and 1-methyl-3-carbamoylpyridinium chloride (VIIIb) exhibited one reduction wave with half-wave potentials near -680 mV, -700 mV, and -800 mV versus the normal hydrogen electrode (N.H.E.) respectively. These are in good agreement with previously reported half-wave potentials for these compounds.\textsuperscript{5,40,44} No prewaves were observed with these compounds in agreement with previous work.\textsuperscript{5,40,44} The reduction wave of 1,1'-ethylenebis(3-carbamoylpyridinium chloride) previously reported to be near -600 mV\textsuperscript{65,67} was only observed at concentrations over 2 x 10^{-3} molar. At lower concentrations a single wave was
observed at approximately -430 mv.* Both the prewave and main wave of 1,1'-methylenebis(3-carbamoylpyridinium chloride) were observed near the previously reported potentials of -180 mv. and -420 mv. respectively. 66 The other pyridinium compounds have not been studied previously.

Only one compound, 1-carbamoylmethyl-3-acetylpyridinium chloride (VIIIj), exhibited two reduction waves at all concentrations. The first wave was independent of pH, as has been found 5,40 for the reduction wave (wave I in Figure 2) of other pyridinium salts. The second wave had a pH dependence of about 35 millivolts per pH unit. Based on the pH dependence, the first wave corresponds to the reduction to the radical (wave I). The reduction occurring at the second wave is not known but may involve reduction to a dihydropyridine. With increasing concentration, the second wave shifts to more negative potentials by 41 millivolts per concentration decade. At concentrations above 10⁻³ M., the plateau of the first wave

* A prewave has been reported for this compound at -380 mv. 66 A difference of 50 mv. between reported half-wave potentials of pyridinium compounds is not uncommon. 40
shifts negatively by a much larger amount, until at high concentrations the two waves merge as shown in Figure 3, preventing an analysis of the first wave being made.

The half-wave potential of wave I (Figure 2, page 25) has been reported to shift to more negative potentials with decreasing concentration\(^43,44\) below \(10^{-3}\) M. A more complex concentration dependence was observed in this study as shown in Figure 9 for 1-methyl-3-carbamoylpyridinium iodide. Similar behaviour was observed with the other pyridinium salts studied. At concentrations below \(10^{-3}\) M, the half-wave potential shifts to more negative potentials with decreasing concentration. The half-wave potential passes through a maximum near \(10^{-3}\) M and then returns to more negative values with increasing concentration. The unusual concentration dependence could be due to a change of mechanism of the electrode reaction, one mechanism operating below \(5 \times 10^{-4}\) M. concentrations and the other operating above \(10^{-3}\) M. concentrations. Other evidence bearing on this will be presented in subsequent sections (Section 3.5.1 and 3.5.3).

The dependence of the limiting current on the height of the mercury column was determined for 1-cyanomethyl-3-carbamoyl-pyridinium chloride. A plot of \(\log i_f\) vs. \(\log h\) was linear with a slope of 0.455, in good agreement with the theoretical dependence of 0.5 for a diffusion controlled reduction process. This result agrees with the findings of previous workers for other pyridinium ions\(^6\).
Figure 8. The effect of changing concentration on the potential of the polarographic reduction of 1-carbamoylmethyl-3-acetylpuridinium chloride

-600 mv.  -600 mv.

2.2 mM

0.20 mM

10.0 mM

100 mv.
Figure 9. The concentration dependence of the polarographic potentials of 1-methyl-3-carbamoylpyridinium iodide.
3.5.1 Analysis of Wave I.

In view of the theoretical treatment by Bonnaterre and Cauquis, a comparison was made between plots of potential, $E$, versus three current functions, $\log \left( \frac{i_d - i}{i} \right)$, $\log \left( \frac{i_d - i}{i} \right)^{2/3}$, and $\log \left( \frac{i_d - i}{i} \right)^{1/2}$. These plots are shown in Figures 10 and 11 for 1-methyl-3-carbamoyl-pyridinium iodide and $\text{MAD}^+$, respectively. A first criterion for comparison is linearity. It is readily apparent that none of the current functions apply over the entire concentration range from $10^{-5}$ to $10^{-2}$ M. Below $10^{-4}$ M, plots of $E$ versus $\log \left( \frac{i_d - i}{i} \right)^{2/3}$ or $\log \left( \frac{i_d - i}{i} \right)^{1/2}$ are linear. At concentrations above $10^{-3}$ M, plots of $E$ versus $\log \left( \frac{i_d - i}{i} \right)$ have only a small curvature near the low-current end of the line. The remaining plots all show considerable curvature.

A second criterion for comparison is slope. (The slope is the change in potential per unit change in the log-current function, $\log \left( \frac{i_d - i}{i} \right)$, etc. and has the units of millivolts.) A slope of 59 mv. is predicted for plots of potential versus $\log \left( \frac{i_d - i}{i} \right)^{1/2}$ or $\log \left( \frac{i_d - i}{i} \right)^{2/3}$. The results of these low-concentration plots, which were the only linear plots, are listed in Table VII. The slopes of these plots are generally higher than predicted which may be due to adsorption effects or may simply be related to the difficulty of getting well resolved polarograms at concentrations approaching the detection limit of the instrument. The plots of the potential versus $\log \left( \frac{i_d - i}{i} \right)^{2/3}$ have slopes closer to the
Figure 10a. Polarographic applied potential versus log\((i_d - i)/i\) for 1-methyl-3-carbamoylpyridinium chloride

0.1 M acetate, pH = 5.6

-0.0157 M
-0.10 M
-2.0 M
-10.0 M
Figure 10b. Polarographic applied potential versus $\log(i_d - i)/(i)^{2/3}$ for 1-methyl-3-carbamoylpyridinium chloride.

0.1 M acetate, pH = 5.6

-925
-950
-975
-1000
-1025
-1050
-1075
-1100
-1125

-1.5 -1.0 -0.5 0.0 0.5 1.0

$\log(i_d - i)/(i)^{2/3}$
Figure 10c. Polarographic applied potential versus $\log(i_d-i)/(i)^{1/2}$ for 1-methyl-3-carbamoylpyridinium chloride.

0.1 M. acetate, pH = 5.6

- 0.0157 mM.
- 0.10 mM.
- 2.0 mM.
- 10.0 mM.
Figure 11a. Polarographic applied potential versus log(\(i_d-i)/(i)\) for nicotinamide adenine dinucleotide

0.1 M acetate, pH = 5.6

- 0.016 mM.
- 0.023 mM.
- 0.18 mM.
- 1.8 mM.
- 9.0 mM.
Figure 11b. Polarographic applied potential versus $\log(i_d-i)/(i)^{2/3}$ for nicotinamide adenine dinucleotide
Figure 11c. Polarographic applied potential versus \( \log(i_d-i)/(i)^{1/2} \) for nicotinamide adenine dinucleotide.

0.1 M. acetate, pH = 5.6

- 0.016 mM.
- 0.023 mM.
- 0.18 mM.
- 1.8 mM.
- 9.0 mM.
TABLE VII

Slopes and Intercepts of Plots of Potential versus 
log \( (i_d - i)/(i)^{2/3} \) and log \( (i_d - i)/(i)^{1/2} \)

![Chemical Structure]

Series I:

<table>
<thead>
<tr>
<th>( R_1 )</th>
<th>Concentration (mM)</th>
<th>( \log(i_d - i)/(i)^{2/3} ) Slope (mv)</th>
<th>( \log(i_d - i)/(i)^{1/2} ) Slope (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{CH}_2\text{CN} )</td>
<td>0.0106</td>
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<td></td>
<td>0.156</td>
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<td>0.248</td>
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<td>82</td>
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<tr>
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<td>0.0141*</td>
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<tr>
<td></td>
<td>0.0475</td>
<td>66</td>
<td>75</td>
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* the same solutions recorded at different current sensitivities
Table VII (cont.)

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<tr>
<th>( R_1 )</th>
<th>concentration (mM)</th>
<th>( E ) against ( \log(i_d - i)/(i)^{2/3} )</th>
<th>( E ) against ( \log(i_d - i)/(i)^{1/2} )</th>
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<tbody>
<tr>
<td>( \text{CH}_2\text{CH}_2\text{OH} )</td>
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Series II:

![Chemical structure]

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<th>( E ) against ( \log(i_d - i)/(i)^{1/2} )</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>log($i_d$ - $i$)</td>
<td>slope (mv)</td>
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<tr>
<td></td>
<td>0.127</td>
<td>74</td>
<td>-642</td>
</tr>
<tr>
<td>O$^-$</td>
<td>0.2</td>
<td>72</td>
<td>-1245</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>70</td>
<td>-1243</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>74</td>
<td>-1231</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>97</td>
<td>-1246</td>
</tr>
</tbody>
</table>

Special Compounds:

**Compound**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_3$</th>
<th>$E$ against $\log(i_d - i)/(i)^{2/3}$</th>
<th>$E$ against $\log(i_d - i)/(i)^{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD$^+$</td>
<td>0.016</td>
<td>63</td>
<td>-637</td>
</tr>
<tr>
<td>(Ia, page 65)</td>
<td>0.023</td>
<td>62</td>
<td>-679</td>
</tr>
<tr>
<td></td>
<td>0.036</td>
<td>71</td>
<td>-666</td>
</tr>
<tr>
<td>NH$_3^+$, pH 4.6</td>
<td>0.020</td>
<td>81</td>
<td>-713</td>
</tr>
<tr>
<td>(II, page 65)</td>
<td>0.030</td>
<td>75</td>
<td>-700</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>83</td>
<td>-693</td>
</tr>
<tr>
<td>pH 5.6</td>
<td>0.018</td>
<td>104</td>
<td>-718</td>
</tr>
<tr>
<td></td>
<td>0.026</td>
<td>84</td>
<td>-702</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>105</td>
<td>-707</td>
</tr>
</tbody>
</table>

\[
\text{CONH}_2
\]

\[
\text{CONH}_2
\]
<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>( \log(i_d - i)/(i)^{2/3} ) Slope (mv)</th>
<th>( \log(i_d - i)/(i)^{1/2} ) Slope (mv)</th>
<th>Intercept (mv)</th>
<th>Intercept (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{H}_2\text{NOC} )</td>
<td>( \text{CONH}_2 )</td>
<td>(.00952)</td>
<td>68</td>
<td>-197</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(.0182)</td>
<td>83</td>
<td>-181</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(.0261)</td>
<td>97</td>
<td>-180</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(.040)</td>
<td>78</td>
<td>-228</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(.20)</td>
<td>76</td>
<td>-201</td>
<td>86</td>
</tr>
<tr>
<td>Wave I'</td>
<td>(.00952)</td>
<td>72</td>
<td>-330</td>
<td>70</td>
<td>-377</td>
</tr>
<tr>
<td></td>
<td>(.0182)</td>
<td>96</td>
<td>-390</td>
<td>104</td>
<td>-361</td>
</tr>
<tr>
<td></td>
<td>(.0261)</td>
<td>70</td>
<td>-428</td>
<td>75</td>
<td>-405</td>
</tr>
<tr>
<td></td>
<td>(.040)</td>
<td>108</td>
<td>-435</td>
<td>120</td>
<td>-408</td>
</tr>
<tr>
<td></td>
<td>(.20)</td>
<td>98</td>
<td>-439</td>
<td>89</td>
<td>-430</td>
</tr>
</tbody>
</table>
theoretical value, indicating equation (18) may be the appropriate equation to use. Table VIII lists the slopes and

\[ E = \varepsilon^\circ + \frac{RT}{F} \ln \left( \frac{i_d - i}{i} \right)^{2/3} \]  

half-wave potentials derived from the linear portions of plots of potential versus \( \log \left( \frac{i_d - i}{i} \right) \) at 2.0 mM. and 10.0 mM. concentrations. The slopes of most of these plots are larger than the theoretical value of 60 mv., indicating that an irreversible electrode reaction is taking place. This irreversibility is not induced mainly by the dimerization reaction since the plots of potential versus \( \log \left( \frac{i_d - i}{i} \right)^{2/3} \) and \( \log \left( \frac{i_d - i}{i} \right)^{1/2} \) showed considerable curvature and equally large slopes.

Thirdly, theory predicts that the intercepts in equations (10), (14), and (18) should be independent of concentration.

\[ E = \varepsilon^\circ + \frac{RT}{\Delta nF} \ln \left( \frac{i_d - i}{i} \right) \]  

\[ E = \varepsilon^\circ + \frac{RT}{F} \ln \left( \frac{i_d - i}{i} \right)^{1/2} \]

This condition is approximately fulfilled by the low concentration plots of potential versus both \( \log \left( \frac{i_d - i}{i} \right)^{2/3} \) and \( \left( \frac{i_d - i}{i} \right)^{1/2} \). The means and standard deviations of the intercepts of the plots are listed in Table IX. The standard deviations range from 2 mv. up to 27 mv. with variations in
### TABLE VIII

Slopes and Half-wave Potentials Obtained by Plotting

Applied Potential Versus log\((i_d - i)/(i)\)

**Series I:**

![Chemical Structure]

<table>
<thead>
<tr>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>2.0 mM. slope (mv)</th>
<th>2.0 mM. Half-wave potential (mv)</th>
<th>10.0 mM. slope (mv)</th>
<th>10.0 mM. Half-wave potential (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CN</td>
<td>74</td>
<td>-554</td>
<td>92</td>
<td>-581</td>
</tr>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;COOCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>74</td>
<td>-657</td>
<td>94</td>
<td>-692</td>
</tr>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;COO(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>76</td>
<td>-668</td>
<td>90</td>
<td>-696</td>
</tr>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;COCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>83</td>
<td>-699</td>
<td>97</td>
<td>-729</td>
</tr>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>77</td>
<td>-657</td>
<td>85</td>
<td>-679</td>
</tr>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>66</td>
<td>-758</td>
<td>83</td>
<td>-780</td>
</tr>
<tr>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>58</td>
<td>-793</td>
<td>84</td>
<td>-813</td>
</tr>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;COO&lt;sup&gt;-&lt;/sup&gt;</td>
<td>90</td>
<td>-826</td>
<td>101</td>
<td>-922</td>
</tr>
</tbody>
</table>

**Series II:**

![Chemical Structure]

<table>
<thead>
<tr>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
<th>2.0 mM. slope (mv)</th>
<th>2.0 mM. Half-wave potential (mv)</th>
<th>10.0 mM. slope (mv)</th>
<th>10.0 mM. Half-wave potential (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>74</td>
<td>-682</td>
<td>83</td>
<td>-710</td>
</tr>
<tr>
<td>CN</td>
<td>67</td>
<td>-509</td>
<td>90</td>
<td>-524</td>
</tr>
<tr>
<td>F</td>
<td>51</td>
<td>-838</td>
<td>67</td>
<td>-869</td>
</tr>
<tr>
<td>H</td>
<td>83</td>
<td>-1029</td>
<td>88</td>
<td>-1056</td>
</tr>
</tbody>
</table>
Table VIII (cont.)

<table>
<thead>
<tr>
<th>R₃</th>
<th>2.0 mM.</th>
<th>10.0 mM.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>slope (mv)</td>
<td>Half-wave potential (mv)</td>
</tr>
<tr>
<td>O⁻</td>
<td>61</td>
<td>-1224</td>
</tr>
</tbody>
</table>

Special compounds:

- \( \text{NAD}^+ \)
  (Ia, page 65)
  - 55 \( \text{mv} \), -683 \( \text{mv} \)
  - 64 \( \text{mv} \), -715 \( \text{mv} \)

- \( \text{NMN}^+ \)
  (II, page 65)
  - 115 \( \text{mv} \), -810 \( \text{mv} \)
  - 103 \( \text{mv} \), -830 \( \text{mv} \)

\[
\begin{align*}
\text{CONH}_2 & \quad \text{CONH}_2 \\
\text{CONH}_2 & \quad \text{CONH}_2
\end{align*}
\]

- 124 \( \text{mv} \), -593 \( \text{mv} \)
  - 129 \( \text{mv} \), 672 \( \text{mv} \)

- 90 \( \text{mv} \), -469 \( \text{mv} \)
### TABLE IX

Mean Intercepts from Plots of Potential versus
\[
\log(i_d - i)/(i)^{2/3} \text{ and } \log(i_d - i)/(i)^{1/2}
\]

**Series I:**

![Chemical Structure]

<table>
<thead>
<tr>
<th>(R_1)</th>
<th>(\log(i_d - i)/(i)^{2/3}) Intercept, (\varepsilon^o) (mv. vs. N.H.E.)</th>
<th>(\log(i_d - i)/(i)^{1/2}) Intercept (mv. vs. N.H.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{CH}_2\text{CN})</td>
<td>-494 ± 16</td>
<td>-471 ± 17</td>
</tr>
<tr>
<td>(\text{CH}_2\text{COOCH}_3)</td>
<td>-610 ± 5</td>
<td>-580 ± 2</td>
</tr>
<tr>
<td>(\text{CH}_2\text{COOCH}(\text{CH}_3)_2)</td>
<td>-629 ± 5</td>
<td>-605 ± 6</td>
</tr>
<tr>
<td>(\text{CH}_2\text{COCH}_3)</td>
<td>-688 ± 12</td>
<td>-661 ± 6</td>
</tr>
<tr>
<td>(\text{CH}_2\text{OCH}_3)</td>
<td>-639 ± 16</td>
<td>-613 ± 15</td>
</tr>
<tr>
<td>(\text{CH}_2\text{CH}_2\text{OH})</td>
<td>-768 ± 5</td>
<td>-746 ± 6</td>
</tr>
<tr>
<td>(\text{CH}_3)</td>
<td>-815 ± 25</td>
<td>-805 ± 5</td>
</tr>
<tr>
<td>(\text{CH}_2\text{COO}^-)</td>
<td>-842</td>
<td>-799</td>
</tr>
</tbody>
</table>

**Series II:**

![Chemical Structure]

<table>
<thead>
<tr>
<th>(R_3)</th>
<th>(\log(i_d - i)/(i)^{2/3}) Intercept, (\varepsilon^o) (mv. vs. N.H.E.)</th>
<th>(\log(i_d - i)/(i)^{1/2}) Intercept (mv. vs. N.H.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{CONH}_2)</td>
<td>-652 ± 8</td>
<td>-628 ± 3</td>
</tr>
<tr>
<td>(\text{CN})</td>
<td>-426 ± 4</td>
<td>-400 ± 10</td>
</tr>
<tr>
<td>(\text{COCH}_3)</td>
<td>-506 ± 4</td>
<td>-480 ± 3</td>
</tr>
<tr>
<td>(\text{F})</td>
<td>-819 ± 4</td>
<td>-799 ± 3</td>
</tr>
</tbody>
</table>
Table IX (cont.)

<table>
<thead>
<tr>
<th>$R_3$</th>
<th>$\log(i_d - i)/(i)^{2/3}$</th>
<th>$\log(i_d - i)/(i)^{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H$</td>
<td>$-1086 \pm 23$</td>
<td>$-1066 \pm 13$</td>
</tr>
<tr>
<td>$O^-$</td>
<td>$-1241 \pm 7$</td>
<td>$-1246 \pm 16$</td>
</tr>
</tbody>
</table>

Special compounds:

- **NAD**<sup>+</sup> (Ia, page 65)
  - $-677 \pm 10$
  - $-649 \pm 12$

- **NMN**<sup>+</sup> (II, page 65)
  - $-703 \pm 8$
  - $-676 \pm 5$

- $H_2 NOC \begin{array}{c} \text{CONH}_2 \\ \text{CONH}_2 \end{array}$
  - $-434 \pm 11$
  - $-408 \pm 14$

- $H_2 NOC \begin{array}{c} \text{CONH}_2 \\ \text{CONH}_2 \end{array}$
  - $-209 \pm 17$
  - $-185 \pm 17$

- Wave I
  - $-409 \pm 27$
  - $-391 \pm 20$
the intercept being random with respect to concentration changes. The use of the charging current compensation, which is necessary to hold the baseline constant at low concentrations, causes shifts in the applied potentials of as much as 100 mv. Metrohm supplies a nomogram to correct for this potential shift but the error could easily reach 15 to 20 mv. at concentrations approaching $10^{-5}$ M.

Based on the preceding analysis, only the low concentration waves are consistent with any of the theoretical equations which might apply for a reversible electrode reaction. The only criterion which distinguishes between plots of potential versus $\log (i_d - i)/(i)^{1/2}$ and $\log (i_d - i)/(i)^{2/3}$ is slope and the observed variations in this quantity make it inconclusive. Mechanistically, there is little to choose between equations (14) and (18), both of which are consistent with reactions (1) and (2) in Scheme 3 reproduced in equation (41).

\begin{equation}
E = \varepsilon^o + \frac{RT}{F} \ln (i_d - i)/(i)^{1/2}
\end{equation}

Equation (14) was derived assuming a rapid dimerization reaction. The dimerization reaction has been found to be
rapid; thus if the dimerization equilibrium is displaced far enough towards products to make this reaction irreversible, as was assumed in the derivation of equation (13), either equation could apply.

\[
E = E^\circ + \frac{RT}{F} \ln\left(\frac{i_d - i}{(i)^{2/3}}\right)
\]

(13)

The intercepts listed in Table IX are plotted against each other in Figure 12. The excellent correlation and slope near unity suggest that the two sets of data are essentially the same and, therefore, subsequent discussions will involve only data derived from plots of potential versus \(\log\left(\frac{i_d - i}{(i)^{2/3}}\right)\).

3.5.2 Prewave Formation

Prewaves were observed in the polarograms of 1-methoxy-methyl-3-carbamoylpyridinium chloride (VIIe), 1-carbomethoxy-methyl-3-carbamoylpyridinium chloride (VIIIf), 1-carbo-i-propoxymethyl-3-carbamoylpyridinium chloride (VIIIg), 1-cyanomethyl-3-carbamoylpyridinium chloride (VIIIh), 1-cyanomethyl-3-carbamoylpyridinium chloride (VIIIh), 1-carbamoylmethyl-3-cyanopyridinium chloride (VIIk), and 1'1'-methylenebis(3-carbamoylpyridinium chloride) (XIXa). At higher concentrations, the prewave wave height becomes independent of concentration as shown in Figure 13. This behaviour is typical of adsorption waves in which the maximum diffusion
Figure 12. Comparison of the intercepts from plots of polarographic potential vs. log\((i_d - i)/(i)^{1/2}\) and log\((i_d - i)/(i)^{2/3}\) for compounds in Series I and Series II.

corr. coeff. = 0.999
slope = 1.01

*see Table X for the corresponding list of compounds
TABLE X
List of Compounds Plotted in Figure 12

<table>
<thead>
<tr>
<th></th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CN</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;COOCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;COOCH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>6.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;COCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>F</td>
</tr>
<tr>
<td>11.</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>12.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;COO&lt;sup&gt;-&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>H</td>
</tr>
</tbody>
</table>
Figure 13. The concentration dependence of the prewave limiting current for the polarographic reduction of 1-carbo-i-propoxymethyl-3-carbamoyl-pyridinium chloride.
current corresponds to the formation of a monolayer of adsorbed material on the electrode surface.\textsuperscript{37,39} Table XI gives the concentration corresponding to the maximum diffusion current of the prewave and the approximate difference between the half-wave potentials of the prewave and main wave for each compound.

The maximum prewave diffusion current of 1-carbo-i-propoxymethyl-3-carbamoylpyridinium chloride was independent of temperature. This behaviour is consistent with a strong adsorption process\textsuperscript{39} in which case the maximum diffusion current would be affected only by factors which affect the size of the mercury drop. In agreement with this, only the prewave is observed at low concentrations for all of the compounds except 1,1'-methylenbis(3-carbamoylpyridinium chloride). At this point it should be recalled that the plots at very low
<table>
<thead>
<tr>
<th>( R_1 )</th>
<th>( R_3 )</th>
<th>Concentration corresponding to the maximum diffusion current (mM)</th>
<th>Potential difference* (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{CH}_2\text{OCH}_3 )</td>
<td>( \text{CONH}_2 )</td>
<td>0.20</td>
<td>100</td>
</tr>
<tr>
<td>( \text{CH}_2\text{COOCH}_3 )</td>
<td>( \text{CONH}_2 )</td>
<td>0.32</td>
<td>90</td>
</tr>
<tr>
<td>( \text{CH}_2\text{COOCH(CH}_3)_2 )</td>
<td>( \text{CONH}_2 )</td>
<td>0.34</td>
<td>150</td>
</tr>
<tr>
<td>( \text{CH}_2\text{CN} )</td>
<td>( \text{CONH}_2 )</td>
<td>0.36</td>
<td>130</td>
</tr>
<tr>
<td>( \text{CH}_2\text{CONH}_2 )</td>
<td>( \text{CN} )</td>
<td>0.49</td>
<td>150</td>
</tr>
<tr>
<td>( \text{H}_2\text{NOC} )</td>
<td>( \text{CONH}_2 )</td>
<td>~0.1</td>
<td>200</td>
</tr>
</tbody>
</table>

*It has not been proven which wave (main wave or prewave), if either, represents the true reduction potential of these compounds. Depending on which wave does, in fact, represent the true reduction potential, errors as large as the potential differences could be introduced.
concentrations (Figures 10 and 11) were consistent with a reversible reduction followed by an irreversible dimerization whereas the plots at high concentration indicated an irreversible reduction (see Section 3.5.1). For compounds exhibiting a prewave, then, it is the prewave which is consistent with reaction (41) and the main wave which exhibits irreversible behaviour. This behaviour is consistent with the adsorption mechanism proposed by Laviron \(^\text{48}\) for the reduction of 3-acetylpyridines (see Section 1.5 Introduction, Electrochemistry) in which the reduction of the bulk of the 3-acetylpyridine is inhibited by a layer of adsorbed reduction product.

The polarographic behaviour of 1,1-methylenebis(3-carbamoylpyridinium chloride) was more complex than the other compounds. At low concentrations, both a prewave and the main wave are seen as shown in Figure 14, in contrast to other pyridinium compounds for which the main wave was not seen at concentrations less than that corresponding to the prewave maximum. The second wave varies between one and two times the height of the first wave in an apparently random way. This variation is probably related to experimental difficulties encountered at such low concentrations. The potential difference

\[ \text{N} \quad R_1 \quad \text{e}^- \quad \begin{array}{c} \text{N} \quad R_1 \\ \vdots \\ \text{N} \quad R_1 \end{array} \quad \begin{array}{c} \text{N} \quad R_1 \\ \vdots \\ \text{N} \quad R_1 \end{array} \quad \text{R}_3 \quad \text{R}_3 \quad \text{R}_3 \quad \text{R}_3 \]
Figure 14. Polarogram of a $1.8 \times 10^{-5}$ M solution of 1,1'-methylenebis-(3-carbamoylpyridinium chloride)
between the prewave and main wave was approximately 200 mV, a larger value than found for any other pyridinium compound. With such a large potential difference, it becomes important to know which wave represents the true reduction potential. Lovecchio has proposed that the first wave corresponds to reduction of adsorbed reactant while the second wave corresponds to reduction of soluble reactant, the latter process possibly being inhibited by adsorbed product. The two waves could not be differentiated on the basis of the potential-log current analyses listed in Table VII, so polarograms were recorded at temperatures between 13° and 50° to see if one wave increased in height relative to the other. If the presence of the two waves is due to a partial adsorption, as suggested, then increasing the temperature should increase the height of the true reduction wave and decrease the wave which is due to adsorption effects. The results of the temperature study are shown in Figure 15. The waves appear to merge as the temperature is increased and the results are too ambiguous to allow the wave that represents the true reduction potential to be identified. Possibly the true reduction potential falls somewhere in between the two waves that are observed.
Figure 15. The effect of temperature change on the polarogram of a $2.3 \times 10^{-5}$ M solution of 1,1'-methylenedibis(3-carbamoylpyridinium chloride)
3.5.3 The Effects of 1-Substituents on Polarography Reduction Potentials.

Initially the effect of substituents on the electrochemical reduction potential was estimated from plots of half-wave potentials, $E_{1/2}$, against Hammett substituent constants, $\sigma$ (see Section 1.6). Conventionally, the reaction constant $\rho$ which is a measure of the effect of substituents on a reaction, is defined by equation (32) using either the equilibrium constant or the rate constant for the reaction. [In practice, $\rho$ is usually found from the slope of a plot of log $K$ (or log $k$) against $\sigma$]. The rate constant, $k_o$, or equilibrium constant, $K$, for a polarographic reduction can, under certain circumstances, be determined from the half-wave potential. If the polarographic wave is described by equation (10), which is the equation for

$$E = E_{1/2} + \frac{RT}{\alpha nF} \ln[(i_d - i)/(i)] \quad (10)$$

\[\text{constant or the rate constant for the reaction.} \]

\[\text{[In practice,} \]

\[\rho \text{ is usually found from the slope of a plot of log } K \text{ (or log } k) \text{ against } \sigma]. \]

\[\text{The rate constant, } k_o, \text{ or equilibrium constant, } K, \text{ for a polarographic reduction can, under certain circumstances, be determined from the half-wave potential. If the polarographic wave is described by equation (10), which is the equation for} \]

\[E = E_{1/2} + \frac{RT}{\alpha nF} \ln[(i_d - i)/(i)] \quad (10) \]

\[\text{The substituent constant, } \sigma, \text{ is an empirical quantity defined from a standard reaction and measures the relative effect of a substituent on the standard reaction. A variety of } \sigma \text{ values exist depending on the structural relationship between the substituent and the reaction centre and on the type of reaction occurring.}^{57,69,76} \]

$\dagger$
a simple polarographic reduction wave, then the half-wave potential can be related to the equilibrium constant, \( K \), [equation (12) and (7)] for a reversible reduction (\( \alpha=1 \), see also Section 1.5) or to the reduction rate constant, \( k_o \) [equation (13)] for an irreversible reduction (\( \alpha<1 \)). For a reversible polarographic reduction, equations (7) and (12) can be combined with equation (32) to give equations (42) and (43). Since it is unlikely that the ratio of the diffusion coefficients, \( D \), will be greatly affected by substituent changes, the second term of equation (43) can be neglected giving equation (44), from which the

\[
\rho = \frac{nF}{2.303RT} \cdot \frac{\Delta E^o}{\Delta \sigma} \tag{42}
\]

\[
\rho = \frac{nF}{2.303RT} \cdot \frac{\Delta E_{1/2}}{\Delta \sigma} + \frac{\Delta \ln(D_{\text{ox}}/D_{\text{red}})^{1/2}}{2.303 \Delta \sigma} \tag{43}
\]

\[
\rho = \frac{nF}{2.303RT} \cdot \frac{\Delta E_{1/2}}{\Delta \sigma} \tag{44}
\]
reaction constant \( \rho \) of a reversible reduction can be determined if the effect of substituents on the half-wave potential, \( \Delta E_{1/2}/\Delta \sigma \), is known. A similar treatment can be applied to an irreversible reduction by combining equations (13) and (32) to yield equation (45). Since the drop time \( T \) of the dropping mercury electrode is under experimental control and the diffusion coefficients would not be expected to change significantly among analogous compounds then the second term of equation (27) can be neglected to give equation (46).

\[
\rho = \frac{\alpha n F}{2.303RT} \cdot \frac{\Delta E_{1/2}}{\Delta \sigma} - \frac{\Delta \log [0.386(T/D)^{1/2}]}{\Delta \sigma} \tag{45}
\]

\[
\rho = \frac{\alpha n F}{2.303RT} \cdot \frac{\Delta E_{1/2}}{\Delta \sigma} \tag{46}
\]

Inspection of equations (44) and (46) reveal that equation (44) (reversible reduction) is simply a special form of equation (46) in which the transfer coefficient \( \alpha \) is equal to 1. The assumptions used in the derivation of equation (44) and (46) were that the polarographic wave must be described by equation (10) (i.e. a plot of \( E \) versus \( \log (i_d - i)/(i) \) must be linear)

\[
E = E_{1/2}^\prime + \frac{RT}{\alpha n F} \ln[(i_d - i)/(i)] \tag{10}
\]

and that the transfer coefficient \( \alpha \) must be approximately independent of substituent effects. If these conditions do not prevail, then a normal Hammett reaction constant \( \rho \) cannot be
calculated directly from the effect of substituents on the half-wave potentials.

The effects of substituents on the half-wave potentials (Table VIII) of the Series I compounds (III) are shown in Figures 16, 17, and 18. Since the half-wave potentials were concentration dependent, (see Section 3.5 and reference 44) the polarograms were all recorded at or near a single concentration for any one correlation (10\(^{-2}\) M. in Figure 16, 2 \times 10^{-3} M. in Figure 17, and 1 \times 10^{-5} to 2 \times 10^{-5} M. in Figure 18). The half-wave potentials are plotted against \(\sigma^*\) since the standard reaction for this substituent constant [equation (33)] has the substituent attached directly to the reaction

\[
\text{RCOOR'} \xrightleftharpoons[k_{\text{CH}_3}^R]{\text{H}^+ \text{ or OH}^-} \text{RCOOH} + \text{R'O}H
\]  

(33)

\[
\sigma^* = \frac{1}{2.48} \left[ \log \left( \frac{k_R}{k_{\text{CH}_3}^R} \right)_{\text{OH}^-} - \log \left( \frac{k_R}{k_{\text{CH}_3}^R} \right)_{\text{H}^+} \right]
\]  

(47)

centre, as is the case with the Series I compounds (III). The values of \(\sigma^*\) used were calculated from Charton's values of
Figure 16. Plot of polarographic half-wave potentials of $10^{-2}$ M solutions of the Series I compounds (Table VIII) against $\sigma^*$ (Table V, page 49).

corr. coeff. = .972

$\Delta E_{1/2}/\Delta \sigma^* = 188$ mv.
Figure 17. Plot of polarographic half-wave potential of $2 \times 10^{-3} \text{ M}$ solutions of the Series I compounds (Table VIII) against $\sigma^*$ (Table V, page 49).

corr. coeff. = 0.978

$\Delta E_{1/2}/\Delta \sigma^* = 162 \text{ mv}$.
Figure 18. Plot of polarographic half-wave potentials of $10^{-5}$ M solutions of the Series I compounds against $\sigma^*$ (Table V, page 49)

$$r = .975$$

$$\Delta E_{1/2}/\Delta \sigma^* = +214 \text{ mV}.$$
\[ \sigma_I \text{ using equation (34)} \] since a more complete list of polar

\[ \sigma^* = \sigma_I / 0.45 \quad (34) \]

\[ X-CH_2COOH \rightleftharpoons X-CH_2COO^- + H^+ \quad (48) \]

\[ \sigma_I = 0.251 \log(K_X/K_H) \quad (49) \]

substituent constants are available from Charton's work than from original \( \sigma^* \) values. The values of \( \sigma^* \) which were used are listed in Table V in Section 3.1.2 (page 49). A value of \( \sigma_I \) is not given by Charton for the carbo-\( i \)-propoxy substituent and so a value of 0.34 has been assumed (\( \sigma^* = .76 \)), identical to the values reported for the carbomethoxy and carboethoxy substituents. At concentrations of \( 10^{-2} \text{ M} \) and \( 2 \times 10^{-3} \text{ M} \), half-wave potentials were reproducible to within 5 mv. for every compound so these potentials are plotted as single points in Figures 16 and 17. Near \( 10^{-5} \text{ M} \), the precision was not always as good and so statistical error limits are shown for each compound in Figure 18. Only one polarogram of the 1-carboxymethyl-3-carbamoylpyridinium ion at \( 10^{-5} \text{ M} \) concentrations was sufficiently distinct for the half-wave potential to be measured (this wave appears as only a shoulder on the background), so the error for this compound has been approximated by the worst error among the other compounds.

See footnote on page Section 3.1.2
Inspection of Figures 16, 17 and 18, reveals a common pattern at all concentrations, the only exception being the carboxy compound at $10^{-2} \text{ M}$. Two compounds consistently fall off of the best least-squares line; the methoxy compound (which has a more positive half-wave potential than would be predicted) and the acetyl compound (which has a more negative half-wave potential than would be predicted). Similar patterns will be seen in the linear free energy relationships of other reactions of the Series I pyridinium salts (III) and dihydro-pyridines (IV).

The slopes of the plots in Figures 16, 17 and 18 are similar to the slope of +220 mv. reported for a plot of three 1-alkylpyridinium salts\textsuperscript{55} but much smaller than the slope of +300 mv. reported for the variations of the half-wave potential of a series of 1-(3' or 4'-substituted phenyl)pyridinium salts (XXVII).\textsuperscript{55} Although the slope of plots of half-wave potential
against $\sigma^*$ vary with the concentration (Figures 16, 17, and 18) reaching a minimum near $2 \times 10^{-3}$ M, this variation does not seem to be large enough to account for the difference between the results of this study and the results from the 1-phenylpyridinium salts (XXVII). Another possible explanation comes from the fact that $\sigma^*$ was used in Figures 16, 17, and 18 and in Zuman's plot of the three 1-alkylpyridinium salts whereas Zuman used $\sigma_m$ and $\sigma_p$ in the plots of the phenylpyridinium salts (XXVII). \textsuperscript{55} Wells has suggested that making $\rho^*$ comparable to $\rho$ for the hydrolysis of esters does not automatically ensure that $\rho^*$ will be comparable to $\rho$ for other reactions. The difference could also be due to differences in experimental conditions which have often caused variations of as much as 50 millivolts in the reported half-wave potentials of pyridinium salts. \textsuperscript{40}

In order to calculate the reaction constant $\rho$ from the slopes of the plots in Figures 16, 17, and 18 using equation (44), the polarographic wave must be described by equation (10)

$$\rho = \frac{\alpha n F}{2.303RT} \cdot \frac{\Delta E_{1/2}}{\Delta \sigma}$$

(44)

$$E = E_{1/2} + \frac{RT}{\alpha n F} \ln[(i_d - i)/(i)]$$

(10)

and the transfer coefficient\textsuperscript{+} $\alpha$ must be independent of

\textsuperscript{+} The transfer coefficient, $\alpha$, can be calculated from the slope of a plot of $E$ vs. $\log(i_d - i)/(i)$ by the equation:

$$1/\alpha = \text{slope} \cdot \frac{nF}{2.303RT}$$
substituent effects. The former requirement is not met by polarographic waves recorded at $10^{-5} \text{ M.}$ and is only approximately met by polarographic waves recorded at $2 \times 10^{-3} \text{ M.}$ and $10^{-2} \text{ M.}$ (see Figures 10a and 11a). The second requirement, that of the transfer coefficient being constant, is not met by all compounds at either $2 \times 10^{-3} \text{ M.}$ or $10^{-2} \text{ M.}$ (Transfer coefficients cannot be calculated for polarographic waves at $10^{-5} \text{ M.}$ since these waves give curved plots of $E$ against $\log(i_d - i)/(i)$).† Despite the failure to meet the second requirement, if average values of the transfer coefficients are calculated for each of the two higher concentrations ($\alpha = 0.8$ at $2 \times 10^{-3} \text{ M.}$ and $\alpha = 0.67$ at $10^{-2} \text{ M.}$) and used in equation (44) then reaction constants $\rho^*$ of +2.1 and +2.2 are

$$\rho = \frac{\alpha nF}{2.303RT} \cdot \frac{\Delta E_{1/2}}{\Delta \sigma}$$

(44)

found for the data at $10^{-2} \text{ M.}$ and $2 \times 10^{-3} \text{ M.}$, respectively. The agreement between the $\rho^*$ values at these two concentrations is striking, considering the approximations that have been used in the calculation, and considering the 15% difference between the slopes, $\Delta E_{1/2}/\Delta \sigma^*$, in Figures 16 and 17, from which the $\rho^*$ values were calculated.

The polarographic waves at low concentrations ($10^{-5} \text{ M.}$) are not described by equation (10), thus preventing the use

†See footnote on previous page.
of equation (44) to determine a reaction constant at this

\[ E = E^{1/2} + \frac{RT}{nF} \ln \left( \frac{i_d - i}{i} \right) \]  \hspace{1cm} (10)

\[ \rho = \frac{\alpha nF}{2.303RT} \cdot \frac{\Delta E^{1/2}}{\Delta \sigma} \]  \hspace{1cm} (44)

congcentration. However, it was found (Section 3.5.1) that the polarographic waves recorded at low concentrations were quite well described by equation (18) (i.e. plots of \( E \) against

\[ E = E^o + \frac{RT}{F} \ln \left( \frac{i_d - i}{i} \right)^{2/3} \]  \hspace{1cm} (18)

\( \log \left( \frac{i_d - i}{i} \right)^{2/3} \) were linear with slopes near the theoretical value of 60 mV.), which is the equation for a one-electron reversible reduction followed by an irreversible dimerization

[reaction (41)]. The intercept, \( E^o \), of equation (18) is

\[ \text{related to the standard reduction potential by equation (19).} \]

In order to determine the reaction constant one would like to use the standard reduction potential for each compound
since this quantity is directly related to the equilibrium constant by equation (7). Combining equations (32) and (7) into (42), the reaction constant can be calculated from the

\[ \lambda_f^2 = \frac{d^2k_d}{D} \]  

(20)

effect of substituents on the standard reduction potential.

To calculate the standard reduction potential from the intercept, \( \varepsilon^o \), of equation (18), equation (19) can be rearranged and combined with equations (16) and (20) to give equation

\[ D^* = \left( \frac{3}{7} \pi D \tau \right)^{1/2} \]  

(16)

(50). The last term of equation (50) is a numerical constant

\[ \varepsilon^o = \varepsilon^o - \frac{RT}{3F} \ln \frac{3/2}{D^{1/2}} k_d - \frac{RT}{3F} \ln \frac{2}{7} \left( \frac{3}{7} \right)^{1/2} \pi^{3/2} \frac{3/2}{F} \]  

(50)
with the value of +216 millivolts when the current in equation (18) is expressed in microamperes. The second term contains the drop time $T$ of the dropping mercury electrode (which is under experimental control), the dimerization rate constant, and the diffusion coefficient. The diffusion coefficients, which can be estimated from the Ilkovic equation [equation 51)] are listed in Table XII. Unfortunately, dimerization

\[ i_d = 607 nCD^{1/2} \tau^{1/6} m^{2/3} \]  

\( (51) \)

- $n =$ number of electrons transferred
- $C =$ concentration of electroactive species (mM)
- $m =$ mercury flow rate (mgm./sec.)
- $D =$ diffusion coefficient (cm$^2$/sec.)
- $\tau =$ drop time (sec.)

rate constants have only been measured for two of the pyridinium radicals, NAD (XIIIa) and 1-methyl-3-carbamoylpyridine (XIIIb).$^{50}$

![Chemical Structures](image-url)
TABLE XII

Diffusion Coefficients of the Pyridinium Ions
Estimated from the Ilkovic Equation

<table>
<thead>
<tr>
<th>R₃</th>
<th>R₂</th>
<th>D x 10⁵ (cm²/sec.)</th>
<th>RT/3F ln(D)¹/² (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₂COO⁻</td>
<td>CONH₂</td>
<td>.89</td>
<td>-50</td>
</tr>
<tr>
<td>CH₃</td>
<td>&quot;</td>
<td>.52</td>
<td>-52</td>
</tr>
<tr>
<td>CH₂CH₂OH</td>
<td>&quot;</td>
<td>.52</td>
<td>-52</td>
</tr>
<tr>
<td>CH₂OCH₃</td>
<td>&quot;</td>
<td>.42</td>
<td>-53</td>
</tr>
<tr>
<td>CH₂COCH₃</td>
<td>&quot;</td>
<td>.42</td>
<td>-53</td>
</tr>
<tr>
<td>CH₂COOCH₃</td>
<td>&quot;</td>
<td>.37</td>
<td>-54</td>
</tr>
<tr>
<td>CH₂COOCH(CH₃)₂</td>
<td>&quot;</td>
<td>.32</td>
<td>-54</td>
</tr>
<tr>
<td>CH₂CN</td>
<td>&quot;</td>
<td>.51</td>
<td>-52</td>
</tr>
<tr>
<td>CH₂CONH₂</td>
<td>&quot;</td>
<td>.46</td>
<td>-53</td>
</tr>
<tr>
<td>CH₂CONH₂</td>
<td>COCH₃</td>
<td>.54</td>
<td>-52</td>
</tr>
<tr>
<td>CH₂CONH₂</td>
<td>CN</td>
<td>.43</td>
<td>-53</td>
</tr>
<tr>
<td>CH₂CONH₂</td>
<td>F</td>
<td>.49</td>
<td>-52</td>
</tr>
<tr>
<td>CH₂CONH₂</td>
<td>H</td>
<td>1.05</td>
<td>-49</td>
</tr>
<tr>
<td>CH₂CONH₂</td>
<td>O⁻</td>
<td>1.17</td>
<td>-49</td>
</tr>
</tbody>
</table>

Special compounds:

H₂NOC

.93 | -50

.23* | -56*
Table XII (cont.)

<table>
<thead>
<tr>
<th></th>
<th>$D \times 10^5$</th>
<th>$\frac{RT}{3F}$</th>
<th>$\ln(D)^{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(cm$^2$sec$^{-1}$)</td>
<td>(mv)</td>
<td></td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>1.97</td>
<td>-46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>.49*</td>
<td>-52*</td>
<td></td>
</tr>
<tr>
<td>NADH$^+$</td>
<td>.21</td>
<td>-56</td>
<td></td>
</tr>
<tr>
<td>NADH$^+$</td>
<td>.78</td>
<td>-50</td>
<td></td>
</tr>
</tbody>
</table>

*Calculated for a two electron reduction*
The diffusion coefficients of NAD$^+$ and 1-methyl-3-carbomoylpyridinium ion (IIIb) in various buffers have been previously reported$^{47,66}$ and average values have been used herein to calculate the values of $-650$ and $-730$ mv. (vs. NHE) respectively, for the standard reduction potentials $E^\circ$ of these two compounds. Since dimerization rate constants are unknown for the remaining compounds and, hence, their standard reduction potentials are not available, a reliable reaction constant $\rho$ based on $E^\circ$ values cannot be obtained.

If equation (50) is combined with equation (42) then

$$
E^\circ = \varepsilon^\circ - \frac{RT}{3F} \ln \frac{T^{3/2} k_d}{D^{1/2}} - \frac{RT}{3F} \ln \frac{2,3}{7} \frac{1}{2} \frac{\pi^{3/2}}{F}
$$

(50)

$$
\rho = \frac{nF}{2.303 RT} \cdot \frac{\Delta E^\circ}{\Delta \varepsilon}
$$

(42)

the reaction constant can be determined from the effect of substituents on the intercept $\varepsilon^\circ$ of equation (18) and on the

$$
E = \varepsilon^\circ + \frac{RT}{F} \ln (i_d - i)/(i)^{2/3}
$$

(18)
dimerization rate constant \( k_d \) by using equation (52) (where the \( n \) in equation (42) has been set equal to 1 and the numerical constant has been eliminated). Equation (52) can be simplified into equation (53) since the drop time \( T \) will be constant for all compounds, and since the diffusion constants, \( D \), do not vary significantly among the NAD\(^+\) model compounds. (In column 4 of Table XII, it can be seen that the variations in the diffusion coefficients result in differences of only a few millivolts in the diffusion coefficient term of equations (50) and (52).)

The second term of equation (53), which is equal to

\[
\rho = \frac{F}{2.303RT} \cdot \frac{\Delta \varepsilon^o}{\Delta \sigma} - \frac{1}{3} \frac{\Delta \log T^{3/2}}{\Delta \sigma} - \frac{\Delta \log [k_d/(D)^{1/2}]}{\Delta \sigma} \tag{52}
\]

one-third the reaction constant, \( \rho^* \), for the dimerization reaction [equations (54) and (55)] cannot be calculated very precisely but can be estimated from the two dimerization rate constants that are known\(^\dagger\) once a \( \sigma^* \) value is estimated for the reaction [equations (54) and (55)] cannot be calculated very precisely but can be estimated from the two dimerization rate constants that are known\(^\dagger\) once a \( \sigma^* \) value is estimated for the reaction centre\(^57\) but with only two rate constants known, it is impossible to know how well the dimerization rate constant is correlated by \( \sigma^* \) and, therefore, whether the estimated value of \( \rho^*_d \) is meaningful.

\(^\dagger\) Usually fairly small reaction constants have been found for radical reactions in which there is no net change in charge at the reaction centre\(^57\) but with only two rate constants known, it is impossible to know how well the dimerization rate constant is correlated by \( \sigma^* \) and, therefore, whether the estimated value of \( \rho^*_d \) is meaningful.
1-substituent of NAD\(^+\). The polarographic half-wave potential of NAD\(^+\) (Ia, page 128) is similar to the half-wave potentials of the 1-carbamoylmethyl(IIIi), 1-acetonyl-(IIIId), 1-methoxymethyl-(IIIe), 1-carbo-i-propoxymethyl-(IIIg) and 1-carbomethoxymethyl compounds (IIIi) of Series I. These substituents all have \(\sigma^*\) substituent constant values near +0.7 and so this value is tentatively assigned to the 5'- (adenosine pyrophosphate)-ribosidyl group (XXIX) attached to
the pyridine ring in NAD$^+$, allowing the reaction constant $\rho_d^*$ of -0.1 to be estimated for the dimerization reaction. This value of $\rho_d^*$ is very low, a situation sometimes found with hydrogen atom abstraction reactions. Equation (53) can now be rewritten as equation (56) in which $\Delta \log k_d/\Delta \sigma^*$ has been replaced by its estimated value of -0.1.

The reaction constant for the electrochemical reduction at low concentration can now be determined from equation (56). The intercept $\epsilon^o$ of equation (18) can be determined from the linear plots of $E$ against $\log[(i_d - i)/(i)]^{2/3}$ and this has been done for each compound (see Table VII and Table IX) at various concentrations between $10^{-5}$ M. and $2 \times 10^{-4}$ M. The values of $\epsilon^o$ for the Series I compounds (III) are plotted against $\sigma^*$ in Figure 19 and give a slope, $\Delta \epsilon^o/\Delta \sigma^*$ of -218 mv. Substituting this into equation (56) gives a reaction constant $\rho^*$ of +3.7 which is significantly different from the value of +2.2 that was found at higher concentrations ($>10^{-3}$ M.). To see if this difference is due to the two different methods of calculating the reaction constant, the half-wave potentials at $10^{-5}$ M. (Figure 12) were used [via equation (44)] to
Figure 19. Plot of polarographic reduction potentials $\varepsilon^o$ (Table IX) of the Series I compounds against $\sigma^*$ (Table V, page 49)

$\text{corr. coeff.} = 0.978$

$\Delta \varepsilon^o/\Delta \sigma^* = 216 \text{ mv.}$

$\rho^* = +3.7$
\[
\rho = \frac{\alpha n F}{2.303 R T} \cdot \frac{\Delta E_{1/2}}{\Delta \sigma} 
\]  
(44)

estimate the reaction constant for the reduction at low concentrations, using an estimated transfer coefficient \(\alpha\) of unity.† The slope, \(\Delta E_{1/2}/\Delta \sigma^*\), of Figure 18 is \(-214\) mv. from which a reaction constant of \(+3.7\) can be calculated [using equation (44)] in good agreement with the value of \(\rho^*\) calculated from equation (56). A \(\rho^*\) value of \(+3.7\) for the polarographic reduction of pyridinium salts [reaction (41)] is also in good agreement with the \(\rho^*\) value of \(+3.8\) reported for the acid dissociation of protonated methyl amines \(^76\) [equation (57)].

\[
\text{RNH}_3^+ \rightleftharpoons \text{RNH}_2^+ + \text{H}^+ 
\]  
(57)

† Unambiguous values of the transfer coefficient, \(\alpha\), cannot be calculated from the slopes of plots of \(E\) vs. \(\log(i_d - i)/(i)\) since these plots are curved for polarographic waves recorded at \(10^{-5}\) M. concentrations. The average slopes of the curves give \(\alpha \approx 1\).
To summarize, then, a reaction constant of +2.2 has been calculated for the polarographic reduction process at high concentrations while a reaction constant of +3.7 has been calculated for the reduction process at low concentrations. Although approximations were used in the calculation of both values, the difference between the two appears to be real. These results imply a dual mechanism for the polarographic reduction, a concept which will be given further consideration in the Discussion.

3.5.4 The Effects of 3-Substituents on the Polarographic Reduction Potentials

The equations developed in Section 3.5.3 for the effect of 1-substituents on the polarographic reduction can also be applied to the 3-substituents. For the reduction occurring at the electrode, [reaction (41)], one would expect \( \sigma_m' \), which
is defined from the ionization of 3-substituted benzoic acids [reaction (58)] to provide the appropriate set of substituent

\[
\begin{align*}
&\text{COOH} \\
\end{align*}
\quad \xrightarrow{R_3} \quad
\begin{align*}
&\text{COO}^- \\
\end{align*}
\quad (58)

constants. The reaction constant can be calculated from the effect of substituents on the half-wave potentials of the Series II compounds (XXII) using equation (44) if two requirements are met: (a) the polarographic waves must be described

\[
\rho = \frac{\alpha n F}{2.303 R T} \cdot \frac{\Delta E_{1/2}}{\Delta \sigma}
\]

by equation (10) and (b) the transfer coefficients \( \alpha \) must be

\[
E = E_{1/2} + \frac{R T}{\alpha n F} \frac{\ln(i_d - i)}{(i)}
\]

approximately the same for all compounds in the series. At concentrations near \( 10^{-5} \text{ M} \), the first requirement is not met for any of the compounds, which eliminates the use of equation (44) for the low-concentration data. At \( 2 \times 10^{-3} \text{ M} \) and \( 10^{-2} \text{ M} \), the first requirement is not met by the unsubstituted,
3-fluoro-, or 3-hydroxy-compounds. Since half-wave potentials could not be determined for the 3-acetyl compound at these higher concentrations (see page 67), only the 3-cyano and 3-carbamoyl compounds are left and different values of the transfer coefficient were found in this work for these two compounds. Thus, for the Series II compounds (XXII), the half-wave potentials cannot be used to determine the reaction constant for the effect of 3-substituents on the polarographic reduction.

\[\text{(III)} \quad \text{(XXII)}\]

The phenolic hydrogen of 1-carbamoylmethyl-3-hydroxypyridinium chloride was found to have a pKa of 4.7. No polarographic wave was observed for the protonated compound (IIIn) but a wave can be observed for the unprotonated compound (XXX) above pH 7. It is this form of the 3-hydroxy compound which is being discussed.

\[\text{(IIIn)} \quad \text{(XXX)}\]
As with the Series I compounds (III), polarograms recorded at concentrations below $2 \times 10^{-4}$ M. are described by equation (18), which is the expression for a reversible reduction followed by an irreversible dimerization. It has been shown in Section 3.5.3 that the reaction constant can be calculated from the effect of substituents on the intercept $E^o$ of equation (18) and on the dimerization rate constant $k_d$ by equation (53).

$$E = E^o + \frac{RT}{F} \ln \left( \frac{i_d - i}{i} \right)^{2/3}$$

(18)

The effect of 3-substituents on the dimerization rate constant is not known, but a reaction constant $\rho^*_d$ of -0.1 can be estimated from the effect of 1-substituents on this reaction. This value will be tentatively used for $\Delta \log k_d/\Delta \sigma_m$, but with the reservation that reaction constants as high as 4 have been observed in hydrogen abstractions from phenols.\textsuperscript{82}

Accepting the value of -0.1 for $\Delta \log k_d/\Delta \sigma_m$, equation (53) can be converted into equation (59) and this equation can

$$\rho_m = \frac{F}{2.303RT} \cdot \frac{\Delta E^o}{\Delta \sigma_m} + .03$$

(59)
now be used to determine the reaction constant for the effect of 3-substituents on the polarographic reduction potential.

The values of the intercept $c^0$ of equation (18) which are listed in Table IX (page 84), are plotted against $\sigma_m$ in Figure 20, with the statistical error limits for each compound shown.

The slope, $\Delta c^0/\Delta \sigma_m$, of +745 mv. gives a reaction constant $\rho_m$ of 10.6 using equation (59) a value which is so large that a reaction constant of 4 for the dimerization reaction would still not be very significant since the correction term is $1/3\rho_d$ or $\approx 1.3$. This reaction constant is much larger than the $\rho^*$ value of +3.7 for the effect of 1-substituents on this reaction and also larger than the fairly substantial value of +5.9 reported$^{78}$ for the acid dissociation constants of

\[
\begin{align*}
\text{R}_3 & \xrightarrow{e^-} \text{R}_3 \quad \rho = +10.8 \\
\text{CH}_2\text{CONH}_2 & \quad (41)
\end{align*}
\]

\[
\begin{align*}
\text{R}_3 & \xrightarrow{} \text{R}_3 + \text{H}^+ \\
\rho & = +5.9 \\
(60) & \quad (78)
\end{align*}
\]

\[
\begin{align*}
\text{R}_3 & \quad + \text{I}^- \quad \xrightarrow{} \text{R}_3 \quad \text{I}^- \\
(61) & \quad (79)
\end{align*}
\]
Figure 20. Plot of the polarographic reduction potentials $\varepsilon^o$ of the Series II compounds against meta-substituent constants $\sigma_m$

$\text{corr. coeff.} = 0.900$

$\Delta \varepsilon^o / \Delta \sigma_m = 628$

$\rho = +10.6$

$\varepsilon^o$ (mV vs. N.H.E.)

$\sigma_m$

Chemical structures with substituents: $-\text{CN}$, $-\text{COCH}_3$, $-\text{CONH}_2$, $-\text{F}$, $-\text{H}$, $-\text{O}$.
protonated pyridines. It is, however, comparable to the ρ of 13.4 obtained by Kosower\textsuperscript{79} for the variation of the charge-transfer absorbance maximum of 3- and 4-substituted-1-alkylpyridinium iodides.

Interestingly, if the Series II polarographic potentials \(\varepsilon^0\) are plotted against \(\sigma_p\), as in Figure 21, instead of against \(\sigma_m\) the correlation is markedly improved.\textsuperscript{†} A slightly larger reaction constant \(\rho\) is found in this case, 12.4 rather than 10.6. The improved correlation would seem to indicate that there is a resonance interaction involving the 3-substituent. Since resonance between 3-substituents and the ring in the pyridinium ion (XXII) is not possible, the resonance interaction must be occurring in the radical product (XXXI).

![Reaction Diagram](image)

A similar interaction has been proposed\textsuperscript{80} between the lone electron and the 3-carbamoyl group of the 1-methyl-3-carbamoylpyridine radical (XXXII) prepared by pulse radiolysis.

\textsuperscript{†} The standard reaction for \(\sigma_p\) substituent constants has a resonance interaction between the substituent and the reaction centre which is not present with the \(\sigma_m\) standard reaction.
Figure 21. Plot of polarographic reduction potentials $\varepsilon^0$ of the Series II compounds against para-substituent constants $\sigma_p^{.81}$

$r = .964$

$\Delta \varepsilon^0 / \Delta \sigma_p$

$\rho_p = 12.4$

\[ R_3 \]

\[ \text{CH}_2\text{CONH}_2 \]

$\text{F}$

$\text{CN}$

$\text{COCH}_3$

$\text{CONH}_2$

$\text{O}^-$

$\text{H}$

$\varepsilon^0$ (mV. vs. N.H.E.)

$\sigma_p$

-500

-600

-700

-800

-900

-1000

-1100

-1200

-4

-2

0

2

4

6
A stabilization of the radical products by electron withdrawing 3-substituents coupled with the destabilization of the positively charged pyridinium ion may be sufficient to explain the extraordinarily large $\rho$ for the effect of the 3-substituents on the polarographic reduction.
3.6 Reduction Potentials of Pyridinium-Dihydropyridine Half-cells

Reduction potentials of pyridinium-dihydropyridine systems were determined potentiometrically using the method developed by Rodkey.\textsuperscript{14} (see Section 5.8.) The relationship between the cell potential and the concentrations of oxidized and reduced compounds is given by the Nernst equation [equation (2)] where $E^*$ is the standard reduction potential of the half-cell.

$$E = E^* - \frac{RT}{nF} \ln \text{[Red]}/\text{[Ox]}$$ \hspace{1cm} (2)\textsuperscript{69}

For the reduction of $\text{NAD}^+$ (Ia), shown in equation (1),

$$\text{NAD}^+ + \text{H}^+ + 2e^- \rightleftharpoons \text{NADH} \quad \text{(1)}$$

at $25^\circ$, equation (2) can be rewritten as in equation (62)\textsuperscript{30}.

The validity of equations (1) and (62) for the $\text{NAD}^+ - \text{NADH}$ half-cell has been amply proven.\textsuperscript{30} Equation (62) can be

$$E = E^* - 29.6 \log \left[ \frac{[\text{NADH}]}{[\text{NAD}^+][\text{H}^+]} \right]$$ \hspace{1cm} (62)
rearranged to equation (63) and then into equation (65), thus allowing the reduction potential at any pH, \( E_{\text{pH}}^{\circ} \), to be determined as the intercept of a plot of cell potential, \( E \), against \( \log([NADH]/[NAD^+] \). The validity of equation (63) has been tested for the NAD\(^+\) model compounds used in this study by plotting the measured cell potential, \( E \), against \( \log([Py^+]/[PyH]) \) as shown in Figures 22 and 23 for 1-methoxy-methyl-3-carbamoylpyridinium chloride (IIIc) and 1-(2'-hydroxy-ethyl)-3-carbamoylpyridinium chloride (IIIc), respectively.

\[
E = E^{\circ} - 29.6(pH) - 29.6 \log([NADH]/[NAD^+]) \tag{63}
\]

\[
E_{\text{pH}}^{\circ} = E^{\circ} - 29.6(pH) \tag{64}
\]

\[
E = E_{\text{pH}}^{\circ} - 29.6 \log([NADH]/[NAD^+]) \tag{65}
\]

\[c: R_1 = CH_2CH_2OH\]

\[e: = CH_2OCH_3\]

(III)

\(\dagger\)The symbols Py\(^+\) and PyH refer to the pyridinium ion and dihydropyridine respectively.
Figure 22. Plot of measured cell potentials against log[Py⁺]/[PyH] for the pyridinium-dihydropyridine half-cell of 1-methoxymethyl-3-carbamoylpyridinium chloride

pH = 8.8; 0.1 M Tris buffer
corr. coeff. = .996
slope = 29.5 mv.
$E^\circ = -357$ mv.

$E$ (mv. v. s. N.H.E.)

-370  -360  -350  -340  -330

$\log[Py⁺]/[PyH]$

-0.4  -0.2  -0.1  0.1  0.3  0.5  0.7
Figure 23. Plot of measured cell potentials against $\log[\text{Py}^+]/[\text{PyH}^{-}]$ for the pyridinium-dihydropyridine half-cell of 1-(2'-hydroxyethyl)-3-carbamoylpyridinium chloride.

$\text{pH} = 9.7$, 0.1 M. glycine buffer
corr. coeff = .967
slope = 29.7 mv.
$E^\circ_{9.7} = -441$ mv.
The slopes of these plots, listed in the third column of Table XIII, are in most cases quite close to the theoretical slope of 29.6 mv., thus confirming that equation (66) applies to the NAD$^+$ model compounds as well as to NAD$^+$. The intercepts of plots of $E$ against log[PyH]/[Py$^+$] give the reduction potentials $E_{\text{pH}}^{\circ'}$ listed in the fifth column of Table XIII. The fourth column of Table XIII lists the pH's at which the potential measurements were made with each compound.

Reduction potentials of biologically important compounds are often quoted at pH 7 (physiological pH) rather than at pH 0 (unit activity of hydrogen ion) but the reduction potentials of many pyridinium salts cannot be directly measured at pH 7 (decomposition reactions of pyridinium ions and dihydropyridine have been discussed in Sections 3.1 through 3.3). Reduction potentials measured at a pH other than 7 can theoretically be corrected to pH 7 by equation (67) which is a modified form of equation (64). The validity of equations (64) and (67) for the NAD$^+$ model compounds used in this study was tested using

\[
E_{\text{pH}}^{\circ'} = E^{\circ} - 29.6(\text{pH}) \quad (64)
\]

\[
E_{7}^{\circ'} = E_{\text{pH}}^{\circ'} + 29.6(7-\text{pH}) \quad (67)
\]
TABLE XIII
Reduction Potentials for Pyridinium/Dihydropyridine Half-cells

\[
\text{R}_1 \text{R}_3^+ + \text{H}^+ + 2e^- \rightleftharpoons \text{R}_1 \text{R}_3 \text{H}_2
\]

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₃</th>
<th>slope of E vs log [PyH]/[Py⁺] (mv)</th>
<th>pH</th>
<th>E°' (mv vs N.H.E.)</th>
<th>E°' (mv vs N.H.E.)</th>
<th>E° (mv vs N.H.E.)</th>
<th>std. dev. (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₂COO⁻</td>
<td>CONH₂</td>
<td>-34.6</td>
<td>10.</td>
<td>-439</td>
<td>-350</td>
<td>-143</td>
<td>5.0</td>
</tr>
<tr>
<td>CH₃</td>
<td>CONH₂</td>
<td>-22.3</td>
<td>10.</td>
<td>-463</td>
<td>-374</td>
<td>-167</td>
<td>7.4</td>
</tr>
<tr>
<td>CH₂CH₂OH</td>
<td>CONH₂</td>
<td>-29.7</td>
<td>9.7</td>
<td>-441</td>
<td>-361</td>
<td>-154</td>
<td>4.1</td>
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<tr>
<td>CH₂OCH₃</td>
<td>CONH₂</td>
<td>-29.5</td>
<td>8.8</td>
<td>-357</td>
<td>-304</td>
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<td>1.1</td>
</tr>
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<td>CH₂COCH₃</td>
<td>CONH₂</td>
<td>-26.2</td>
<td>8.8</td>
<td>-381</td>
<td>-328</td>
<td>-121</td>
<td>4.5</td>
</tr>
<tr>
<td>CH₂COOCH(CH₃)₂</td>
<td>CONH₂</td>
<td>-24.7</td>
<td>7.0</td>
<td>-318</td>
<td>-318</td>
<td>-111</td>
<td>2.9</td>
</tr>
<tr>
<td>CH₂CN</td>
<td>CONH₂</td>
<td>-28.3</td>
<td>5.6</td>
<td>-223</td>
<td>-263</td>
<td>56</td>
<td>1.7</td>
</tr>
<tr>
<td>CH₂CONH₂</td>
<td>CONH₂</td>
<td>-29.2</td>
<td>9.2</td>
<td>-385</td>
<td>-320</td>
<td>-113</td>
<td>1.6</td>
</tr>
<tr>
<td>CH₂CONH₂</td>
<td>COCH₃</td>
<td>-27.3</td>
<td>8.8</td>
<td>-331</td>
<td>-278</td>
<td>-71</td>
<td>1.6</td>
</tr>
<tr>
<td>CH₂CONH₂</td>
<td>CN</td>
<td>-31.9</td>
<td>7.0</td>
<td>-225</td>
<td>-225</td>
<td>-18</td>
<td>3.7</td>
</tr>
</tbody>
</table>
### Table XIII (cont.)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Slope of $E$ vs log$[PyH]/[Py^+]$ (mv)</th>
<th>pH</th>
<th>$E^\circ_{PH}$ (mv vs N.H.E.)</th>
<th>$E^\circ_7$ (mv vs N.H.E.)</th>
<th>$E^\circ$ (mv vs N.H.E.)</th>
<th>std. dev. (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD$^+$ (Ia, page 128)</td>
<td>-</td>
<td>8.8</td>
<td>-365</td>
<td>-312</td>
<td>-105</td>
<td>5.7</td>
</tr>
<tr>
<td>NMN$^+$ (II, page 128)</td>
<td>30.3</td>
<td>8.9</td>
<td>-358</td>
<td>-302</td>
<td>-95</td>
<td>4.5</td>
</tr>
<tr>
<td>H$_2$NOC-</td>
<td>-</td>
<td>6.9</td>
<td>-279</td>
<td>-282</td>
<td>-</td>
<td>15.</td>
</tr>
</tbody>
</table>
l-carbamoylmethyl-3-carbamoylpyridinium chloride (IIIi)
between pH 7.7 and 9.6 as shown in Figure 24. The triangles

\[
\begin{align*}
&\text{ONH,} \\
&\text{CH}_2\text{CONH}_2
\end{align*}
\]

(IIIi)

represent potentials measured in glycine buffer, the diamonds
potentials measured in Tris buffer. Potentials measured at
pH's greater than 9.8 tended to be much less reproducible than
at lower pH's and were therefore not included. (This problem
was encountered again in the measurement of the reduction
potentials of l-carboxymethyl-(IIIa), l-methyl-(IIb), and
1-(2'-hydroxyethyl)-3-carbamoylpyridinium ions which had to
be made between pH 9.7 and 10.4). The reduction potentials

\[
\begin{align*}
&\text{CONH}_2 \\
&\text{N} \\
&\text{R}_1
\end{align*}
\]

(III)

a: \( R_1 = \text{CH}_2\text{COO}^- \)
b: \( = \text{CH}_3 \)
c: \( = \text{CH}_2\text{CH}_2\text{OH} \)

at each pH, \( E^0' \), were calculated from equation (63) and
plotted against pH in Figure 24, from which a pH dependence,
Figure 24. The pH dependence of the reduction potential, $E^o_{pH}$, of 1-carbamoylmethyl-3-carbamoylpyridinium chloride.
\[ E_{\text{pH}}^0 = E - 29.6 \log[\text{PyH}]/[\text{Py}^+] \] (66)

\[ \Delta E_{\text{pH}}^0/\Delta \text{pH}, \text{ of } 30.5 \text{ mV. per pH unit was found, in good agreement with the theoretical dependence of 29.6 mV. per pH unit.} \]

Having confirmed that the pH dependence of the potentiometric reduction potentials is as expected, equations (64) and (67) can be used to calculate the standard reduction potential,

\[ E^0 = E_{\text{pH}}^0 + 29.6(\text{pH}) \] (64)

\[ E_7^0 = E_{\text{pH}}^0 + 29.6(\text{pH}-7) \] (67)

\( E^0 \), and the reduction potential at pH 7, \( E_7^0 \). These values are listed in the seventh and eighth columns of Table XIII respectively.

The last column of Table XIII lists the standard deviations of the reduction potentials of each compound determined in the following way. Equation (66) was used to calculate a value of \( E_{\text{pH}}^0 \) from each measured cell potential, \( E \), and a standard deviation was then calculated from the collection of \( E_{\text{pH}}^0 \) values. This method gives a better estimate of the error in the reduction potentials than would be obtained from the standard deviations of the measured cell potentials about the
best least-squares line for each compound.

The reduction potential of \( \text{NAD}^+ \) (III) has been determined previously\(^{14,32} \) so this compound was not studied extensively. The potential was first determined in this study by an oxidative titration of \( \text{NADH} \) (IV), similar to the method used by Rodkey\(^ {14} \). Throughout the oxidative titration the calculated reduction potential (using equation (65)) become more positive,

\[
E_{pH}^{\circ} = E - 29.6 \log[\text{NADH}]/[\text{NAD}^+] \tag{65}
\]

as shown in Table XIV and when the final solution was assayed for \( \text{NAD}^+ \) polarographically, it was found that the solution contained two pyridinium salts in approximately a 50:50 mixture with the second pyridinium ion appearing at a slightly more negative polarographic reduction potential than \( \text{NAD}^+ \).
<table>
<thead>
<tr>
<th>[NADH] (mM)</th>
<th>[NAD(^+)] (mM)</th>
<th>E (mV)</th>
<th>E(^o)' (mV) (_{PH})</th>
<th>E(^o)' (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.26</td>
<td>0.40</td>
<td>-392</td>
<td>-370</td>
<td>-320</td>
</tr>
<tr>
<td>1.67</td>
<td>0.93</td>
<td>-368</td>
<td>-361</td>
<td>-311</td>
</tr>
<tr>
<td>1.11</td>
<td>1.54</td>
<td>-355</td>
<td>-359</td>
<td>-309</td>
</tr>
<tr>
<td>0.53</td>
<td>2.02</td>
<td>-334</td>
<td>-351</td>
<td>-301</td>
</tr>
</tbody>
</table>
Since the NAD$^+$ sample was found to be homogeneous in polarographic studies at pH 5.6, it was concluded that the NAD$^+$ and/or the NADH must be decomposing at the higher pH of 8.7, where the potentiometric measurements were made. In an attempt to measure the NAD$^+$/NADH potential before decomposition became significant, single solutions were prepared containing both NAD$^+$ and NADH (method of mixtures) and the contents were analyzed as soon as possible after the measured cell potential had reached a constant value. The polarographic assay for NAD$^+$ showed that approximately 15% of the NAD$^+$ had again been converted to another pyridinium compound in the approximately 2 hours that the crystals had been dissolved in the buffer. Values of -309 mv. and -312 mv. were calculated$^+$ for the reduction potential at pH 7, $E^\circ_7$, from the measured cell potentials of these solutions but since only the total dihydropyridine concentration and not the concentration of NADH can be determined these values can be considered as no better than rough estimates. Despite this the values are in fair agreement with previously reported potentials for this system.

When solutions containing NAD$^+$ and NADH were left overnight, the cell potentials slowly drifted to more positive

$^+$Since the concentration of NADH itself could not be determined, the total pyridinium and dihydropyridine concentrations were used in equation (63) to calculate $E^\circ_7$. 
potentials and, after the solutions were assayed for pyridinium and dihydropyridine compounds, reduction potentials, $E^0'$, of approximately -300 mv. were calculated [equations (65) and (67)]. Since the polarographic assay showed that the solution contained only one pyridinium ion, this reduction potential probably represents the value of $E^0'_7$ for the decomposition product of NAD. This potentiometric reduction potential of -300 mv. and the polarographic reduction potential being slightly more negative than for NAD$^+$ are consistent with the decomposition product being the mononucleotide, NMN (II), arising from hydrolysis of the pyrophosphate bond in NAD$^+$ (Ia).

\[\text{H}_2\text{O, pH 8.7}\]

\[\text{Ia} \rightarrow \text{II} + \text{II}^+\]
Since the decomposition of NAD$^+$ (Ia) at pH 3.8 causes the measured cell potential to drift to more positive potentials, then the estimated value of $-312$ mV. for $E^\circ_7'$ is probably a few millivolts more positive than the true reduction potential of NAD$^+$. This puts $E^\circ_7'$ very close to the reduction potentials determined for 1-carbo-ι-propanoylmethyl- and 1-carbamoylmethyl-3-carbamoylpyridinium chlorides. The reduction potential of nicotinamide mononucleotide (II) is about 10 to 15 mV. more positive than that for NAD$^+$, falling very close to the reduction potential of 1-methoxymethyl-3-carbamoylpyridinium chloride.

The reduction potential of 1,1'-ethylenebis(3-carbamoylpyridinium chloride) (XIXb) has been estimated in this work for the two electron reduction of one ring. There is

\[
\begin{align*}
\text{H}_2\text{NOC} & \quad \text{CONH}_2 \\
\text{(XIXb)} & \\
+ \quad \text{H}^+ & + \quad 2\text{e}^- \\
\text{H}_2\text{NOC} & \quad \text{CONH}_2 \\
\text{(XXXII)}
\end{align*}
\]
some doubt as to the accuracy of the value found since compound XXXII could not be recovered during attempted syntheses. For the potentiometric determinations, XXXII was prepared in situ by reduction of XIXb with sodium dithionite. The concentrations of both XIXb and XXXII were measured spectrophotometrically, but in order to do this, extinction coefficients of both compounds had to be determined. An extinction coefficient for XIXb of 9.02 mM$^{-1}$cm$^{-1}$ at 265 nm. was found which is in good agreement with the value of 8.99 reported by Craig et al. A solution of XIXb was prepared and partially reduced by the addition of small amounts of sodium dithionite. The concentration of XXXII was determined after each addition of dithionite from the decrease in absorbance at 265 nm. The concurrent increase in absorbance at 340 nm. was then used to calculate an extinction coefficient of 12.5 for XXXII at this wavelength. Since there was no noticeable absorbance peak near 290 nm. in these solutions, it was assumed that the acid catalyzed decomposition (see section 3.1) of the dihydropyridine ring of XXXII was not occurring to a significant extent. The extinction coefficient of 12.5 mM$^{-1}$cm$^{-1}$ for XXXII is a value of 6.25 mM$^{-1}$cm$^{-1}$ per pyridine ring which is comparable to the extinction coefficients found in this work for other 3-carbamoyl-1,4-dihydropyridines at this wavelength (5.5 to 6.5 mM$^{-1}$cm$^{-1}$). Unfortunately, there is no way of distinguishing between XXXII and the fully reduced compound (XXXIII) although it is probably significant that during dithionite titrations of XIXb the acid catalyzed
decomposition of the dihydropyridine ring became noticeable only after approximately 1 equivalent of dithionite had been added. On the basis of inductive effects (see section 3.1) one would expect XXXIII to undergo acid decomposition more readily than the 1,4-dihydropyridine ring of XXXII so it seems that reduction of the second ring does not reach significant levels until the first ring has been almost fully reduced. Thus the observed potentials are probably reasonably close to the true reduction potential of XIXb.

3.6.1 Substituent Effects at the 1-position

The reaction constant \( \rho \) is a measure of the effect of substituents on a reaction and for a reversible reaction can be determined from the effect of substituents on the equilibrium constant [equation (32)]. For an oxidation-reduction

\[
\rho = \frac{\Delta \log K}{\Delta \sigma} \quad (32)
\]
reaction, the equilibrium constant is related to the standard reduction potential, $E^o$, by equation (7). Combining equations

$$E^o = \frac{RT}{nF} \ln K$$  \hspace{1cm} (7)

(32) and (7) into equation (42) allows the reaction constant $\rho$ to be determined from the effect of substituents on the standard reduction potential.

The standard reduction potentials (listed in Table XIII) for the Series I compounds (III) are plotted against $\sigma^*$ in Figure 25. The error limits shown for each are the standard deviations listed in Table XIII. As was observed with the polarographic reduction, the carboxymethyl- and methoxymethy1-compounds have more positive reduction potentials than would be expected. The best least-squares line (excluding the
Standard Reduction Potential, $E^\circ$ (mv. vs. N.H.E.)

Figure 25. Plot of standard reduction potentials for the pyridinium-
dihydropyridine half-cells of the Series I compounds against $\sigma^*$ (Table IV)

$\Delta E^\circ/\Delta \sigma^* = 83$ mv.

$\rho^* = +2.8$

corr. coeff. $= 0.98$ (excl. -CH$_2$COOH)
1-carboxymethyl compound has a slope, $\Delta \bar{\rho}^\circ / A \sigma^*$, of +83 mv. from which a $\rho^*$ of +2.8 can be calculated using equation (42). This value is considerably smaller than the $\rho^*$ of +3.8 reported for the acid dissociation of protonated methyl amines [equation (57)], a reaction in which one would expect a

$$\begin{align*}
\text{CONH}_2^- + H^+ + 2e^- & \rightleftharpoons \text{CONH}_2^-
\end{align*}$$

(68)

$$R-N^+H_3 \rightleftharpoons R-NH_2 + H^+$$

(57)

similar substituent effect since both reaction (68) and (57) have substituents attached to a positively charged quaternary nitrogen atom which is transformed into an uncharged nitrogen in the products.

### 3.6.2 Substituent Effects at the 3-position

The data is not really sufficient to allow a proper analysis of the effect of the 3-substituent on the reaction shown in equation (68). Of the original six compounds in Series II (XXIII to XXIN) the Py$^+$PyH reduction potentials of only the 3-carbamoyl-, 3-acetyl-, and 3-cyano-compounds could be measured. At a pH as high as 10.5, 1-carbamoylmethyl-3-fluoro-1,4-dihydropyridine was decomposed (see Section 3.2)
too rapidly for potentiometric measurements to be made, and the reduced forms of the 3-hydroxy and 3-proto compounds could not be prepared.

\[
\text{XXII} \quad \text{XXXIV}
\]

\[
\begin{align*}
R_3 + H^+ + 2e^- & \rightleftharpoons \text{R}_3 \text{CH}_2\text{CONH}_2 \\
(68)
\end{align*}
\]

\[
\begin{align*}
i: & \quad R_3 = \text{CONH}_2 \\
j: & \quad \text{COCH}_3 \\
k: & \quad \text{CN} \\
l: & \quad \text{F} \\
m: & \quad \text{H} \\
n: & \quad \text{OH}
\end{align*}
\]

When the standard reduction potentials of the three Series II compounds are plotted against \(\sigma_m\) a slope, \(\Delta\epsilon^\circ/\Delta\sigma_m\), of +276 mv. (corr. coeff. = .986) is found from which a \(\rho_m\) of +9.3 can be calculated using equation (42).

\[
\rho = \frac{nF}{2.303RT} \cdot \frac{\Delta\epsilon^\circ}{\Delta\sigma} \quad (42)
\]

As was found with the polarographic reduction potentials, the effect of the 3-substituents on the potentiometric reduction potentials is better correlated by \(\sigma_p\) (corr. coeff. = .9994) giving a slope
of +317 mv. from which a reaction constant of 10.7 can be calculated, a slightly larger value than is calculated from the $\rho_m$ plot. The values are both considerably larger than the already substantial reaction constant of 5.9 reported\textsuperscript{77} for the acid dissociation of protonated pyridines [reaction (53)]

\[ \text{R, } \text{N} \text{CH}_2\text{CONH}_2 + \text{H}^+ + 2e^- \rightleftharpoons \text{R, } \text{N} \text{CH}_2\text{CONH}_2 \]  

(68)

\(\rho = +9.3\) to +10.7

but are quite similar to the $\rho$ of +10.8 to +12.4 found in this work for the effect of 3-substituents on the polarographic reduction of pyridinium ions, [equation (41)].

\[ \text{R, } \text{N} \text{CH}_2\text{CONH}_2 + e^- \rightleftharpoons \text{R, } \text{N} \text{CH}_2\text{CONH}_2 \]  

(41)

\(\rho = +10.8\) to +12.4
3.7 The Oxidation of Dihydropyridines by Flavins

The oxidation by flavins [equation (69)] is one of the more facile oxidation-reduction reactions of 1,4-dihydropyridines. The rate constants can readily be determined anaerobically by following the rate of reduction of the flavin in the presence of excess dihydropyridine, or aerobically by following the rate of disappearance of the 340 nanometer dihydropyridine absorbance band in the presence of a catalytic amount of flavin. In the latter method, the flavin is rapidly reoxidized by oxygen. Both methods have been used previously to study the oxidation of various dihydropyridines by various flavins at various temperatures. The former method has been used exclusively in this work.
The rate constants for the oxidation of most of the dihydropyridines were found using equation (70) for a second-order irreversible reaction by plotting the quantity on the left side of equation (70) against time as shown in Figure 26.

The slope of this plot is then the second-order rate constant, \( k_2 \). The quantity \( X \) in equation (70) was calculated from the absorbance at 450 nanometers, which is due solely to the oxidized and reduced flavin, using equation (71).

\[
X = \frac{(A_0 - A)}{l(\varepsilon_{\text{reac}} - \varepsilon_{\text{prod}})}
\]

where \( A = \) absorbance at time
\( A_0 = \) initial absorbance
\( l = \) path length of the absorbance cell
\( \varepsilon_{\text{reac}}, \varepsilon_{\text{prod}} = \) extinction coefficients of reactant (oxidized flavin) and product (reduced flavin) respectively.
Figure 26. The determination of the second order rate constant for the oxidation of NADH by riboflavin from a plot of \( \log\left(\frac{1}{a_0 - b_0}\right) \) against time.

\[ k_2 = 2.303 \times \text{slope} = 0.032 \text{ M}^{-1}\text{min}^{-1} \]
The curvature at longer times is probably due to reoxidation of the flavin by oxygen leaking into the cell.

The rate of reaction of FNM with 1-carbamoylmethyl-3-cyano-1,4-dihydropyridine (Vk) was determined from pseudo-first order kinetics, shown in Figure 27, since the dihydropyridine was present in an excess of more than 100 times the concentration of FNM. The pseudo-first order rate constant, \( k_{\text{obs}} \), can be found from a plot of \( \ln(A - A_\infty) \) against time as shown below. The curvature at longer times is probably due to reoxidation of the flavin by oxygen leaking into the cell. Since the concentration of dihydropyridine was so much larger than that of the flavin one can write equation (72). The

\[
- \frac{d[F1]}{dt} = k_2 [F1][PyH] = k_{\text{obs}} [F1] \tag{72}
\]

where \( k_{\text{obs}} = k_2 [PyH] \) \tag{73}

concentration of oxidized flavin, [F1], can be found from the absorbance by rearranging (23) to give (26) and (74).
Figure 27. Determination of the second order rate constant for the oxidation of 1-carbamoylmethyl-3-cyano-1,4-dihydropyridine by FNM from a pseudo-first order plot of $\ln(A - A_\infty)$ against time.

\[
\frac{d[A]}{dt} = -k_2[FM] = k_{obs}\cdot[A]_0
\]

\[k_{obs} = 0.350 \text{ molarity}^{-1} \text{ min}^{-1}\]
\[
\frac{\Delta}{x} = \varepsilon_{\text{react}} C_{\text{react}} + \varepsilon_{\text{prod}} C_{\text{prod}} \tag{23}
\]

\[
C_{\text{react}} = \frac{(A/\ell) - \varepsilon_{\text{prod}} C_{\text{initial}}}{\varepsilon_{\text{react}} - \varepsilon_{\text{prod}}} \tag{25}
\]

Substitution of equation (74) into (62) gives (28) which

\[
A_\infty = \varepsilon_{\text{prod}} C_{\text{initial}} \tag{26}
\]

\[
C_{\text{react}} = \frac{A - A_\infty}{\ell (\varepsilon_{\text{react}} - \varepsilon_{\text{prod}})}
\]

or

\[
[\text{Fl}] = \frac{A - A_\infty}{\ell (\varepsilon_{\text{Fl}} - \varepsilon_{\text{FlH}_2})} \tag{74}
\]

integrates to equation (29). The second order rate constant

\[
- \frac{d[A - A_\infty]}{dt} = k_{\text{obs}} [A - A_\infty] \tag{28}
\]

\[
\ln(A - A_\infty) = k_{\text{obs}} t + \text{const.} \tag{29}
\]

can then be found from \( k_{\text{obs}} \) using equation (73).

The rate of oxidation of NADH (XXI) by flavins has been reported by various authors.\textsuperscript{20,22-24,26} Suelter and Metzler\textsuperscript{20} found a second order rate constant of 0.75 M\(^{-1}\)·sec\(^{-1}\) for the aerobic oxidation of NADH by riboflavin (Xa) near pH 7. Rate constants for the same oxidation using FMN (Xb) have been
determined aerobically\textsuperscript{22} and anaerobically\textsuperscript{23,24} at pH 7 and range from 0.092 M\textsuperscript{-1} sec\textsuperscript{-1} to 1.1 M\textsuperscript{-1} sec\textsuperscript{-1}, after being corrected to 25°C. using an activation energy of 8.3 kcal/mole.\textsuperscript{23}

Bruice found a rate constant of 0.43 M\textsuperscript{-1} sec\textsuperscript{-1} for the oxidation of NADH at pH 7.7 by lumiflavin (X\textsubscript{d}), a flavin which had
previously been shown to react with 1-propyl-3-carbamoyl-1,4-
dihydropyridine (XX) at the same rate as does riboflavin.  

\[
\text{CO}_2\text{H}
\]

(XX)

The diverse rate constants reported in the literature do not facilitate comparisons with the present work. Several authors\textsuperscript{22,24,26} have also determined the rate constants for the oxidation of both 1-propyl-3-carbamoyl-1,4-dihydropyridine and NADH under similar conditions but the ratios of these two rate constants vary from 65 to 160, again a discouragingly wide range. As a result, only order-of-magnitude comparisons can be made between this study and previous work.

The second-order rate constants determined in this study are listed in Table XV. NADH was oxidized by riboflavin at a rate of $0.53 \text{ M}^{-1} \text{sec}^{-1}$, a value which is reasonably consistent with previous rates using riboflavin\textsuperscript{20} and lumiflavin.\textsuperscript{26} At pH 8.1, FMN and riboflavin were equally effective at oxidizing 1-acetonyl-3-carbamoyl-1,4-dihydropyridine. The literature is very confusing on this point since reports have FMN reacting both 50% faster\textsuperscript{20} and 40% slower\textsuperscript{19} than riboflavin at pH 7. Dihydronicotinamide mononucleotide (NMNH, XXXV) was
TABLE XV

Second Order Rate Constants for the Oxidation of Dihydropyridines by Flavins

\[
\begin{align*}
\text{R}_1 & \quad \text{R}_3 & \quad \text{Flavin} & \quad \text{pH} & \quad k_2 (M^{-1} \text{-sec}^{-1}) \\
\text{CH}_3 & \quad \text{CONH}_2 & \quad \text{Rfl} & \quad 9.8-10.3 & \quad 64 & \quad \pm 13. \\
\text{CH}_2\text{CH}_2\text{OH} & \quad \text{CONH}_2 & \quad \text{Rfl} & \quad 9.8 & \quad 21 & \quad \pm 2.6 \\
\text{CH}_2\text{OCH}_3 & \quad \text{CONH}_2 & \quad \text{Rfl} & \quad 8.1-8.7 & \quad 0.607 & \quad \pm 0.019 \\
\text{CH}_2\text{COCH}_3 & \quad \text{CONH}_2 & \quad \text{Rfl} & \quad 8.1 & \quad 3.70 & \quad \pm 0.24 \\
& & \quad \text{FMN} & & \quad 3.70 & \quad \pm 0.23 \\
\text{CH}_2\text{COOCH}_3 & \quad \text{CONH}_2 & \quad \text{FMN} & \quad 7.8 & \quad 1.99 & \quad \pm 0.18 \\
\text{CH}_2\text{CN} & \quad \text{CONH}_2 & \quad \text{FMN} & \quad 8.2 & \quad 0.150 & \quad \pm 0.022 \\
\text{CH}_2\text{CONH}_2 & \quad \text{CONH}_2 & \quad \text{FMN} & \quad 7.8 & \quad 2.97 & \quad \pm 0.36 \\
& & & & \quad 9.4 & \quad 2.98 & \quad \pm 0.09 \\
\text{CH}_2\text{CONH}_2 & \quad \text{COCH}_3 & \quad \text{Rfl} & \quad 8.1 & \quad 0.548 & \quad \pm 0.019 \\
\text{CH}_2\text{CONH}_2 & \quad \text{CN} & \quad \text{FMN} & \quad 6.9 & \quad .0449 & \quad \pm .0032 \\
\text{Special Compounds:} & & & & & \\
\text{NADH (XXIV)} & \quad \text{Rfl} & & \quad 8.1 & \quad .53 \\
\text{NMNH (XXXV)} & \quad \text{FMN} & & \quad 8.1 & \quad .249 & \quad \pm .012
\end{align*}
\]
oxidized at approximately half the rate of NADH at pH 3.1.

\[
\text{XXXV}
\]

Although the absolute rate constants are very different, McCormick et al.\(^{22}\) have found a similar 2:1 ratio for the oxidation rates of NADH and NMNH. Riboflavin oxidizes 1-methyl-3-carbamoyl-1,4-dihydropyridine at 150 times faster than NADH, which is comparable to the 65 to 160 fold rate enhancements of 1-propyl-3-carbamoyl-1,4-dihydropyridine over NADH reported previously.\(^{22,24,26}\) The remaining dihydropyridines have not been studied previously.

The effect of substituents on reaction (69) can be

\[
\text{XXXVI}
\]
measured via the reaction constant, $\rho$, which is determined from equation (35) using a plot of $\log k$ against Hammett substituent constants $\sigma$. The logarithms of the rate constants for the Series I compounds (IV) are plotted against the polar substituent constants $\sigma^*$, (listed in Table V, page 49) in Figure 28. As was found in the linear free energy relationships of the polarographic and potentiometric reduction potentials, the 1-methoxymethyl compound falls off the line formed by the other compounds. Interestingly, the oxidation rate of NADH is quite close to that of the 1-methoxymethyl compound. Excluding the point for 1-methoxymethyl-3-carbamoyl-1,4-dihydropyridine (Ve), the correlation is quite good (corr.
Figure 28. Plot of the logarithm of the second order rate constants for the oxidation of the series I dihydropyridines by flavins against $\sigma^*$ (Table IV, page 43).

$corr. \text{ coeff.} = 0.992$ (excl. $\text{CH}_2\text{OCH}_3$)

$\rho^* = \frac{\Delta \log k_2}{\Delta \sigma^*} = -1.9$
coeff. = 0.992), giving a slope (equivalent to $\rho^*$) of -1.9. This value is approximately one-half of the magnitude of found for the polarographic (one-electron) reduction ($\rho^* = +3.7$) and approximately three-fourths of the magnitude of $\rho^*$ found for the potentiometric (two-electron) reduction ($\rho^* = +2.8$).

The Series II compounds for which rate constants could be determined, the 3-carbamoyl, 3-acetyl-, and 3-cyano-compounds (XXIV i-k), lead to reaction constant ($\rho$) values of -5.4

$\log k_2$ is plotted against $\sigma_m$ and $\sigma_p$ respectively. As with both the polarographic and potentiometric reductions, the $\rho$ derived from the para-substituent constants is slightly larger in magnitude and has a slightly better correlation coefficient.

![Diagram](image-url)
4. DISCUSSION

4.1 Polarographic Reduction Mechanisms

The generally accepted mechanism for the electrochemical reduction of pyridinium salts (VIII) is shown in Scheme 3. The portion of this scheme which corresponds to the first reduction wave (wave I in Figure 2) is reproduced in reaction (41). The pyridinium salt (VIII) undergoes a reversible one-electron reduction to give the pyridine radical (XV) which rapidly dimerizes in an irreversible reaction to give XVI.
An equation has been derived to describe the polarographic wave when a mechanism as shown in reaction (41) is operating (equation 18). The validity of this equation in the polarographic reduction of pyridinium ions was tested in this work by plotting the potential, $E$, against $\log(\frac{i_d - i}{i})^{2/3}$ for polarographic waves at various concentrations between $10^{-5}$ M. and $10^{-2}$ M. Polarographic waves recorded at concentrations below $5 \times 10^{-4}$ M. were reasonably well described by equation (18), (Plots of $E$ vs. $\log(\frac{i_d - i}{i})^{2/3}$ were linear with slopes near the theoretical value of 60 mv.) in agreement

$$E = E^o + \frac{RT}{F} \ln(\frac{i_d - i}{i})^{2/3}$$  \hspace{1cm} (18)
with the mechanism shown in equation (41). Thus it can be concluded that this mechanism is operating (i.e., the electrode reaction is reversible) at pyridinium ion (VIII) concentrations less than \(5 \times 10^{-4}\) M.

At concentrations above \(10^{-3}\) M, the polarographic waves are not described by equation (18), but instead give near-linear plots when the potential, \(E\), is plotted against \(\log (i_d - i)/(i)\).\(^\dagger\) The slopes of these latter plots are significantly larger than the theoretical slope for a reversible reduction (60 mv.) indicating an irreversible electrode reaction. This transition from a reversible electrode

\[ E = E_{1/2} + \frac{RT}{\alpha F} \ln \left( \frac{i_d - i}{i} \right) \]  

\(^\dagger\) A linear plot of \(E\) vs. \(\log (i_d - i)/(i)\) is indicative of a reversible or irreversible electrode reaction which is unaffected by previous or subsequent chemical reactions.
reaction (conc. < $5 \times 10^{-6}$ M.) to an irreversible electrode reaction (conc. > $10^{-3}$ M.) corresponds to the maximum height of the prewave (i.e. a monolayer of material has been adsorbed on the electrode) for compounds which exhibited a prewave. Various studies\textsuperscript{5, 39, 40, 47, 66} have demonstrated that the dimeric products (XVI) of the reduction are strongly adsorbed on the electrode and that this adsorbed layer can inhibit the electrode reaction.\textsuperscript{39} Thus, after a monolayer of dimeric product (XVI) has been adsorbed, the reduction of the bulk of the pyridinium ion is inhibited sufficiently that the electrode reaction becomes rate-determining. Apparently, the dimeric product (XVI) is adsorbed in a multi-layer fashion since increasing concentration (from $2 \times 10^{-3}$ M. to $10^{-2}$ M.) causes a further retardation of the electrode reaction, with a resulting increase in slope of a plot of applied potential $E$ against $\log (i_{d} - i)/(i)$.

The concept of a reversible electrode reaction at low concentrations (less than $5 \times 10^{-4}$ M.) of pyridinium ion and
an irreversible electrode reaction at high concentrations (greater than $10^{-3}$ M) of pyridinium ion is also supported by the effects of 1-substituents on the polarographic reduction potential. At low concentrations, a reaction constant, $p^*$, of +3.7 was found in this work. The similarity between this value and the $p^*$ of +3.8 reported by Wells for the acid dissociation of protonated methylamines, reaction (57), is consistent with a near-unit change in the charge on the ring nitrogen in reaction (41). However, at high concentrations

$$\text{R-NH}_3^+ \rightleftharpoons \text{R-NH}_2^+ + \text{H}^+$$ (57)

(greater than $10^{-3}$ M), a reaction constant $p^*$ of only +2.1

---

$^*$The reaction constant is a measure of the effect of substituents on a reaction and is determined from $p = (\Delta \log k/\Delta \sigma)$ where $\sigma$ is a set of empirical substituent constants.
is found for the polarographic reduction of pyridinium ions (III). The lower reaction constant implies a change in the charge on the ring nitrogen of less than one unit, consistent with the rate-controlling electrode reaction shown in reaction (75).

\[
\text{ONH}_2
\]

\[
\text{R}_1
\]

\[
\text{CONH}_2
\]

(III)

transition state

\[
\rho^* = +2.1
\]

To summarize, the electrode reaction has been found to be reversible as shown in reaction (41) at concentrations of pyridinium ion less than \(5 \times 10^{-4}\) M. In the vicinity of \(10^{-3}\) M, the surface of the electrode becomes saturated by a monolayer of dimeric product (XXXVI). This adsorption process appears to occur with all of the pyridinium salts investigated, even though only about half of the compounds exhibited adsorption prewaves. The layer of adsorbed dimeric product (XXXVI) inhibits the electrode reaction sufficiently that the equilibrium concentrations of pyridinium ion (III) and pyridine radical (XIII)
are not attained at the electrode surface (i.e. the electrode reaction becomes irreversible). As the concentration of pyridinium ion is increased, the dimeric product (XXXVI) is adsorbed in a multilayer fashion, with a resulting increase in the inhibition of the electrode reaction.

Since the electrode reaction is rate controlling at concentrations above $10^{-3}$ M., the reaction constant $\rho^*$ of +2.1 can be considered a measure of the degree of electron transfer in the transition state of reaction (75). At lower concentrations (less than $5 \times 10^{-4}$ M.) the reaction constant $\rho^*$ of +3.7 for the polarographic reduction supports a unit change in the charge on the ring nitrogen, as would be expected for the reversible reduction shown in reaction (41).

\[
\begin{align*}
\text{(III)} & \quad \text{CONH}_2 \quad \text{e}^- \quad \text{CONH}_2\text{H}_2\text{NO}_2 \quad \text{CONH}_2 \\
\text{(XIII)} & \quad \text{CONH}_2 \quad \text{H}_2\text{NO}_2 \quad \text{CONH}_2 \\
\text{(XXXVI)} & \quad \text{CONH}_2 \end{align*}
\]
4.2 The Effects of Substituents on the Reduction of Pyridinium Ions

Pyridinium ions (VIII) can be reduced to both one-

\[
\begin{align*}
\text{N} & \quad \text{R}_1 \\
\text{R}_1 & \quad \text{(VIII)}
\end{align*}
\]

electron and two-electron products. The one-electron reduction shown in reaction (41) can be observed polarographically and the reduction potentials for this reaction will, in the ensuing discussion, be referred to as polarographic reduction potentials in order to distinguish them from the reduction potentials of the two-electron reduction shown in reaction (68), which are measured potentiometrically and hence will be called potentiometric reduction potentials. Substituent effects have been reported in the literature for several reactions.
which would be expected to parallel the substituent effects in reactions (41) and (68). Two of these are reactions (60) and the previously discussed reaction (57)\textsuperscript{76}, the acid dissociation reaction of protonated pyridines and primary amines, respectively. The 3-substituents, $R_3$, in reaction (60) are analogous to the 3-substituents, $R_3$, of the NAD$^+$ model compounds (VIII) and the substituents in reaction (57)

\[
\begin{align*}
\text{Pyridinium ion} + H^+ + 2e^- & \rightleftharpoons \text{Pyridine} \\
\text{NAD}^+ & \rightleftharpoons \text{NADH} + H^+ \\
R-NH_3^+ & \rightleftharpoons R-NH_2 + H^+
\end{align*}
\]
Lindquist and Cordes\textsuperscript{15} have determined a reaction constant, $\rho^*$, of +3.7 for the equilibrium of the addition of cyanide ion to 1-substituted-3-carbamoylpyridinium ions, reaction (76), a reaction which is very similar to the two-electron reduction of pyridinium ions shown in reaction (68).

Kosower has reported a $\rho$ of +13.4 for the charge transfer complex formation of pyridinium iodides, reaction (61), a reaction which is somewhat analogous to the polarographic (one-electron) reduction.

The reaction constants of the foregoing reactions are summarized in Table XVI and the reactions are reproduced here for comparison. The effects of the 1-substituents will be considered first.

Three reactions, the acid dissociation of primary amines,\textsuperscript{76} the addition of cyanide to pyridinium ions,\textsuperscript{15} and the reversible polarographic (one-electron) reduction of pyridinium ions (this work) have nearly identical values of reaction constants $\rho^*$. Unexpectedly, the potentiometric (two-electron) reduction was found to have a much smaller $\rho^*$-value,
\[ R{-}NH_3^+ \rightleftharpoons R{-}NH_2 + H^+ \]  

(57)

\[ \text{PhCONH}_2 + \text{CN} \rightleftharpoons \text{PhCNCONH}_2 \]  

(76)

\[ \text{Ph}^+ \rightleftharpoons \text{Ph} + H^+ \]  

(60)

\[ \text{Ph}^+ + I^- \rightleftharpoons \text{PhI}^- \]  

(61)

\[ \text{Ph}^+ + e^- \rightleftharpoons \text{Ph} \]  

(41)

\[ \text{Ph}^+ + H^+ + 2e^- \rightleftharpoons \text{Ph} \]  

(68)
Table XVI

Summary of Reaction Constants of Reduction Reactions of pyridinium ions and analogous reactions.

<table>
<thead>
<tr>
<th>Substituentposition</th>
<th>Substituent constant scale</th>
<th>Reaction constant</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid dissociation of primary amines (57)</td>
<td>-</td>
<td>$\sigma^*$</td>
<td>+3.3</td>
</tr>
<tr>
<td>Cyanide addition to pyridinium ions (76) (equilibrium)</td>
<td>1</td>
<td>$\sigma^*$</td>
<td>+3.7</td>
</tr>
<tr>
<td>Acid dissociation of pyridines (60)</td>
<td>3</td>
<td>$\sigma_m$</td>
<td>+5.9</td>
</tr>
<tr>
<td>Polarographic (1-electron) reduction of pyridinium ions (41)</td>
<td>1</td>
<td>$\sigma^*$</td>
<td>+3.7</td>
</tr>
<tr>
<td>Potentiometric (2-electron) reduction of pyridinium ions (68)</td>
<td>1</td>
<td>$\sigma^*$</td>
<td>+2.8</td>
</tr>
<tr>
<td>Charge-transfer complex formation (61)</td>
<td>3 &amp; 4</td>
<td>$\sigma_m$ &amp; $\sigma_p$</td>
<td>+13.4</td>
</tr>
</tbody>
</table>
only +2.5, compared to the values near +3.7 for the effects of 1-substituents on the other equilibria.

It was surprising to find that the $\rho^*$ values for reactions (68) and (76) differ since the substrate is the same in the two cases. The difference in the $\rho^*$ values must reflect different effects of substituents on the stability of the products. Equations (68) and (76) can be combined to give

\begin{align*}
\text{Equation (68)}: & \quad \text{H}^+ + 2e^- \\
\text{Equation (76)}: & \quad \text{CN}^- \\
\end{align*}

the hypothetical reaction shown in equation (77), the reaction

\begin{align*}
\text{Equation (77)}: & \quad \text{H}^+ + 2e^- \\
\end{align*}
constant of which can be considered to be a measure of the relative effects of the 1-substituents on the stabilities of IV and XXXVII. The equilibrium constant of reaction (77), $K_{Ex}$, is related to the equilibrium constants of reaction (63), $K_{Red}$, and reaction (76), $K_{CN}$, by equation (78). Hence, the reaction constant, $\rho_{Ex}^*$, for reaction (77) can be found from

$$K_{Ex} = \frac{K_{CN}}{K_{Red}}$$

(78)

equation (79). The values of +3.7 and +2.8 for $\rho_{CN}^*$ and $\rho_{Red}^*$ respectively give a value of +0.9 for $\rho_{Ex}^*$. The positive value of $\rho_{Ex}^*$ for reaction (77) implies that electron withdrawing 1-substituents stabilize the 4-cyano compounds (XXXVII) while electron donating 1-substituents stabilize IV.

At first glance, it seems unreasonable that electron-withdrawing substituents should have a greater stabilizing effect on the compound (XXXVII) which already contains an extra electron-withdrawing group in the ring.

The answer may lie in a resonance interaction, shown in equation (80) which is possible between the ring nitrogen and the 3-carbamoyl group of 1,4-dihydropyridines. This
resonance leads to the same charge distribution as arises from the electronic transition, equation (81), in 1,4-dihydro-
pyridines. The ease of the electronic transition (i.e. the $\lambda_{\text{max}}$ of the absorbance peak) is, therefore, a reasonable measure of the contribution XXXIX could make to the overall stability of the 1,4-dihydropyridine. In general, electron-withdrawing substituents at the 1-position tend to shift the absorbance peak to shorter wavelengths, corresponding to a higher energy electronic transition, as one would expect since such substituents would tend to destabilize structure XXXIX. Absorbance peaks of 1,4-dihydropyridines (IV) appear at approximately 15 nm, longer wavelengths than do the
absorbance peaks of their 4-cyano counterparts (XXXVII), meaning the electronic transition in equation (81) is easier with IV than with XXXVII. Therefore, there is probably a greater contribution from the resonance structure (XXXIX) to the stability of IV than to the stability of the 4-cyano compounds (XXXVII). Electron-donating substituents at the 1-position will stabilize structure XXXIX and will thereby stabilize IV to a greater extent than XXXVII.

The 3-substituents were found to have a much greater effect on reactions (41) and (68) than would be expected from a comparison with reaction (60). In these three reactions
the 3-substituents should affect the stabilities of the pyridinium reactants to the same extent, hence, the different reaction constants must be due to relative amounts of stabilization of the products (XL, XXXIV, XLI) by 3-substituents. The 3-substituents would not be expected to have any great effect on the stabilities of the pyridines (XLI) so the larger reaction constants for reactions (41) and (68) must be due to a stabilization of the pyridine radical (XL) and the 1,4-dihydropyridines (XXXIV) by electron-withdrawing substituents. It is probably significant that the effects of 3-substituents
on reactions (41) and (68) were found to be correlated better by para-substituent constants $\sigma_p$ than by meta-substituent constants $\sigma_m$ whereas the acid dissociation of protonated pyridines, reaction (58), is correlated better with $\sigma_m$. The improved correlations with $\sigma_p$ would indicate the presence of a resonance interaction between the 3-substituent and the ring$^\dagger$ of the pyridine radical (XL) and the 1,4-dihydropyridine (XXXIV), an interaction which one would not expect to find with the pyridines (XLI). Just such an interaction was proposed earlier to explain the unexpectedly lower reaction constant for the effect of 1-substituents on the potentiometric (two-electron) reduction of pyridinium ions reaction (68). The contribution

$$\begin{align*}
\text{CONH}_2 + H^+ + 2e^- & \rightleftharpoons \text{CONH}_2 \\
& \text{(68)}
\end{align*}$$

$^\dagger$The para-substituent constants, $\sigma_p$, contain a resonance contribution which is much less significant in meta-substituent constants, $\sigma_m$. Taft$^7$ has proposed a separation of $\sigma_p$ and $\sigma_m$ into inductive and resonance terms: $\sigma_p = \sigma_I + \sigma_R$ and $\sigma_m = \sigma_I + 1/3 \sigma_R$ where $\sigma_I$ and $\sigma_R$ are inductive and resonant contributions respectively.
from the resonance form (XXXIX) of the dihydropyridine product

(IV) would be enhanced by electron-withdrawing 3-substituents which can participate in this resonance (i.e. CONH$_2$, COCH$_3$, and, to some extent, CN) but would be non-existent for groups which are incapable of participating in such resonance (i.e. F, H, or OH). Thus the 3-carbamoyl, 3-acetyl and 3-cyano analogues of (IV), by stabilizing structure (XXXIX) or its analogues will contribute to the overall stability of the 1,4-dihydropyridine (IV) and make the reduction easier.

A resonance structure analogous to XXXIX can also be written for the pyridine radical. (In structure (XLII) the

(XLII)  (XLIII)  (XLIV)
electron has been arbitrarily placed at the 4-position of the pyridine ring. Resonance structures can be written which also place the lone electron on C-2 and C-6). The existence of interactions between the 3-substituent and the lone electron as in

\[
\text{CONH}_2
\]

exist, have been proposed by Bruhlmann and Mayon\(^8\) for the 1-methyl-3-carbamoylpyridine radical (XLV) so there is little doubt that the resonance structures (XLIII) and (XLIV) do contribute to the overall stability of the pyridine radical. Quite a large contribution is probably made by XLIV because of the presence of the aromatic ring. However, the effects of 1-substituents on the polarographic reduction were consistent with a low charge density on the ring nitrogen whereas the contribution of structures XLIII and XLIV place a partial positive charge on the ring nitrogen. Probably the partial positive charge is effectively neutralized by the induction of electron density towards the ring nitrogen in structures XLVI and XLVII.

To summarize then, the effects of 1- and 3-substituents on the polarographic (one-electron) and potentiometric
(two-electron) reductions of pyridinium ions can be explained by considering resonance interactions between the 3-substituent and the ring in the reduction products. These resonance interactions are summarized in Schemes 4 and 5. (All of the possible resonance structures of the pyridine radical have not been shown. The missing structures simply result in shifting the lone electron around the pyridine ring.)
4.3 The Effects of Substituents in Dihydropyridines on the Rates of Oxidation by Flavins

With regard to substituent effects, the \( \rho \) value for the potentiometric (two-electron) reduction of substituted pyridinium ions and for the equilibrium between a flavin and substituted 1,4-dihydropyridines must be identical. This can be shown to be so by the following derivation, equations (82) through (91).

\[
\text{Py}^+ + \text{FlH} \rightleftharpoons \text{PyH} + \text{Fl}
\]  

\( (82) \)

\[
K_{eq} = \frac{[\text{PyH}][\text{Fl}]}{[\text{Py}^+][\text{FlH}]}
\]  

\( (83) \)

\[
\text{Py}^+ + \text{H}^+ + 2e^- \rightleftharpoons \text{PyH}
\]  

\( (84) \)

\[
K_{Py} = \frac{[\text{PyH}]}{[\text{Py}^+][\text{H}^+]} \]

\( (85) \)

\[
\text{Fl} + \text{H}^+ + 2e^- \rightleftharpoons \text{FlH}^-
\]  

\( (86) \)

\[
K_{Fl} = \frac{[\text{FlH}^-]}{[\text{Fl}][\text{H}^+]} \]

Substituents on the pyridine ring will affect only reactions (82) and (84). Therefore one can write equations (83) through (90), where \( \rho \) is zero since substituents on the pyridine ring will not affect the reduction potential of the flavin.
\[ \eta_{Eg} = \frac{\Delta \log K_{Eg}}{\Delta \sigma} = \frac{\Delta \log [PyH]/[Py^+]}{\Delta \sigma} + \frac{\Delta \log [Fl]/[FlH^-]}{\Delta \sigma} \]  

(88)

\[ \rho_{Py} = \frac{\Delta \log K_{Py}}{\Delta \sigma} = \frac{\Delta \log [PyH]/[Py^+][H^+]}{\Delta \sigma} \]  

(89)

\[ \rho_{Fl} = \frac{\Delta \log K_{Fl}}{\Delta \sigma} = \frac{\Delta \log [FlH^+]/[Fl][H^+]}{\Delta \sigma} = 0 \]  

(90)

Combining equations (88), (89), and (90) gives the identity in equation (91). Thus the reaction constant \( \rho^* \) for the effect of 1-substituents on the equilibrium between flavins (X) and 1,4-dihydropyridines (V), reaction (69), is -2.8, equal in

\[ \rho_{Eg} = \rho_{Py} - \rho_{Fl} = \rho_{Py} \]  

(91)

of 1-substituents on the equilibrium between flavins (X) and 1,4-dihydropyridines (V), reaction (69), is -2.8, equal in
magnitude to $\rho^*$ for the potentiometric (two-electron) reduction of pyridinium ions, but opposite in sign since the reaction with flavins (69) has been written for the oxidation of dihydropyridine (V) rather than the reduction of pyridinium ion (III). The effect of 1-substituents on the forward reaction in equation (69) has been found in this study to give a reaction constant $\rho^* = -1.9$. The reaction constants for the equilibrium and the forward and backward rates are related by equation (92) arising from a rearrangement of the familiar equation (93). Using the reaction constants found in this work for the equilibrium ($\rho_{\text{Eq}}^* = -2.0$) and the rate of oxidation of dihydropyridines by flavins ($\rho_{\text{for}}^* = -1.9$) it is possible to calculate a reaction constant $\rho_{\text{rev}}^*$ of +0.9 for the reverse reaction (reduction of pyridinium ions by the flavins). This value of $\rho^*$ is quite different from the $\rho^*$ of +2.2 found by Lindquist and Cordes for the rate of addition of cyanide to pyridinium ions, reaction (76), a reaction which is somewhat analogous to the two-electron reduction of pyridinium ions. Indeed, very similar values of $\rho$ have been reported for the cyanide addition to
benzaldehydes and the sodium borohydride reduction of acetophenones. However, there are certainly mechanistic differences between cyanide addition and hydride transfer not the least of which is the fact that the hydride ion is not a free ion in solution, as is the cyanide ion, but is transferred directly from one molecule to the other. Thus the degree of bond formation in the transition state of a cyanide addition reaction is dependent partially on the solvation of the free

\[ \text{(76)} \]
cyanide ion and partially on the molecule to which the cyanide is adding, whereas the degree of hydride transfer in the transition state would depend mostly on the stability of the two molecules between which the hydride is being transferred. Thus, although it seems surprising that the reaction constant for the rate of addition of cyanide ions to pyridinium ions should be so much larger ($\rho^* = +2.2$) than the reaction constant for the rate of reduction of pyridinium ions by flavins ($\rho^* = +0.9$) found in this study it is not totally inconsistent with a hydride transfer from reduced flavin to pyridinium ions.

Somewhat surprisingly, the 1-substituents have almost the same effect on the acid-decomposition of 1,4-dihydropyridines ($\rho^* = -2.0$ to $-2.6$, see Section 3.1) as they do on the oxidation of dihydropyridines by flavins ($\rho^* = -1.9$). In view of
the relatively poor agreement between the reaction constants for the addition of cyanide ($\rho^* = +2.2$) to pyridinium ions and the reduction by flavins ($\rho^* = +0.9$) of pyridinium ions, it is tempting to formulate a covalent intermediate such as (XLVIII) as an alternative to a hydride reduction. A similar intermediate was proposed by Dunn\textsuperscript{85} for the reduction of aldehydes by 1,4-dihydropyridines. The intermediate (XLVIII), unfortunately does not explain the deuterium isotope effects ($k_H/k_D = 3.16$) observed by Suelter and Metzler\textsuperscript{20} during the oxidation of 1-propyl-3-carbamoyl-4-deutro-1,4-dihydropyridine (XLIX) since the proton transfers would have to take place in a fast reaction after the rate determining formation of the
intermediate (XLVIII). It is also very difficult to envisage any way to form this intermediate in the reverse reaction.

\[
\begin{align*}
\text{H - D} & \quad \text{CONH}_2 \\
\text{C}_3\text{H}_8 & \quad \text{CONH}_2
\end{align*}
\]

(XLIX)

starting with pyridinium ions and reduced flavin since this would require a nucleophilic attack at the 3-position of the pyridinium ring, a reaction which is very uncharacteristic of pyridinium ions. The hydride-transfer mechanism, which has considerable support, appears to best explain all the available evidence, although the reaction constants.
determined in this study seem to indicate that the mechanism may be more complex than this.
4.4 Comparisons of 1,1'-Alkylenebis(3-carbamoylpyridinium) compounds with the Series I Compounds

The Series I compounds (III and IV) have been used to determine the inductive effect exerted by 1-substituents on selected reactions of pyridinium ions (III) and dihydro-pyridines (IV). These results can now be utilized to evaluate the effects of more complex substituents at the 1-position, particularly substituents which may be capable of interacting with the pyridine ring by means other than induction through the ring nitrogen. One such substituent would be a second pyridinium ring. Batzold found evidence of charge transfer stacking when indoles were added to polymers containing pyridinium rings attached to a polystyrene backbone. The compounds used in this study (XIX) contained only two pyridinium rings separated by one or two methylene groups. Before the effects of a second pyridinium ring on the reactions of the first pyridinium ring can be evaluated, the inductive substituent effects of the groups XXV and XXVI must be known. Values of $\sigma^*$ of approximately 1.7 and 1.0 were determined.
Compound XIXa, at a concentration of $10^{-2}$ M., exhibited one polarographic reduction wave with a half-wave potential of -470 mv. (vs. N.H.E.). The half-wave potential that would be expected on the basis of inductive effects alone ($\sigma^* = 1.7$) is -450 mv. At $2 \times 10^{-3}$ M., compound XIXa exhibited both a prewave and a main wave but since the correlation of the Series I compounds (III) at $2 \times 10^{-3}$ M. (Section 5.3.5) used the half-wave potentials of the main wave, then only the main wave should be used for comparison. The main reduction wave appeared at a potential of approximately -430 mv. but, on the basis of inductive effects only ($\sigma^* = 1.7$) would be
predicted at a potential of -515 mv. At concentrations below 5 x 10^{-4} M., the polarographic reduction potential has been determined in this work as the intercept, $E^\circ$, of a plot of applied potential, $E$, against $\log(i_d - i)/(i)^{2/3}$ [equation (18)]. However, at concentrations below 5 x 10^{-4} M. this compound (XIXa) exhibited two polarographic reduction waves, one of which had a reduction potential, $E^\circ$, of -209 mv. and the other of which had a reduction potential of -409 mv. The enormous potential difference (>220 mv.) between the first wave ($E^\circ = -209$ mv.) and the reduction waves at higher concentrations would seem to rule out the use of this reduction potential in comparisons with the Series I correlations since with no Series I pyridinium ion was the difference between the half-wave potential, $E_{1/2}$, at 2 x $10^{-3}$ M. and $E^\circ$ more than 60 mv. The second wave ($E^\circ = -409$ mv.) is a much more reasonable 20 mv. more positive than the half-wave potential of the polarographic wave at 2 x $10^{-3}$ M. From the inductive effect ($\sigma^* = 1.7$) a reduction potential, $E^\circ$, of -417 mv. would be predicted which is in good agreement with
the value of -409 mv. observed. The predicted and observed reduction potentials of XIX are summarized in Table XVII.

\[
\begin{align*}
a: & \quad n = 1 \\
b: & \quad n = 2
\end{align*}
\]

Only at a concentration of \(2 \times 10^{-3} \text{ M.}\) is there a significant difference between the observed reduction potentials and those predicted on the basis of oxidation effects. Thus the proximity of the two pyridinium ions appears to have no special effect on the polarographic reduction of XIXa.

The results for the polarographic reduction potentials of XIXb, listed in Table XVII, are much less definitive. At a concentration of \(10^{-2} \text{ M.}\), the observed reduction potential is approximately 90 mv. more negative than the potential of -583 mv. predicted from inductive effects (\(\sigma^* = 1.0\)), whereas at \(2 \times 10^{-3} \text{ M.}\), the observed potential of -595 mv. is fairly close to the predicted potential of -630 mv. At concentrations below \(5 \times 10^{-4} \text{ M.}\), the observed reduction potential of -434 mv. is 135 mv. more positive than would be predicted from inductive effects (\(\sigma^* = 1.0\)). The more negative observed potential at a concentration of \(10^{-2} \text{ M.}\) is probably due to an extraordinarily large inhibition of the electron transfer
Table XVII

Predicted and Observed Polarographic Reduction Potentials of the 1,1'-alkylenebis(3-carbamoylpyridinium) Compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>concentration (M)</th>
<th>reduction observed potentials from inductive effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Compound 1" /></td>
<td>$10^{-2}$</td>
<td>-450</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^{-3}$</td>
<td>-515</td>
</tr>
<tr>
<td></td>
<td>$&lt;5 \times 10^{-4}$</td>
<td>-417</td>
</tr>
<tr>
<td><img src="image2.png" alt="Compound 2" /></td>
<td>$10^{-2}$</td>
<td>-583</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^{-3}$</td>
<td>-623</td>
</tr>
<tr>
<td></td>
<td>$&lt;5 \times 10^{-4}$</td>
<td>-569</td>
</tr>
</tbody>
</table>
process with this compound, which is also reflected in the very large slope of 125 mV. from plots of applied potential, \( E \), against \( \log(i_d - i)/(i) \) [equation (10)]. The more positive potential observed at concentrations less than \( 5 \times 10^{-4} \) M. may indicate a non-inductive interaction between the two rings which is stabilizing the product and thus making the reduction easier. But the results at the higher concentrations do not corroborate this and the potential observed at concentrations below \( 5 \times 10^{-4} \) M. may result from adsorption effects instead. The polarographic results for XIXb are too inconclusive to determine if any non-inductive interactions are operating between the two rings.

\[
E = E_{1/2} + \frac{RT}{a n F} \ln\left(\frac{i_d - i}{i}\right) \tag{10}
\]

The slopes of \( E \) against \( \log(i_d - i)/(i) \) for most compounds were between 70 and 90 mV.
A reduction potential has been estimated in this work for the reduction of one ring of XIXb to a dihydropyridine. Compound XXXII was not isolated but was prepared in situ from XIXb by dithionite reduction and was assayed spectrophotometrically (the method by which the extinction coefficient was determined is given in Section 3.6). The reduction potential was estimated to be approximately -280 mv. at pH 7. A $\sigma^*$ value of 1.0 for the inductive effect of the unreduced pyridinium ring gives a predicted potentiometric reduction potential at pH 7, $E^{\circ'}$, of -287 mv. which is in good agreement with the observed reduction potential of -280 mv.

Thus it would appear that the proximity of the two pyridine rings in XIXa and XIXb has no effect on the oxidation-
reduction behaviour of these compounds that cannot be accounted for simply on the basis of inductive effects.
4.5 Comparison of NAD$^+$ and NMN$^+$ with the Series I Compounds

Before the effects of the ribosidyl group on the properties of NAD$^+$ (Ia) and NMN$^+$ (II) can be evaluated, the inductive effect of the ribose group must first be estimated. Intuitively, one would expect it to be determined primarily by the presence of oxygen in the ring and therefore to be...
quite similar to the inductive effect of the methoxymethyl group. The properties of NAD\(^+\), NMN\(^+\) and 1-methoxymethyl-3-carbamoylpyridinium chloride (VIIId) are summarized in Table XVIII. However, the inductive effect of the ribose group

![Diagram of ribose group]

will also contain a contribution from the hydroxyl group on C-2' which should exert an inductive effect quite similar to that of the hydroxethyl group. In this work\(^a\) a value

![Diagram of substituted pyridine]

(VIIId)

of 0.55 has been used for the methoxymethyl substituent and 0.11 for the hydroxyethyl substituent. Combining these two values gives a predicted substituent constant of 0.66 for the ribose group. Hence the reduction potentials and rate constants for NAD and NMN are also compared in Table XVIII with values predicted from the correlations of the Series I compounds (III) by assuming a value of 0.66 for the \(\sigma^*\)-value of the ribosidyl group. It should be noted that a different

\(^a\)see Table V, page 49
### Table XVIII

Selected Properties of NAD⁺, NMN⁺, and 1-methoxymethyl-3-carbamoylpyridinium chloride.

<table>
<thead>
<tr>
<th></th>
<th>NAD⁺ (Ia)</th>
<th>NMN⁺ (II)</th>
<th>MMCPy⁺ (VIIIe)</th>
<th>Predicted from ( G^* = 0.66 ) for ribose group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polarographic reduction potential at ( 10^{-2} ) M. (mv)</td>
<td>-715</td>
<td>-830</td>
<td>-679</td>
<td>-698</td>
</tr>
<tr>
<td>Polarographic reduction potential at ( 2 \times 10^{-3} ) M. (mv)</td>
<td>-683</td>
<td>-810</td>
<td>-657</td>
<td>-684</td>
</tr>
<tr>
<td>Polarographic reduction potential at ( &lt;5 \times 10^{-4} ) M. (mv)</td>
<td>-677</td>
<td>-703</td>
<td>-639</td>
<td>-642</td>
</tr>
<tr>
<td>Potentiometric standard reduction potential (mv)</td>
<td>-105 (this work)</td>
<td>-1043</td>
<td>-95</td>
<td>-97</td>
</tr>
<tr>
<td>Rate of oxidation of dihydropyridine by flavin (M⁻¹·sec⁻¹)</td>
<td>.53</td>
<td>.25</td>
<td>.61</td>
<td>2.4</td>
</tr>
<tr>
<td>Rate of acid-decomposition of dihydropyridine by H₃O⁺ (M⁻¹·sec⁻¹)</td>
<td>10.2</td>
<td>7.7</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>Rate of acid-decomposition of dihydropyridine by acetic acid (M⁻¹·sec⁻¹) x 10³</td>
<td>.95</td>
<td>.90</td>
<td>7.7</td>
<td>5.9</td>
</tr>
</tbody>
</table>
σ-value can be calculated for the ribose group from the 
σ-value of 0.66 reported by Wells\textsuperscript{76} for the methoxymethyl group.

In general, the properties of NAD\textsuperscript{+} (Ia) and NMN\textsuperscript{+} (II) 
investigated in this work are in fair agreement with those of 
l-methoxymethyl-3-carbamoylpuridinium chloride (VIIIe) and 
with those predicted assuming a σ*-value of 0.66 for the 
ribosyl group. The polarographic reduction potentials of:
NAD$^+$ were approximately 30 mv. more negative than those of 1-methoxymethyl-3-carbamoylpyridinium chloride at all concentrations and 0 to 30 mv. more negative than would be predicted from $\sigma^*$ equals 0.66. The polarographic reduction of NMN$^+$ appears to be inhibited to a greater extent by adsorption of the dimeric product (XVI) (plots of E against $\log(i_d - i)/(i)$ have slopes greater than 100 mv., see Sections 3.5.1 and 4.1) than is NAD$^+$, which is the probable cause of the very negative polarographic reduction potentials found for this compound at concentrations above $10^{-3}$ M. At concentrations below $5 \times 10^{-4}$ M., the polarographic reduction potential of NMN$^+$ is approximately 25 mv. more negative than that of NAD$^+$ and about 60 mv. more negative than would be predicted from either the polarographic reduction potential of 1-methoxymethyl-3-carbamoylpyridinium chloride or from a $\sigma^*$-value of 0.66. The 0 to 30 mv. difference between the polarographic reduction potentials of NAD$^+$ and the predicted potentials may reflect an effect of the
adenine (L) moiety toward making the pyridinium ring more difficult to reduce. The difference is so small, however, that it may not be significant.

The potentiometric (two-electron) reduction potential of NAD$^+$ agrees quite well with the value predicted using a $\sigma^*$-value of 0.66, whereas the potentiometric reduction potential of NMN$^+$ agrees quite well with the slightly lower reduction potential found for 1-methoxymethyl-3-carbamoylpyridinium chloride. The agreement between NMN$^+$ (II) and 1-methoxymethyl-3-carbamoylpyridinium chloride (VIIIe) would be expected on the basis of inductive effects. The potentiometric reduction potential of NAD$^+$ (Ia) is approximately 10 mv. more negative
than those of NMN$^+$ and 1-methoxymethyl-3-carbamoylpyridinium chloride, which again may reflect an effect of the adenine group (L) toward making the pyridinium ring more difficult to reduce.

The rates of oxidation of the corresponding dihydro-pyridines of NAD$^+$ and NMN$^+$ again are in good agreement with that of the 1-methoxymethyl model compound. Surprisingly, these rates are all considerably smaller than the oxidation rate predicted using a σ-value of 0.66. A similar effect was observed by Lindquist and Cordes$^{15}$ in their study of the rates and equilibria of cyanide addition to pyridinium ions. They observed that NAD$^+$ had a much larger affinity for cyanide ion than would be expected on the basis of the rate of the addition reaction. In this work, it has been found that the rate of oxidation of NADH by flavins is much slower than would be expected on the basis of its standard reduction potential. Since similar values of the oxidation rate and standard reduction potential were also found for NMNH (LI) and 1-methoxymethyl-3-carbamoyl-1,4-dihydropyridine (Ve) then the
slower rates are probably related in some way to the presence of the oxygen atom on the \( \alpha \)-carbon of the 1-substituent. The two-fold rate enhancement of 1-methoxymethyl-3-carbamoyl-1,4-dihydropyridine (Ve) over NMNH (LI) may reflect steric crowding between the larger ribosidyl group and the approaching flavin (X). Regardless, the two-fold rate enhancement of
NADH (XXV) over NMNH (LI) probably reflects a weak stabilization of the transition state by the adenine moiety.

The only reaction in which NAD$^+$ and NMN$^+$ did not behave very similarly to 1-methoxymethyl-3-carbamoylpypyridinium chloride was in the acid decomposition of the corresponding dihydro compounds, in which NADH and NMNH reacted much more slowly than did 1-methoxymethyl-3-carbamoyl-1,4-dihydropyridine or than would be predicted from a $\sigma^*$-value of 0.66.

On the basis of the polarographic reduction potentials, potentiometric reduction potentials, and rates of oxidation of the corresponding dihydropyridines by flavins, there appears to be no significant effect from the adenine portion of the NAD$^+$ molecule on the oxidation-reduction properties of this coenzyme. There may be a very small stabilization of the pyridinium ring by the adenine resulting in the slightly more negative reduction potentials of NAD$^+$ and slightly faster oxidation rate of NADH. The formation of a charge transfer complex between the adenine and the pyridinium rings of NAD$^+$ has been proposed by Cilento and Schreier, but the results of this study show that the effects of such a complex on the oxidation-reduction properties of NAD$^+$ are very small at best.
5. SUGGESTIONS FOR FURTHER RESEARCH

In some areas, this work has posed as many questions as it has answered. Although the adenine moiety of NAD⁺ (Ia)

![Chemical Structure](image)

was found to have very little effect on the oxidation-reduction properties of this molecule, the presence of oxygen in the ribose ring appears to be responsible for the slower rates of oxidation of NADH and NMNH by flavins. It would be of interest to know (1) if this slower rate also occurs with 1-alkoxymethyl substituents as was found with the 1-methoxymethyl group in this study, (2) if any other hetero-atoms attached to the α-carbon of the 1-substituent can effect a similar rate retardation and (3) how the oxygen of the 1-substituent interacts with the dihydropyridine ring or transition state to slow the reaction rate.

The two-electron reduction of pyridinium ions shown in reaction (68), was found to have a reaction constant of only +2.8 compared with reaction constants of +3.7 found in this work and elsewhere¹⁵,⁷⁶ for three analogous reactions.
The difference was attributed to a resonance interaction between the ring-nitrogen and the 3-carbamoyl group of IV.

To further test this hypothesis, it would be interesting to determine a reaction constant for the two-electron reduction of a series of dihydropyridines such as 1-substituted-3-chloro-1,4-dihydropyridines (LII) for which the resonance interaction could be eliminated.
Further studies into 1,1'-alkylenebis(3-carbamoyl-pyridinium) compounds would be useful as possible models of enzymatic NAD reactions. The two compounds of this type used in this work (XIXa and b) exhibited no direct interactions between the two rings but this may have been due to an inability of the two rings to align properly in these two specific compounds which have very short methylene bridges. The reduction potentials of XIX compounds having longer methylene bridges may be affected by direct interactions between the two rings, particularly between the two rings of the half-reduced form XXXII. The rates of oxidation of both XXXII and XXXIII
compounds by flavins could also be of interest as model enzymatic reactions.

(XXXIII)
6. EXPERIMENTAL

6.1 Buffers

The water used throughout this work was distilled in a Manesty 00BE glass still. Solutions which were to be used in the dry box were boiled and flushed with nitrogen while cooling.

Buffers were prepared from commercially obtained reagent grade chemicals; the following methods were used.

Acetate buffers:

The required amount of sodium acetate crystals to make a 1.0 M. solution was weighed into a flask. The volume of 1.0 M. perchloric acid required to give the desired pH was added by pipette. Water was then added up to the required volume. Acetate buffers were prepared at pH's 5.65, 4.6 and 3.4. Solutions of 0.1 M. buffers were prepared by dilution of the 1.0 M. buffers.

Tris(hydroxymethyl)aminomethane (Tris) buffers:

The required amount of Tris crystals to make a 1.0 M. (or 0.1 M.) solution was weighed into a flask. The amount of sodium perchlorate or sodium chloride required to prepare a solution of the proper ionic strength (1.0 or 0.1) was then weighed into the flask. The desired pH was obtained by adding 1.0 M. perchloric acid (or hydrochloric acid) by pipette.
Water was added up to the final volume. Tris buffers were prepared at pH's 7.1, 8.1, 8.7, 9.3, and 9.4.

**Ethylenediamine Buffers:**

The required amount of ethylenediamine dihydrochloride to make a 0.05 M. solution was weighed into a flask. The volume of 0.1 M. or 1.0 M. sodium hydroxide required to obtain the desired pH was added by pipette and water was added up to the final volume. Ethylenediamine buffers were prepared at pH's of 7.0 and 10.4.

**Glycine Buffers:**

The required amount of glycine crystals to make an 0.1 M. solution was weighed into a flask. The volume of 0.1 M. or 1.0 M. sodium hydroxide required to obtain the desired pH was added by pipette. Water was then added up to the final volume. Glycine buffers were prepared at pH's of 3.3, 3.2, 3.5, 9.3, and 10.3.
6.2 Oxygen-free Work

The dry box used for oxygen-free work was a Model HE-43-2 fitted with a Model HE-493 Dri-Train purchased from Vacuum Atmospheres Corporation. High purity helium gas was used to supply the inert atmosphere. (High purity Nitrogen and "oxygen-free" Nitrogen were found to be unsuitable because of their oxygen content). The gas inlet system was modified as in Figure 29 so that incoming gas was forced through the Dri-Train before entering the box. Copper tubing was used to connect the helium tank to the gas inlet on the dry box.

Materials to be transferred to the dry box were placed in the antechamber and the antechamber evacuated and refilled from the dry box through valve A (see Figure 29) twice before the door between the antechamber and the dry box was opened. During the evacuation procedure, fresh helium gas was slowly introduced to the dry box through valve B to replenish that used to refill the antechamber.
Figure 29. Dry-box and accessories
6.3 Synthesis of Quaternary Pyridinium Salts

Quaternary pyridinium salts were prepared from the corresponding pyridines and alkyl halides. Methyl chloroacetate was prepared from chloroacetic acid and methanol by the method of Clinton and Laskowski. Isopropyl chloroacetate was also prepared by the method of Clinton and Laskowski by using 2-propanol in place of methanol. All of the pyridines and the other alkyl halides were obtained commercially and used without further purification.

The general method for preparing the pyridinium salts consisted of mixing 30 millimoles each of the pyridine and alkyl halide in 15 mls. of solvent (usually acetone or acetone/dimethylformamide) and refluxing for one day. The crystals were filtered, washed with acetone and dried under aspirator vacuum. Individual variations on this scheme are noted below.

1-Acetonyl-3-carbamoylpyridinium chloride:

Forty-five millimoles of chloroacetone (5.0 mls) with 30 millimoles of nicotinamide (3.66 grams), 8.0 mls. of dimethylformamide, and 10.0 mls. of acetone yielded 6.3 grams (98%) of crude product after refluxing for two days. The crude product was dissolved in methanol (8 mls/gram) at 50° and filtered. Acetone was added at 50° until the solution turned cloudy. The product recrystallized upon cooling to -15° (m.p.: 202-3° dec.)
Elemental analysis:

calculated: C: 50.35; H: 5.13; N: 13.05
found: C: 50.42; H: 5.10; N: 13.26.

1-Carboxothoxymethyl-3-carbamoylpyridinium chloride:

Thirty-seven millimoles of methyl chloroacetate (4.0 grams), 30 millimoles of nicotinamide (3.66 grams), 6.0 mls. of dimethylformamide and 12.0 mls of acetone yielded 6.3 grams (91%) of crude product after refluxing for one day. The crude product was dissolved in methanol (5 mls./gram) at 50° and filtered. Acetone was added at 50° until the solution turned cloudy. The product recrystallized upon cooling. (m.p.: 165-6° dec).

Elemental analysis:

calculated: C: 46.85; H: 4.77; N: 12.15
found: C: 46.64; H: 5.06; N: 11.80.

1-Carbo-i-propoxymethyl-3-carbamoylpyridinium chloride:

Thirty millimoles of nicotinamide (3.66 grams), 40 millimoles of i-propylchloroacetate (5.5 grams), 5.0 mls. of dimethylformamide, and 10.0 mls. of acetone were refluxed for one day, yielding 7.14 grams (92%) of crude product. The crude product was dissolved in methanol (5 mls/gram) at 50-60° and filtered. Acetone was added until recrystallization began. The recrystallization was completed upon cooling. (m.p.: 202-4° dec)
Elemental analysis:

calculated:  C: 51.06;  H: 5.80;  N: 10.83
found:    C: 50.89;  H: 5.69;  N: 10.88.

l-Carbamoylmethyl-3-acetylpyridinium chloride:
Thirty millimoles each of chloroacetamide (2.80 grams) and 3-acetylpyridine (3.63 grams) were mixed with 5.0 mls. of dimethylformamide and 10.0 mls. of acetone and refluxed for one day. A yield of 5.2 grams (81%) of crude product was obtained. The crude product was dissolved in refluxing methanol (12 mls/gram) and filtered. Acetone was added until crystallization began (18 mls/gram) and the solution was allowed to cool, yielding fine needle-like crystals. (m.p.: 214-214.5° dec.)

Elemental analysis:

calculated:  C: 50.35;  H: 5.13;  N: 13.05
found:    C: 50.33;  H: 5.00;  N: 12.87.

l-Carbamoylmethyl-3-carbamoylpyridinium chloride:
A yield of 6.14 grams (95%) of crude product was obtained when 3.0 mls. of dimethylformamide, 15.0 mls. of acetone and 30.0 millimoles each of nicotinamide (3.66 grams) and chloroacetamide (2.80 grams) were refluxed for one day. The crude product was dissolved in water (2.5 mls/gram) at 65° and filtered. Ethanol was added until the solution became cloudy. On cooling, white star-like crystals formed. (m.p.: 213-4° dec.)
Elemental analysis:

calculated:  C: 44.5; H: 4.6; N: 19.5
found:  C: 44.63; H: 4.85; N: 19.46.

1-Carbamoylmethyl-3-fluoropyridinium chloride:

Four mls. of dimethylformamide, thirteen mls. of acetone, thirty millimoles each of chloroacetamide (2.80 grams) and 3-fluoropyridine (2.91 grams), and a catalytic amount of sodium iodide were refluxed for three days. The red oil which separated was crystallized by seeding or by cooling the solution below 0° and stirring the oil into the acetone layer. The yield was 3.92 grams (68%). The crude product was dissolved in refluxing ethanol (5 mls/gram) and filtered. Acetone was added until the solution turned cloudy (8 mls/gram). The product recrystallized upon cooling. (m.p.: 160-2° dec.).

Elemental analysis:

calculated:  C: 44.09; H: 4.20; N: 14.70
found:  C: 43.80; H: 4.17; N: 14.67.

1-Carbamoylmethyl-3-cyanopyridinium chloride:

Thirty millimoles each of nicotinonitrile (3.04 grams) and chloroacetamide (2.80 grams), a catalytic amount of sodium iodide, and 15 mls. of acetone were refluxed for two days, yielding 3.8 grams (64%) of crude product. The crude product was recrystallized from 100% ethanol (80 mls/gram). (m.p.: 193-6° dec.)
Elemental analysis:

  calculated:  C: 48.61;  H: 4.05;  N: 21.27
  found:  C: 48.74;  H: 4.10;  N: 21.23.

1-Carbamoylmethylpyridinium chloride:

Thirty millimoles of chloroacetamide (2.80 grams), 5.0 mls. of pyridine, and 15 mls. of acetone, after refluxing for one day, yielded 4.5 grams (87%) of crude product. The crude material was dissolved in refluxing methanol (10 mls/gram) and filtered. Acetone (30 mls/gram) was added slowly and the solution allowed to cool, yielding needle-like crystals. (m.p.: 206-206.5° dec.)

Elemental analysis:

  calculated:  C: 48.70;  H: 5.22;  N: 16.23
  found:  C: 48.82;  H: 5.08;  N: 15.96.

1-Carbamoylmethyl-3-hydroxypyridinium chloride:

Three millimoles each of 3-hydroxypyridine and chloroacetamide in 15.0 mls. of acetone were refluxed for one day. The crude crystals were filtered, washed with acetone, dried under aspirator vacuum and recrystallized from 100% ethanol to give white needles (m.p.: 197-9° dec.)

Elemental analysis:

  calculated:  C: 44.56;  H: 4.78;  N: 14.85
  found:  C: 44.73,  H: 4.68;  N: 14.80.
To confirm that alkylation occurred at the ring nitrogen rather than at the 3-hydroxyl group, the U.V. spectrum of this product was compared with the spectra of 1-methyl-3-hydroxypyridinium chloride\(^9\) in acidic and basic solutions. The agreement does confirm N-alkylation, as expected.

<table>
<thead>
<tr>
<th>pH</th>
<th>1-methyl-3-hydroxy pyridinium chloride</th>
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<tr>
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<td>289</td>
</tr>
<tr>
<td>13</td>
<td>245</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td></td>
<td>313</td>
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</tbody>
</table>

1-Carboxymethyl-3-carbamoylpyridinium monohydrate:

Thirty millimoles each of nicotinamide (3.66 grams) and sodium chloroacetate (3.50 grams) (or sodium iodacetate) were allowed to react in 20 mls. of refluxing methanol for one day. After cooling the reaction mixture to below 0°, the crude product was filtered and washed with acetone. The crude product was dissolved in water (5 mls/gram), which was heated on a steam bath. This solution was then filtered, ethanol (15 mls/gram) was added and recrystallization took place upon cooling. (m.p.: 200-3° dec., lit. m.p.: 204-6°,\(^9\) 206-8°\(^9\))

1-Cyanomethyl-3-carbamoylpyridinium chloride:

Six mls. of dimethylformamide, 12 mls. of acetone, 30 millimoles of nicotinamide (3.66 grams), and 44 millimoles of chloroacetonitrile (3.32 grams) yielded 5.1 grams (86%) of
crude product after refluxing for 2 days. The crude crystals were dissolved in water (5 mls/gram), which was heated on a steam bath and ethanol was added until the solution turned cloudy (4 mls/gram). Upon cooling, the product recrystallized as a fine, yellow, crystalline powder. (m.p.: 236-8° dec.)

Elemental analysis:
- calculated: C: 48.6; H: 4.1; N: 21.3

1,1'-Ethylenebis(3-carbamoylpyridinium chloride):

Thirty millimoles of nicotinamide (3.66 grams), 10.0 mls. of ethylene dichloride, a catalytic amount of sodium iodide, 15.0 mls. of acetone and 5.0 mls. of dimethylformamide were heated to 90-100° in a pressure bottle for six days. The resulting crystals were filtered, washed with acetone and dried under aspirator vacuum. The crude product was dissolved into refluxing water, containing decolourizing carbon and the solution filtered and allowed to cool. The crystals were recovered and recrystallized a second time from water to give very pale beige crystals (m.p.: 297° dec.) lit. m.p.: 285°, 96°, 300°; λ\text{max} (ε) = 265 (9090); lit. λ\text{max} (ε) = 265 (8990)\text{°}.

1-(2'-Hydroxyethyl)-3-carbamoylpyridinium chloride:

Thirty millimoles of nicotinamide (3.66 grams), 40 millimoles of 2-chloroethanol (3.22 grams), 5 mls. of
dimethylformamide and 10.0 mls. of acetone were refluxed for two days, yielding 2.4 grams (40%) of crude product. The crude product was recrystallized from 100% ethanol to give fluffy white crystals (m.p.: 195°; lit. m.p.: 195.5-196°, 182°).  

1-Methoxymethyl-3-carbamoylpyridinium chloride:  

Thirty millimoles of nicotinamide (3.66 grams) were dissolved in 110 mls. of acetone at room temperature. Three mls. of chloromethyl methyl ether were added dropwise to the stirred solution and stirring continued for two hours. The recovered product needed no further purification. (m.p.: 123-5°)  

Elemental analysis:  

Calculated: C: 47.41; H: 5.43; N: 13.83  

Found: C: 47.20, H: 5.61; N: 14.00.  

All attempts at recrystallization resulted in decomposition to nicotinamide.  

1-Methyl-3-carbamoylpyridinium iodide:  

The preparation follows the method used by Suelter and Metzler for 1-n-propyl nicotinamide iodide. Sixty-four millimoles of nicotinamide (7.80 grams), 4.0 mls. of methyl iodide, and 27 mls. of methanol were refluxed for four hours, yielding 13.7 grams (81%) of crude product. The crude material was recrystallized from 100% ethanol, yielding fine
feather-like crystals. (m.p.: 207-210° dec.; lit m.p.: 204°)

1,1'-Methylenebis(3-carbamoylpyridinium) chloride iodide:

Thirty millimoles each of nicotinamide (3.66 grams) and chloroiodomethane (5.30 grams) in 15.0 mls of acetone were refluxed for two days. The resulting crystals were filtered, washed with acetone and dried under aspirator vacuum. The crude product was recrystallized from water to give bright yellow crystals (m.p.: 218-219° dec.)

Elemental analysis:
- calculated: C: 37.10; H: 3.33; N: 13.32, Cl: 8.41; I: 30.20

This compound was the only product recovered during attempts to synthesize 1-chloromethyl-3-carbamoylpyridinium iodide, even when a five-fold excess of chloroiodomethane was used.

1,1'-Methylenebis(3-carbamoylpyridinium chloride):

Thirty millimoles of nicotinamide (3.66 grams), 10.0 mls. of methylene chloride, a catalytic amount of sodium iodide, 12.0 mls. of acetone and 5.0 mls. of dimethylformamide were heated in a pressure bottle at 90-95° for five days. The 45% yield of crude crystals were filtered, washed with acetone and dried under aspirator vacuum. The crude product was dissolved in refluxing water, treated with decolourizing
carbon and recrystallized. The crystals were recrystallized a second time from water (m.p.: 192-3° dec.)

Elemental analysis:

- calculated for monohydrate: C: 44.95; H: 4.61; N: 16.13
- found: C: 44.78; H: 4.70; N: 15.66.

The proton resonance spectrum of this compound was almost identical with that of 1,1'-methylenebis(3-carbamoylpyridinium) chloride iodide, thus confirming the structure assigned (XIXa).

![Chemical structure of compound](image)

**Chemical shift (no. of hydrogen)**

<table>
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<tr>
<th></th>
<th>H1</th>
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<td>9.85(1)</td>
<td>9.2(1)</td>
<td>8.5(1)</td>
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<td>Dichloride salt</td>
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<td>9.9(1)</td>
<td>9.2(1)</td>
<td>8.5(1)</td>
<td>9.6(1)</td>
</tr>
</tbody>
</table>
6.4 Synthesis of 1,4-dihydropyridines.

The 1,4-dihydropyridines were prepared by the classical method of reduction of the corresponding pyridinium salts with sodium dithionite. The pyridinium salts were used directly as recovered from their preparative reaction mixtures (vide ante).

The general method consisted of dissolving 10.0 millimoles of the pyridinium salt in 15.0 mls. of water. High purity nitrogen was bubbled through this solution for at least ten minutes before the reaction was begun and continued throughout the reaction. Sodium dithionite (2.61 grams, approximately 15 millimoles) and sodium or potassium carbonate (20 millimoles) were mixed and added portionwise over a five minute period. For dihydropyridines which are particularly sensitive to acid (e.g. 1-methyl-3-carbamoyl-1,4-dihydropyridine), the carbonate was replaced by 35 millimoles of sodium or potassium hydroxide. This was added to the reaction mixture as needed to maintain the pH greater than 8. After addition of the sodium dithionite and the base, the reaction mixture was stirred until it turned to a bright yellow colour or until crystallization occurred. Crystals were filtered, washed with small portions of water, and dried under aspirator vacuum. Products which did not crystallize were recovered by continuous extraction with methylene chloride which had been stored over potassium hydroxide. The methylene chloride extract was concentrated from about 200 mls.
to about 20 mls. on a rotary evaporator. The remaining solvent was evaporated under aspirator vacuum in a flask fitted with a nitrogen bleed. The nitrogen bleed was necessary in order to recover a crystalline product; without it, evaporation of the remaining methylene chloride left only a yellow oil. Individual variations on this general method are noted below.

1-Acetonyl-3-carbamoyl-1,4-dihydropyridine:

Ten millimoles (2.14 grams) of crude 1-acetonyl-3-carbamoylpyridinium chloride were dissolved in 15 mls. of water. Potassium carbonate (1.66 grams, 12 millimoles) and sodium dithionite (2.08 grams, 12 millimoles) were mixed and added portionwise. The mixture was stirred under nitrogen for 30 minutes, then extracted overnight with methylene chloride. The extract was concentrated to about 20 mls. on a rotary evaporator and the remaining solvent was evaporated in vacuo in a flask fitted with a nitrogen bleed. The crude product was recrystallized from ethyl acetate (m.p.: 110-21° dec.)

Elemental analysis:

calculated:  C: 60.00; H: 6.67; N: 15.56
found:  C: 60.33; H: 6.63; N: 15.20.
1-Carbomethoxymethyl-3-carbamoyl-1,4-dihydropyridine:

Ten millimoles (2.30 grams) of crude 1-carbomethoxy-methyl-3-carbamoylpyridinium chloride were dissolved in 20 mls. of water. Potassium carbonate (1.66 grams, 12 millimoles) and sodium dithionite (2.08 grams, 12 millimoles) were mixed and added portionwise. The mixture was stirred under nitrogen for 15 minutes. The resulting crystals were filtered and washed with small portions of water. The crude product was recrystallized from benzene to give yellow needles, which were stable in air for a few months. (m.p.: 128-132° dec.)

Elemental analysis:

calculated:  C; 55.12; H: 6.12; N: 14.29


1-Carbo-i-propoxymethyl-3-carbamoyl-1,4-dihydropyridine:

Ten millimoles (2.58 grams) of 1-carbo-i-propoxymethyl-3-carbamoylpyridinium chloride were dissolved in 15 mls. of water. Sodium dithionite (2.61 grams, 15 millimoles, and potassium carbonate (2.76 grams, 20 millimoles) were mixed and added portionwise. After stirring for about five minutes, a red oil separated. The mixture was extracted continuously overnight with 150 mls. of methylene chloride. The extract was reduced to about 20 mls. on a rotary evaporator, then evaporated to dryness in vacuo in a flask fitted with a nitrogen bleed. The crude product was recrystallized from
ethyl acetate (20 mls/gram), being allowed to cool under nitrogen. The crystals appear to require several days of exposure to air for noticeable decomposition. (m.p.: 112-4° dec.)

Elemental analysis:
- calculated: C: 51.06; H: 5.80; N: 10.83
- found: C: 50.89; H: 5.69; N: 10.88.

1-Carbamoylmethyl-3-acetyl-1,4-dihydropyridine:

Ten millimoles (2.14 grams) of crude 1-carbamoylmethyl-3-acetylpseudridinium chloride were dissolved in 15 mls. of water. Sodium dithionite (2.61 grams, 15 millimoles) and potassium carbonate (2.76 grams, 20 millimoles) were mixed and added portionwise. The mixture was stirred under nitrogen for 20 minutes before filtering. The filtrate was extracted continuously for three days with 150 mls. of methylene chloride, the extract evaporated to about 20 mls. on a rotary evaporator, then to dryness in vacuo in a flask fitted with a nitrogen bleed, yielding 1.16 grams of crude product (64%). The crude material was recrystallized from ethyl acetate (160 mls/gram), cooling under nitrogen, to give fine yellow needles, which are stable for several days in air. (m.p.: 162-3° dec.)

Elemental analysis:
- calculated: C: 60.00; H: 6.67; N: 15.56
- found: C: 60.31; H: 6.80; N: 15.30.
1-Carbamoylmethyl-3-carbamoyl-1,4-dihydropyridine:

Ten millimoles (2.15 grams) of 1-carbamoylmethyl-3-carbamoylpyridinium chloride were dissolved in 20 mls. of water. Sodium dithionite (3.48 grams, 20 millimoles) and potassium carbonate (2.76 grams, 20 millimoles) were mixed and added portionwise. The mixture was stirred under nitrogen for ten minutes and the crude product was filtered, washed with 30 mls. of cold water, and dried under aspirator vacuum, yielding 1.65 grams (91%). The crude crystals were recrystallized from water (30 mls/gram) giving an overall 66% yield of yellow, rod-like crystals, which are stable indefinitely in the presence of air at room temperature. (m.p.: 179-182° dec.)

Elemental analysis:

    calculated:  C; 53.0; H: 6.1; N: 23.2
    found:      C: 52.79; H: 6.21; N: 23.05.

1-Carbamoylmethyl-3-cyano-1,4-dihydropyridine:

Five millimoles (1.0 grams) of crude 1-carbamoylmethyl-3-cyanopyridinium chloride were dissolved in 7 mls. of water and the solution was flushed with nitrogen gas. Sodium dithionite (2.0 grams) and potassium carbonate (1.38 grams) powders were mixed and added portionwise to the stirred solution. Crystals formed within about two minutes after the final addition of reagents. The mixture was stirred under nitrogen in a cold water bath for one hour. The
crystals were filtered, washed with small portions of water and dried overnight under aspirator vacuum. The filtrate and washings were extracted overnight with methylene chloride. The methylene chloride extract was evaporated to dryness on a rotary evaporator. The former crystals were combined with the extraction product and recrystallized from benzene (100 mls/gram). A yield of 70 milligrams (approximately 10%) of pure product were obtained. (m.p. = 139-139.5°C)

Elemental analysis:

- calculated: C: 58.39; H: 5.52; N: 25.77
- found: C: 58.16; H: 5.72; N: 25.45.

1-Carboxymethyl-3-fluoro-1,4-dihydropyridine:

Five millimoles (0.95 grams) of 1-carboxymethyl-3-fluoropyridinium chloride was dissolved in 7 mls. of water. The solution was flushed with nitrogen. Sodium dithionite (1.3 grams) and potassium hydroxide (0.9 grams) were added in alternate portions, the pH being maintained greater than ten. Crystals formed within a couple of minutes and were filtered immediately. If the crystals were stirred in solution for more than ten minutes, they redissolved. Continuous extraction of the filtrate with methylene chloride yielded no product. Presumably, the dihydropyridine is so susceptible to acid catalyzed decomposition that the product must be recovered immediately even at a pH as high as ten. The crude product was recrystallized from benzene (40 mls/gram) giving a 10% yield of pure product. (m.p.: 116.5-118° dec.)
Elemental analysis:

calculated: C: 53.85; H: 5.77; N: 17.95
found: C: 53.88; H: 5.80; N: 17.94.

1-Cyanomethyl-3-carbamoyl-1,4-dihydropyridine:

One gram (5 millimoles) of 1-cyanomethyl-3-carbamoyl-pyridinium chloride was dissolved in 12 mls. of water. Potassium carbonate (1.38 grams, 10 millimoles) and sodium dithionite (1.74 grams, 10 millimoles) were mixed and added portionwise. The mixture was stirred for 30 minutes before the product began to crystallize and then for another 30 minutes after crystallization had begun. The crystals were filtered and washed with small portions of cold water. The crude crystals were dissolved in refluxing methylene chloride and filtered. Petroleum ether was added until crystallization began. The solution was cooled under nitrogen to yield fluffy, pale yellow crystals. (m.p.: 127-130° dec.)

Elemental analysis:

calculated: C: 58.89; H: 5.52; N: 25.77
found: C: 58.72; H: 5.68; N: 25.75.

1-Methoxymethyl-3-carbamoyl-1,4-dihydropyridine:

Ten millimoles (2.02 grams) of 1-methoxymethyl-3-carbamoylpyridinium chloride were dissolved in 15 mls. of water. Sodium dithionite (2.61 grams, 15 millimoles) and potassium carbonate (2.76 grams, 20 millimoles) were mixed and added portionwise. The mixture was stirred under
nitrogen for five minutes, then extracted continuously over-
night with 150 mls. of methylene chloride. The extract was
evaporated to about 20 mls. on a rotary evaporator. The re-
mainning solvent was evaporated under vacuum in a flask
fitted with a nitrogen bleed to yield 1.16 grams (70%) of pale
yellow crystals. The crude crystals were recrystallized
twice from ethyl acetate. The recrystallization flask must
be scraped with a glass rod before sealing the solution under
nitrogen or recrystallization will not occur. The pure
crystals are stable for only a few days in the presence of
air. (m.p.: 106-7° dec.)

Elemental analysis:
calculated:  C: 57.14; H: 7.14; N: 16.67
found:  C: 57.40; H: 7.07; N: 16.66.

1-(2'-Hydroxyethyl)-3-carbamoyl-1,4-dihydropyridine:

Ten millimoles (2.02 grams) of crude 1-(2'-hydroxy-
ethyl)-3-carbamoylpyridinium chloride were dissolved in 15 mls.
of water. Sodium dithionite (2.61 grams, 15 millimoles) were
added alternately. The mixture was stirred under nitrogen for
five minutes, then extracted continuously overnight with 150
mls. of methylene chloride. The extract was evaporated to
about 20 mls on a rotary evaporator. The crystals were fil-
tered, dried, and stored under nitrogen until they were
transferred to the dry box. Less than a day's exposure to air
is sufficient to cause noticeable discoloration of the crystals.
Elemental analysis:

- Calculated: C: 57.14; H: 7.14; N: 16.67
- Found: C: 56.97; H: 6.96; N: 16.53.

1-Methyl-3-carbamoyl-1,4-dihydropyridine:

Ten millimoles (2.64 grams) of crude 1-methyl-3-carbamoyl-
pyridinium iodide were dissolved in 15 mls. of water and the
solution flushed with nitrogen. Sodium dithionite (2.61
grams, 15 millimoles) and potassium hydroxide (1.9 grams, 35
millimoles) were added in alternate portions while the pH was
maintained above 8. After the solution had turned a bright
yellow (about one-half hour), it was extracted continuously
overnight with approximately 200 mls. of methylene chloride.
The extract was evaporated to approximately 20 mls. on a
rotary evaporator and the remaining solvent was evaporated
in vacuo in a flask fitted with a nitrogen bleed. A yield of
0.4 grams of bright yellow crystals was obtained (m.p.: 73.5-
78° dec.; lit m.p.: 84°, 33 85.3-86.8°98); \(\lambda_{\text{max}}\) (\(\varepsilon\)) = 360
(7820); lit \(\lambda_{\text{max}}\) (\(\varepsilon\)) = 355 (6680)98).

Attempts to recrystallize the crude product were unsuccessful.
The lower extinction coefficient in the literature was recorded
in water, where this compound decomposes even in the absence
of any added acid as buffer. The spectrum of this compound
was recorded at pH 9.8 in this study to slow down the decompo-
sition reaction, but even at this pH a decomposition of a
few percent an hour is observable.
6.5 Kinetics

Kinetic runs were followed by visible or u.v. absorption spectroscopy. Reactions which had half-lives greater than five minutes were followed on a Cary 16 spectrophotometer. Whenever the absorbance was not being read, the sample cell was removed from the path of the light beam. Reactions which had half-lives less than five minutes were followed on a Bausch and Lomb Spectronic 505 recording spectrophotometer. A continuous recording was made of the absorbance at a single wavelength and times were marked along this recording.

6.5.1 Acid-catalyzed Decompositions of 1,4-dihydropyridines

The rate of decomposition of 1,4-dihydropyridines in acid was measured by following the loss of dihydropyridine absorbance in the region between 300 and 390 nanometers. The decomposition is first order and, therefore, initial concentrations need not be known. The buffers used were 1.0 M. and 0.1 M. Tris at pH 7.1 and 1.0 M. and 0.1 M. acetate at pH's 5.65, 4.6 and 3.4. Buffer solutions were thermally equilibrated for at least one-half hour before use. Solutions in 1.0 M. buffers were prepared by dissolving crystals of the dihydropyridine in the buffer solution. The timer was started. An 0.1 cm. absorbance cell was filled with this solution and placed in the thermostated cell compartment of the spectrophotometer.
Solutions in 0.1 M. buffers were prepared in either of the two following ways. For slower reactions, 5.0 mls. of a 1.0 M. solution, prepared as above, were immediately diluted to 50 mls. with 1.0 M. sodium perchlorate. A 1.0 cm. absorbance cell was filled with this diluted solution and placed in a second thermostated compartment in the Cary 16 spectrophotometer. For reactions in 1.0 M. buffer which had a half-life of less than five minutes, the 0.1 M. solution was prepared directly. Crystals of the dihydropyridine were dissolved in 1.0 M. sodium perchlorate. Five mls. of a 1.0 M. buffer were added, the timer started, and the solution made up to 50 mls. with 1.0 M. sodium perchlorate. An 0.1 cm. absorbance cell was filled with the sample solution and placed in the thermostated cell compartment of the appropriate spectrophotometer.

The reaction mixtures were analyzed within a few hours after the decomposition was begun. The pH was measured on a Radiometer 26 pH Meter standardized at pH 4 and 7 or 7 and 10. The concentration of acetic acid was determined by titration to a phenolphthalein endpoint with a standard sodium hydroxide solution. The concentration of protonated Tris was determined by titration to pH 10.5 with a standard sodium hydroxide solution.
6.5.2 Reaction Between Flavins and 1,4-Dihydropyridines

The rate of the reaction between riboflavin or flavin mononucleotide and 1,4-dihydropyridines was determined by following the loss of absorbance in the region between 430 and 470 nanometers due to the reduction of the flavin. The dihydropyridine was present in excess in all cases. A stock solution of each of the reactants was prepared and thermally equilibrated in the dry box. The reactants were mixed in either of the two following ways.

For reactions with half-lives greater than five minutes, the reactants were mixed in the dry box. The timer was started immediately and as quickly as possible, a 1.0 cm. absorbance cell was filled to overflowing with the reaction mixture and sealed with a silicone rubber stopper and a rubber septum. The double seal was necessary to keep the cell free of oxygen for at least two hours. The cell was removed from the dry box and placed in a thermostated compartment of the Cary 16. Absorbance readings were taken until the absorbance remained unchanged for at least one-half hour. Too much time was required to fill the absorbance cell and remove it from the dry box for this method to be used with reactions having half-lives less than five minutes. For these reactions, the required volume of flavin stock solution was placed in the absorbance cell in the dry box and the cell was sealed with a silicone rubber stopper and rubber septum. A syringe needle was inserted into the space over the flavin solution in the cell. The top of this needle was then
sealed into a test tube as in Figure 30. This arrangement allowed the pressure to be relieved when the dihydropyridine solution was injected into the cell and, since the needle was never exposed to the atmosphere, it could not act as an avenue for oxygen to diffuse into the cell. Using this method, the absorbance cell remained free of oxygen for a longer time than when the cell was flushed with high-purity nitrogen while the dihydropyridine solution was being injected.

The required volume of dihydropyridine stock solution was measured into a syringe in the dry box and a small volume of dihydropyridine stock solution was placed in a test tube to act as a back-up supply. The test tube was sealed with a rubber septum and the needle of the syringe containing the dihydropyridine solution was inserted through the septum. The syringe was removed from the dry box in this way to prevent oxygen from entering the syringe before the solutions were mixed.

The syringe and absorbance cell apparatus was removed from the box. The absorbance cell was placed in the thermostated compartment of the Bausch and Lomb spectrophotometer, then the dihydropyridine solution was injected into the cell and the timer was started as the cell contents were mixed. The recording of the absorbance was continued until no change was evident over a period of at least 15 minutes. Reoxidation of flavin, caused by oxygen leaking into the cell, was noticeable within an hour of mixing the solutions but this was
Figure 30. Syringe and absorbance cell apparatus for oxygen-free work
longer than the reaction time for any of the solutions prepared in this way.

Stock solutions of riboflavin (Xa) and flavin mononucleotide were prepared by dissolving approximately 7.1 milligrams of sample in 75 to 100 mls. of buffer in a red coloured Kimax "Ray-sorb" glass volumetric flask in the dry box. The riboflavin required at least one-half hour of stirring to completely dissolve. The concentration of the stock solution was determined as soon as possible by measuring the absorbance of the solution at a minimum of three wavelengths.

\[
\text{CH}_2\text{OR} \\
\text{CHOH} \\
\text{CHOH} \\
\text{CHOH} \\
\text{CHOH} \\
\text{N} \quad \text{N} \quad \text{N} \quad \text{O} \\
\text{O}
\]

Stock solutions of 1,4-dihydropyridines were prepared by dissolving the required amount of dihydropyridine crystals in 25 mls. of buffer in the dry box. The amount of dihydropyridine was chosen such that the reactions would have half-lives between three and thirty minutes. The concentration
of the stock solution was determined by measuring the absorbance of the solution at at least three wavelengths. The initial concentrations of the reactants were then calculated from the concentrations of the stock solutions using the appropriate dilutions.
6.6 Polarography

All polarograms were recorded on a Metrohm E 261 Polarograph. The drop time was regulated at 0.166 seconds by a Metrohm drop controller attached to the dropping mercury electrode. The flow constants of the capillary were: a mercury flow rate (m) of 3.25 milligrams/second and a natural drop time of 2.5 seconds. A scan rate of 0.1 volts/minute was used. The potentials were measured against a silver-silver chloride-saturated potassium chloride reference electrode, for which a value of 222 mv. was used to refer the observed polarographic potentials to the standard hydrogen electrode. The Polarecord E 261 is fitted with a charging current compensation which was adjusted for each polarogram to obtain a base line which was as close as possible to horizontal.

The buffer for most compounds was 0.1 M. acetic acid-sodium acetate at a pH of 5.65. The ionic strength was adjusted to 0.1 with sodium perchlorate. Sodium perchlorate was the preferred salt since more nucleophilic salts, such as chloride, formed addition products with some of the more easily reduced pyridinium salts. Lower pH buffers were used for 1-carbamoylmethyl-3-cyanopyridinium chloride (pH = 4.6) and methylene bis(3-carbamoyl-pyridinium chloride) (pH = 3.4) to prevent the reaction of these compounds with hydroxide ion.

Test solutions were prepared by either dissolving a known weight of pyridinium salt or diluting a stock solution
of pyridinium salt in a pipetted volume of buffer in a water-jacketed polarography vessel thermostated at 25°. These test solutions were then deoxygenated by bubbling high purity nitrogen through the solution for at least ten minutes. The nitrogen flow was diverted to pass over the solution while the polarogram was recorded, a period of five to seven minutes.

In order to carry out a complete analysis of the polarographic waves, the current must be measured at several potentials along the wave. The following two methods were used to determine the current, i, and the limiting current, i_d. If the wave was sufficiently isolated from all other waves that both the base line and plateau were linear, as in Figure 31a, then these linear portions were extrapolated to more negative and more positive potentials, respectively. At any given potential, the limiting current (i_d) was measured as the distance between the extrapolated base and plateau lines and the current (i) as the distance between the polarographic wave and the extrapolated base line. Currents were measured from approximately 3% to 97% of the limiting current.

When a second wave appeared within about 200 millivolts of the measured wave, as in Figure 31b the plateau region could not be extrapolated to more positive potentials. A single value for the limiting current was measured at the inflection point between the two waves. The current was measured as before. The presence of the second wave causes
Figure 31. Sample polarograms
a distortion of the plateau region of the measured wave so currents were only measured up to 85% of the limiting current.
6.7 Potentiometry

Potentiometric measurements were made in the dry box under a helium atmosphere. Potentials were recorded on a Beckman Model G pH meter. The instrument was calibrated periodically with an unbuffered solution containing 0.1 M. each of potassium ferrocyanide and potassium ferricyanide. The cell potential was measured with a platinum electrode referred to a saturated potassium chloride-calomel electrode (Figure 32). The cell contents were assumed to have reached equilibrium when potentials recorded an hour apart were constant to within one millivolt.

Rodkey\textsuperscript{14,31,32} has successfully used a flavoenzyme xanthine oxidase to accelerate equilibrium between NAD and a potential mediator such as benzyl viologen. The same mediator system was used in this study to measure NAD solutions and tried with some NAD models. The xanthine oxidase was purchased from Sigma Corp. as a suspension in 2.3 M. ammonium sulphate. The specific enzymatic activity quoted by Sigma was used as a guide when determining the volume of suspension to use each time. Three units of enzyme were prepared for each potentiometry cell, where one unit is the amount required to convert one micromole of xanthine to uric acid per minute at pH 7.5 and 25°. The suspension was centrifuged, the supernatant liquid discarded and the solid transferred to the dry box. The desired amounts of NAD\textsuperscript{+}, NADH, and benzyl viologen were weighed into the potentiometry cell and 25 mls. of buffer
Figure 32. Potentiometry cell.
were added. The total concentration of NAD$^+$ and NADH was between $1 \times 10^{-3}$ and $5 \times 10^{-3}$ M., with the benzyl viologen concentration being five to ten percent of this. One to two mls. of this solution were then used to dissolve the xanthine oxidase and transfer it into the potentiometry cell.

The xanthine oxidase-benzyl viologen mediator system worked well with NAD but gave erratic results with NAD models. A solution containing oxidized and reduced 1-methoxymethyl-3-carbamoylpyridine and benzyl viologen was prepared as above for NAD. The solution was split into two cells and three units of xanthine oxidase were added to each. After equilibrium was apparently reached (the cell potentials were constant for at least one hour), the two cell potentials differed by 50 mv. In other attempts with 1-methoxymethyl-3-carbamoylpyridinium, 1-carbamoylmethyl-3-carbamoylpyridinium and 1-methyl-3-carbamoylpyridinium ions the cell potentials did not respond in a systematic way with changes in the pyridinium-to-dihydropyridine ratio.

Flavins were tested as possible mediators for the NAD models since riboflavin and FMN are readily reduced by 1,4-dihydropyridines and are electromotively active. The reduction potentials of flavins are more positive than any of the NAD models tested, in some cases by as much as 200 mv. but despite this, the measured potentials showed very little scatter. Methyl viologen was also added as a co-mediator to solutions of 1-methyl-3-carbamoyl pyridinium, 1-carboxymethyl-3-carbamoylpyridinium and 1-(2'-hydroxyethyl)-3-carbamoyl-
pyridinium since the cell potentials of the solutions near pH 10 were within 50 mv. of the reduction potential of methyl viologen. Methyl viologen, benzyl viologen, methyl red, and alizarin blue S did not react directly with 1,4-dihydropyridines and, therefore, riboflavin must still be used to catalyze the reduction of methyl viologen by 1,4-dihydropyridines. The necessity of using riboflavin as a catalyst may negate any possible benefits derived from using the methyl viologen since the cell potentials of identical solutions were similar whether methyl viologen was present or not.

To ensure that the flavins were giving correct potential measurements, the method was compared with the xanthine oxidase/benzyl viologen mediator system in the measurement of NAD$^+$ potentials. Identical solutions containing NAD$^+$ and NADH were prepared. Benzyl viologen and xanthine oxidase were added to one solution and riboflavin was added to the other. The riboflavin solution required over twelve hours for the potential to reach a constant value, after which time the measured potentials of the two solutions agreed to within 5 mv.

In general, the oxidation-reduction potentials of the NAD models were determined by the method of mixtures. The desired quantities of a substituted pyridinium salt and the corresponding dihydropyridine were weighed into a Metrohm polarography cell fitted with a water jacket. The weight of pyridinium salt and dihydropyridine were chosen to give a total concentration between $10^{-3}$ and $10^{-2}$ M. Two to four milligrams
of riboflavin or FMN (and approximately one-half milligram of methyl viologen, when used) were weighed into the cell. Riboflavin was used when the pH was greater than 7.5. At pH's less than 7.5, the reduced riboflavin precipitated significantly, leaving too little riboflavin in solution for it to act effectively as a potential mediator so FMN was used instead. (A concentration of flavin of five to ten percent of the total pyridinium-dihydropyridine concentration was required.) Twenty-five mls. of buffer were added to the cell and the crystals were dissolved by stirring the solution for 15 to 20 minutes.

Buffers were chosen to prevent, where possible, the decomposition of pyridinium ions while minimizing the acid catalyzed decomposition of the dihydropyridine. The potentials of NAD$^+$, NMN$^+$, 1-carbamoylmethyl-3-carbamoylpyridinium ion (IIIi), 1-acetonyl-3-carbamoylpyridinium ion (IIIe) and 1-methoxymethyl-3-carbamoylpyridinium ion (IIIId) could be determined between pH 8 and 10. Acid-catalyzed decomposition of the dihydropyridines prevented potentiometric measurements being made of 1-(2'-hydroxyethyl)-3-carbamoylpyridinium ion (iii$c$) below pH 9.5 or 1-methyl-3-carbamoylpyridinium (IIIb) and 1-carboxymethyl-3-carbamoylpyridinium ions (IIIa) below pH 10. The oxidized forms of 1-cyanomethyl-3-carbamoylpyridinium (IIIh), 1-carbamoylmethyl-3-cyanopyridinium (IIIk) and 1-carbamoylmethyl-3-acetylpyridinium (IIIj) ions react sufficiently rapidly with base to prevent their potentials
\[
\begin{align*}
&\text{III} \\
&\text{a: } R_1 = \text{CH}_2\text{COO}^- \\
&\text{b: } \text{CH}_3 \\
&\text{c: } \text{CH}_2\text{CH}_2\text{OH} \\
&\text{d: } \text{CH}_2\text{OCH}_3 \\
&\text{e: } \text{CH}_2\text{COCH}_3 \\
&\text{f: } \text{CH}_2\text{COOCH}_3 \\
&\text{g: } \text{CH}_2\text{COOCH(CH}_3\text{)}_2 \\
&\text{h: } \text{CH}_2\text{CN} \\
&\text{i: } \text{CH}_2\text{CONH}_2 \\
&\text{j: } \text{CH}_2\text{CONH}_2 \\
&\text{k: } \text{CH}_2\text{CONH}_2 \\
&\text{l: } \text{CH}_2\text{CONH}_2 \\
&\text{m: } \text{CH}_2\text{CONH}_2 \\
&\text{n: } \text{CH}_2\text{CONH}_2 \\
&\text{R}_3 = \text{CONH}_2 \\
&\text{V} \\
&\text{R}_1 \\
\end{align*}
\]
from being measured at pH's greater than 6.0, 7.5, and 9.5, respectively. At the two lower pH's there is some unavoidable decomposition of l-cyanomethyl-3-carbamoyl-1,4-dihydropyridine (Vh) and l-carbamoylmethyl-3-cyano-1,4-dihydropyridine (Vk). The ester group of the l-carbomethoxymethyl-3-carbamoylpyridinium ion (IIIf) was so rapidly hydrolyzed by base that it was only stable in acidic media where the corresponding dihydropyridine l-carbomethoxymethyl-3-carbamoyl-1,4-dihydropyridine (Vf) underwent rapid acid-catalyzed decomposition. Thus it was impossible to determine the reduction potential of this compound. The more sterically hindered ester of the l-carbo-i-propoxymethyl-3-carbamoylpyridinium ion (IIIg) was sufficiently stable at pH 7 to allow potential determinations but even at this pH, 30% of the ester had been hydrolyzed to the l-carboxymethyl compound (IIIa) and 20-30% of the dihydropyridine material had decomposed after eight hours. It was assumed that only one dihydropyridine compound was present, l-carbo-i-propoxymethyl-3-carbamoyl-1,4-dihydropyridine (Vg). With a reduction potential difference of approximately 60 millivolts between the l-carbo-i-propoxymethyl compound and the l-carboxymethyl compound and near equal concentrations of the two pyridinium ions, only one to two percent of the total dihydropyridine concentration should be in the form of l-carboxymethyl-3-carbamoyl-1,4-dihydropyridine (Va). The lability of the ester group was utilized for the
preparation of l-carboxymethyl-3-carbamoyl-1,4-dihydropyridine
since at high pH, the ester of l-carbomethoxymethyl-3-carbamoyl-
1,4-dihydropyridine (Vf) is rapidly hydrolyzed and the product
l-carboxymethyl-3-carbamoyl-1,4-dihydropyridine (Va) is de-
composed only very slowly.

Equilibrium as determined by a constant cell potential
was generally reached within three to eight hours. Those
pyridinium ions with less negative reduction potentials tended
to reach equilibrium the quickest. After the cell potential
was measured, the solutions were analyzed to determine the
final concentrations of pyridinium salt and dihydropyridine
and the pH. A sample of the potentiometry solution was
placed in an 0.1 cm. absorbance cell in the dry box. The
remaining solution was placed in an erlenmeyer flask sealed
with a rubber septum.

As quickly as possible after removal from the dry box,
the absorbance of the solution was read on the Cary 16 spectro-
photometer at two wavelengths between 440 and 460 nm. and
at a minimum of three wavelengths between 300 and 400 nm.
These wavelength regions include the absorbance maxima of
dihydropyridine and oxidized flavins. The concentrations
of dihydropyridine and oxidized and reduced flavin were cal-
culated by solving the systems of simultaneous linear equations
containing the absorbance data. At least five different
absorbance combinations were used and the resulting values
averaged to give the concentration values used in subsequent
calculations. When necessitated by high dihydropyridine concentrations (greater than $5 \times 10^{-3}$ M.), the potentiometric solutions were diluted with buffer before the absorbance was read.

The concentration of pyridinium salt in the potentiometry solution was determined by polarography. A calibration line was calculated by the method of least squares from at least seven concentration-diffusion current pairs. The calibration lines were generally linear between $10^{-2}$ and $10^{-4}$ M. although the lines did not pass through the origin. The potentiometry solution was removed from the dry box sealed in an erlenmeyer flask. The polarogram was recorded within two minutes after pouring the erlenmeyer contents into the polarography cell. The analysis must be carried out quickly to minimize the flavin-catalyzed air oxidation of dihydropyridine to the pyridinium ion which is being assayed. After recording the polarogram, the pH of the solution was determined. Except when xanthine oxidase was used, the pH of a particular buffer never varied by more than .05 pH units.

Pyridinium salts have an absorbance maximum between 260 and 270 nm. and attempts were made to use direct spectrophotometric determinations of these compounds. Only 1-acetonyl-3-carbamoylpyridinium and 1-carbamoylmethyl-3-acetylpyridinium ions could be assayed in this way. The results for these two compounds agreed with the polarographic results to within ten percent. The product of acid catalyzed
decomposition of 1,4-dihydropyridines exhibits a high absorbance maximum near 290 nm.\textsuperscript{1,9,10,71} which interferes with the direct spectrophotometric determinations of other pyridinium compounds.

The success of Rodkey\textsuperscript{14,31,32} with the cyanide adduct as an assay method for NAD\textsuperscript{+} inspired attempts to use this method for NAD\textsuperscript{+} models. Success was limited to 1-methoxy-methyl-3-carbamoylpyridinium ions (III\textsubscript{d}) and 1-acetonyl-3-carbamoylpyridinium ions (III\textsubscript{e}) since substituted pyridines having much more negative reduction potentials than NAD were not converted quantitatively to 4-cyano-1,4-dihydropyridines in aqueous cyanide solutions and the cyanide adducts of 1-cyanomethyl-3-carbamoylpyridinium and 1-carbamoyl methyl-3-acetylpyridinium ions, which both have more positive reduction potentials than NAD\textsuperscript{+} underwent significant decomposition over a 15 minute period at pH 5.6 and 8.1 respectively, the pH's at which the potentiometry of these compounds was carried out. In the few cases in which the method was used successfully, it confirmed the concentration determined by polarography.

The polarographic determination of the pyridinium ion held another advantage over the two spectrophotometric methods which were unable to detect decompositions which left the pyridinium ring intact. If the reduction potentials of the original pyridinium ion and its decomposition product are sufficiently different, as was often the case, then the two concentrations can be determined by polarography. One
commonly met decomposition reaction was the hydrolysis of the 3-carbamoyl group at pH's greater than 10. The 3-carboxy product had a half-wave potential between 200 and 300 mv. more negative than the wave due to the 3-carbamoyl compound. The ester groups of 1-carbomethoxymethyl-3-carbamoylpyridinium (IIIf) and 1-carbo-i-propoxymethyl-3-carbamoylpyridinium (II Ig) ions underwent significant base-catalyzed hydrolysis at a pH as low as 7 and the resulting 1-carboxymethyl-3-carbamoylpyridinium ion (IIIa) was easily distinguished from the original esters on the polarograms.
6.8 Absorbance Spectra

Absorbance spectra were recorded on a Cary 16 spectrophotometer between 220 and 520 nm, at 10 nm intervals. Spectral determinations were repeated until the extinction coefficients near \( \lambda_{\text{max}} \) were reproducible to within 3%. The values of \( \lambda_{\text{max}} \) and the corresponding extinction coefficients of most of the compounds used in this study are listed in Table XIX. The spectra were recorded in water, except those of 1-methyl-3-carbamoyl-1,4-dihydropyridine (Vb), 1-(2'-hydroxyethyl)-3-carbamoyl-1,4-dihydropyridine (Vc), 1-carboxymethyl-3-carbamoyl-1,4-dihydropyridine (Va), and 1-carbamoylmethyl-3-fluoro-1,4-dihydropyridine (Vl), which decompose rapidly in pure water and therefore were recorded in pH 10 buffer. Typical spectra of a pyridinium salt and a dihydropyridine are shown in Figure 33. The spectrum of 1-carbamoylmethyl-3-fluoro-1,4-dihydropyridine was so unlike the other spectra that it is shown separately in Figure 34.
Table XIX

$\lambda_{\text{max}}$ and Extinction Coefficients of Compounds Used in this Work

<table>
<thead>
<tr>
<th>$R_1$</th>
<th>$R_3$</th>
<th>$\lambda_{\text{max}}$ $(\text{M}^{-1}\text{cm}^{-1})$</th>
<th>$\lambda_{\text{max}}$ $(\text{M}^{-1}\text{cm}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_2$COO$^-$</td>
<td>CONH$_2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>CONH$_2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CH$_2$CH$_2$OH</td>
<td>CONH$_2$</td>
<td>265</td>
<td>4340</td>
</tr>
<tr>
<td>CH$_2$OCH$_3$</td>
<td>CONH$_2$</td>
<td>264</td>
<td>$\sim$4500</td>
</tr>
<tr>
<td>CH$_2$COCH$_3$</td>
<td>CONH$_2$</td>
<td>266</td>
<td>4860</td>
</tr>
<tr>
<td>CH$_2$COOCH$_3$</td>
<td>CONH$_2$</td>
<td>266</td>
<td>4750</td>
</tr>
<tr>
<td>CH$_2$COOCH(CH$_3$)$_2$</td>
<td>CONH$_2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CH$_2$CN</td>
<td>CONH$_2$</td>
<td>265</td>
<td>4900</td>
</tr>
<tr>
<td>CH$_2$CONH$_2$</td>
<td>CONH$_2$</td>
<td>267</td>
<td>4800</td>
</tr>
<tr>
<td>CH$_2$CONH$_2$</td>
<td>COCH$_3$</td>
<td>267.5</td>
<td>4450</td>
</tr>
<tr>
<td>CH$_2$CONH$_2$</td>
<td>CN</td>
<td>269.5</td>
<td>4670</td>
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<tr>
<td>CH$_2$CONH$_2$</td>
<td>F</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^+$ were not measured.

See Figure 34
Figure 33. Typical Spectra of a Pyridinium ion and a 1,4-dihydropyridine

- 1-(2'-hydroxyethyl)-3-carbamoyl-1,4-dihydropyridine
- 1-(2'-hydroxyethyl)-3-carbamoyl-pyridinium chloride
Figure 34. U.V. absorbance spectrum of 1-carbamoylmethyl-3-fluoro-1,4-dihydropyridine
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