Mechanism of oxidation of reduced pyridine nucleotide analogs by riboflavin

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MECHANISM OF OXIDATION OF REDUCED
PYRIDINE NUCLEOTIDE ANALOGS BY RIBOFLAVIN

by

Clarence Henry Suelter

A Dissertation Submitted to the
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Ames, Iowa
1959
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INTRODUCTION

Riboflavin in its coenzyme forms functions in many biological oxidation processes. One of the reactions catalyzed by riboflavin containing enzymes is the oxidation of reduced diphosphopyridine nucleotide (DPNH). This is a particularly important reaction since riboflavin and diphosphopyridine nucleotide are two of the electron carriers of the respiratory sequence of mitochondria. Associated with electron transport through this sequence is the phosphorylation of adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) which was first quantitatively described by Belitzer (1). There are at least 3 moles of ATP formed during the transfer of electrons from substrate through the respiratory sequence to O₂, one mole of which is believed to be formed by the oxidation of DPNH with riboflavin.

Despite the great advances which have been made in recent years in elucidating the mechanism of various reactions involving ATP formation, there is little known concerning the detailed mechanisms involved in the formation of ATP coupled to electron transport in the respiratory sequence. The purpose of this study, therefore, was to determine the mechanism by which riboflavin oxidizes DPNH in the hope that aside from the fundamental chemistry of riboflavin oxidations, some information could be obtained concerning the phosphorylation coupled to this reaction.
Since much success has been realized recently through the use of model non-enzymatic reactions to explain certain enzymatic processes, this reaction was studied non-enzymatically. However, because of the complexity of the DPNH molecule (Figure 1), a model compound, 1-propyl-1,4-dihydronicotinamide (NPrNH),

was employed in most of this study. This analog has an absorption maximum at 360 μm (Figure 2) which corresponds to the absorption maximum of DPNH occurring at 340 μm.

One sidelight of this problem which should not be neglected is the age old question as to whether an oxidation is mediated via two 1-electron transfers or a simultaneous 2-electron transfer. This is a difficult question and Ball (2) has aptly commented that the decision as to whether an oxidation is a 1-electron or a 2-electron transfer seemed to depend on which was in vogue at the time. This will be discussed briefly in a later section.
Figure 1. Reduced diphosphopyridine nucleotide
Figure 2. Absorption spectrum

•—•—• Nicotinamide-l-propochloride
○—○—○ l-propyl-1,4-dihydronicotinamide
The experimental part of this study was designed to answer typical questions such as: what is the order of the reaction, the effect of solvent, the effect of pH, effect of different buffers, the effect of metal ions and the rate limiting step? Analogs of riboflavin and other analogs of DPNH besides NPrNH were prepared in order to determine how different substituents effect the rate of oxidation. The possibility of a bimolecular complex being formed between NPrNH and riboflavin prior to oxidation was also explored.
Reduced Diposphopyridine Nucleotide and Its Analogs

Reduced diposphopyridine nucleotide (DPNH) was first isolated by Harden and Young (3, 4) in 1906. However, it was not until 1935 that nicotinamide was first shown to be a constituent of this molecule by Euler et al. (5, 6). This was an important observation since Warburg et al. (7), working with triphosphopyridine nucleotide (TPN), soon demonstrated that this moiety was responsible for electron transport. When the coenzyme was reduced chemically with dithionite, one mole of hydrogen was taken up and an enzymatically active product was isolated. Reduction with H₂/Pt resulted in an uptake of 3 moles of hydrogen producing an enzymatically inactive product. In the latter case the pyridine ring of the coenzyme was reduced to a piperidine ring. No other part of the molecule was reduced. To further substantiate the conclusion that nicotinamide was responsible for electron transport, it was shown that reduction of nicotinamide 1-methochloride with dithionite resulted in a product with an absorption maximum at a wavelength of 350 mμ corresponding to the peak at 340 mμ for the reduced coenzyme.

On the basis of the properties of the reduced N-methyl nicotinamide compared with the properties of other ortho or para dihydro-pyridine compounds known at that time, Karrer et al.
sometime later concluded that reduction took place in a position α to the nitrogen of the pyridine ring. The exact structure of the coenzyme remained unquestioned then until 1953 when Pullman (9) undertook a study to determine whether this reduction took place at the "2" or the "6" position. Upon enzymatic reoxidation of DPNH which was chemically reduced with dithionite in D₂O and isolation of nicotinamide, it was shown that the 2 and 6 pyridone obtained from the methyl betaine still contained the same amount of deuterium. It was, therefore, suggested that reduction took place at the "4" position. This observation has since been confirmed by several workers (Mauzerall and Westheimer, 10; Loewus et al., 11; Rafter and Colowick, 12; Hutton and Westheimer, 13). Traber and Karrer (14) have now given an excellent review of their previous work which led to the wrong conclusion.

Another interesting property of DPNH noted early in its history is the instability in acid solution. Haas (15) was the first to give a clear description of this instability by noting the changes in the absorption spectrum of DPNH in acid. The addition of acid caused the absorption band at 340 mμ to disappear with the formation of a new band at 300 mμ. Upon further acidification this band was destroyed, but it remained stable in the presence of bisulfite. Karrer and Stare (16) obtained a non-crystalline, very hygroscopic substance from several n-alkyl dihydronicotinamide derivatives upon treatment with HCl or H₂SO₃. These products contained Cl⁻ or HSO₃⁻.
which could be easily hydrolyzed. It was suggested that addition took place across a double bond. Since these products could not be obtained in crystalline form, their structures were never elucidated.

Renewed interest in this acid modification reaction was aroused after a report of Rafter et al. (17) that glyceraldehyde-3-phosphate dehydrogenase catalyzed a reaction with DPNH resulting in a product identical with that of the primary product produced from DPNH and acid. This modification was catalyzed by pyrophosphate, citrate and ortho phosphate ions. Acetate had no effect in the reaction. Efforts with P$^{32}$ to show incorporation of HPO$_4^{2-}$ into the molecule were fruitless. It was later shown by Chaykin et al. (18) that this enzymatically modified DPNH, which was called DPNH-X, was not identical to the acid modified product since the absorption maximum occurred at 265 m$\mu$ rather than 290 m$\mu$. When DPNH-X was treated with acid, however, a spectrum similar to that of the acid modified product resulted.

Particularly interesting in this regard was the observation of Meinhardt et al. (19) that DPNH-X but not the acid modified product could be converted to DPNH with an enzyme from yeast. ATP and Mg$^{++}$ ion were required for this reaction. Despite the very interesting implications of this observation, this reaction has not been studied further.

Because of these observations which were made in Krebs' Laboratory, Anderson and Berkelhammer (20) initiated a study
of this acid modification reaction and finally succeeded in isolating two products from the reaction of 1-benzyl-1,4-dihydro-3-acetyl pyridine with HCl. Of the two compounds isolated, the structure of I was proven by direct synthesis.

\[ \text{I} \]

Compound II, which was a dimer, was suggested to have the following structure:

\[ \text{II} \]
The reoxidation of either of these two products to the original quaternary salt was not demonstrated.

Wallenfels et al. (21) have also reported the isolation of addition products of DPNH analogs. Particularly interesting is the observation that both the 1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide and the l-(2,6-dichlorobenzyl)-1,6-dihydronicotinamide gave the same addition product with H₂SO₃ in methanol. A rearrangement was invoked with the suggestion that the most stable product was isolated. However, addition of thiophenol and 2,4-dinitrophenylsulfenyl chloride to the 2 isomers gave two products. The structure of the H₂SO₃ adduct was suggested to be

![Chemical structure](image)

but no experimental evidence was offered. The structure of the other addition products was not determined.

Also pertinent for this review is the non-enzymatic oxidation of DPNH or its model compounds. Dolin (22) reported
that 2,6-dichlorophenol indophenol rapidly oxidized DPNH at pH 5.4 but the rate was very slow at pH 7. Ferricyanide reacted at about the same rate. Singer and Kearney (23) studied the oxidation of DPNH by riboflavin. This will be discussed in greater detail on page 23. Schellenberg and Hellerman (24) reported that spirocyclohexylporphyrexide

![Chemical structure](image)

and porphyrindine,

![Chemical structure](image)

obligate 1-electron acceptors, oxidized DPNH instantaneously at pH 4.6 and 8. Fenton's reagent reacted rapidly at pH 4.5 to 5. Ferricyanide, methylene blue, 2,6-dichlorophenol indophenol and riboflavin also oxidized DPNH but at a slower rate, however, the latter three reacted more rapidly in the lower pH
range. The DPN produced in all these oxidations was enzymatically active. It was suggested from these data that DPNH is oxidized more efficiently only by free radical reagents and other molecules which are capable of functioning as 1-electron acceptors.

Burton and Lamborg (25) recently reported that hydrogen peroxide would not oxidize DPNH except in the presence of metal ions. Cu++, Co++ and Ni++ in that order were shown to be effective catalysts. The product of this reaction was shown by chromatography on a Dowex-1-formate column to be DPN.

Concerning DPNH analogs Karrer et al. had previously reported that they could be oxidized by methylene blue (8), indigo sulfonate (26), 2,6-dichlorophenol indophenol (27) and riboflavin (28). Leach (29) also reported that several dyes would oxidize reduced nicotinamide methochloride. However, the nicotinamide methochloride was reduced electrolytically and it has now been shown by Stein and Stiassny (30) that this reduction leads to a reduced analog which is not identical with the product of the dithionite reduction. Two products have been isolated by Paiss and Stein (31) from the electrolytic reduction, a dimer and the 1,6-dihydro or 1,2-dihydro derivatives. Because of the uncertainty then as to whether the ortho or para dihydro derivative was formed, Leach (32) studied the kinetics of the oxidation of reduced N-methyl acridine
since this compound could only be reduced at the para position. The ionic strength, according to Leach, had a negligible effect on the rate but it increased rapidly as the pH decreased. Through a mathematical analysis of the kinetics it was concluded that N-methyl acridan is oxidized by way of its conjugate acid. Since an ionic mechanism for oxidation would generate 2 positive charges on the N-methyl acridan, it was assumed that the oxidation was a free radical reaction.

Later Mauzerall and Westheimer (10) studied the oxidation of several 1-substituted dihydronicotinamides. It was noted that these compounds were oxidized by \( \alpha, \alpha \)-diphenyl-\( \beta \) -picryl hydrazyl in ethanol or benzene, and by alloxan, quinone, phenanthraquinone and malachite green in aqueous solution. Chloral, acetone, biacetyl and pyruvamide would not function as oxidizing agents. The oxidation of deuterated 1-benzyl-1, 4-dihydronicotinamide by malachite green resulted in a direct transfer of deuterium to malachite green. The isotope effect for this oxidation was estimated to be \( 4.5 \pm 0.5 \).
In an extension of this work, Abeles et al. (33) studied the oxidation of 1-benzyl-1,4-dihydronicotinamide with thio-benzophenol. A direct hydrogen transfer from the DPNH analog was again demonstrated with an estimated isotope effect between 4 and 5. The reaction was largely independent of pH but increased with increasing solvent polarity. Electron donating substituents on the thiketone decreased the rate whereas electron withdrawing substituents accelerated it. These data were all interpreted in terms of an ionic mechanism in which a hydride ion was transferred in the rate controlling step.

Recently Wallenfels and Gellrich (34) reported the oxidation of DPNH analogs with several quinones. In accordance with the observations of Abeles et al. (33) the electron withdrawing substituents on the pyridine ring reduced the rate of oxidation. The rate of the oxidation by 2,6-dichlorophenol indophenol, which was given a more detailed analysis, became more rapid at low pH's as was observed by Leach. In contrast to the conclusion of Leach et al. (32), it was concluded that the oxidation was mediated via a hydride ion transfer. The increased rate at low pH was postulated to be due to the formation of protonated indophenol rather than protonation of the reduced DPNH analog since the protonated indophenol should have a much stronger affinity for the hydride ion.

In a study of the oxidation of dihydronaphthalene by quinones, Braude (35) concluded that this oxidation was also a hydride ion transfer. The rate of oxidation increased at
lower pH, which was attributed to the formation of the conjugate acid of the quinone. It was noted that the reactivity of the conjugate acid must exceed that of the quinone by several powers of ten, for the concentration of the conjugate acid present when the increased rate was first noted was extremely small.

Wallenfels and Gellrich (36) have recently reported that reductive titration of DPNH analogs with chromium II acetate, a one electron reducing agent, leads to a dimerized product. This was explained by assuming that the initially formed radical was very reactive and, hence, rapidly dimerized. However, when 1-(2,6-dichlorobenzyl)-isonicotinamide

\[
\begin{align*}
\text{NH}_2 \\
\text{C}=\text{O} \\
\text{C} & \text{H} \\
\text{N} & \text{H} \\
\text{C} & \text{H}_2 \\
\text{C} & \text{H}_2 \\
\text{C} & \text{H}_2 \\
\text{C} & \text{H}_2
\end{align*}
\]

is reduced with chromium II acetate, a resonance stabilized free radical is obtained. These observations formed the basis for the postulation of Wallenfels and Gellrich (36) that oxidation and reduction of DPNH and DPN or its analogs must occur by an ionic mechanism.
The question as to whether DPNH or its analogs are oxidized via an ionic or free radical mechanism must, however, remain unanswered. One-electron oxidizing agents will oxidize these compounds presumably via a free radical, but detailed studies of the mechanism of these oxidations are not available. All evidence for other oxidizing agents substantiates an ionic mechanism.

Riboflavin

Riboflavin (vitamin B$_2$) was first obtained from milk in 1879 by Blyth (37) and, hence, the German word "Lactoflavin" which is still used today reflects its first source. However, it was some 44 years later that Kuhn et al. (38) next described a procedure for extracting the flavin from egg white. It was then described as "one component of the vitamin B$_2$ complex". The compound was noted to undergo a facile and reversible reduction-oxidation i.e. it took up one mole of hydrogen when reduced with H$_2$/PtO and then could easily be reoxidized to the yellow form by simply shaking with air. In a span of two years after Kuhn et al. (38) made his first isolation, the correct structure of riboflavin was confirmed by Karrer et al. (39) by its complete synthesis.

Prior to this time, however, Warburg and Christian (40) had already isolated a yellow enzyme from yeast. This proved to be a major step in the understanding of the biological function of riboflavin, for this yellow color was soon shown by
Warburg and Christian (41) to be due to riboflavin.

The chemical name for riboflavin is 6,7-dimethyl-9-(D-1'-ribityl)-isoalloxazine.

The molecule has one dissociable hydrogen at the "3" position, the pK of which was first determined by Kuhn and Moruzzi (42). For this determination advantage was taken of its brilliant yellow-green fluorescence and a value of 10.2 was reported. The pK for protonation of the isoalloxazine ring was also determined fluorometrically. A pK of 2 was reported which disagrees with a value of -0.2 reported later by Michaelis et al. (43). Michaelis determined the pK spectrophotometrically by measuring the absorption at 495 m\(\mu\) in various concentrations of HCl ranging from 0 M to 10.7 M. Their data indicated 50% ionization somewhere between 1 and 2 M HCl and so a value of -0.2 was used by them without claiming much accuracy for it. Weber (44) has stated that this value should be more accurate than 2, since the life time of the excited state of riboflavin was shorter in acid which could lead to a consider-
able error in the pK determined fluorometrically. The pK for the reduced riboflavin was reported by Michaelis (43) to be 6.1.

Since riboflavin was already known to function in biological oxidative-reductive processes, the first studies of the reactions of this compound were concerned with this problem. The red intermediate obtained when riboflavin was reduced in strong mineral acid (10% HCl) was first reported by Kuhn and Wagner-Jauregg (45). This intermediate has a strong absorption band at 490 m\(\mu\). Analogous with the interpretation previously applied to several phenazine compounds by Michaelis and Hill (46), it was suggested that the red form was a semiquinone radical. Michaelis et al. (43) in substantiating and extending these observations reported that the semiquinone was red below pH 0, but only a trace orange pink at pH 1. However, it was their impression that at higher pH, the semiquinone was still present but in another state of ionization hence exhibiting another color. After Michaelis et al. (43) succeeded in isolating the semiquinone as large red plates, Kuhn and Strobele (47) reported the isolation of several different dimeric, partially reduced products. Later Michaelis and Schwarzenbach (48) after a careful analyses of their reductive titration curves of riboflavin could find no evidence for any of these molecular complexes in solution except the dimeric semiquinone.
Recently Beinert (49) has investigated the spectrum of riboflavin at different oxidation levels. The monomeric semiquinone was reported to have a characteristic absorption band at 565 m\(\mu\) between pH 2 and 7. The principal absorption of the dimeric form occurred between 700 and 1100 m\(\mu\). One interesting observation was the detection of the dimeric form even at pH 11.8. No evidence for other dimeric forms was observed.

If this red intermediate is a semiquinone, then it is apparent that riboflavin can exist as a fairly stable free radical. However, the conclusion that riboflavin always functions as a 1-electron oxidizing agent seems to have no justification. The mere fact that this semiquinone can be formed in solution does not indicate that the molecule functions as a 1-electron oxidizing agent since there is no evidence that precludes the possibility of disproportionation. The experiment in which equal amounts of dihydro-riboflavin and riboflavin are mixed under anaerobic conditions to determine whether the semiquinone can be formed by disproportionation has not been done.

Other studies of the oxidation-reduction of riboflavin were those concerned with the oxidation of DPNH and its analogs. As noted earlier in this review Karrer et al. (28) in 1937 had already demonstrated that riboflavin acted as a catalyst in the air oxidation of several DPNH analogs. Singer and Kearney (23) and Schellenberg and Hellerman (24) have since reported
that riboflavin would oxidize DPNH non-enzymatically. Of these the study made by Singer and Kearney (23) is of particular interest. It was observed that the oxidation of DPNH by riboflavin was first order in riboflavin and DPNH. The dihydro-riboflavin produced during the reaction was immediately reoxidized by the dissolved O₂ so that 1st order kinetics were observed. The rate of this reaction was observed to increase with decreasing pH.

Riboflavin has also been shown to catalyze the photo-oxidation of several amines. Recently Frisell et al. (50) reported the study of the photo-oxidation of glycine, methyl glycine, dimethyl glycine and several other amines. The significance that these reactions may have in the biological field is not known, except for the possibility that they may be important in the plant kingdom.

Another very interesting facet of riboflavin chemistry which may be pertinent for this work is the seemingly wide variety of compounds with which it forms bimolecular complexes. The first suggestion that riboflavin may form such complexes was made by Weber (44), based on the fact that several compounds quenched the fluorescence of riboflavin. That this quenching was not due to a collision phenomenon was demonstrated by showing that a lower temperature increased the amount of quenching. If the quenching were due to intermolecular collisions, one would expect a lower temperature to decrease quenching. The following compounds were shown to
form complexes: adenine, adenosine, hypoxanthine, caffeine, thymine, cytosine, hydroquinone and catechol.

Yagi and Matsuoka (51) have reported a change in the absorption spectrum of riboflavin from 300 to 500 m\(\mu\) in the presence of phenol. The concentrations of phenol used ranged from 0.1 to 0.6 M in a 2.66 \(\times\) 10\(^{-5}\) M riboflavin solution. This reaction was found to be bimolecular. Because of the discrepancy in the dissociation constant calculated from spectral and fluorescent data, it was postulated that hydrogen bonding as well as complex formation were responsible for the spectral differences. However, the large concentrations of phenol used makes this interpretation of these results somewhat questionable.

Harbury and Foley (52) have described a more thorough study of this complexing phenomenon. It was suggested, based on the fact that 3-methyl riboflavin formed as strong a complex as riboflavin, that a molecular charge transfer may be an important feature of complex formation and that hydrogen bonding was not significant. In the author's opinion such an interpretation appears incorrect, since the methyl group should increase the electron density of the ring and hence increase hydrogen bonding.

At about this same time Isenberg and Szent-Gyorgyi (53) found that a red color was formed when tryptophan was mixed with riboflavin in solution. The approximate absorption maximum was 500 m\(\mu\), which is quite similar to the semiquinoid form of riboflavin in 1 M HCl. This observation led them to
suggest that tryptophan stabilizes a semiquinone at a neutral pH through some type of complex.

Dolin (54) has observed that after addition of excess DPNH to a flavoprotein peroxidase, a new broad absorption band appears in the region of 520-600 mμ. A riboflavin-DPNH complex was postulated.

Grabe (55, 56) also, on the basis of theoretical calculations, postulated a DPNH-riboflavin complex (Figure 3). One feature of this interaction is that the hydrogen which is transferred to riboflavin during oxidation of DPNH or its analog falls adjacent to the nitrogen at the "10" position of riboflavin and can thus be transferred directly. As the reaction proceeds through the transition state, a positive charge will develop on the nitrogen of the pyridine ring which is adjacent to the carbonyl at the "2" position of riboflavin. If this position of riboflavin accumulates an increased net negative charge during the transition state, then a positive charge on the nitrogen of the complexed nicotinamide moiety will exert a beneficial effect. This simultaneous series of events should aid immensely in the oxidation.

The wide variety of compounds with which riboflavin complexes indicates that this may be an important feature of riboflavin chemistry. The possibility, therefore, exists that a bimolecular complex is formed between DPNH and riboflavin prior to oxidation.
Figure 3. Diagram of Grabe's DPNH-riboflavin complex (55, 56)
$R_1 = \text{-RIBOSE-PHOSPHATE-PHOSPHATE-RIBOSE-ADENINE}$

$R_2 = \text{-CH}_2\text{-C-\text{-C-}}\text{-C-}}\text{-C-}}\text{-H}$
Models for Phosphorylation Coupled to Oxidation

Several model reactions have been proposed to demonstrate phosphorylation coupled to oxidation. The first mechanism was proposed by Wessels (57) and involved phosphorylated vitamin K (Figure 4). This mechanism, in essence, was also proposed by Harrison (58), Wieland and Patterman (59) and Clark et al. (60). None of these authors suggest a mechanism for the formation of the hydroquinone phosphate, which in the reviewer's opinion would probably involve a great expenditure of energy.

Wieland and Patterman (59) and Clark et al. (60) have oxidized the hydroquinone phosphate non-enzymatically with simultaneous phosphorylation. In the first case the hydroquinone phosphate was oxidized in an alcoholic solution by iodine with the accompanied phosphorylation of the alcohol. In the latter case the hydroquinone phosphate was oxidized with bromine in the presence of phosphate to form pyrophosphate.

Recently Grabe (55, 56) has proposed the mechanism (Figure 5) which involves riboflavin and DPNH. This represents a very interesting postulate, but mechanistically is an example of the same theories proposed earlier (57, 58, 59, 60). An excellent review of the mechanisms proposed for oxidative phosphorylation has been written by Slater (61).
Figure 4. Wessel's mechanism for phosphorylation coupled to oxidation (57)
CH₂-CH=C-R

+ ATP → CH₃

ADP → CH₂-CH=C-R + 2H

- 2H

H₃PO₄ → HO-PO²⁻

CH₃

R = -(CH₂)₃CH(CH₂)₃CH(CH₂)₃CHCH₃
Figure 5. Grabe's mechanism for phosphorylation coupled to the oxidation of DPNH by riboflavin (55, 56)
\[
\text{DPNH} + \text{RIBOFLAVIN} \xrightarrow{\text{H}_2\text{PO}_4^-} \text{DPN} + \]

\[
\begin{align*}
\text{R} = \text{CH}_2\text{C} & \text{C} \text{C} \text{C} \text{C} \text{H} \\
\text{OH} & \text{OH} \text{OH} \text{OH} \text{OH}
\end{align*}
\]
EXPERIMENTAL

Methods

Kinetics

The reaction rates were measured through the use of a linear recorder (Varian Associates, Model G-10) attached to a Model DU Beckman Spectrophotometer which was equipped with a thermostated cell compartment. In a typical experiment the buffer, riboflavin solution (usually $10^{-4}$ M) and other components which were previously equilibrated to the desired temperature (25°C unless otherwise stated) were pipetted into a standard 3 ml rectangular silica cell and the volume brought to 3 ml with H$_2$O. To initiate the reaction, 0.1 ml of the DPNH analog (usually $3 \times 10^{-3}$ M) was added with Boyer and Segal's (62) adder-mixer and the optical density recorded at 360 m$m\mu$. Using this technique measurements could be obtained within 8-10 seconds after addition of the reduced analog. For each series of experiments (conducted on the same day) a fresh solution of the DPNH analog was prepared with water adjusted to pH 8-10. A fresh riboflavin solution was also prepared for each day's determination. The pH of each reaction mixture was determined routinely with a Beckman pH meter.
Deuterium analysis

The complete procedure including combustion apparatus, purification of water samples, and analysis of water samples for deuterium oxide is elegantly described by Warkentin (63). The water samples were purified by a modification of the method of Keston et al. (64). The falling drop method described by Kirshenbaum (65) was employed for the determination of the amount of deuterium oxide in the water samples.

Ionization constants for riboflavin

The pK's for riboflavin and its various derivatives were determined spectrophotometrically. Approximately 10^{-4} M solutions of riboflavin or its derivatives were prepared in 0.1 ionic strength buffers except at high acid concentrations where the ionic strength exceeded 0.1. The following buffers were used: pH 0-2, HCl; pH 3-5, formate; pH 5-6.5, acetate; pH 7-8.5, triethanolamine; pH 8.5-10, ethanolamine or borate; pH 10.5-12, ethylamine or carbonate. For the pK of protonation (pK_{1}) the optical density was recorded at 400 and 450 m\(\mu\) and at 340 and 360 m\(\mu\) for pK_{2}. The pH of each solution was determined with a Beckman pH meter. All values were fit to a theoretical curve for the ionization, the mid-point of which determined the pK. In case of pK_{1}, pH measurements could only be made to 0 and hence the limiting optical densities needed for calculation of the theoretical ionization curve had to be
assumed, except for 6,7-dimethyl-9-formylmethylisocarboxazidine. The limiting optical densities which had to be assumed were determined by trial and error until a theoretical curve was obtained which fit the data best. The accuracy of the $pK_1$ values is difficult to estimate. However, since the $a_H$'s for the cation at 390 m$\mu$ (Table 1, p. 51) are essentially identical, it is estimated that the ranges listed for the $pK_1$ are reasonably correct.

**Oxidation of $\beta$-$\delta$-tritiated reduced diphosphopyridine nucleotide ($\beta$-DPNH(T))**

Fifteen and nine tenths mg of $\beta$-DPNH(T) was placed into 0.5 ml of 0.2 ionic strength imidazole buffer pH 7. To this solution was added 3 ml of $4 \times 10^{-4}$ M riboflavin. The reaction mixture was allowed to stand from 24-36 hours. After this time, the pH was adjusted to 2 with 6 N HCl and the DPN was precipitated with 5 volumes of cold acetone. After 12-24 hours, the solution was centrifuged at 4$^\circ$C. The precipitate was placed in 10 ml H$_2$O, adjusted to pH 8, and placed on a Dowex-1-formate column 14 cm long and 1 cm in diameter. The eluate with the highest optical density at 260 m$\mu$ was used for the tritium assay. Determined spectrophotometrically, 9.6 mg of tritiated diphosphopyridine nucleotide (DPN(T)) was isolated.
Oxidation of $\alpha$-H-tritiated reduced diphenolphosphoridinenucleotide ($\alpha$-DPNH(T))

To one of the 250 ml round bottom flasks containing the lyophylized $\alpha$-DPNH(T) as described in materials section was added 5 ml H$_2$O. Two ml of this solution was added to 3 ml of riboflavin (15 mg/100 ml) and the pH adjusted to 7. After 36 hours at room temperature the reaction mixture was adjusted to pH 2, 6 volumes of cold acetone (-30°C) were added and then allowed to remain at -20°C for 8 hours. The procedure for isolation of DPNH(T) is the same as that described under oxidation of $\beta$-DPNH(T) (p. 35). Ten mg of DPNH(T) was obtained as determined spectrophotometrically.

Oxidation of $\alpha$-DPNH(T) by alcohol dehydrogenase

Three ml of the diluted $\alpha$-DPNH(T) preparation as described under oxidation of $\alpha$-DPNH(T) by riboflavin (p. 36) was adjusted to pH 7.4. To this solution was added 0.05 ml of alcohol dehydrogenase (100 mg/ml) (Sigma Chem. Co.) and 0.3 ml of 2% redistilled acetaldehyde as described by Fisher et al. (66). The reaction was allowed to proceed for 45 minutes at which time HCl was added to obtain a pH of 2. Six volumes of cold acetone were added and the solution was stored at -20°C for 8 hours. The procedure for isolation of DPNH(T) is the same as that described under oxidation of $\beta$-DPNH(T) (p. 35). Analyzed spectrophotometrically 13.7 mg of DPNH(T) was obtained.
Tritium assay

One ml of the solution of DPN(T) which was to be assayed was diluted to 25 ml and the optical density at 260 m\(\mu\) determined. The mg of DPN(T)/ml of original solution was calculated from the molar absorbancy (18 \(\times\) 10\(^3\) lt/mole-cm) assuming a molecular weight of 663. One ml of the original solution was then placed on a previously weighed (\(\pm\) 0.005 mg) Model GC-12 sample pan (Nuclear Chicago Corp.). This pan has 4 concentric annules and the sample was contained in the area enclosed by these annules. They were then dried under an infra red heat lamp, reweighed and counted with a gas flow G-M tube. All counts were made to a 2\% deviation as outlined by Aronoff (67, p. 61) and corrected for self absorption as described by Gage (68, p. 57). The samples usually ranged in thickness from 0.280 to 0.500 mg/cm\(^2\) and the correction for self absorption then ranged from 53 to 70\%. Since no standard source was available for tritium, the G-M tube was standardized against a standard C\(^{14}\) source. The amount of tritium present was reported as disintegration/min/mg DPN.

Materials

Nicotinamide-1-propochloride

This compound was prepared by the method which Holman and Wiegand (69) described for the 1-methochloride. Eighteen
and one-half gm of nicotinamide (Eastman Organic) in 30 ml of absolute methanol was refluxed with 27.5 gm n-propyl iodide (Eastman Organic) for 10 hours. The resulting quaternary iodide was converted to the chloride salt with a small excess of freshly prepared silver chloride. The compound was recrystallized from absolute ethanol. Yield 18.4 gm (60%); melting pt. 192-194°; \( a_M = 3.94 \times 10^3 \text{l/mole-cm at 265 m} \mu \).

**1-propyl-1,4-dihydronicotinamide**

Nicotinamide-1-propochloride was reduced by the procedure of Karrer et al. (8) for the preparation of 1-methyl-1,4-dihydronicotinamide. A solution of 4.3 gm of nicotinamide-1-propochloride in 50 ml of \( \text{H}_2\text{O} \) was made alkaline by the addition of 15 gm of anhydrous \( \text{Na}_2\text{CO}_3 \). The solution was then placed in an ice bath and sodium dithionite (\( \text{Na}_2\text{S}_2\text{O}_4 \)) (14 gm) was added in small portions over a period of ten minutes. The reaction was then stirred and \( \text{SO}_2 \) removed by a stream of \( \text{N}_2 \) for 2 hours. The reduced analog was extracted with several portions of \( \text{CHCl}_3 \) totaling 500 ml. The \( \text{CHCl}_3 \) was removed in vacuum. The product was recrystallized as needed from reagent grade ether containing 0.5% (by volume) water. The reduced analog darkened rapidly when kept at room temperature and, hence, was stored in a desiccator at -25°C. The compound melted at 91-91.5°, reported by Mauzerall and Westheimer (10) 91-92°. \( a_M = 7.06 \times 10^3 \text{l/mole-cm at 360 m} \mu \text{ in H}_2\text{O} \), reported (20) 7.15 \times 10^3 \text{ in ethanol.}
$^4$-deuterated-$l$-propyl-$l,4$-dihydronicotinamide ($NPrNH(D)$)

To 3 gm of nicotinamide-$l$-propochloride in 80 ml of 99.5% D$_2$O (Liquid Carbonic) at ca 4°C was added 960 mg of KCN. The solution was placed in a refrigerator (4°C) for 2 hours during which time it became quite dark in color. Hydrochloric acid (6N) was then added dropwise with vigorous agitation until a pH of 7 was attained. During the latter operation and for an additional 20 minutes, N$_2$ was bubbled through the solution to remove all HCN. The preparation was evaporated to dryness in vacuum and then taken up in 25 ml of 99.5% D$_2$O for reduction. This solution was made basic by addition of 3.75 gm Na$_2$CO$_3$. It was then reduced after placing it in an ice bath by adding 8 gm of sodium dithionite in small portions over a ten minute period. Nitrogen was then bubbled through the solution for 2 hours. The precipitated NPrNH was filtered and the eluate was extracted with several portions of CHCl$_3$ totaling 300 ml. The CHCl$_3$ was removed in vacuum and the NPrNH(D) recrystallized from reagent grade ether containing 0.5% H$_2$O by volume.

$l$-benzyl-$3$-acetyl-$l,4$-dihydropyridine

This dihydropyridine was prepared by reduction of $l$-benzyl-$3$-acetyl pyridinium chloride (20) as described by Anderson and Berkelhammer (20). The purified compound melted at 63-66°, reported 61-67° (20). $\alpha_M = 1.04 \times 10^4$ at 380 m\mu.
in 10% ethanol, reported (20) $\alpha_M = 1.04 \times 10^4$ at 371$\mu$ in ethanol.

1-benzyl-1,4-dihydronicotinamide

Following the procedure of Anderson and Borkelhammer (20), nicotinamide-1-benzyl chloride (20) was reduced to give the desired product which melted at 110-112$^\circ$, reported (20) 110-114$^\circ$. $\alpha_M = 7.40 \times 10^4$ at 360 m$\mu$ in 12% ethanol, reported (20) $\alpha_M = 7.42 \times 10^4$ at 355 m$\mu$ in ethanol.

Riboflavin

Riboflavin was purchased from the Merck Chemical Co. It was recrystallized twice from 2 N acetic acid and stored in a desiccator over Mg(ClO$_4$)$_2$ in the dark. $\alpha_M = 1.22 \times 10^4$ at 450 m$\mu$ and 1.06 $\times 10^4$ at 375 m$\mu$.

Riboflavin-5'-phosphate

This compound was obtained gratis from the Sigma Chem. Co. It was used without further purification. $\alpha_M = 1.12 \times 10^4$ at 450 m$\mu$.

Lumiflavin

The procedures and relative quantities of intermediates used for the preparation of lumiflavin were those described by Hemmerich et al. (70). The product was recrystallized from 2 N acetic acid. Calcd. for C$_{13}$H$_{12}$N$_4$O$_2$: C, 60.93; H, 4.72;
3-methyl lumiflavin

3-methyl lumiflavin was synthesized by the same method used for the preparation of lumiflavin. A suspension of 3 gm of N-methyl-3,4-dimethyl-6-(p-carboxyphenyl) azo aniline (70) and 2.6 gm of 3-methyl barbituric acid (71) was refluxed in 10 cc of glacial acetic acid and 30 cc of n-butanol for 5 hours. 1-methyl lumiflavin is not formed under these conditions. An equal volume of ether was added to the reaction mixture and the red orange precipitate was removed by filtration. 3-methyl lumiflavin was recrystallized from anhydrous methyl alcohol. Yield 1 gm; melting pt. 284-285°dec.;

\[ a_M = 3.96 \times 10^4 \text{ at } 265 \text{ m\(\mu\)}, \]  
\[ 9.90 \times 10^3 \text{ at } 370 \text{ m\(\mu\)} \text{ and} \]  
\[ 1.21 \times 10^4 \text{ at } 450 \text{ m\(\mu\)}. \]

Anal. Calcd. for C_{14}N_{14}O_2: C, 62.21; H, 5.22; Found: C, 62.17; H, 5.30.

6,7-dimethyl-9-formylmethylisocarboxazide

The synthesis of this compound was accomplished by the procedure of Fall and Petering (72). The compound which was recrystallized from absolute methanol and dried under vacuum at 78°C for 5 hours contained 1 molecule of methanol and decomposed at 270-271°, reported (72) 270.5-271°. \[ a_M = 2.66 \times 10^4 \text{ at } 270 \text{ m\(\mu\)}, \]  
\[ 9.21 \times 10^3 \text{ at } 375 \text{ m\(\mu\)} \text{ and} \]  
\[ 1.11 \times 10^4 \text{ at } 450 \text{ m\(\mu\)}. \]

Calcd. for C_{14}H_{12}N_{14}O_3-CH_3OH: C, 56.95; H, 5.10; Found: C, 57.04; H, 4.99.
Tritiated DPN

The procedure for preparation of tritiated DPN is adapted from that used by San Pietro (73) for the incorporation of deuterium at the "4" position of the nicotinamide moiety. To 700 mg of DPN·4 H₂O dissolved in 8 ml of 1.25 M KCN was added 2 ml of tritiated H₂O containing approximately 1 mc/ml. Necessary precautions were observed at all times to eliminate inhalation of any tritiated compounds. After this solution had incubated for 2.5 hours at room temperature, 6 N HCl was added with rapid agitation until pH 7 was attained. Nitrogen was then passed through the solution for 20-25 minutes to remove all HCN. The preparation was then adjusted to pH 2 as determined with pH paper and divided equally among four 50 ml polypropylene tubes. Forty to forty-five ml of cold acetone were added to each tube which were then capped and placed in a cold box at -18°C for 24 hours. After this time they were centrifuged, the precipitate was washed 4 times with acetone and stored in a desiccator over Mg(ClO₄)₂ at -30°C. The DPN(T) was purified on a Dowex-1-formate column as described by Kornberg and Pricer (74, p. 22).

The eluate from the column containing the DPN was lyophilized. The lyopholysate was redissolved in a small amount of H₂O, adjusted to pH 2 and reprecipitated with 5 volumes of cold acetone. Yield 418 mg containing 437 ± 5 dis/min/mg of tritium activity against a C¹⁴ standard source. In order to
demonstrate that all the tritium activity was in the nicotinamide moiety, DPN(T) was hydrolyzed and the nicotinamide was isolated as described by Marcus et al. (75). To 21.2 mg of DPN(T) and 21.3 mg of nicotinamide in 16 ml H$_2$O were added 552 mg of KH$_2$PO$_4$. The solution was adjusted to pH 9.2, and placed in a boiling water bath for 12 minutes. The cooled preparation was then adjusted to pH 7.1 and extracted continuously for 8 hours with reagent grade ether in a Kutscher-Steudel extractor. The nicotinamide was recrystallized twice from benzene. The nicotinamide after correction for dilution contained $2.89 \pm 0.13 \times 10^5$ dis/min/m mole. The DPN(T) contained $2.89 \pm 0.03 \times 10^5$ dis/min/m mole.

$\alpha$-DPNH(T)

The procedure used for the preparation of $\alpha$-tritiated DPNH was adapted from Loewus et al. (76). Two samples were prepared as follows. To 5 ml of water were added 26 mg Na$_2$HAsO$_4$•7H$_2$O, 20 mg cysteine HCl, 600 mg tris (hydroxymethyl) aminomethane, 0.1 ml conc HCl, 50 mg of DPN(T) and 1.8 ml of a solution of fructose-1,6-diphosphate (To 700 mg of the Mg salt of fructose-1,6-diphosphate dihydrate and 500 mg of 8 hydroxy quinoline were added 4 ml H$_2$O, adjusted to pH 8.5 with 5 N KOH and centrifuged. The clear supernatant was used directly.). After adjusting the final solution to a pH of 8.4-8.5, 0.2 ml of glyceraldehyde-3-phosphate dehydrogenase (50 mg/ml) (Sigma Chemical Co.) and 0.5 ml of aldolase
(10 mg/ml) (Sigma Chemical Co.) were added. The reaction was complete in 25 minutes. The preparation was then placed in a 250 ml round bottom flask, lypholyzed and stored in a desiccator over Mg(ClO₄)₂ at -30°C. Each flask contained 24 mg DPNH(T) as determined spectrophotometrically.

**β-DPNH(T)**

β-DPNH(T) was made by the method of Fisher et al. (66). To 90 mg of DPN(T) was added 2.5 ml tris (hydroxymethyl) aminomethane buffer (3 gm tris (hydroxymethyl) aminomethane in 40 cc H₂O adjusted to pH 9.5-9.6), 0.3 ml 95% ethanol and 0.3 ml alcohol dehydrogenase solution (0.1 ml alcohol dehydrogenase suspension (Sigma Chemical Co.) (30 mg/ml) in 4.9 ml of gelatin phosphate buffer (0.01 M phosphate, pH 7.4, 0.1% gelatin)). The reaction was complete in 20 minutes. It was then heated in a boiling water bath for 1.5-2 minutes and cooled rapidly. Five ml of absolute ethanol (-15°-20°C) were added and the pH adjusted between 7.3 to 7.4 with 2 N HCl with vigorous stirring. Absolute ethanol was then added until a total volume of 35 ml was attained and the suspension was placed in a refrigerator at -20°C for 24 hours. The suspension was then centrifuged, washed with cold 50/50 ethanol-ether, and finally with cold ether before storing in a desiccator over Mg(ClO₄)₂ at -30°C. Yield 65 mg.
RESULTS AND DISCUSSION

Oxidation of l-propyl-l,4-dihydronicotinamide
with Riboflavin

**Kinetics**

In studying the related oxidation of reduced DPN by riboflavin, Singer and Kearney (23) noted that the dihydro-riboflavin formed during the reaction was immediately re-oxidized by dissolved oxygen. This observation simplifies the kinetic study of oxidation reactions of riboflavin in aqueous solutions because the reactions need not be performed under difficultly attained anaerobic conditions. However, since riboflavin was found to oxidize NPrNH about 160 times more rapidly than DPNH at pH 6.65 in triethanolamine, it was necessary to reconsider this question. The following observed facts indicated that the reoxidation of dihydro-riboflavin by oxygen is not rate limiting in the reaction with NPrNH. First, the absorption of riboflavin at 450 m\(\mu\) does not change during a reaction. A comparison of the spectrum of dihydro-riboflavin given by Beinert (49) with oxidized riboflavin indicates that the formation of significant concentration of dihydro-riboflavin could be detected at this wavelength. Second, the rate of oxidation of NPrNH is the same whether done in a solution which is equilibrated with the atmosphere or one which has
been aerated with pure O₂. The concentration of O₂ in these solutions is estimated to be $2.5 \times 10^{-4}$ M and $2.4 \times 10^{-3}$ M respectively.

Singer and Kearney (23) had previously reported that the oxidation of DPNH by riboflavin was 1st order in both DPNH and riboflavin. The oxidation of NPrNH by riboflavin is also found to obey the same kinetics. When the log of the 1st order rate constant ($k_1$) is plotted against the log of the riboflavin concentration (Figure 6), a straight line with a slope of 1 is obtained. Therefore,

$$\frac{-d(NPrNH)}{dt} = k(NPrNH)(Rb)$$

and, since the riboflavin concentration does not change under aerobic conditions

$$k_2 = \frac{k_1}{(Rb)}$$

where $k_2$ = second order rate constant

$k_1$ = observed 1st order rate constant

(Rb) = concentration of riboflavin

The rate of oxidation versus pH

The effect of pH on this reaction is presented graphically in Figure 7. In the pH range 7-12, the rate of the reaction falls on the calculated theoretical line for the ionization of
Figure 6. Log of 1st order rate constant (sec$^{-1}$) for oxidation of NPrNH versus log of riboflavin concentration
LOG RATE $k_i$
Figure 7. The 2nd order rate constant (lt/mole-sec) for oxidation of NPrNH by riboflavin versus pH

Nitrogen base buffers, 0.1 ionic strength, triethanolamine, pH 5-8.5; ethanolamine, pH 8.5-10.5; ethylamine, pH 10.5-12

Oxy-acid buffers, 0.1 ionic strength, acetate, pH 5-6.5; phosphate, pH 6.5-8.5; borate, pH 8.5-10.5; carbonate, pH 10.5-12
riboflavin. The pK of riboflavin was determined to be 9.95 (Table 1). Since the rate of oxidation approaches zero at pH 12, the riboflavin anion either will not oxidize NPrNH or reacts at an extremely slow rate. Further confirmation of this conclusion can be noted from Table 2. At pH 10, where one-half the riboflavin exists as the anion, 3-methyl lumiflavin which has no ionizable hydrogen, oxidizes NPrNH twice as fast as riboflavin. If one assumes that the rate controlling step in this reaction is the removal of a hydride ion from NPrNH, then the negative charge on the riboflavin would inhibit the oxidation as observed.

At lower pH values the rates must be corrected for the acid-catalyzed decomposition of NPrNH. At pH 5 and in 10^-4 M riboflavin, this correction amounts to 37% of the total rate in acetate buffers and 7% of the total rate in amine buffers.

Table 1. Dissociation constants for riboflavin and riboflavin derivatives

<table>
<thead>
<tr>
<th>Derivative</th>
<th>pK_1</th>
<th>Calcd. a_M for cation at 390 m(\mu)</th>
<th>pK_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>0.12 ± 0.03</td>
<td>1.89 x 10^4</td>
<td>9.95</td>
</tr>
<tr>
<td>Riboflavin-5'-phosphate</td>
<td>0.05 ± 0.02</td>
<td>1.90 x 10^4</td>
<td>10.32</td>
</tr>
<tr>
<td>3-methyl lumiflavin</td>
<td>0.18 ± 0.03</td>
<td>2.20 x 10^4</td>
<td>-</td>
</tr>
<tr>
<td>6,7-dimethyl-9-formyl-methylisalloxazine</td>
<td>3.50 ± 0.02</td>
<td>1.82 x 10^4</td>
<td>a</td>
</tr>
</tbody>
</table>

*This compound is unstable at high pH
Because of this decomposition, it becomes increasingly difficult to obtain reliable data at low pH. Nevertheless, the results leave no doubt that the rate of oxidation is increased greatly at lower pH, since the rate at pH 3.7, which is not given in Figure 7, is estimated to be 400 ± 19 lt/mole-sec.

This increased reaction rate at low pH might result from a more rapid reaction of either the protonated form of riboflavin (pK = 0.12) (Table 1) with NPrNH, or of the protonated form of NPrNH with neutral riboflavin. The latter possibility is unlikely. When the decay curve of NPrNH at various pH's down to pH 3 is extrapolated to zero time, the same absorption at 360 m\(\mu\) is observed. Further, since the decomposition of DPNH analogs is acid catalyzed, and if the mechanism depicted for this reaction (Reaction A, Figure 16, p. 80) is correct, then one would predict that protonation of the ring nitrogen of NPrNH would inhibit this reaction. Such inhibition is not observed. The pK of this ring nitrogen, therefore, must be below pH 1.5.

A third possible explanation for the increased rate at low pH is that a general acid catalysis of the oxidation occurs. The increased rate of oxidation in phosphate buffer as compared to the amine buffers (Figure 7) may be due to general acid catalysis by \(H_2PO_4^-\). However, if it is assumed that the rate limiting step in the oxidation is the transfer of the hydride ion, then in order to have a general acid catalysis, the proton from \(H_2PO_4^-\) or any other general acid
must be complexed with riboflavin prior to the oxidation. If this is the case, then the spectrum of riboflavin in a concentrated phosphate buffer should indicate formation of the riboflavin cation. When the spectrum of riboflavin was observed in 2 M phosphate buffer pH 6, a difference spectrum was obtained which was similar to the spectrum of the riboflavin cation, however, the differences were small and a thorough study was not made.

Rates of oxidation by various flavins

The rates of oxidation of NPrNH by several flavins are compared in Table 2. Of particular interest is the high rate of oxidation by 6,7-dimethyl-9-formylmethylisoalloxazine (Structure A, Figure 8). This increased rate may be due to an interaction of the carbonyl group with the nitrogen at the "1" position as indicated in Structure B (Figure 8). In support of this interaction is the fact that the pK for protonation of 6,7-dimethyl-9-formylmethylisoalloxazine is 3.5 compared to 0.12 for riboflavin (Table 1). The spectrum of the cation formed by protonation is essentially the same as the spectrum of the riboflavin cation (Figure 9). Structure C (Figure 8) is suggested for the cation of the 9-formylmethyl compound.

An explanation for the increased rate of oxidation by riboflavin -5'-phosphate is not readily apparent. There may be an acid catalysis by the hydrogen of the ribityl phosphate as noted with riboflavin in phosphate buffer. At pH 10.2 this
Figure 8. 6,7-dimethyl-9-formylmethylisoalloxazine
A. Neutral form
B. Suggested structure for the reduced compound
C. Suggested structure for the cation
Figure 9. Riboflavin spectra

- - - - Riboflavin anion

x---x--- Neutral form

——— Calculated for riboflavin cation
Table 2. Comparison of rates of oxidation by various riboflavin derivatives

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Rate&lt;sup&gt;a&lt;/sup&gt; at pH 6.8 phosphate 0.1</th>
<th>Rate&lt;sup&gt;a&lt;/sup&gt; at pH 10.2 borate 0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Riboflavin-5'-phosphate</td>
<td>1.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Lumiflavin (5,6,9-trimethyl-isoalloxazine)</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>3-methyl lumiflavin</td>
<td>0.8</td>
<td>2.0</td>
</tr>
<tr>
<td>6,7-dimethyl-9-formylmethyl-isoalloxazine</td>
<td>2</td>
<td>b</td>
</tr>
<tr>
<td>Flavin adenine dinucleotide</td>
<td>0.44</td>
<td>c</td>
</tr>
</tbody>
</table>

<sup>a</sup>The rates given are relative rates with riboflavin given a rate of 1 at pH 6.8 and 10.2

<sup>b</sup>Unstable

<sup>c</sup>Not measured

Hydrogen is not available and a slower rate is observed. Again the doubly charged ribityl phosphate anion present at this pH may exert an effect because of its proximity to the isoalloxazine nucleus. Molecular models do not rule out these suggestions.

Substitution of the hydrogen at the "3" position by a methyl group produced only a small decrease in the rate of oxidation at pH 6.8, and, as mentioned earlier, the rate does
not decrease at pH 10.2.

The slow rate of oxidation by flavin adenine dinucleotide is ascribed to the formation of an internal complex between riboflavin and adenine as described by Weber (44). The complexed riboflavin will not function as an oxidizing agent as shown by the fact that the rate of oxidation of NPrNH in the presence of caffeine, a known complexing agent of riboflavin, is also reduced. In $9.65 \times 10^{-3}$ M caffeine the second order rate constant was decreased by 12%.

**Effects of polarity and ionic strength of the reaction medium**

The rate of oxidation is greatly reduced as the concentration of ethanol in the reaction mixture is increased (Figure 10). The pH of the ethanolic solutions were obtained with a Beckman pH meter which was standardized against 0.001 M HCl at the various ethanol concentrations used. This standard solution was assigned a pH of 3. As predicted then the rate of oxidation increases with increasing ionic strength (Figure 11). It is, therefore, concluded that the transition state for this oxidation must be more polar than either of the two reactants.

**Relative rates of oxidation of DPNH and DPNH analogs**

From the data (Table 3), it is concluded that the stronger the electron withdrawing property of a group attached to the dihydro ring, the slower the rate. These same
Figure 10. Second order rate constant (lt/mole-sec) for oxidation of NPrNH by riboflavin versus percent ethanol by volume in 0.1 ionic strength phosphate buffer, pH 6.3 at 20°C
Figure 11. Second order rate constant (lt/mole-sec) for oxidation of NPrNH by riboflavin in tris (hydroxy-methyl) aminomethane buffer pH 6.85 versus ionic strength.
Table 3. Comparison of rate of oxidation of DPNH and DPNH analogs by riboflavin

<table>
<thead>
<tr>
<th>DPNH analog</th>
<th>$k_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>triethanolamine 0.1 M, pH 6.65</td>
<td></td>
</tr>
<tr>
<td>1-propyl-1,4-dihydronicotinamide</td>
<td>118 l/mole-sec</td>
</tr>
<tr>
<td>1-benzyl-1,4-dihydronicotinamide</td>
<td>29 l/mole-sec</td>
</tr>
<tr>
<td>1-benzyl-3-acetyl-1,4-dihydronicotinamide</td>
<td>3.7 l/mole-sec</td>
</tr>
<tr>
<td>DPNH</td>
<td>0.75 l/mole-sec</td>
</tr>
</tbody>
</table>

Observations were observed by Abeles et al. (33) and Wallenfels and Gellrich (34) during the oxidation of DPNH analogs by other oxidizing agents. These substituent directing effects are as would be predicted if the oxidation were effected by removal of a hydride ion.

**Effect of metal ions**

Most metal ions ($\text{Cu}^+$, $\text{Cu}^{2+}$, $\text{Fe}^{2+}$, $\text{Mg}^{2+}$, $\text{Mn}^{2+}$, $\text{Co}^{2+}$) either have no effect or exhibit a small inhibitory effect. However, $\text{Ag}^+$ is a strong inhibitor. The rate in presence of $1 \times 10^{-4} \text{ M } \text{Ag}^+$ being decreased by a factor of 10. Silver was reported by Weber (44) to form a red complex with riboflavin.

**Deuterium isotope effect**

As noted in the experimental section (p. 34), in order
to analyze the deuterated NPrNH for deuterium, the compound was first combusted with dry O₂ and the water from the combustion train was collected in a dry ice trap. This water sample after purification was analyzed in the falling drop apparatus and found to have a reciprocal drop time of 1.40 ± 0.04 x 10⁻² sec⁻¹ corresponding to 1.41 ± 0.01 deuterium atoms/mole NPrNH. Therefore, 0.4 of the compound was dideuterated and 0.6 was monodeuterated.

When this material was oxidized with riboflavin, the rate was not constant but became slower as the oxidation proceeded indicating that the protiated derivative was being selectively oxidized. By plotting the log of the concentration observed during a reaction, versus time, it was found that the last portion of the decay curve fell on a straight line which extrapolated to zero time to give the theoretical starting concentration of dideuterated product. The second order rate constant calculated from this plot was 37.1 ± 0.6 l/mole-sec in pH 7 tris (hydroxymethyl) aminomethane buffer, 0.1 ionic strength. The rate for the diprotiated product is 117 l/mole-sec, and hence $\frac{k_2 HZO}{k_2 D_2} = 3.16 ± 0.05$. It is, therefore, concluded that a hydrogen bond is being broken in the rate limiting step of the oxidation.

Oxidation of Tritiated Reduced Diphosphopyridine Nucleotide with Riboflavin

Since riboflavin has been shown to form bimolecular com-
Figure 12. Calibration curve of kerosene-bromobenzene mixture in the falling-drop apparatus at 27.8°C
plexes with a variety of compounds, the question arose as to whether such a complex could be formed between NPrNH or DPNH prior to oxidation. Two attempts were made to investigate this possibility.

Bimolecular complex formation can usually be detected spectrally; however, the direct examination of the spectrum of riboflavin or NPrNH in the presence of each other is obviously not possible. In order to examine the spectrum of either of these compounds in the same solution, NPrNH was placed in varying concentrations of riboflavin-5'-phosphate and the observed decay at several wavelengths extrapolated to zero time. Small differences in the molar absorbancy of NPrNH at 360 m\(\mu\) were observed. No change in the absorption spectrum of riboflavin at 450 m\(\mu\) was noted. Bimolecular complexing with riboflavin usually changes the absorption spectrum of riboflavin at 450 m\(\mu\).

If a DPNH-riboflavin complex was formed prior to oxidation, then, because of the stereo-chemistry of the nicotinamide-ribose linkage in DPNH and other asymmetric centers in ribose, one might expect a stereo-specific oxidation as is observed enzymatically (Vennesland, 77). The two reduced isomers of DPNH(T) (\(\alpha\)-DPNH(T) and \(\beta\)-DPNH(T)) were, therefore, made and reoxidized with riboflavin. When either the \(\alpha\)-DPNH(T) or \(\beta\)-DPNH(T) was oxidized with riboflavin, 77% and 81% of the activity was retained (Table 4). It, therefore, appears that non-enzymatically riboflavin has no preference for the \(\alpha\)-H or
Table 4. Oxidation of tritiated DPNH(T)

<table>
<thead>
<tr>
<th>Labeled DPNH</th>
<th>Oxidizing agent</th>
<th>Activity of DPN dis/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN(T)</td>
<td>before reduction</td>
<td>437 ± 5</td>
</tr>
<tr>
<td>β-DPNH(T)</td>
<td>riboflavin</td>
<td>355 ± 10</td>
</tr>
<tr>
<td>α-DPNH(T)</td>
<td>riboflavin</td>
<td>338 ± 13</td>
</tr>
<tr>
<td>α-DPNH(T)</td>
<td>alcohol dehydrogenase</td>
<td>51 ± 10</td>
</tr>
</tbody>
</table>

the β-H of DPNH during oxidation. It is believed that the kinetic isotope effect as demonstrated with NPrNH(D), accounts for the fact that only 23% and 19% of the tritium activity was removed and not 50% as would be expected. Oxidation of α-DPNH(T) with alcohol dehydrogenase removed 89% of the tritium activity.

The question then as to whether a bimolecular complex is formed prior to oxidation cannot be answered at this time. The data presented, however, do not support nor do they exclude the possibility of such a mechanism. However, it is felt that if a complex is formed prior to oxidation, the extent of this interaction must be quite small. Further experimentation is necessary.
Other Reactions of 1-propyl-1,4-dihydronicotinamide

When NPrNH is placed into an acidic solution, the absorption at 360 m\(\mu\) is rapidly lost and a new absorption band appears at 300 m\(\mu\) (Figure 13) as was discussed earlier in this thesis (p. 9). In analyzing the kinetics of this reaction by plotting the log of the 1st order rate constant versus pH (Figure 14) a line with a slope of 1 was obtained with the nitrogen base buffers (nicotinamide, pyridine and histidine) indicating an uptake of 1 proton during the reaction. This observation is confirmed by Anderson and Berkelhammer's (20) demonstration that 1-benzyl-3-acetyl-1,4-dihydropyridine took up one mole \(\text{H}_2\text{O}\) in acid. However, when the log of the rate in formate, acetate and phosphate buffers was plotted versus pH, a line with a slope of 0.57 was realized (Figure 14). Later evidence by Gardner et al. (78) is now available which suggests that this increased effect is due to a general acid and a general base catalysis. Since nitrogen base buffers do not exhibit this general base catalysis, it is suggested, therefore, that phosphate, acetate and formate may actually add to the reduced analog. This is an extremely facile reaction for even at pH 7 in 0.2 M phosphate buffers at 25°C, the half life for the reaction is 46 sec. Other experiments designed to demonstrate addition of phosphate are not complete.

The substituents on the reduced pyridine ring also exert
Figure 13. Absorption spectrum

- - - - - 1-propyl-1,4-dihydronicotinamide

o - o - o Acid catalyzed decomposition product
Figure 14. Log 1st order rate constant (sec$^{-1}$) in

▲▲▲ Nitrogen base buffers

○○○ Oxy-acid buffers
a large effect on the rate of this reaction (Table 5). The

Table 5. Comparison of rate of acid catalyzed decomposition of DPNH analogs

<table>
<thead>
<tr>
<th>DPNH analog</th>
<th>$k_1 \text{ sec}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-propyl-1,4-dihydro-nicotinamide</td>
<td>$5.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>1-benzyl-1,4-dihydro-nicotinamide</td>
<td>$1.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>1-benzyl-3-acetyl-1,4-dihydropyridine</td>
<td>$6.7 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

direction of this effect is exactly the same as that observed in the oxidation with riboflavin i.e. those substituents which tend to decrease the electron density of the ring decrease the rate and vice versa.

The spectrum of the decomposition product of NPrNH in phosphate buffer, pH 7 is shown in Figure 15 (Spectrum 3). After heating the reaction for 1 hour at 50°C, a new product is formed (Spectrum 1, Figure 15). When hydroxylamine is added to this reaction mixture, the absorption at 290 m\(\mu\) is rapidly lost, with formation of a new product absorbing at 260 m\(\mu\) (Spectrum 2, Figure 15). This same spectrum was obtained by Rafter (79) after addition of hydroxylamine to the
Figure 15. Absorption spectrum of

1. Phosphate catalyzed decomposition product of NPrNH after heating for 1 hour at 50°C

2. Phosphate catalyzed decomposition product of NPrNH after reaction with hydroxylamine

3. Phosphate catalyzed decomposition product of NPrNH
decomposition product obtained from 1-methyl-1,4-dihydro-
nicotinamide and HCl.

Interesting enough, however, is the observation that
the primary addition product of NPrNH and phosphate can be
oxidized by riboflavin. The product of this oxidation had an
absorption maximum at 265 m\(\mu\) and chromatographically (Johnson
and Lin, 80) was identical to pure nicotinamide-1-propochloride.

The mechanisms (Figure 16) are suggested to account for
these observations. The structure in brackets demonstrates
the 6-membered ring which can be drawn with phosphate, acetate
or formate to explain why the oxy-acids but not the nitrogen
bases can exert both general acid and general base catalysis.
The addition product of reaction A which is shown with phos­
phate may then react by pathway B or C. Oxidation with ribo-
flavin by reaction B may be visualized to yield a product which
would eliminate phosphate via reaction G to obtain the oxidized
analog. However, sufficient evidence is not available to rule
out the reversibility of reaction A, in which case the reduced
analog would be oxidized directly. Pathway C is the hydroly­
sis of the assumed phosphate addition product to give an open
chain aldehyde which would be in equilibrium with the closed
ring structure shown by reaction D. Either of these two
forms then could possibly be oxidized by riboflavin to yield
the oxidized analog via reaction F.

The significance of these reactions is at present unknown.
However, if phosphate does add to the analog, then the possi-
Figure 16. Suggested mechanisms for reactions observed with NPrNH
RIBOFLAVIN

\[ \text{RIBOFLAVIN} + \text{H}_2\text{PO}_4^- \rightarrow \text{A} \]

\[ \text{B} \rightarrow \text{C} \]

\[ \text{D} \rightarrow \text{E} \]

\[ \text{F} \]

\[ \text{G} \]

\[ \text{R} = -\text{CH}_2-\text{CH}_2-\text{CH}_3 \]
bility exists that a mechanism is available here for phosphorylation of ADP coupled to electron transport in the mitochondria. Whether oxidation of the phosphate addition product (Reaction B, Figure 16) by riboflavin yields a high energy phosphate is at present unknown. If this were the case, then the ring opening reaction as shown by Reaction C (Figure 16) would be greatly favored over loss of inorganic phosphate. The O—P bond in the product of Reaction B (Figure 16) would then be the high energy bond. The possibility exists, however, that the phosphate addition product itself is extremely unstable and can thus act as a phosphorylating agent (Reaction C, Figure 16). Analogous to the previous case, the same O—P bond would constitute the high energy linkage. In both of these cases one could imagine a substituent of the enzyme molecule which would aid in polarizing the carbonyl of the carboxamide group and the electron withdrawing tendency of the nicotinamide ribose linkage would all favor the instability of the O—P bond. Experiments are now underway to test these possibilities.
The mechanism for the oxidation of DPNH by riboflavin has been studied non-enzymatically. Because of the complexity of the DPNH molecule, an analog of DPNH, 1-propyl-1, 4-dihydronicotinamide (NPrNH) was used. The dihydro-riboflavin formed during the oxidation of NPrNH was immediately reoxidized by the oxygen in solution so that 1st order kinetics were observed under aerobic conditions. The reaction was also 1st order with respect to riboflavin.

The rate of the reaction increases with increasing ionic strength and is decreased in non-polar solvents. The riboflavin anion (pK 9.95) does not function as an oxidizing agent, whereas, the cation (pK 0.12) appears to be a potent oxidizing agent. Substituents on the pyridine ring of the reduced analog which decrease the electron density of the ring, retard the rate of oxidation by riboflavin. Substituents on the riboflavin ring which increase the electron density of the ring also retard the rate. The deuterium isotope \( \frac{k_2}{H_2} \) was found to be \( 3.16 \pm 0.05 \). Attempts to demonstrate a bimolecular complex between DPNH or NPrNH and riboflavin were not successful. These data are all interpreted in terms of an ionic mechanism in which a hydride ion is removed from NPrNH in the rate limiting step. The addition of a proton in the final step is a rapid reaction, since 3-methyl lumiflavin at pH 10.2 oxidizes NPrNH at 92% of the rate observed at pH 7.
By analyzing the kinetics of the acid catalyzed decomposition of NPrNH in nitrogen base buffers, it was shown that 1 proton was taken up during the reaction. The rate in oxy-acid buffers was more rapid than in nitrogen base buffers. Because of this catalytic effect, it is postulated that phosphate, acetate or formate actually add to the pyridine nucleus of NPrNH. The decomposition product when formed in phosphate buffer can then be reoxidized with riboflavin. Chromatographically the product of this oxidation was found to be identical to the oxidized analog, nicotinamide-1-propochloride. The mechanism and implications of these reactions are discussed.
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