THE OXIDATION OF A REDUCED PYRIDINE NUCLEOTIDE ANALOG BY FLAVINS

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SUMMARY

The kinetics of the non-enzymic hydrogen transfer from 1-propyl-1,4-dihydronicotinamide to riboflavin have been studied. The riboflavin anion does not react, but the cation formed by protonation of the isoalloxazine nucleus is estimated to react 10^4 times faster than neutral riboflavin. Oxidation rates with several other flavins are compared.

Substituents on the ring nitrogen of the dihydronicotinamide which decrease the electron density of the ring cause a decrease in the oxidation rate.

Oxidation is more rapid the more polar the solvent, and the higher the ionic strength.

The rate of oxidation of the 4-deuterated 1-propyl-1,4-dihydronicotinamide is 3.2 times slower than that of the diprotiated form. Thus a hydrogen atom is removed in the rate limiting step of the oxidation.

The results are consistent with oxidation by a hydride ion transfer mechanism.

INTRODUCTION

Although riboflavin (I) in its coenzyme forms functions in many biological oxidation processes, little is known about the detailed mechanisms of these oxidation–reduction reactions. Riboflavin can be reduced to a stable semiquinone[1–3] and it is often assumed that the enzyme reactions involving flavin coenzymes are 1-electron transfer reactions. However, a hydride ion transfer mechanism may be just as plausible. The exact site at which hydrogen is transferred onto the flavin from the substrate is also uncertain. The possible importance of molecular complex formation prior to hydrogen transfer[4,5] needs clarification.

Of the many riboflavin dependent reactions, the oxidation of DPNH by riboflavin-containing enzymes of the electron transport chain of mitochondria is of special interest. The phosphorylation of adenosine diphosphate to adenosine triphosphate appears to be intimately coupled to this oxidation reaction. We have undertaken a study of the non-enzymic oxidation of DPNH by riboflavin in the hope of answering

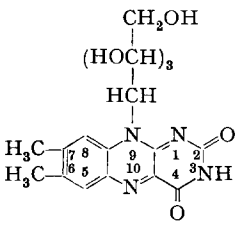
Abbreviations: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; NPrNH, 1-propyl-1,4-dihydronicotinamide.

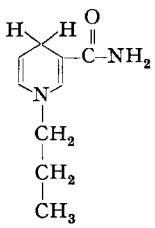
* From the Ph. D. thesis of Clarence H. Suelter, Iowa State University of Science and Technology, 1959.

some questions about the detailed reaction mechanism, and of perhaps gaining some insight relevant to the mechanism of oxidative phosphorylation.

The non-enzymic oxidation of DPNH and DPNH analogs by riboflavin occurs readily at room temperatures[6-8] SINGER AND KEARNEY[6] report that the reaction is first order in both DPNH and in riboflavin, and that the rate of reaction increases with decreasing pH.

Because of the complexity of the DPNH molecule, we have used an analog, NPrNH (II) in most of our experiments. This paper is a report of some of the details of the oxidation of NPrNH by riboflavin. Possible mechanisms for the reaction will be discussed.





I. Riboflavin(6,7-dimethyl-9-ribitylisoalloxazine) II. N-propyl-1,4-dihydronicotinamide(NPrNH)

METHODS AND MATERIALS

Kinetics

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 thermostatic cell compartment. In a typical experiment the buffer, riboflavin solution and other components which were previously equilibrated to the desired temperature were pipetted into a standard 3 ml rectangular silica cell and the volume was brought to 3 ml with H_2O. To initiate the reaction, 0.1 ml of the DPNH analog was added with an adder-mixer[9] and the O.D. was recorded at 360 mμ against a blank containing all reactants except NPrNH. Using this technique, measurements could be obtained within 8–10 sec 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 determinations. The pH of each reaction mixture was determined with a Beckman pH meter.

Deuterium analysis

The deuterium content of the [4-^{2}H]NPrNH was determined by burning the compound in dry oxygen, isolating the water produced, purifying[10] and analyzing for deuterium by the falling drop method[11]. The exact procedures and equipment are described by WARKENTIN[12].

Ionization constants

The apparent acidic dissociation constants of riboflavin and its analogs were

determined spectrophotometrically. Measurements were conducted on approx. 10^{-4} *M* flavin solutions 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 determination of K_1 (dissociation of riboflavin cation) absorbancies were measured at 400 and 450 mμ, and for determination of K_2 at 340 and 360 mμ. The experimental data were fitted with a theoretical titration curve, the midpoint of which determined the pK value. Since pK_1 is very low (except for 6,7-dimethyl-9-formylmethylisoalloxazine) and pH measurements could be made only to 0, the limiting absorbancies of the flavin cations needed for calculation of the theoretical titration curve had to be assumed. These assumed absorbancies were adjusted by trial and error until the theoretical curve was obtained which fit the data best.

MATERIALS

Nicotinamide-1-propochloride was prepared by the method which HOLMAN AND WIEGAND[13] describe for the 1-methochloride. 18.5 g of nicotinamide (Eastman Organic) in 30 ml of absolute methanol was refluxed with 27.5 g *n*-propyl iodide (Eastman Organic) for 10 h. 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 g (60 %); m.p. 192–194°; a_M $3.94 \cdot 10^3$ l/mole-cm at 265 mμ.

1-Propyl-1,4-dihydronicotinamide

Following a procedure of KARRER *et al.*[14], a solution of 4.3 g of nicotinamide-1-propochloride in 50 ml of H_2O was made alkaline by the addition of 15 g of anhydrous Na_2CO_3. The solution was placed in an ice bath and 14 g of sodium dithionite ($Na_2S_2O_4$) was added in small portions over a period of 10 min. The reaction was then stirred and SO_2 was removed by a stream of N_2 for 2 h. The reduced product was extracted with several portions of $CHCl_3$ totaling 500 ml. The $CHCl_3$ was removed *in vacuo* and the product was recrystallized as needed from reagent grade ether containing 0.5 % (by volume) water. The compound darkened rapidly when kept at room temperature, and hence, was stored in a desiccator at —25°. The compound melted at 91–91.5°, reported [15] 91–92°. a_M, $7.06 \cdot 10^3$ l/mole-cm at 360 mμ in H_2O, reported[16] $7.15 \cdot 10^3$ in ethanol.

[4-^{2}H]NPrNH

To 3 g of nicotinamide-1-propochloride in 80 ml of 99.5 % D_2O (Liquid Carbonic) at approx. 4° was added 960 mg of KCN. The solution was placed in a refrigerator (4°) for 2 h during which time it became quite dark in color. Hydrochloric acid (6 *N*) was then added dropwise with vigorous agitation until pH 7 was attained. During the latter operation and for an additional twenty minutes, N_2 was bubbled through the solution to remove all HCN. The preparation was evaporated to dryness *in vacuo* and was then taken up in 25 ml of 99.5 % D_2O for reduction. This solution was made basic by addition of 3.75 g Na_2CO_3. It was then reduced after placing it in an ice bath by the addition of 8 g of sodium dithionite in small portions over a 10-min period.

Nitrogen was bubbled through the solution for 2 h, the precipitated NPrNH was filtered off and additional NPrNH was extracted from the filtrate with several portions of $CHCl_3$ totaling 300 ml. The $CHCl_3$ was removed *in vacuo* and the [4-^{2}H]NPrNH was recrystallized from reagent grade ether containing 0.5 % H_2O by volume.

1-benzyl-3-acetyl-1,4-dihydropyridine was prepared by reduction of 1-benzyl-3-acetyl pyridinium chloride as described by ANDERSON AND BERKELHAMMER[16]. a_M, $1.04 \cdot 10^4$ at 380 mμ (λ_{max}) in 10 % ethanol, reported[16] a_M, $1.04 \cdot 10^4$ at 371 mμ (λ_{max}) in ethanol.

1-benzyl-1,4-dihydronicotinamide was prepared by reduction of nicotinamide-1-benzyl chloride[16]. m.p. 110–112°, reported[16] 110–114°; a_M, $7.40 \cdot 10^4$ at 360 mμ in 12 % ethanol, reported[16] a_M, $7.42 \cdot 10^4$ at 355 mμ in ethanol.

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. a_M, $1.22 \cdot 10^4$ at 450 mμ and $1.06 \cdot 10^4$ at 375 mμ. Riboflavin-5'-phosphate, donated by the Sigma Chem. Co., was used without further purification. a_M, $1.12 \cdot 10^4$ at 450 mμ. Isoriboflavin (5,6-dimethyl-9-ribitylisoalloxazine) was used as obtained from the California Corporation for Biochemical Research.

Lumiflavin was prepared according to the procedure of HEMMERICH *et al.*[17]. The product was recrystallized from 2 *N* acetic acid. Calcd. for $C_{13}H_{12}N_4O_2$: C, 60.93; H, 4.72 %; Found: C, 60.67; H, 4.85 %.

3-Methyllumiflavin was synthesized by the same method used for the preparation of lumiflavin. A suspension of 3 g of N-methyl-3,4-dimethyl-6-(*p*-carboxyphenyl)-azoaniline[17] and 2.6 g of 3-methylbarbituric acid[18] was refluxed in 10 ml of glacial acetic acid and 30 ml of *n*-butanol for 5 h. 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-methyllumiflavin was recrystallized from anhydrous methyl alcohol. Yield 1 g; m.p. 284–285° dec.; a_M, $3.96 \cdot 10^4$ at 265 mμ, $9.90 \cdot 10^3$ at 370 mμ and $1.21 \cdot 10^4$ at 450 mμ. 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-formylmethylisoalloxazine was synthesized by the procedure of FALL AND PETERING[19]. The compound which was recrystallized from absolute methanol and dried under vacuum at 78° for 5 h contained 1 molecule of methanol and decomposed at 270.5–271°. a_M, $2.66 \cdot 10^4$ at 270 mμ, $9.21 \cdot 10^3$ at 375 mμ and $1.11 \cdot 10^4$ at 450 mμ. Calcd. for $C_{14}H_{12}N_4O_3 \cdot CH_3OH$: C, 56.95; H, 5.10 %; Found: C, 57.04; H, 4.99 %.

RESULTS

Kinetics

In studying the related oxidation of reduced DPN by riboflavin, SINGER AND KEARNEY[6] noted that the dihydroriboflavin formed during the reaction was immediately reoxidized by dissolved oxygen. This observation simplifies the kinetic study of oxidation reactions of riboflavin in aqueous solution because the reactions need not be performed under difficultly attaine danaerobic conditions. However, since riboflavin oxidized NPrNH about 160 times more rapidly than DPNH at pH 6.65 in triethanolamine, it is necessary to reconsider this point. The following facts indicate that the reoxidation of reduced riboflavin by oxygen is not rate-limiting in the reaction with NPrNH.

1. The absorption of riboflavin at 450 mμ does not change during a reaction. A comparison of the spectra of dihydroriboflavin[3] and oxidized riboflavin indicates that the formation of a significant concentration of dihydroriboflavin could be detected at this wavelength.

2. The rate of the oxidation of NPrNH is the same whether done in a solution which is equilibrated with the atmosphere or in one which has been aerated with pure O_2. The concentration of O_2 in these solutions is estimated to be $2.6\ 10^{-4}\ M$ and $1.23 \cdot 10^{-3}\ M$ respectively.

3. The 2nd order rate constant obtained in an anaerobic solution was identical to that obtained in an aerobic solution*.

Since dihydroriboflavin is immediately reoxidized by oxygen, the measured rate is that of the oxidation of NPrNH by riboflavin. The observed kinetics are first order with respect to NPrNH under all conditions tested and the first order rate constant k_1 varies in direct proportion to the riboflavin concentration (Fig. 1). Therefore, a second order rate constant, k_2 can be computed by equation 1.

$$-\frac{d(NPrNH)}{dt} = k_1(NPrNH) = k_2(NPrNH)\ (riboflavin) \qquad (1)$$

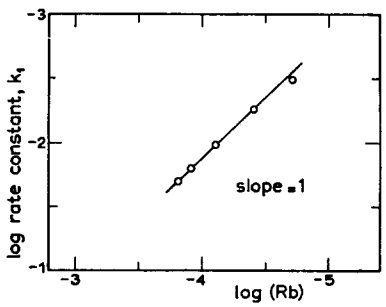


Fig. 1. Logarithm of the first order rate constant, k_1 (sec^{-1}) for the oxidation of NPrNH versus the logarithm of the riboflavin concentration, 25°.

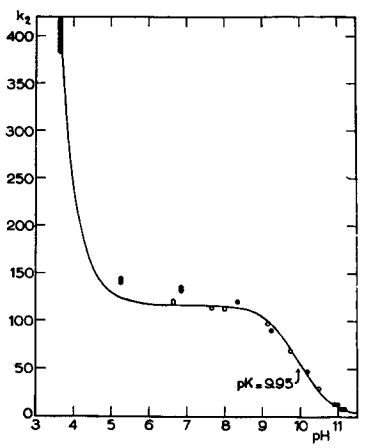


Fig. 2. The second order rate constant, k_2 ($l \cdot mole^{-1}\ sec^{-1}$ for oxidation of NPrNH by riboflavin versus pH, 0.1 ionic strength, 25°. ○, Nitrogen base buffers: pH 5–8.5, triethanolamine; pH 8.5–10.5, ethanolamine; pH 10.5–12, ethylamine. ●, Oxy-acid buffers: pH 3.6–6.5, acetic acid; pH 6.5–8.5, phosphoric acid; pH 8.5–10.5, boric acid; pH 10.5–12, bicarbonate.

The rate of oxidation versus *pH*

The second order rate constant k_2 is plotted against pH in Fig. 2. Above pH 7 the points fit the curve (solid line) which has been calculated upon the assumption that only the neutral form of riboflavin reacts at a measurable rate. This curve is governed by the dissociation of the hydrogen on position 3 of the flavin ring system with a pK of 9.95 (Others have reported values of 9.8 (see ref. 20) and 10.2 (see ref. 21).

Since the rate of the oxidation approaches zero at pH 12, the riboflavin anion either will not oxidize NPrNH or reacts at an extremely slow rate. Further confirma-

* We are indebted to Mr. D. CARR for the measurement of the rate constant in anaerobic solutions.

tion of this conclusion is found in the fact that the rate of oxidation by 3-methyllumiflavin, which has no ionizable hydrogen, is just as large at pH 10.2 as at pH 6.8 (Table I).

At lower pH values the loss of NPrNH through oxidation by riboflavin must be corrected for the acid-catalyzed decomposition of NPrNH. At pH 5 (10^{-4} M riboflavin) this correction amounts to 37 % of the total rate in acetate buffer and 7.2 %

TABLE I

COMPARISON OF RATES OF OXIDATION OF NPrNH AND pK VALUES FOR VARIOUS RIBOFLAVIN DERIVATIVES

Oxidant	*Rate* at pH 6.8*	*Rate at pH 10.2*	*pK_1***	*pK_2*
Riboflavin	1	0.35	0.12	9.95
Riboflavin-5′-phosphate	1.5	0.3	0.05	10.32
Isoriboflavin (5,6-dimethyl-9-ribityl-isoalloxazine)	2	0.8	— 1	10.0
Lumiflavin (6,7,9-trimethylisoalloxazine)	0.9	0.3		
3-methyllumiflavin	0.8	0.7	0.18	
6,7-dimethyl-9-formylmethylisoalloxazine	2	***	3.50	***
Flavin adenine dinucleotide	0.44			

* The rates given are relative with riboflavin assigned a value of 1 at pH 6.8 phosphate buffer, 0.1 ionic strength. Rates at pH 10.2 were measured in borate buffer, 0.1 ionic strength.

** Apparent acid dissociation constants, pK_1 for the protonated isoalloxazine nucleus and pK_2 for the dissociation of the hydrogen on position 3 (see METHODS).

*** Unstable at high pH.

of the total rate in amine buffers. 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 as the pH is lowered. A similar acid catalysis has been reported in the oxidation of N-2,6-dichlorobenzyl-1,4-dihydronicotinamide[22] and N-methyl acridan[23] by 2,6-dichlorophenol indophenol and in the oxidation of N-2,6-dichlorobenzyl-1,4-dihydronicotinamide[20] and dihydrobenzene[24] by quinones.

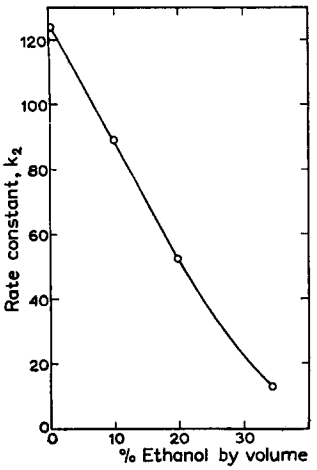
The increased reaction rate at low pH might result from a more rapid reaction of either the protonated form of riboflavin (pK = 0.12) with NPrNH, or of a protonated form of NPrNH with neutral riboflavin. The latter possibility is unlikely. When the decay curve of NPrNH at various pH values down to pH 3 is extrapolated to zero time, the same O.D. is observed, hence, the pK of the ring nitrogen of NPrNH is probably below pH 1.5. On this basis we estimate that the second order rate constant for the protonated riboflavin is $1 \cdot 10^6$, nearly 10^4 times greater than that for neutral riboflavin.

A third possible explanation for the increased reaction rate at low pH is that a general acid catalysis of the oxidation occurs. The increased rate of oxidation in phosphate buffer at pH 7 over that in amine buffers (Fig. 2) might result from general acid catalysis by $H_2PO_4^-$.

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

The rate of oxidation is greatly reduced as the concentration of ethanol is increased (Fig. 3). The pH of the ethanolic solution 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. The rate of oxidation increases with increasing ionic strength (Fig. 4), as would be expected for an ionic mechanism.



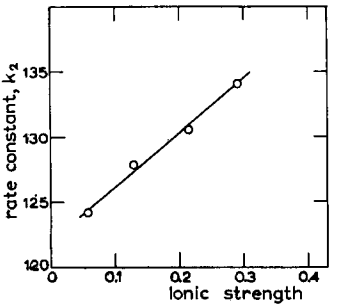


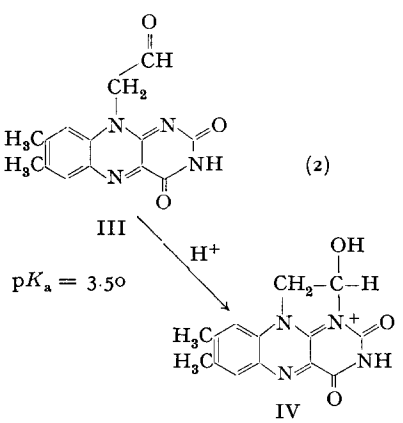
Fig. 4. Rate of oxidation of NPrNH by riboflavin in tris(hydroxymethyl)aminomethane buffer, pH 6.85, *versus* ionic strength.

Fig. 3. Rate of oxidation of NPrNH by riboflavin, *versus* ethanol concentration, 0.1 ionic strength phosphate buffer, pH 6.3, 20°.

From the measurements at various temperatures from 20 to 49°, the Arrhenius activation energy was estimated to be 4.7 ± 0.8 kcal/mole.

Effect of flavin structure on rates of oxidation

The rates of oxidation of NPrNH by several flavins are compared in Table I. Of particular interest is the high reactivity of 6,7-dimethyl-9-formylmethylisoalloxazine (III). This increased rate may be due to an interaction of the carbonyl



group with the No. 1 nitrogen. Such interaction would be favored in the cation (IV), as indicated in eqn. 2. In support of this interaction is the high pK of 3.5 for the protonated form of 6,7-dimethyl-9-formylmethylisoalloxazine. The spectrum of the cation formed by portonation is essentially the same for both flavins (Fig. 5).

The cause for the increased rate of oxidation by riboflavin-5'-phosphate is uncertain, but may also be an acid catalysis by the protonated phosphate group. At pH 10 this hydrogen is not available and a slower rate is observed. Again the doubly charged ribityl phosphate anion may exert an effect because of its proximity to the isoalloxazine nucleus, both on the reaction rate and on pK_2.

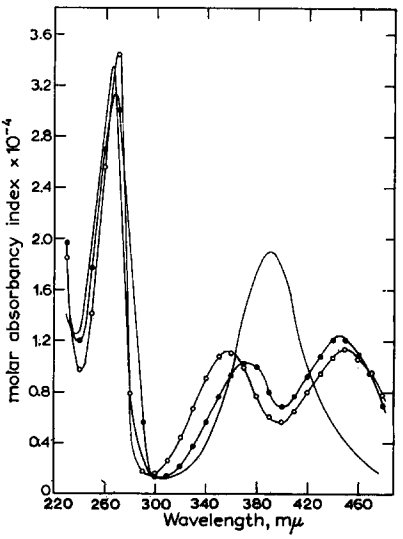


Fig. 5. Riboflavin spectra: O, anion; ●, neutral; —, calculated for riboflavin cation, from data at low pH and estimated pK value.

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[25]. The complexed riboflavin either will not function as an oxidizing agent or reacts at a slower rate. In the presence of caffeine ($9.7 \cdot 10^{-3}$ M) a known complexing agent of riboflavin, the 2nd order rate constant for oxidation of NPrNH by riboflavin was decreased by 12 %.

Neither replacement of the ribityl group by a methyl group to give lumiflavin nor substitution of the No. 3 hydrogen of lumiflavin by a methyl group have significant effects on the reaction rate at pH 6.8. However, as mentioned on page 27, with 3-methyl lumiflavin as an oxidant, the rate does not decrease at pH 10.2.

We cannot offer any certain explanations for the increased rate observed with isoriboflavin and the low pK_1 (Table I). The high catalytic activity of this compound has been reported previously by SINGER AND KEARNEY[6].

Relative rates of oxidation of DPNH and DPNH analogs

From the data in Table II, which compares rates of oxidation for NPrNH, DPNH and two other analogs, we conclude that the stronger the electron withdrawing property of a group attached to the dihydropyridine ring, the slower the rate of oxidation. WALLENFELS AND GELLRICH[22] have noted the same substituent effect during the oxidation of other DPNH analogs by 2,6-dichlorophenol indophenol.

TABLE II

COMPARISON OF RATE OF OXIDATION OF DPNH AND DPNH ANALOGS BY RIBOFLAVIN

Triethanolamine buffer, pH 6.65.

Compound	k_2, 0.1 μ
1 propyl-1,4-dihydronicotinamide	118 l/mole-sec
1 benzyl-1,4-dihydronicotinamide	29 l/mole-sec
1 benzyl-3-acetyl-1,4-dihydropyridine	3.7 l/mole-sec
DPNH	0.75 l/mole-sec

TABLE III

COMPARISON OF RATE OF OXIDATION OF DIPROTIATED, MONODEUTERATED AND DIDEUTERATED NPrNH, pH 7, TRIS(HYDROXYMETHYL)AMINOMETHANE BUFFER, 0.1 IONIC STRENGTH

Reactant	k_2 *l/mole-sec*
Diprotiated NPrNH	117
Monodeuterated NPrNH	106
Dideuterated NPrNH	37

Deuterium isotope effect

When the 4-deuterated-1-propyl-1,4-dihydro-nicotinamide which contained 1.41 deuterium atoms/mole was oxidized by riboflavin, the rate was not constant, but became slower as the oxidation proceeded indicating that the protiated derivative was being selectively oxidized. When the log of the 360 mμ absorbancy during a reaction was plotted versus time (Fig. 6), it was found that the last portion of the

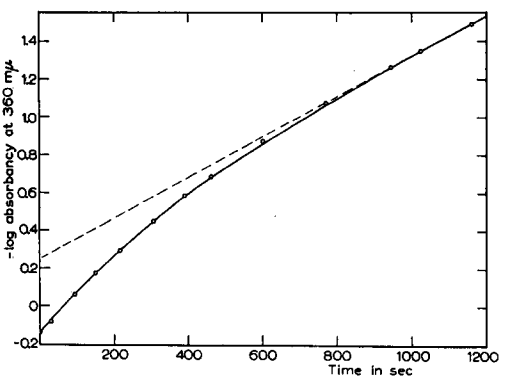


Fig. 6. Absorbancy at 360 mμ *versus* time during the oxidation of deuterium labelled NPrNH by riboflavin. Riboflavin concentration = $6.7 \cdot 10^{-5}$ *M*, tris(hydroxymethyl)aminomethane buffer, 0.1 ionic strength, pH 7, 25°.

decay curve fell on a straight line which when extrapolated to zero time gave the theoretical starting concentration of dideuterated reactant. The second order rate constants for oxidation of the deuterated derivatives are compared in Table III.

The theoretical decay curve calculated with eqn. 3 agrees well with the experimental points (Fig. 6).

$$C_T = C_{HD}\, e^{-k_{HD}t} + C_{DD}\, e^{-k_{DD}t} \qquad (3)$$

C_T = total concentration of monodeuterated and dideuterated NPrNH at time t, (C_T = Absorbancy at 360 mμ/7.06·10^3), C_{HD} = initial concentration of monodeuterated NPrNH, C_{DD} = initial concentration of dideuterated NPrNH, and k_{HD}, k_{DD} = first order rate constants for oxidation of the monodeuterated and dideuterated product respectively. The deuterium isotope effect k_{HH}/k_{DD} is 3.16 $\pm$ 0.05.

Effect of metal ions

Most metal ions tested (Cu^{++}, Cu^{+}, Zn^{++}, Mg^{++}, Mn^{++}, Fe^{++}, Co^{++}) either have no effect or exhibit a small inhibitory action on the reaction. However, Ag^{+} is a strong inhibitor, the rate in the presence of $1\cdot10^{-4}$ M Ag^{+} being decreased by a factor of 10. Silver is known to form a red complex of high stability with riboflavin[25].

Effect of light

Two recent reports[26,27] indicate that the oxidation of reduced DPN by flavins is promoted by light. Careful measurements were made in this laboratory (by Mr. D. CARR) in which the reactants were kept in total darkness prior to and during the reaction, until the O.D. measurement was made. Rate constants for the oxidation of both NPrNH and DPNH by riboflavin were the same under these conditions as when the sample was continuously in the spectrophotometer beam, or intermittantly exposed to dim laboratory light. We believe, therefore that photochemical processes are not responsible for any of the results reported here.

DISCUSSION

The results presented can all be explained on the basis of an oxidation by hydride ion transfer. The riboflavin anion does not react since the negative charge on the molecule repels the further addition of a negative hydride ion. The doubly charged ribityl phosphate anion of riboflavin-5'-phosphate is believed to cause the decreased rate observed with this flavin at pH 10.2. The increased rate obtained with 6,7-dimethyl-9-formylmethylisoalloxazine indicates that the positive charge associated with the polarized carbonyl group exerts a definite effect, which is substantiated by the differences in the pK of protonation between riboflavin and the formyl-methyl analog. This effect is greatly magnified by protonation of the isoalloxazine nucleus. The cation is estimated to react 10^4 times faster than riboflavin. The positively charged isoalloxazine nucleus is apparently an ideal acceptor for the negative hydride ion. Substituents on the pyridine moiety of the DPNH analog which decrease the electron density of the ring cause a decrease in the rate as expected for an ionic mechanism.

The rate of oxidation is more rapid the more polar the solvent indicating that the transition state for the reaction is more polar than the reactants. Similar observations and conclusions have been offered by ABELES *et al.*[28] for the oxidation of DPNH analogs by thiobenzophenone, and by BRAUDE *et al.* who studied oxidation of dihydrobenzene by quinones[24].

An alternative mechanism has been advanced by SCHELLENBERG AND HELLERMAN[29] who have studied the oxidation of DPNH by free radical reagents of high oxidation potential. They conclude that two one-electron transfer steps are probably involved in the oxidation of DPNH by flavins. However, neither these experiments, nor the demonstration of a measurable concentration of semiquinone molecules in an active enzyme[30] rule out a hydride ion transfer mechanism. It is also clear that it

would be difficult to distinguish between a hydride ion transfer and the slow transfer of a hydrogen atom occurring in a complex and followed by a rapid electron transfer.

Since the rate of oxidation of [4-^{2}H]NPrNH is 3.16 times slower than that of the diprotiated form, the hydrogen transfer step must be rate limiting.

No definite proof of molecular complex formation prior to hydrogen transfer was obtained during the course of this work. However, the low (4–5 kcal/mole) activation energy suggests that formation of such a complex with a negative enthalpy change may be involved. The formation of a weak complex with a negative enthalpy of formation between riboflavin phosphate and DPNH is suggested by spectrophotometric data of ISENBERG AND SZENT-GYÖRGYI[31] taken at —78°.

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