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Kinetics and Mechanism of Oxidation–Reduction Reactions between Pyridine Nucleotides and Flavins*

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ABSTRACT: The oxidation of reduced pyridine nucleotides or analog by equimolar flavin or flavinyl peptide was measured to determine the influences of molecular interactions between the amino acid residue and flavin prosthetic group and between the latter and the pyridine nucleotide. For peptides with substitution at position N-10 of the flavin, the rates follow alanyl ≫ phenylalanyl > tyrosyl > tryptophanyl. This order varies inversely with the degree of intramolecular complexing between the given amino acid and the flavin portion. Increased temperature slightly increases the rates. The relative rates with flavins and flavinyl peptides are governed by the size and charge: those with 2-methylene groups in the side chain > those with 5; flavinylphenylalanine with 5 methylenes > FMN > FAD; also flavinylphenylalanine with 5 methylenes > ω-carboxypentylflavin; methyl esters of flavinylphenylalanines > the acid peptides; and the esters of flavin acids ≫ the free acids. The rates for flavin acids follow 2 > 5 > 4 > 3 methylenes which indicates that the carboxylate function of a side chain with 3 methylenes has the greatest negative-charge effect near flavin N-5. For a given flavin derivative, 1-propyl-1,4-dihydronicotinamide reacts fastest, whereas reduced α-nicotinamide–adenine dinucleotide (α-NADH) reacts faster than the more folded β-NADH. For the tightly complexed flavinyltryptophans and -tyrosines, the rates follow reduced β-nicotinamide mononucleotide (β-NMNH$_2$) > β-NADPH > β-NADH. The opposite order was observed for the weakly complexed flavinylphenylalanines and flavin acid with 5 methylenes. These orders are governed by size, charge, and conformation. Decrease in solvent polarity markedly decreases the rate of the above redox reactions. Generally similar results were observed for the N-3 series as with N-10 flavins. The relative rates are again governed by charge as well as size: phenylalanyl peptide > carboxymethyllumiflavin; ethyl esters of ω-carboxyalkylriboflavins > the acids; and the rates for the ethyl esters follow 1 > 2 > 4 > 5 methylenes, but the opposite order was found with the free acids. Riboflavin reacts slower than both ω-carboxypentylriboflavin and its ester, but faster than 3-methylriboflavin. These results indicate that the 3-methyl group may exert steric hindrance to the reaction, whereas a carboxyl or carbonylethoxy group may disallow steric impedance from the ribityl side chain. Overall, the results confirm a hydride ion mechanism with initial addition at the flavin N-5.

Of many flavin coenzyme dependent reactions, the oxidation–reduction couple between reduced pyridine nucleotide and flavoprotein is of special interest. Suelter and Metzler (1960) first proposed a hydride ion transfer mechanism for the nonenzymic oxidation of 1-propyl-1,4-dihydronicotinamide (NPrNH$_2$),[1] a reduced pyridine nucleotide analog, by riboflavin. However, the exact site at which hydrogen is transferred onto the flavin from the substrate has not been certain.

Certain flavins with aromatic amino acids attached at the N-10 position show significantly altered light-absorbing and fluorescence properties (Föry *et al.*, 1968; MacKenzie *et al.*, 1969). The particular intramolecular interaction in these flavinyl peptides has recently been investigated by proton magnetic resonance spectroscopy (Föry *et al.*, 1970). It became of interest to determine how much the interactions

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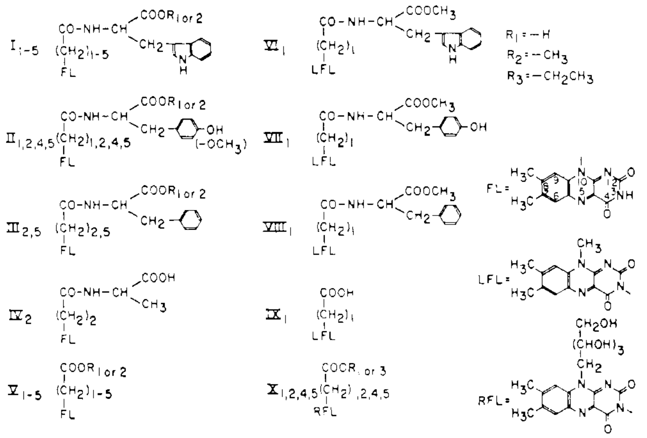
[1] Abbreviation used is: NPrNH$_2$, 1-propyl-1,4-dihydronicotinamide.

observed within these peptides and their overall physical properties effect significant alteration in redox potential. The results could then serve as the basis for further understanding of the redox properties and reactions of the flavins in close proximity to amino acid residues in functional flavoproteins.

To examine further the proposed ionic mechanism of flavin reduction by reduced pyridine nucleotide and to determine more exactly the position of the flavin where initial hydride ion addition takes place, flavins substituted at the N-10 and N-3 positions were synthesized as models of flavoproteins. In addition to the previously prepared flavinyl peptides, the following new compounds were synthesized: N-10 methyl esters of ω-carboxyalkylflavins, N-3 ω-carboxyalkylriboflavins, and the corresponding ethyl esters with 1, 2, 4, and 5 methylene groups in the alkyl chains. The structures of all of these flavin derivatives are shown in Chart I.

CHART I



The reduced pyridine nucleotides and analog studied are α- and β-NADH, β-NADPH, β-$NMNH_2$, and $NPrNH_2$.

Experimental Section

Materials. REDUCED PYRIDINE NUCLEOTIDES AND ANALOG. β-NADH, β-NADPH, α-NADH, and β-NMN were purchased from Sigma Chemical Co. β-$NMNH_2$ was prepared by dithionite reduction of β-NMN following the procedure used by Lehninger (1956) for the similar preparation of NADH. $NPrNH_2$ was synthesized according to Suelter and Metzler (1960).

10-ω-Carboxyalkylflavins, Derived Flavinyl Peptides, and Methyl Esters of Both. Carboxyalkylflavins (V_{1-5}, R = R_1) and the derived peptide methyl esters (I, II, III, R = R_2) with 1 to 5 methylene groups were obtained by previously published methods (Föry *et al.*, 1968). *O*-Methyltyrosine derivatives (II, R = R_1) were prepared according to MacKenzie *et al.* (1969). The syntheses of flavinyltryptophan ($I_{2,5}$, R = R_1), -tyrosine ($II_{2,5}$, R = R_1), -phenylalanine ($III_{2,5}$, R = R_1), and -alanine (IV_2) were described recently (Föry *et al.*, 1970). The methyl esters of flavinylphenylalanine ($III_{2,5}$, R = R_2) and ω-carboxyethyl- (V_2, R = R_2) and ω-carboxypentylflavins (V_5, R = R_2) were prepared in the usual manner by reacting the corresponding acids with methanol under reflux in the presence of a catalytic amount of hydrochloric acid. These esters moved as single spots in 1-butanol–2 N ammonium hydroxide–ethanol (3:1:1, v/v) upon thin-layer chromatograms.

3-Carboxymethyllumiflavin and the derived flavinyl peptide methyl esters (IX_1, VI_1, VII_1, $VIII_1$) were synthesized as described previously by Föry *et al.* (1968).

3-ω-Carboxyalkylriboflavins and the Corresponding Ethyl Esters ($X_{1,2,4,5}$, R = R_1 and R_3). The esters and the acids in 70 and 50% overall yields were synthesized approximately according to the method (Föry, 1967) used to prepare the corresponding ester and acid forms of 3-carboxymethylriboflavin. For these new compounds, 5 mole equiv of ω-bromoalkyl acid ethyl ester (Eastman Organic Chemicals) was reacted with stirring for 20 hr at room temperature with 1 mole equiv of 2′,3′,4′,5′-tetraacetylriboflavin in anhydrous *N,N*-dimethylformamide containing 5 mole equiv of anhydrous potassium carbonate. Solvent was removed by evaporation under reduced pressure with warming, the residue dissolved in methylene chloride and washed successively with 1 N acetic acid, water, 1 N sodium bicarbonate, again with water, the organic phase was dried over anhydrous sodium sulfate, and solvent was removed to obtain the 3-alkoxycarbonylalkyl-*O*-tetraacetylriboflavin. This material was dissolved in absolute ethanol to which a catalytic amount of *p*-toluenesulfonic acid was added. The solution was refluxed for 20 hr to deacetylate the ribityl side chain, the volume reduced to one-half, and the crude 3-alkoxycarbonylalkylriboflavin allowed to precipitate in the cold. This 3-carboxyalklyflavin ethyl ester was purified by redissolving in hot ethanol, filtering off biflavin impurities formed during the initial combination, and crystallizing. The ester was dissolved in 6 N hydrochloric acid, refluxed 2 hr, and evaporated thoroughly to dryness for the crude 3-carboxyalkylriboflavin. The acid was dissolved in a minimal volume of saturated sodium bicarbonate, treated with a little Norit A, filtered, and precipitated by addition of concentrated hydrochloric acid to pH near 0. Purities of both esters and final acids were checked by thin-layer chromatography. Small amounts of the carboxyethyl ester were obtained by paper chromatography with ascending chloroform–acetone (1:1, v/v). In addition, elemental analyses of the long-chain compounds were performed by Schwarzkopf Microanalytical Laboratory of Woodside, N.Y. *Anal.* Calcd for carboxybutyl ester ($C_{24}H_{32}N_4O_8 \cdot H_2O$): C, 54.8; H, 6.6; N, 10.7. Found: C, 55.2; H, 6.5; N, 10.8. Calcd for carboxypentyl ester ($C_{25}H_{34}N_4O_8 \cdot H_2O$): C, 56.0; H, 6.8; N, 10.4. Found: C, 56.4; H, 6.7; N, 10.6. Calcd for carboxybutyl acid ($C_{22}H_{28}N_4O_8 \cdot H_2O$): C, 53.4; H, 6.1; N, 11.3. Found: C, 53.7; H, 5.9; N, 11.3. Calcd for carboxypentyl acid ($C_{23}H_{30}N_4O_8 \cdot H_2O$): C, 54.3; H, 6.3; N, 11.0. Found: C, 54.4; H, 6.1; N, 11.0.

Riboflavin, FMN, and FAD were obtained from Sigma Chemical Co. 3-Methylriboflavin was supplied by Merck Sharp and Dohme Research Laboratories. Lumiflavin and 3-ethyllumiflavin were prepared by the procedures of Hemmerich *et al.* (1956, 1964).

Methods

Potentiometric measurements of the oxidation–reduction potentials of flavins and flavinyl peptides were done using sodium hydrosulfite in the usual manner (see, *e.g.*, Draper

TABLE I: Oxidation–Reduction Potentials of Flavin Analogs Expressed Relative to the Hydrogen Electrode.

Number of Methylenes	E_m' (mV) of Flavins and Flavinyl Peptides[a]			
	−COOH (V)	−Trp (I)	−Tyr (II)	−OMeTyr (II)
1	−205	−152	−152	−152
2	−200	−192	−190	
3	−210	−212		
4	−217	−214	−212	
5	−217	−216	−215	−215

[a] For all compounds, R = R_1.

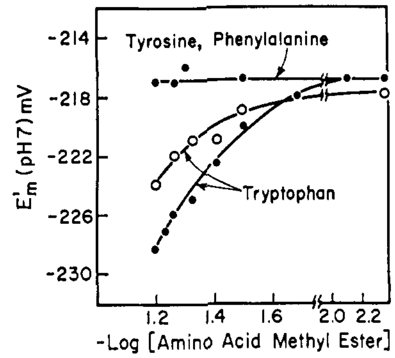


FIGURE 1: The effects of the methyl esters of L-phenylalanine, L-tyrosine, and L-tryptophan on the oxidation–reduction potential of 10-ω-carboxypentylflavin (●) and flavinyltryptophan with five methylenes (○).

and Ingraham, 1968). All flavins (10^{-4} M) in 0.1 M sodium phosphate buffer, pH 7, containing 20% (v/v) ethanol, were titrated at 25° in the dark. The titration vessel was a jacketed flat-bottomed tube, inside dimensions 1.5 × 10 cm, sealed with a rubber stopper. A Radiometer PK149 platinum–calomel combination electrode was inserted through the stopper. The electrode was constructed with a capillary tube which was used for the admission of a stream of argon. A syringe needle inserted through the stopper served as a gas outlet. The solution was stirred by means of a magnetic stir bar in conjunction with the bubbling of gas. Titrations were performed with a Radiometer TTT1C titrator and SBU1a syringe buret equipped with a 1-ml syringe. The titrant, 4 mM sodium hydrosulfite in 0.1 M sodium phosphate, pH 7, was stored in a sealed vessel under a stream of argon. The syringe was fitted with a fine polyethylene tubing which was inserted through the septum of this vessel in order to fill the syringe without the admission of air. After insertion of the tubing through a hole in the stopper of the titration vessel, and bubbling argon for 10 min, the titration was started. The oxidation–reduction potentials of free flavins were also checked with a Heath Model EUW-401 polarograph.

Reaction rates were determined by the decrease in absorbance of reduced pyridine nucleotides at 340 mμ or $NPrNH_2$ at 360 mμ through the use of a Model DU Beckman spectrophotometer (slower reactions) or a Gilford Model 2000 recording spectrophotometer (faster reactions). The cell compartment was thermostated with a Haake Model F constant-temperature circulator. Unless otherwise stated, all measurements were conducted in a 1.0-cm path-length cell containing equimolar solutions (5×10^{-5} M) of the flavin and reduced pyridine nucleotide or $NPrNH_2$, generally in aerobic 0.05 M sodium phosphate buffer, pH 7.0, at 25°. The absorbance of the reaction mixture was recorded against both reductant and flavin controls, and any measurable change was calibrated from the sample readings before values of absorbance were calculated.

Flavin concentration was checked by the absorbance at 450 mμ where flavins have a maximal absorption with a molar extinction coefficient of approximately 12×10^3. For each series of experiments conducted on the same day, fresh solutions of both flavins and reductants were prepared. A Wang calculator (Wang Laboratories, Inc., Tewksbury, Mass.) program, CAL 360-STAT-4, was used for routine determination of initial rates from ln *A vs.* time plots. The program provides a least-square regression analysis of the plots.

Results

Oxidation–Reduction Potentials of N-10 ω-Carboxyalkylflavins and Methyl Esters of the Corresponding Flavinyl Peptides. High concentrations of tryptophan methyl ester cause the value of the measured oxidation–reduction potential (E_m') of the ω-carboxypentylflavin and flavinyltryptophan with 5 methylenes to become more negative (Figure 1). Neither the methyl ester of tyrosine nor that of phenylalanine cause such an effect in the same concentrations.

The values of E_m' for the flavin analogs are shown in Table I and can be compared with that of riboflavin near −200 mV under these conditions. The oxidation–reduction potentials of the free flavins become slightly more positive as the number of methylene groups in the side chain decreases from 5 to 1. All peptides with 1 methylene show markedly more positive E_m' values than the corresponding free flavin, and no difference is observed between peptides of tryptophan or tyrosine. Also such a peptide of phenylalanine has an oxidation–reduction potential of −154 mV. That all these aromatic amino acid moieties of these flavinyl peptides do not significantly affect E_m' is further substantiated by the fact that a similar value was obtained for the *N*-hexyl amide of this 10-carboxymethylflavin. The more positive redox potential of the flavinyl peptides with but 1 methylene is a consequence of the increased electron deficiency of the isoalloxazine ring system induced by the proximity of the amide carbonyl group.

The same E_m' values for the free flavin were obtained polarographically, but anomalous results were obtained with the peptides, probably due to different degrees of adsorption of these much less soluble compounds on the mercury electrode.

Kinetics. As reported earlier (Singer and Kearney, 1950), the observation that the dihydroriboflavin formed during the oxidation of reduced NADH by riboflavin was reoxidized immediately by oxygen simplified the kinetic study in aerobic, aqueous solution. Suelter and Metzler (1960) gave further

TABLE II: Pseudo-First-Order Rate Constants for the Reactions of N-10-Substituted Flavins with Reduced Pyridine Nucleotides and Analog.[a]

	Reduced Pyridine Nucleotides and Analog				
Flavins[b]	β-NADH[c]	β-NADPH	β-$NMNH_2$	α-NADH[c]	$NPrNH_2$
None	0.19	0.52	1.13	0.66	31
I_5	1.33	1.66	1.97	2.85	192
I_2	1.42	1.98	2.30	3.01	211
II_5	1.58	2.08	2.88	3.28	223
II_2	2.05	2.50	3.07	4.54	237
III_5	5.88	4.08	3.75	6.60	340
III_2	6.20	5.81	4.12	7.67	388
IV_2					485
V_5	5.03	3.83	3.12	5.62	337
V_4					299
V_3					225
V_2			3.99		374
FAD	1.25	1.42	1.83		169
FMN	3.54	3.56	3.10		249

[a] Rate constants are expressed as $k_1 \times 10^3$ min^{-1}. [b] For flavins I, II, III, and V, R = R_1. [c] α and β indicate the configurations of the glycosidic linkage.

evidence that the reoxidation of reduced riboflavin by oxygen is not rate-limiting in the reaction with 1-propyl-1,4-dihydronicotinamide ($NPrNH_2$), and the observed kinetics are first order with respect to $NPrNH_2$. The rate constant, k_1, varies in direct proportion to the riboflavin concentration. Based on these facts, a general rate expression for the present oxidation-reduction reactions can be formulated as follows:

$$\frac{-d[PHN_2]}{dt} = k_1[PHN_2] = k_2[PHN_2][FL]$$

where PNH_2 is the reduced pyridine nucleotide or analog, and k_2 is the second-order rate constant. The rate constants listed in all tables, then, are pseudo first order for k_1.

Effects of Size and Charge on Oxidation of Reduced Pyridine Nucleotides or Analog by N-10 Flavin Derivatives. With respect to a specific reduced pyridine nucleotide or $NPrNH_2$, the rates for flavinyl peptides follow alanyl ≫ phenylalanyl > tyrosyl > tryptophanyl. Rate constants for N-10 flavins studied are shown in Tables II and III. With no exception, flavins and flavinyl peptides which have 2 methylene groups in the side chain react faster than those with 5. The same size factor also operates in the following rate order: flavinylphenylalanine (III_5, R = R_1) > FMN > FAD. However, flavin (V_5, R = R_1), though less bulky, reacts slower than the peptide (III_5, R = R_1). Furthermore, the rates for the methyl esters of flavins ($V_{2,5}$, R = R_2) and flavinylphenylalanines ($III_{2,5}$, R = R_2) are greater than those for the corresponding acids ($V_{2,5}$, $III_{2,5}$, R = R_1). The relative rates for flavin acids (V_{2-5}, R = R_1) follow the decreasing order 2 > 5 > 4 > 3 methylene groups in the side chain.

TABLE III: Effect of Solvent Polarity on the Rate Constants of $NPrNH_2$–Flavin Reactions.[a]

	% of Ethanol in Buffer		
Flavins	0[b]	25	50
Control[c]	3.1	0.7	0.26
I_5, R = R_1	19.2	6.8	1.82
II_5, R = R_1	22.3	7.2	2.26
III_5, R = R_1	34.0	10.5	3.11
III_5, R = R_2		12.0	
III_2, R = R_1	38.8	12.7	
III_2, R = R_2		20.7	
V_5, R = R_1	33.7	9.8	
V_5, R = R_2		20.4	
V_2, R = R_1	37.4	11.4	
V_2, R = R_2		56.6	

[a] Rate constants are expressed as $k_1 \times 10^2$ min^{-1}. Experiments were performed at 25°. [b] The data were taken in part from the $NPrNH_2$ column of Table II. [c] The solution contains no flavin but $NPrNH_2$.

For a given flavinyl peptide of tryptophan or tyrosine ($I_{2,5}$ or $II_{2,5}$, R = R_1), rates for reduced pyridine nucleotides and analog are in the following order: $NPrNH_2$ ≫ α-NADH > β-$NMNH_2$ > β-NADPH > β-NADH. However, a different order was observed for flavinylphenylalanines ($III_{2,5}$, R = R_1) and flavin (V_5, R = R_1) with which rates follow $NPrNH_2$ ≫ α-NADH > β-NADH > β-NADPH > β-$NMNH_2$. Finally, the oxidation rates of reduced pyridine nucleotides and $NPrNH_2$ in the absence of flavins under aerobic conditions are also compared in Table II. The ratio of rates is 1 (β-NADH):2.7 (β-NADPH):3.5 (α-NADH):5.9 (β-$NMNH_2$): 163 ($NPrNH_2$).

Effects of Polarity and Temperature of the Reaction Medium on Oxidation of $NPrNH_2$ by N-10 Flavin Derivatives. The results in Table III also show that the rate of oxidation is greatly reduced as the concentration of ethanol is increased from 0 to 50% (v/v). This is consistent with the results obtained by Suelter and Metzler (1960).

The three longest flavinyl peptides (I_5, II_5, III_5, R = R_1) together with $NPrNH_2$ were chosen for a study of the temperature effect. Upon change from 15 to 35°, the rates of $NPrNH_2$ oxidation are enhanced 1.2 to 1.4 times with each peptide, but this is no more than that seen in the absence of flavin.

Effects of Size and Charge on Oxidation of $NPrNH_2$ by N-3 Lumiflavin and Riboflavin Derivatives. Due to lower solubility of the methyl esters of 3-flavinyltryptophan (VI_1), -tyrosine (VII_1), and -phenylalanine ($VIII_1$) in aqueous medium, 3.5% *N*,*N*-dimethylformamide buffer (v/v) was used. It can be seen from the data in Table IV that the rates for these peptide methyl esters follow the same order observed in the N-10 series: $VIII_1$ > VII_1 > VI_1. However, the rate constant with ω-carboxymethyllumiflavin (IX_1), obtained in buffer alone, seems to be smaller than that with the bulkier flavinylphenylalanine methyl ester ($VIII_1$).

Data in Table IV also summarize the kinetic studies with riboflavin and lumiflavin, and their derivatives, 3-ω-carboxyalkylriboflavins and the corresponding ethyl esters ($X_{1,2,4,5}$, $R = R_1$ and R_3), in 2% *N,N*-dimethylformamide buffer solution. As noted in N-10 flavin derivatives, the relative rates for the ethyl esters (X, $R = R_3$) and riboflavin are in the following decreasing order: $X_1 > X_2 > X_4 > X_5 >$ riboflavin, and for the acids (X, $R = R_1$) and riboflavin: $X_5 >$ riboflavin $> X_4 > X_2 > X_1$. Again, the rates of the ethyl esters are greater than those of the acids; the rate ratio of ester to acid has the following order: 3.5 (X_1) > 2.4 (X_2) > 2.1 (X_4) > 1.4 (X_5). Finally, the rate ratios of lumiflavin, 3-ethyllumiflavin, and 3-methylriboflavin to riboflavin were found to be 1.2, 0.7, and 0.7, respectively. The first ratio is not in agreement with the 0.9 reported by Suelter and Metzler (1960), but the second one complements the value (0.8) which they obtained for 3-methyllumiflavin.

Discussion

The tight interaction between aromatic amino acids and flavin, which results in greater than 95% loss of flavin fluorescence in the cases of the flavinyltryptophans (MacKenzie *et al.*, 1969), does not cause a change in the measured oxidation–reduction potential, E_m'. However, such a change is observed in the intermolecular case where much less fluorescence quenching occurs.[2] If free flavin is more readily reduced than that complexed, an alteration in the ratio of $[F_{ox}]/[F_{red}]$ leads to a decrease in the E_m' value according to the Nernst equation. The addition of tryptophan to the flavinyltryptophan peptide causes a change in E_m' (*cf.* Figure 1), because free tryptophan can associate more avidly with the oxidized flavinyl peptide. As the change in E_m' of the flavinyl peptide is not greater than that observed with the free flavin, a 1:1 complex must be involved.

From the kinetic data obtained with N-10 flavins, the rates for flavinyl peptides are more rapid with the smaller and less complexed amino acid moieties. Flavin compounds with two methylene groups react faster than those with five. Also, FAD is the slowest in the N-10 series, and FMN is slower than flavinylphenylalanine (III_5, $R = R_1$). Based on such data and the similar redox potentials for both short- and long-chain flavins, it is evident that size must be a governing factor. However, the rates for the bulkier methyl esters of ω-carboxyalkylflavins ($V_{2,5}$, $R = R_2$) and phenylalanyl peptides ($III_{2,5}$, $R = R_2$) are much greater than their corresponding acids ($R = R_1$). These results indicate that influence of charge in these cases is even greater than that of size, and the negative charge on the carboxylate function must exert a repelling effect on an attacking species of like charge. As these effects are seen with $NPrNH_2$, as well as the reduced pyridine nucleotides, the repulsion cannot be only due to the anionic phosphate residues of the latter, but to the actual form of hydrogen passed as immediate reductant. These effects of charge establish that a hydride ion transfer mechanism operates. The effect of converting acids into methyl esters upon rate is greater for flavins than for flavinyl peptides and greatest for the flavin with shortest alkyl chain. Evidently, closer proximity of the carboxyl group of flavins around the flavin electrophilic center causes a larger retarding effect on rate. The optimal chain length for the carboxyl group to exert the greatest effect upon the approaching hydride ion is with three methylene functions. These findings are in accord with the results from the proton magnetic resonance study (Föry *et al.*, 1970), which indicated that these flavins have a vertically stacked conformation wherein the side chain is folded back on the flavin ring system, and strongly suggest that the initial hydride ion addition must take place at the flavin N-5 position. A recent report on the photochemistry of riboflavin (Moore and Baylor, 1969) showed that photobleaching of all isoalloxazines with N-10 alcoholic type side chains of three or more carbon atoms produces cyclic intermediates which are formed by attachment of the side chain to N-5. These results complement the present findings.

TABLE IV: Pseudo-First-Order Rate Constants for the Reactions of N-3-Substituted Flavins with $NPrNH_2$.[a]

Flavins	% of Dimethylformamide in Buffer		
	0	2.0	3.5
None	3.1	2.9	2.4
VI_1			20.5
VII_1			22.3
$VIII_1$			30.0
IX_1	26.8		
X_1, $R = R_1$		25.4	
X_1, $R = R_3$		80.5	
X_2, $R = R_1$		28.4	
X_2, $R = R_3$		67.4	
X_4, $R = R_1$		29.8	
X_4, $R = R_3$		62.9	
X_5, $R = R_1$		35.2	
X_5, $R = R_3$		48.4	
Riboflavin	32.7	30.3	
3-Methylriboflavin		22.2	
Lumiflavin		35.8	
3-Ethyllumiflavin		21.1	

[a] Rate constants are expressed as $k_1 \times 10^2$ min^{-1}.

The results of the quantum-mechanical calculations of the electronic characteristics of the oxidized isoalloxazine ring (Pullman and Pullman, 1963) indicate that both the electronic charge and the free valence are greater at N-1 than at N-5. Furthermore, experiments (Suelter and Metzler, 1960; Michaelis *et al.*, 1936) confirm the prediction from the calculation of the corresponding basicity parameters of N-1 and N-5 that the most basic center of riboflavin is N-1. All these situations suggest that the negative hydride ion should add preferentially to the more positive N-5. This is confirmed experimentally by this study.

That an ionic mechanism must account for the present results is further substantiated by the observations that change in solvent polarity affects the rate of oxidation–reduction. A radical mechanism including flavin semiquinone as intermediate would not be so affected by change in polarity and, therefore, would be unlikely.

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[2] Tryptophan methyl ester (10^{-2} M) causes a 50% decrease in fluorescence of 2×10^{-5} M flavin.

The influence of size, charge, and conformation on rate, which prevails in the flavin series, was also observed throughout the reduced pyridine nucleotides and analog. In the absence of flavin, *i.e.*, with O_2 as sole oxidant, the rates observed with the smaller, less complexed species, *e.g.*, β-NMNH$_2$ and especially NPrNH$_2$, are faster than those with the nucleotides wherein the dihydronicotinamide ring is less accessible. In the much faster reactions with a given flavin or flavinyl peptide in the presence of O_2, NPrNH$_2$, the smallest of the present reductants, again reacts fastest. In comparison with α-NADH, the somewhat slower rate with natural β-NADH may be explained on the basis that there is more intramolecular interaction between the pyridine and adenine moieties in the β isomer at pH 7.0 (Sarma *et al.*, 1968). For tryptophan or tyrosyl peptides ($I_{2,5}$, $II_{2,5}$, $R = R_1$), both of which are intramolecularly tightly complexed, the rates for the reduced pyridine nucleotides follow β-NMNH$_2$ > β-NADPH > β-NADH, since β-NMNH$_2$ is the smallest, whereas β-NADH has a more folded conformation than β-NADPH. However, for weakly complexed flavinylphenylalanines ($III_{2,5}$, $R = R_1$) or flavin (V_5, $R = R_1$), the rate order is the opposite with β-NADH > β-NADPH > β-NMNH$_2$. This is in the order of decreasing intramolecular interaction of the reduced pyridine nucleotides. Apparently, the phosphate group of β-NMNH$_2$ has more degrees of freedom than those of β-NADH or β-NADPH. This may give rise to a larger retarding effect during the approach of β-NMNH$_2$ to the flavins.

With regard to the N-3 lumiflavin and riboflavin derivatives, generally similar results as with the N-10 series were obtained. In comparison with the flavinyl peptides and methyl esters and 3-ethyllumiflavin, lumiflavin itself reacts much faster. This is mainly the size effect. However, k_1 of ω-carboxymethyllumiflavin (IX_1), obtained in buffer alone, seems to be smaller than that of the bulkier flavinylphenylalanine methyl ester ($VIII_1$). As mentioned before for the N-10 derivatives, this implies that the influence of charge dominates over that of the molecular size in the N-3 compounds as well. Moreover, the relative rates for 3-ω-carboxyalkylriboflavin ethyl esters (X, $R = R_3$) are mainly governed by the size, and that for the corresponding acids (X, $R = R_1$) are again determined by the proximity of the carboxyl group to N-5. This order of the latter suggests that the carboxyl groups on longer chains are less folded back onto N-5. The rates with the esters exceed those with the corresponding acids, again supporting the significance of the charge effect in an ionic mechanism. Whereas the greatest effect from a chain of 3 methylenes was found for the N-10 series, the shortest N-3 acid with 1 methylene group experiences the greatest effect of carboxyl group upon rate. These differences imply a different orientation of the side chain in overlapping with the flavin nucleus.

Somewhat surprisingly, riboflavin itself reacts slower than both 3-ω-carboxypentylriboflavin (X_5, $R = R_1$) and its ethyl ester ($R = R_3$) but faster than 3-methylriboflavin. These results allude to the conclusion that the 3-methyl may exert some steric hindrance, but this may be subordinate with a carboxyl or carbonylethoxy group which may additionally disallow steric impedance from the ribityl side chain. Finally, the fact that riboflavin reacts slower than lumiflavin can be explained not only by the effect of size but also that of the polarity on the polyhydroxyribityl chain and the conformation of the molecule.

In summary, all of the foregoing results confirm the hydride ion transfer mechanism in the oxidation of reduced pyridine nucleotide coenzymes by flavins. It is also clear, based on theoretical calculations and past and present experimental data, that the hydride ion addition must occur at the flavin N-5 position.

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