York, N.Y.
Lowenstein, M. A., and Birnboim, M. H. (1975), *Biopolymers 14,* 419.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.
Pecora, R. (1964), *J. Chem. Phys. 40,* 1604.
Pecora, R. (1965), *J. Chem. Phys. 43,* 1562.
Pusey, R. N., Koppel, D. E., and Schaefer, D. W. (1974), *Biochemistry 13,* 952.
Rimai, L., Hickmott, J. T., Jr., Cole, T., and Carew, E. B. (1970), *Biophys. J. 10,* 20.
Sata, T., Havel, R. J., and Jones, A. L. (1972), *J. Lipid Res. 13,* 757.
Schumaker, V. N. (1967), *Adv. Biol. Med. Phys. 11,* 245.
Tanford, C. (1961), Physical Chemistry of Macromolecules, Wiley, New York, N.Y.

Chemical and Enzymatic Properties of Riboflavin Analogues†

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ABSTRACT: The chemical and enzymatic properties of 26 analogues of riboflavin are presented. These analogues include both endo- and exocyclically substituted isoalloxazines with redox potentials from −370 to −128 mV. Physical and chemical data such as the electronic absorption spectra, pK_as, and redox potentials of the analogues are presented and are discussed with respect to preferred tautomeric and resonance forms. Like riboflavin, most of the analogues are shown to be catalytic oxidants of dihydro-5-deazaflavins. Analogue binding to egg white binding apoprotein has been quantitated and serves to determine the origins of binding site specificity for this protein. Nearly all of the analogues that possess D-ribityl groups are found to be processed to the FAD level by the flavokinase/FAD synthetase system of *Brevibacterium ammoniagenes.* Most extensively studied are the reactivities of the analogues with the NAD(P)H:flavin oxidoreductase of *Beneckea harveyi.* Many of the analogues are substrates in this enzymatic redox reaction, and a linear free energy–rate relation (log V_{max} vs. E_0' of the analogue) is seen that parallels similar relationships in the nonenzymatic oxidation of dihydro-5-deazaflavins. This suggests a common mechanism for the reactions of such diverse flavins as riboflavin, 5-deazariboflavin, and 1-deazariboflavin.

In the past few years there has been increasing interest in the preparation of isosteric replacements for enzyme substrates and coenzymes. Such molecules have potential both as therapeutic agents and as models with which to probe the mechanisms of enzyme catalysis (Rivlin, 1975; Singer, 1976). Several analogues of the flavin coenzymes have been shown to have antivitamin activity (Lambooy, 1975; Lambooy & Shaffner, 1977; Chu & Bardos, 1977; Otani, 1976). One class of such flavin analogues involves exocyclic substitutions (particularly at N-10) that do not alter the chemistry of the isoalloxazine ring system, so that their in vivo activity is based on steric, not chemical, changes. Another class involves isosteric substitutions that alter the coenzyme redox chemistry. In this paper we explore these two categories with a series of riboflavin analogues recently synthesized by Rogers and colleagues (Ashton et al., 1977; Graham et al., 1977). Several of these have already been shown to be potent anticoccidial agents (Graham et al., 1977).

In order to use these analogues as probes of flavin redox chemistry and, perhaps, to understand the bases of their in vivo activities, we have surveyed the elementary chemistry (pK_as, redox potentials, behavior in a redox model system reaction) of the analogues. We also present results of assays with the analogues in three in vitro flavoprotein systems: the egg white binding protein, the flavokinase and FAD synthetase from *Brevibacterium ammoniagenes,* and the NAD(P)H:flavin oxidoreductase from *Beneckea harveyi.* Much of this information is self-explanatory and is presented in Tables I and II; the observations of particular interest as well as correlations between the chemical and enzymatic properties of the analogues are presented in the Results and Discussion.

Experimental Section

Materials

The flavin analogues were prepared or purchased as follows (numbering as in Tables I and II[1]): **1,** Shunk et al. (1952); **2, 6, 12b,** Graham et al. (1977); **4, 10,** from Sigma Chemical; **5,** Shunk et al. (1955); **8,** from **10** by snake venom phosphodiesterase cleavage; **9,** Yagi (1971); **11,** Berezovskii & Rodionova, (1958); **12,** a generous gift of Dr. Shiao-Chun Tu; **13, 14, 18,** Ashton et al. (1977); **15,** O'Brien et al. (1970); **20,** Yoneda et al. (1976), with a modified preparation of the intermediate 6-(*N*-D-ribityl-3,4-xylidino)uracil (W. T. Ashton, R. D. Brown, & R. L. Tolman, submitted for publication); **21a, 21d, 21e,** from **3** and an excess of the appropriate amine by heating at 100 °C in dimethylformamide for several hours.

7,8-Didemethyl-8-chlororiboflavin (**3**) was prepared by the method of Haley & Lambooy (1954) for 8-demethyl-8-chlororiboflavin. 2,4-Dichloronitrobenzene and D-ribitylamine

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were reacted in hot pyridine to give a 53% yield of 5-chloro-2-nitro-N-D-ribitylaniline, mp 185–186 °C (MeOH). Catalytic reduction (Pt, CH_3CO_2H) of the latter compound and condensation with alloxan gave a 68% yield of **3** as an orange powder, mp 245 °C (dec) (CH_3CO_2H–H_2O). Anal. Calcd for $C_{15}H_{15}ClN_4O_6$: C, 47.07; H, 3.95; N, 14.64. Found: C, 47.28; H, 4.29; N, 14.99.

10-[(2,6-Dichlorophenyl)methyl]-7,8-dimethylbenzo[g]pteridine-2,4(3H,10H)-dione (**7**). 3,4-Dinitro-*o*-xylene and 2,6-dichlorobenzylamine were reacted using the procedure of Foery et al. (1968) to give an 80% yield of 4,5-dimethyl-2-nitro-*N*-(2,6-dichlorobenzyl)aniline, mp 117–119 °C (C_2H_5OH). Reduction and condensation with alloxan using the same procedure as for **3** gave a 55% yield of **7** as an orange powder, mp 308–310 °C (CH_3CO_2H). Anal. Calcd for $C_{19}H_{14}Cl_2N_4O_2$: C, 56.87; H, 3.52; N, 13.96. Found: C, 57.12; H, 3.45; N, 13.63.

10-(2-Hydroxyethyl)-7,8-dimethylpyrimido[4,5-b]quinoline-2,4(3H,10H)-dione (**16**). A mixture of 8.09 g of 2-(3,4-xylidino)ethanol (Lattes & Verdier, 1965), 2.40 g of 6-chlorouracil (Cresswell & Wood, 1960), and 63 mL of H_2O was heated under reflux for 17 h. The cooled reaction mixture was basified with 16 mL of 2.5 N NaOH and extracted twice with CH_2Cl_2. The pH of the aqueous phase was adjusted to 2.8 with 6 N HCl, and the copious precipitate was filtered, washed with water, dried and recrystallized from CH_3OH–H_2O to give 3.43 g (76%) of 6-[*N*-(2-hydroxyethyl)-3,4-xylidino]uracil, mp 248–249 °C. A 2.92-g sample of the latter compound was dissolved in 127 mL of hot pyridine and cooled to room temperature and 1.2 mL of acetic anhydride was added. After 30 min at room temperature and 2 min at 50 °C, the reaction mixture was evaporated in vacuo. The residue was suspended in water, filtered, washed with water, and dried to give 2.98 g (89%) of 6-[*N*-(2-acetoxyethyl)-3,4-xylidino]uracil, mp 209–210.5 °C ($CHCl_3$–benzene). A mixture of 2.79 g of the latter compound, 27.5 mL of triethylorthoformate, 7 mL of Me_2SO, and 189 mg of *p*-toluenesulfonic acid was heated at 120 °C in a N_2 atmosphere for 2 h. The cooled reaction mixture was diluted with 70 mL of acetone. The yellow precipitate was filtered, washed several times with $CHCl_3$, and dried to give 0.75 g (26%) of 10-(2-acetoxyethyl)-7,8-dimethyl-5-deazaisoalloxazine, mp 252–255 °C (dec). The latter compound (0.74 g) was suspended in 10 mL of H_2O and 50% aqueous NaOH was added dropwise until the solid dissolved (final pH 12.7). After 10 min at room temperature, the pH of the solution was adjusted to 5.1 with 10% aqueous CH_3CO_2H. The thick suspension was diluted with 40 mL of H_2O, cooled to 0 °C for 1 h, and filtered. The yellow solid was washed with H_2O and acetone and dried to give 0.60 g (92%) of **16**, mp >320 °C. Anal. Calcd for $C_{15}H_{15}N_3O_3 \cdot 0.25H_2O$: C, 62.17; H, 5.39; N, 14.50. Found: C, 62.35; H, 5.48; N, 14.44.

1-Deoxy-1-[3,4-dihydro-5,7,8-trimethyl-2,4-dioxopyrimido[4,5-b]quinolin-10(2H)-yl]-D-ribitol (**17**) was prepared by treatment of 1-deoxy-1-[(3,4-dimethylphenyl-(1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidinyl)amino]-D-ribitol tetraacetate with acetyl chloride and $SnCl_4$ followed by deacetylation (W. T. Ashton, R. D. Brown, & R. L. Tolman, submitted for publication).

8-Demethyl-8-hydroxyriboflavin (**19**) was prepared in three steps from 5-amino-*o*-cresol, ribose, and violuric acid by the procedure of Kasai et al. (1975) for roseoflavin except that H_2O instead of CH_3OH was used as solvent in the violuric acid step. The compound was obtained in 12% overall yield as a copper colored powder, mp 296–298 °C (dec). Anal. Calcd for $C_{16}H_{18}N_4O_7 \cdot 0.5H_2O$: C, 49.64; H, 4.95; N, 14.47. Found: C, 49.80; H, 4.81; N, 14.41.

8-Demethyl-8-(methylthio)riboflavin (**22**). Methanethiol gas was passed into a suspension of 0.84 g of 57% sodium hydride dispersion in 20 mL of DMF[1] with cooling in an ice bath until H_2 evolution ceased. To the clear solution was added 3.21 g of 5-chloro-4-methyl-2-nitro-*N*-D-ribitylaniline (Haley & Lambooy, 1954), and the mixture was heated at 60 °C in a N_2 atmosphere for 3 h. The dark orange reaction mixture was poured into 100 mL of ice water, and the orange solid was filtered, washed with water, dried, and recrystallized from C_2H_5OH–H_2O to give 2.2 g (66%) of 4-methyl-5-methylthio-2-nitro-*N*-D-ribitylaniline as orange needles, mp 128–130 °C. Reduction and condensation with alloxan using the same procedure as for **3** gave a 70% yield of **22** as orange crystals, mp 280–282 °C (dec) (3 N HCl). Anal. Calcd for $C_{17}H_{20}N_4O_6S$: C, 49.99; H, 4.94; N, 13.72. Found: C, 50.39; H, 4.96; N, 13.55.

8-Demethyl-8-(propylamino)riboflavin (**21c**). A mixture of 300 mg of 8-chloro-8-demethylriboflavin (Haley & Lambooy, 1954), 3 mL of *n*-propylamine, and 3 mL of DMF was heated at 80 °C for 18 h in a N_2 atmosphere. The deep red reaction mixture was evaporated in vacuo. The orange crystalline residue was triturated with ether, filtered, and stirred with 30 mL of CH_3OH for 2 h to give 160 mg (48%) of **21c** as red-orange crystals, mp 259–263 °C (dec). Anal. Calcd for $C_{19}H_{25}N_5O_6 \cdot H_2O$: C, 52.17; H, 6.22; N, 16.01. Found: C, 52.11; H, 5.92; N, 15.66.

When judged necessary after thin-layer chromatography, analogues were purified by absorptive chromatography on Bio-Gel P_2 with water as eluent.

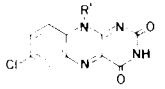
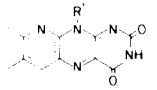
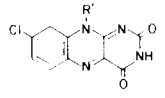
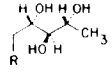
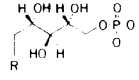
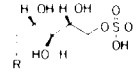
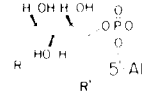
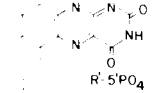
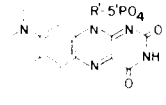
All other materials, including the proteins described, were prepared as in Spencer et al. (1976) and Fisher et al. (1976).

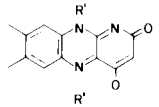
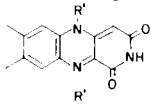
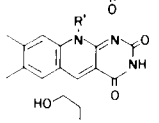
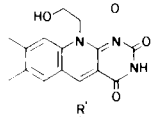
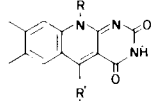
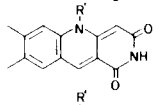
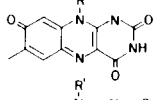
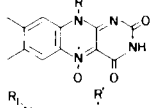
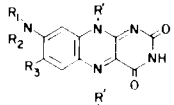
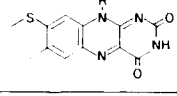
Methods

pK_a Values. Analogues in a buffer of phosphate, pyrophosphate, and acetate (10 mM each) were titrated with 2 M HCl and 2 M KOH with absorption spectra recorded at each pH. Spectral changes were fitted to the Henderson–Hasselbach equation to obtain the pK_as. Spectra and pH values of

[1] The names of the analogues as numbered in Tables I and II, according to current Chemical Abstracts index usage, are as follows: **1,** 7-chloro-7,8-didemethylriboflavin; **2,** 1-deoxy-1-(3,4-dihydro-7,8-dimethyl-2,4-dioxopyrido[3,2-*g*]pteridin-10(2*H*)-yl)-D-ribitol; **3,** 7,8-didemethyl-8-chlororiboflavin; **4,** riboflavin; **5,** 5′-deoxyriboflavin; **6,** 10-[(3-chlorophenyl)methyl]-7,8-dimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione; **7,** 10-[(2,6-dichlorophenyl)methyl]-7,8-dimethylbenzo[*g*]pteridine-2,4-(3*H*,10*H*)-dione; **8,** riboflavin 5′-(dihydrogen phosphate); **9,** riboflavin 5′-(hydrogen sulfate); **10,** riboflavin 5′-(trihydrogen diphosphate) 5′ → 5′-ester with adenosine; **11,** 6-methylriboflavin; **12,** 8-demethyl-8-(dimethylamino)riboflavin 5′-(dihydrogen phosphate); **13,** 1-deoxy-1-(4-hydroxy-7,8-dimethyl-2-oxopyrido[2,3-*b*]quinoxalin-10(2*H*)-yl)-D-ribitol; **14,** 1-deoxy-1-(2,3-dihydro-7,8-dimethyl-1,3-dioxopyrido[3,4-*b*]-quinoxalin-5(1*H*)-yl)-D-ribitol; **15,** 1-deoxy-1-(3,4-dihydro-7,8-dimethyl-2,4-dioxopyrimido[4,5-*b*]quinolin-10(2*H*)-yl)-D-ribitol; **16,** 10-(2-hydroxyethyl)-7,8-dimethylpyrimido[4,5-*b*]quinoline-2,4(3*H*,-10*H*)-dione; **17,** 1-deoxy-1-(3,4-dihydro-5,7,8-trimethyl-2,4-dioxopyrimido[4,5-*b*]quinolin-10(2*H*)-yl)-D-ribitol; **18,** 1-deoxy-1-(2,3-dihydro-7,8-dimethyl-1,3-dioxobenzo[*b*][1,6]naphthyridin-5(1*H*)-yl)-D-ribitol; **19,** 8-demethyl-8-hydroxyriboflavin; **20,** riboflavin 5-oxide; **21a,** 7,8-didemethyl-8-aminoriboflavin; **21b,** 8-demethyl-8-(methylamino)riboflavin; **21c,** 8-demethyl-8-(propylamino)riboflavin; **21d,** 7,8-didemethyl-8-[(6-aminohexyl)amino]riboflavin; **21e,** 7,8-didemethyl-8-(dimethylamino)riboflavin; **22,** 8-demethyl-8-(methylthio)riboflavin. Familiar names are presented in Table I. Abbreviations used are: RF, riboflavin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Fl_{ox}, oxidized flavin (general); FlH^-, dihydroflavin anion (general); FlH·, flavin semiquinone. "Natural" or "parent" flavin refers to riboflavin, FMN, or FAD; DMF, dimethylformamide.

TABLE I: Properties of the Flavin Isosteres.

No.	Structure	Name	E_0' (mV)	λ_{max}[a] (nm)	pK_{a,FlH_2}	Oxidation of 5-dRFH$^-$ (M^{-1} s^{-1})	Oxidation of 1,5-did-RFH$^-$ (M^{-1} s^{-1})	K_D, binding protein (M)	Flavokinase, FAD synthetase substrate	*B. harveyi* oxidoreductase V_{max} rel	*B. harveyi* oxidoreductase K_m (μM)	*B. harveyi* oxidoreductase K_I (μM)	*B. harveyi* oxidoreductase V_H/V_D
1		7-Chloro	−128	447 (9.8), 334 (7.3)		2330	13 500	1.2×10^{-7}	+	2.3	2.6		1.4
2		9-Aza	−135	437 (21.2), 313 (6.5)	5.95	2200	22 700	1.3×10^{-8}	++	0.35	0.5		2.4
3		8-Chloro	−144	432 (12.1), 356 (8.6)	6.04	1070	7 600	3.3×10^{-7}		0.31	0.4		3.3
4		Riboflavin	−208[a]	445 (12.5),[c] 373 (10.6)	6.45	680	4 500	1.3×10^{-9} [d]	++	≡1.0	0.27		4.8
5		5′-Deoxy	−208	445 (12.5), 373 (10.6)		800		3.8×10^{-8}	−	0.75	0.3		3.0
6		10-(3-Chloro-benzyl)		440 (12), 357 (19)				7.4×10^{-9}	−	0.033	0.02		4.6
7		10-(2,6-Di-chlorobenzyl)		446, 377				8×10^{-8}					
8		FMN	−216[a]	445 (12.5),[c] 373 (10.4)		560		5.6×10^{-7}		1.0	0.7		4.3
9		5′-Sulfate		445 (12.5), 373 (10.5)				1.1×10^{-7}	−	0.75	0.7		4.7
10		FAD	−219[b]	450 (11.3),[c] 375 (9.3)		360		$>10^{-6}$					
11		6-Methyl	−219	447 (11.5), 396 (19.2)		620		1.5×10^{-9}	+	0.13	0.07		6.5
12		RoseoFMN	−222[e]	505 (32.8),[e] 314 (6.8)				1.8×10^{-6}		0.001			

13		3-Deaza	−240	421 (20.0)	6.48	1.3	110	1.1×10^{-8}	+[f]	0.004		14	
14		1-Deaza	−280	535 (6.8), 365 (4.0)	5.6	50	510	1.6×10^{-9}	++	0.024	0.02	0.04	2.5
15		5-Deaza	−311	397 (12.5), 338 (12.2)	7.2	22[g]	720	4.6×10^{-9}	++	0.0076	7	13	3.8
16		10-Hydroxy-ethyl-5-deaza		397 (12.5), 338 (12.2)				1.5×10^{-7}	−	0.013		2	
17		5-Methyl-5-deaza		394 (12), 335 (13)	7.16			5.2×10^{-9}	++	<0.0005		2	
18		1,5-Dideaza	−370	476 (9.5), 344 (4.2)		5		1.9×10^{-8}		<0.001		2	
19		8-Hydroxy		472 (41.0),[h] 300 (10.7)		13		3.5×10^{-6}	−	<0.0005		0.04	
20		5-Oxide		462 (9.3), 369 (9.0), 327 (7.4)				$<10^{-6}$	++	0.21			
21a–e		8-Alkylamino[i]		488–505 (−40.),[i] 305–318 (10–20)		5–18[i]		*i*	*i*	<0.0005		0.002–0.035[i]	
22		8-Methylthio		475 (35.5)		500		7.9×10^{-9}		0.45	0.2		3.7

[a] Values in parentheses are ϵ (mM^{-1} cm^{-1}). [b] Loach (1968). [c] Beinert (1960). [d] Becvar (1973). [e] Otani (1976). [f] Rapidly converted to 3-deazaFMN; no 3-deazaFAD evident. [g] pH 9, 0 °C (Spencer et al., 1976). [h] Ghisla & Mayhew (1976). [i] See Table II.

TABLE II: Properties of the 8-Amino Analogues.

No.	R_1[a]	R_2[a]	R_3[a]	Name	λ_{max}[b] (nm)	Oxidation of 5-deaza-RFH^- (M^{-1} s^{-1})	K_d, binding protein (M)	K_i, *B. harveyi* oxido-reductase (nM)
21a	H	H	H	8-Amino	501 (42.3), 312 (20)	8	6.6×10^{-7}	15
21b	H	CH_3	CH_3	8-Methylamino	488 (41.1), 305 (10.5)	5	1.1×10^{-8}	7
21c	H	$CH_2CH_2CH_3$	CH_3	8-Propylamino	488 (41), 305 (10)	18	7.4×10^{-7}	2
21d	H	$(CH_2)_6NH_3^+$	H	8-Aminohexylamino	493, 313	8	$>3 \times 10^{-4}$	35
21e	CH_3	CH_3	H	8-Dimethylamino	505 (41.9), 318 (13.7)	5	3.8×10^{-8}	15

[a] See Table I for structure. [b] Values in parentheses are ϵ (M^{-1} cm^{-1}).

solutions of dihydro analogues were obtained in cells maintained under an argon atmosphere and with a slight excess of sodium dithionite present.

Redox Potentials. Solutions of approximately equimolar analogue and redox indicator in 0.1 M KP_i (pH 7.0, 25 °C) were maintained under an argon atmosphere and titrated to a series of redox equilibrium positions with sodium dithionite (reductant) and oxygen (oxidant). Visible absorption spectra were recorded at each equilibrium position, and from these spectra the concentrations of oxidized and reduced analogue and redox indicator were calculated. Application of the Nernst equation then allowed calculation of the E_0' of the analogue. The redox indicators used were indigo disulfonate ($E_0' = -116$ mV: Edmondson & Singer, 1973), diethylsafranin (−251 mV; Loach, 1968), anthraquinone-2-sulfonate (−232 mV; by titration with riboflavin, $E_0' = -208$ mV; Loach, 1968), and NADH (−320 mV; Loach, 1968). The equilibria with NADH were catalyzed by the *B. harveyi* oxidoreductase at pH 8.3 and have been corrected to pH 7.0 (Fisher, 1976; Spencer et al., 1977a). The redox potential of 1,5-dideazariboflavin was beyond the range of accurate equilibration with these indicators and was therefore estimated from the bimolecular rate constants for reduction of 5-deazariboflavin by dihydro-1,5-dideazariboflavin and reduction of 1,5-dideazariboflavin by dihydro-5-deazariboflavin.

Catalytic Oxidation of Dihydro-5-deazaflavins. These assays were performed in 0.1 M KPP_i, pH 8.3, at 30 °C. Oxidation of dihydro-5-deazariboflavin was monitored by appearance of oxidized 5-deazariboflavin at 397 nm; oxidation of dihydro-1,5-dideazariboflavin was similarly monitored at 476 nm. Dihydro-5-deazariboflavin was prepared by sodium borohydride reduction (Spencer et al., 1976), and dihydro-1,5-dideazariboflavin by sodium borohydride reduction in dimethylformamide.

Analogue Binding to Egg White Binding Protein. The dissociation constants of most of the analogues were determined by the quenching of their fluorescence on titration with binding apoprotein. Scatchard analysis of the data allowed calculation of both dissociation constants and accurate extinction coefficients (assuming 1:1 binding; Becvar, 1973). Those analogues that are nonfluorescent (1-deazariboflavin, 3-deazariboflavin), very weakly fluorescent (1,5-dideazariboflavin, 8-aminoriboflavin, 8-methylaminoriboflavin), or that have fluorescence spectra that do not overlap that of riboflavin (5-deazariboflavin, 5-methyl-5-deazariboflavin, 10-hydroxyethyl-5-deazaisoalloxazine) were assayed by the technique of ligand competition. In these assays holo binding protein (with 1 mol of riboflavin bound per mol of protein) was titrated with the analogue, and the concentration of riboflavin released in competition with the analogue measured by its fluorescence (445 nm excitation, 530 nm emission). Equilibria were rapidly established as expected from the kinetic data of Becvar (1973) ($t_{1/2}$ for riboflavin release = 13 s). From these data the dissociation constant for the analogue was readily computed (Becvar, 1973). All titrations were in 0.1 M KP_i, pH 7.0, at ambient temperature.

Flavokinase and FAD Synthetase. These activities were estimated by analogue fluorescence on thin-layer chromatograms after incubation with the enzymes, as in Spencer et al. (1976). The activities of the nonfluorescent analogues were estimated by purification of their FMN or FAD analogues on anion-exchange supports after incubation with the enzymes. Thin-layer chromatography of analogue products before and after treatment with snake venom phosphodiesterase established their structures as FMN or FAD analogues. Flavokinase activity with [2-^{14}C]riboflavin as substrate was assayed by spotting reaction aliquots for paper chromatography with water as solvent. In this system labeled FMN and FAD migrate together, so that FAD synthetase activity is not measured.

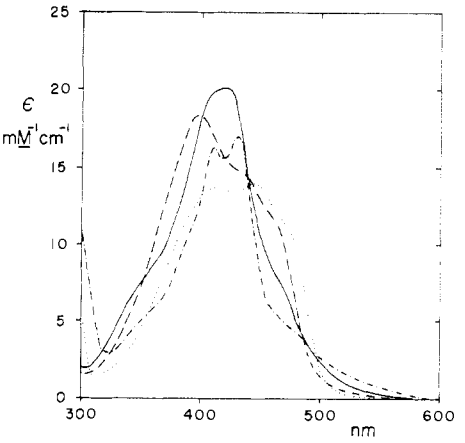


FIGURE 1: Spectra of 3-deazariboflavin: (—) anionic, pH 6.9; (- - -) neutral, pH 4.8; (- · · · -) anionic bound to the egg white binding protein, pH 9.9; (· · ·) neutral bound to the egg white binding protein, pH 7.3.

NAD(P)H:Flavin Oxidoreductase. This enzyme was assayed by monitoring dihydronicotinamide oxidation at 340 nm. Assays were in 0.1 M KPP_i, pH 8.6, 30 °C, except for the 8-hydroxyriboflavin pH studies which used a buffer of P_i, PP_i, and acetate, 10 mM each, at 30 °C.

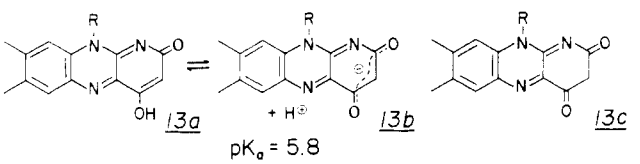
Instrumentation. A Radiometer 28 or Beckman 3500 meter was used to measure pH. Absorbance spectra and kinetics were performed with a Gilford 222 or Perkin-Elmer 200 spectrophotometer. A Perkin-Elmer MPF-4 fluorometer, uncorrected, in ratio mode, was used for fluorescence spectra and assays.

Results and Discussion

Chemical Properties of the Analogues

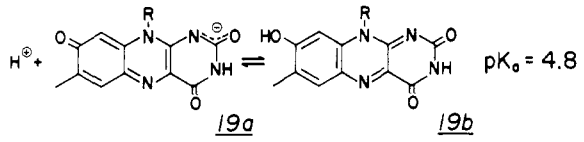
Unusual Tautomeric and Resonance Forms. Two of the flavin analogues (in the oxidized state) exist in tautomeric forms at neutral pH that are not tautomerically analogous to riboflavin. Both of these have pK_a values near neutrality that are manifested in dramatic changes in their visible absorption spectra, offering easily assayable and complementary probes of flavoprotein active site acid–base chemistry.

3-Deazariboflavin (**13**) has a pK_a of 5.8 for deprotonation to its monoanion. The spectra of the neutral and anionic forms are shown in Figure 1; neither has any visible fluorescence. Based on this ionization and the NMR observation of Ashton et al. (1977) that the hydrogen at C-3 integrates to a single proton, we suggest that **13a** and **13b** represent neutral and anionic 3-deazariboflavin, and that the 2,4-dione tautomer **13c** is not a major contributor.



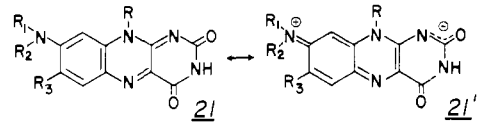
The second of the analogues with an accessible pK_a is 8-hydroxyriboflavin (**19**), which has been well characterized by Ghisla & Mayhew (1976). The pK_a at 4.8 corresponds to proton dissociation from O^8 and a concomitant shift from the phenolic tautomer **19b** to the paraquinoid tautomer **19a.** As might be expected, it is only the phenolic tautomer that pos-

sesses redox activity in flavoenzyme systems (Ghisla & Mayhew, 1976; vide infra).



19a 19b

All of the 8-amino substituted analogues in Table II (**21a–e**) are neutral species between pH 2 and 10. There is, however, a striking similarity between the absorption spectra of these analogues and that of the paraquinoid anion of 8-hydroxyriboflavin (**19a**) (Tables I and II). This suggests a substantial contribution by the zwitterionic resonance form **21′** to the 8-alkylamino analogues. Such a contribution could explain the low redox activity of the 8-alkylamino analogues relative to the other analogues.



21 21′

pK$_a$ Values of Dihydroflavin Analogues. Several of the analogues were reduced with an excess of sodium dithionite and acid–base titrated under an argon atmosphere. On titration from acidic to basic pH each of the dihydro analogues shows loss of an absorbance shoulder (420 to 480 nm) that allows calculation of its pK_a value (Table I). For dihydroriboflavin and dihydro-5-deazariboflavin, this pK_a has been assigned to proton dissociation from N-1 (Blankenhorn, 1976; Spencer et al., 1976), and by inference the same ionization is assigned to the dihydro-9-aza, dihydro-8-chloro, and dihydro-5-methyl-5-deaza analogues.

Oxygen Reactivity of the Dihydro Analogues. We note here the qualitative observations that all of the analogues are reduced by sodium dithionite, and all of the dihydro analogues *except* those with carbon at position 5 are readily oxidized by molecular oxygen. This supports the suggestions of Hemmerich (1976) and Bruice (1976) that it is the antiaromaticity and/or N-5–N-10 orbital splitting of the central dihydropyrazine ring of dihydroflavins that is essential for a rapid reaction between singlet dihydroflavins and triplet molecular oxygen. Kemal et al. (1977) have recently examined the kinetics of dihydroflavin oxidation by oxygen in detail.

Redox Potentials. Determination of the redox potentials of the analogues provides a basis for correlating and understanding their redox reactions. In titrations with redox indicators (see Methods), each analogue showed a slope ($dE'/d \log[FlH_2]/[Fl_{ox}]$) of nearly 30 mV, indicating that each was a net two-electron redox reaction.

Those analogues for which redox potentials could not be determined are also of interest. The two N^{10}-benzyl analogues (**6** and **7**) were too insoluble for accurate aqueous titration, and in any case should have redox potentials not far from that of riboflavin. All of the other analogues in this category did not equilibrate with the redox indicators used. For 5-methyl-5-deazariboflavin (**17**) this probably reflects excessive steric hindrance at position 5, the presumed site of electron entry (Spencer et al., 1976). For the 8-alkylamino- (**21a–e**), 8-hydroxy- (**19**), and 8-methylthioriboflavins (**22**), there may be a kinetic barrier to redox reactions due to a substantial contribution from the paraquinoid resonance form (**19a, 21′**).

Catalytic Oxidation of Dihydro-5-deazaflavins. Though dihydro-5-deazariboflavin and dihydro-1,5-dideazariboflavin

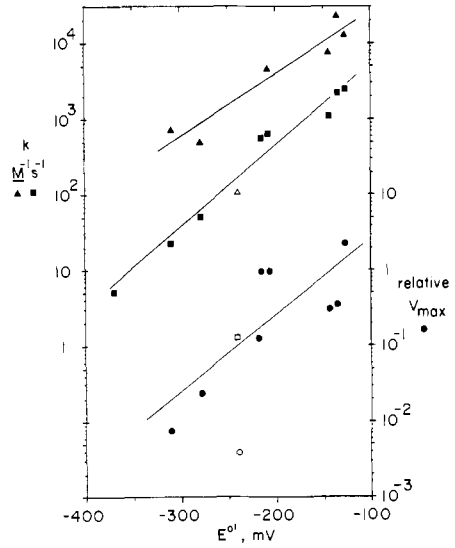


FIGURE 2: From the data of Table I, the rates of dihydro-1,5-dideazariboflavin oxidation (▲, left ordinate) and dihydro-5-deazariboflavin oxidation (■, left ordinate) by the flavin analogues are plotted vs. the redox potentials of the analogues. The relative V_{max} values for the oxidation of NADH by the analogues and the *B. harveyi* oxidoreductase (●, right ordinate) are also plotted vs. these redox potentials. The flavin analogues used are the following, from left to right within each series: (●) **15, 14, 4, 3, 2, 1**; (■) **18, 15, 14, 8, 4, 3, 2, 1**; (●) **15, 14, 11, 8, 4, 3, 2, 1.** The open symbols indicate the rates with 3-deazariboflavin, which were not included in the least-squares calculation to give the correlation lines shown.

are essentially inert to oxidation by molecular oxygen, both are readily oxidized by most of the other analogues. The reactions involved are shown in eq 1:

$$5\text{-deazaFlH}^- + Fl_{ox} \rightleftharpoons 5\text{-deazaFl}_{ox} + FlH^- \quad (1)$$

(fast, O_2)

in aerobic buffer the rate of oxidation of FlH^- exceeds the rate of its production, so that the flavin remains oxidized in the steady state and is only required in catalytic quantities. Under the conditions employed ([5-deazaFlH$^-$] ~ 10^{-4} M, [Fl_{ox}] ~ 10^{-5} M), the oxidation of 5-deazaFlH$^-$ is pseudo-first-order in both 5-deazaFlH$^-$ and Fl_{ox}, and the derived second-order rate constants are listed in Table I. These values are plotted (as log k) vs. the redox potential of the catalytic analogue in Figure 2. The linear free energy relations that these data imply are discussed later in conjunction with the enzymatic oxidation of NADH. The disproportionation between oxidized and reduced 5-deazariboflavin has been measured by radioactive label exchange (Spencer et al., 1976), and this rate correlates well with the rate of oxidation of dihydro-5-deazariboflavin by the other analogues.[2]

Protein Binding and Enzymatic Properties of the Analogues

Riboflavin Binding Protein. This protein, which may serve in vitamin storage in the avian egg, is readily purified to homogeneity from hen egg white, and the apoprotein (required for study of analogue binding) is prepared in quantitative yield by ion-exchange chromatography at pH 3.5 (Becvar, 1973). The kinetics and thermodynamics of the association of several

[2] 5-Deazariboflavin disproportionation was measured at 0 °C (Spencer et al., 1976) and the other reactions were at 30 °C. The correlation is close, nevertheless, since these reactions have small enthalpies of activation (Blankenhorn, 1976).

TABLE III: pK_a Values of Analogues Free and Bound to the Egg White Binding Protein.

Analogue	Ionization position	pK_a Free	pK_a Bound
8-Hydroxyriboflavin	O^{8a}	4.8[a]	8.0
3-Deazariboflavin	O^4 or O^2	5.8	8.7
Dihydro-1-deazariboflavin	O^2 or O^4 [b]	5.6	7.9
Dihydro-5-deazariboflavin	N^1 [c]	7.2[c]	9

[a] Ghisla & Mayhew, 1976. [b] Spencer et al., 1977a. [c] Blankenhorn, 1976; Spencer et al., 1976.

analogues have been investigated by Becvar (1973), who showed that the protein has a reasonably broad tolerance for structurally modified alloxazines and isoalloxazines, with riboflavin the most tightly bound of all those tested. These features prompted our use of this protein to assay the association of these newer analogues with a flavin binding site. Dissociation constants for the analogues are listed in Table I and are discussed below with respect to substitutions at N-10, then C-7 and C-8, positions 6 and 5; and finally general site hydrophobicity as expressed in bound analogue pK_a values.

The progressively poorer binding of 5′-deoxyriboflavin (**5**), riboflavin 5′-sulfate (**9**, a monoanion), and FMN (**8**, a dianion) suggests the existence of a hydrogen bond to the 5′ position, and discrimination against explicit charge at this position. The poor binding of FAD probably reflects intolerance of the charge and bulk of the adenosyl group. That the ribityl side chain is not essential for binding, however, is shown by the avid binding of 10-(3-chlorobenzyl)flavin (**6**); the binding energy lost with specific ribityl protein contacts may be compensated for by partitioning of this very hydrophobic analogue out of the aqueous solvent.

That there is a distinct steric restriction at position 8 is indicated by the dramatic increase in K_d from 8-methylaminoriboflavin (**21b**, Table II) to 8-propylamino- and 8-aminohexylaminoriboflavins (**21c,d**). This is in general agreement with Becvar (1973) and provides a contrast to the bacterial flavodoxins, in which positions 7 and 8 are exposed to solvent (Mayhew & Ludwig, 1975) and bind flavins with bulky 8α substituents tightly (Oestreicher et al., 1976). Further comparisons of analogue binding between the flavodoxins and the egg white protein should prove interesting.

The tight binding of both 6-methylriboflavin (**11**) and 5-methyl-5-deazariboflavin (**17**) shows that this region of the binding site is relatively open. This is somewhat surprising, since the binding protein stabilizes the neutral "blue" semiquinone of riboflavin (Spencer et al., 1977a; Massey & Hemmerich, 1977) which has been presumed to require a strong hydrogen bond to position 5 (Hemmerich, 1976). Further studies of this region are under way with a series of N^5-alkylriboflavins.

The vibronic resolution of the riboflavin spectrum on its association with the binding protein suggests that the site is hydrophobic (Becvar, 1973), and similar spectral shifts are seen on binding of most of the analogues (e.g., Figure 1). This hydrophobicity is also expressed in the preference of the binding site for neutral over charged species in all cases examined; this is quantitated by the changes in pK_a of the analogues listed in Table III. The magnitude of these pK_a changes shows that each of the neutral analogues is bound 60- to 1600-fold tighter than the anion. That this preference indicates a hydrophobic site rather than specific protonation of the bound anions by a nearby acid residue is suggested by the multiple sites of protonation observed. Blankenhorn (1978) has recently reached the same conclusions and finds that one tyrosine and one tryptophan are essential for flavin binding.

Flavokinase and FAD Synthetase. These two enzymatic activities, partially purified from *Brevibacterium ammoniagenes* (Spencer et al., 1976), are responsible for the 5′ phosphorylation of riboflavin to FMN followed by the adenylylation of FMN to FAD. The qualitative ability of the analogues to serve as substrates for these enzymes is indicated in Table I; not surprisingly a 5′-hydroxyl is an absolute requirement but beyond this there is a broad substrate specificity. In this lack of specificity with respect to the flavin the bacterial enzymes are similar to the yeast and rat liver enzymes (McCormick, 1962, 1964; Kearney, 1952). At the present stage of copurification of these enzymes, FAD synthetase activity exceeds that of flavokinase, so that, with riboflavin as substrate, FAD accumulates as product from a low steady-state concentration of FMN. With the single exception of 3-deazariboflavin this is also the case for all of the analogues which are substrates. 3-DeazaFMN is apparently not a substrate for the FAD synthetase, since 3-deazaFMN accumulates as product and 3-deazaFAD is not formed.

Quantitative assay of the flavokinase is feasible with [2-^{14}C]riboflavin as substrate. The enzyme has a K_m for riboflavin of approximately 5 μM and shows substrate inhibition at concentrations of riboflavin over 20 μM. Three of the analogues that lack the 5′-hydroxyl group (and are therefore not substrates) were assayed as competitive inhibitors of the flavokinase. The results show that these are all potent inhibitors: for 5′-deoxyriboflavin, $K_i = 20\ \mu$M; for 10-(3-chlorobenzyl)flavin, $K_i = 0.9\ \mu$M; and for 10-(2,6-dichlorobenzyl)flavin, $K_i \simeq 1.5\ \mu$M (all vs. riboflavin as substrate). The very low K_i values for the N^{10}-benzyl analogues suggest a hydrophobic active site.

Since this flavokinase and FAD synthetase system is bacterial, it is risky to infer from it the properties of the avian and mammalian enzyme counterparts. However, it is possible that in the antimetabolite activities of some of these analogues (Graham et al., 1977; Otani, 1976) two very different loci of action are involved. Those analogues that lack the 5′-hydroxyl group may exert their effects by inhibiting the conversion of natural riboflavin to FMN and FAD, while those analogues with a ribityl side chain may be efficiently converted to FMN and FAD analogues, and then exert their in vivo effects after incorporation into redox-active flavoenzymes. The tight binding to various apoflavoenzymes of some of the analogues in in vitro studies (Hersh & Jorns, 1975; Fisher et al., 1976; Ghisla & Mayhew, 1976; Spencer et al., 1977b) together with their diminished redox activity suggests that this latter form of in vivo inhibition could be effective and functionally irreversible.

NAD(P)H:Flavin Oxidoreductase. This enzyme from the luminescent marine bacterium *Beneckea harveyi* catalyzes the reaction of eq 2

$$Fl_{ox} + NAD(P)H \rightleftharpoons NAD(P)^+ + FlH^- \quad \xleftarrow{O_2\ (nonenzymatic)} \tag{2}$$

and has the physiological role of supplying luciferase (the light emitting enzyme) with dihydroFMN, one of its substrates. The advantage of the oxidoreductase in flavin analogue studies is that it is nearly unique in catalyzing a flavin redox reaction with the flavin as *substrate* rather than as tightly bound coenzyme; in addition it accepts flavins at the riboflavin, as well as FMN, level. This obviates the microscale 5′ phosphorylation of the analogues. Riboflavin, 5-deazariboflavin, and 1-dea-

zariboflavin have been extensively studied as substrates for this enzyme (Fisher et al., 1976; Spencer et al., 1977b). Our preparation of the enzyme is extensively characterized in Fisher (1976); it is devoid of the NADPH-specific oxidoreductase purified by Jablonski & DeLuca (1977) as evidenced by its unique Michaelis constants and inability to bind to blue dextran.

Since most of the dihydro analogues are rapidly oxidized by oxygen, assay of the enzyme in aerobic buffer proceeds exclusively toward NAD(P)H oxidation. The results of assays in this direction are presented in the last columns of Tables I and II. Notable trends and exceptions in these data are discussed below for each of the four measured parameters: V_{max}, K_m (flavin), K_i (vs. riboflavin), and V_H/V_D (the ratio of maximum velocities obtained with NADH and [4*R*-^{2}H]-NADH as substrates).

V_{max}. The substantial isotope effects on V_{max} (vide infra) show that it is hydrogen transfer in the flavin reduction step that is largely rate determining in enzyme turnover. Thus, the redox chemistry of a given analogue may be expected to play a large role in determining its V_{max} with this enzyme. This expectation increases the value of the oxidoreductase in the examination of redox mechanisms as is borne out in the correlation between V_{max} and the analogue redox potentials (Figure 2). The oxidoreductase contrasts with those flavoenzymes which have a physical step that is rate determining (e.g., product release with D-amino acid oxidase) and also with those flavoenzymes for which the catalytic competence on flavin analogue substitution displays no obvious correlation with the chemical properties of the flavin analogues (e.g., pyridoxamine oxidase (Kazarinoff & McCormick, 1975) and bacterial luciferase (Mitchell & Hastings, 1969)).

The V_{max} data of Tables I and II show that the 8-hydroxy- and 8-alkylamino analogues have negligible activity with the oxidoreductase. We have suggested that this is due to their existence as tautomers or resonance forms not analogous to riboflavin. The ability of 8-hydroxyriboflavin to serve as an enzyme substrate as a function of pH supports this view, since the V_{max} data (not shown) indicate that 8-hydroxyriboflavin becomes a measurable substrate for the oxidoreductase as the solution pH is lowered. The data can be fit by the Henderson–Hasselbach function fit to the pK_a of 8-hydroxyriboflavin (4.8; Ghisla & Mayhew, 1976) and a V_{max} at 100% phenolic tautomer (**19b**) 0.068 that of riboflavin. The fit of the V_{max} data to such a line suggests that the paraquinoid (**19a**) to phenolic (**19b**) tautomeric change represents a switch between redox inert and active forms.[3] Ghisla & Mayhew (1976) support this hypothesis with their observation that 8-hydroxyFMN:flavodoxin is the only one of their several 8-hydroxyflavin-reconstituted flavoenzymes that has catalytic activity and is also the only one to stabilize the phenolic tautomer.

The importance of position 5 of the isoalloxazine ring as the site of electron entry is shown by the negligible activity of 5-methyl-5-deazariboflavin with the enzyme. This analogue binds at the active site (K_i = 2 μM), but the methyl group at C-5 creates a barrier to reduction that is not present with 5-deazariboflavin.

K_m and K_i. With the kinetic mechanism of the oxidoreductase still uncertain (Gerlo & Charlier, 1975; Michaliszyn et al., 1977), it is not known which rate constants determine K_m for a flavin or K_i for an inert flavin analogue. Nonetheless some trends may be seen.

For many of the analogues, V_{max} and K_m correlate in that the V_{max}/K_m ratio is approximately the same over a factor of 100 change in V_{max}. This suggests that the flavin, once bound at the active site, is more likely to be reduced than released (without having reacted) back to solvent.[4] Such an inference illustrates the value of the analogues as probes of kinetic, as well as chemical, enzyme mechanism.

While hydroxy and amino substituents pose barriers to reduction of analogues **19** and **21a–e**, they do not inhibit binding at the oxidoreductase active site, as shown by these analogues' very low K_i values. Since the K_i of 8-aminohexylaminoriboflavin (**21d**) is comparable to those of the other 8-alkylamino analogues, the oxidoreductase may have this portion of the bound flavin accessible to solvent and unhindered, like the flavodoxins but unlike the egg white binding protein.

V_H/V_D. The isotope effect of 4.8 at V_{max} in riboflavin reduction shows that the dihydronicotinamide oxidation–flavin reduction step is significantly rate determining in catalysis. That the isotope effect in flavin analogue reduction remains between 2.4 and 6.5 over two orders of magnitude change in V_{max} further establishes both the 4*R* chirality of the dihydronicotinamide and the partial rate limitation of hydrogen transfer in analogue turnover.

Conclusions

Both the nonenzymatic and the enzymatic (oxidoreductase) reactions compared in Figure 2 display comparable correlations in linear free energy–rate profiles. Since hydrogen transfer occurs in the rate-determining transition states for both types of reactions over the whole range of flavin redox potential levels examined,[5] it is reasonable to propose a commonality of redox chemistry in the rate-determining steps. The 5-deazaflavins correlate with other flavins in these profiles, supporting our earlier contention (Fisher et al., 1976) that 5-deazaflavins can be valid probes of flavoenzyme mechanisms.[6] With a common mechanism in mind, it is still difficult to pin down how hydrogen and electrons are transferred, i.e., whether the hydrogen species in flight in the redox steps is a hydride ion or a hydrogen atom.

The transferring hydrogen cannot be a proton, at least not a freely dissociating one, because nonenzymatic experiments show retention of labeled hydrogen by the partner molecule undergoing reduction. Brüstlein & Bruice (1972) demonstrated direct hydrogen transfer between a dihydronicotinamide and a 5-deazaisoalloxazine without exchange of protons from solvent. Spencer et al. (1976) showed that [5-^{3}H]-dihydro-5-deazariboflavin and 5-deazariboflavin disproportionate in aqueous buffer with complete retention of label. This latter observation is more pertinent since this reaction is included in Figure 2 and found to correlate well with the other dihydro-5-deazariboflavin redox reactions.

The commonly held view for nicotinamide–flavin redox

[3] This is only the simplest explanation. The instability of both enzyme and NADH below pH 4 prohibits the assignment of an apparent pK_a based on the enzyme kinetics alone, and the availability of the enzyme in only catalytic quantities does not permit measurement of the pK_a of 8-hydroxyriboflavin bound at the active site.

[4] In its simplest representation $K_m = (k_{off} + k_{cat})/k_{on}$. Therefore k_{cat}/K_m can be independent of k_{cat} only if $k_{cat} \gg k_{off}$.

[5] This is inferred from the data of Table II and Spencer et al. (1976): for riboflavin oxidation of dideuterio-5-deazariboflavin, k_H/k_D = 6.6, and for 5-deazariboflavin oxidation of [5-^{3}H]dihydro-5-deazariboflavin, $k_H/k_T > 7$ when extrapolated to complete tritium substitution.

[6] The reactions here and in Fisher et al. (1976) are examples of flavin *reductive half-reactions*. Flavoenzyme oxidative half-reactions often involve sequential one electron transfers to oxygen or iron-containing coenzymes, and in such reactions dihydro-5-deazaflavins are clearly *not* kinetically comparable to dihydroflavins.

chemistry (enzymatic or nonenzymatic) has been hydride transfer. Recently, Blankenhorn (1976) has investigated the oxidation of a series of dihydronicotinamide analogues (including dihydro-5-deazaflavins) by lumiflavin and finds a linear free energy–rate relationship with a slope ($(d \log k/d E)^{-1}$) of 30 mV. He argues that these data support the transfer of a hydride ion (i.e., hydrogen accompanied by *two* electrons) in the rate-determining step. Unfortunately, flavin substituents alter the free energy of the hydrogen transfer transition state, so that the slopes of flavin analogue data range from 90 to 120 mV (Figure 2) and 140 mV (Blankenhorn, 1975) and are not directly amenable to such interpretation about electron stoichiometry in the redox step. Indeed, they cast some doubt on the simple expectation that the slopes should either be 30 mV ($2e^-$) or 60 mV ($1e^-$) in the redox step. An indirect argument for hydride ion transfer in these reactions is also suggested by the very low redox potentials for formation of 5-deazaflavin and nicotinamide semiquinones, −650 and −730 mV, respectively (Blankenhorn, 1976), which may preclude the ready formation of such radicals required in a hydrogen atom transfer mechanism.

Nonetheless, the possibility of hydrogen atom transfer in these reactions cannot be excluded. Bruice and colleagues have emphasized the one-electron nature of carbonyl-producing flavin redox reactions (Williams & Bruice, 1976; Chan & Bruice, 1977; Bruice & Taulane, 1976), and the one-electron chemistry of dihydropyridines has been recently explored more fully (Dittmer et al., 1976; Wong & Frey, 1976; Kill & Widdowson, 1976; Okamoto et al., 1977; Tuazon & Johnson, 1977). Thus while we conclude that flavins, 5-deazaflavins, 1-deazaflavins, and the other analogues of Figure 3 share a common electron transfer mechanism in these reactions, details of that electron transfer mechanism remain uncertain.

Regardless of mechanism, it is useful to classify the analogues of Tables I and II with respect to their chemical and enzymatic reactivities, and propose how analogues in each category may behave in both in vitro enzymatic systems and in in vivo antimetabolite studies. First, several of the analogues may be expected to duplicate the one- and two-electron redox chemistry of the parent flavins, and by possessing the N^{10}-D-ribityl side chain be good isosteric replacements for the natural flavins. These include riboflavin itself, 7-chloro-, 9-aza-, 8-chloro-, and 1-deazariboflavins. All of these (if necessary as FMN or FAD analogues) may be expected to replace the natural coenzymes and be functional in catalysis, though 1-deazaflavins with their low redox potential may reduce turnover rates for many flavoenzymes. Whether or not these analogues will prove functional in all classes of flavoenzymes (oxidases, dehydrogenases, monooxygenases) remains to be determined. Monooxygenases are of particular interest since they may be readily "uncoupled" to serve only as NAD(P)H oxidases; recent studies in this laboratory have shown that 1-deazaFAD- and 9-azaFAD-reconstituted orcinol hydroxylase are apparently totally uncoupled.[7]

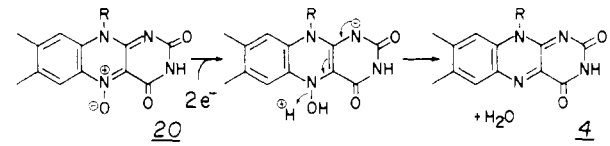
Secondly, several of the analogues have essentially unaltered isoalloxazine systems but may have steric prohibitions to enzymatic activity; in this category are all the analogues that lack the complete D-ribityl side chain and, perhaps, 6-methylriboflavin. These are of less interest for in vitro mechanistic studies but may be potent inhibitors of flavoenzymes in vivo. Specifically the hydrophobic N^{10}-benzyl analogues may inhibit those flavoenzymes with hydrophobic active sites and some of the many flavoenzymes that are membrane associated.

[7] A. Cheung, Y. Cheung, & C. Walsh, unpublished observations.

Third, those analogues with electron donating substituents at position 8 (**12, 19, 21a–e**) are quite inert to several biological reductants (Tables I and II and Ghisla & Mayhew, 1976). These analogues may be good steric replacements for natural flavins but not catalytic substitutes. Perhaps for this reason they are among the most potent anticoccidial agents (Graham et al., 1977) and the naturally occurring roseoflavin is found to be an effective antimetabolite (Otani, 1976). Also in this category we include 3-deazariboflavin, which, denoted by the open symbols in Figure 3, is considerably less reactive than its redox potential might suggest. Though it has an electrostatic barrier to complex formation and oxidation of dihydro-5-deazaflavins (both are anions at pH 8.3), the origins of its poor reactivity are more complex since 3-deazariboflavin is no more active below its pK_a than above with NADH and the *B. harveyi* oxidoreductase.

The 5-deazaflavins make up the fourth category. 5-DeazaFMN and 5-deazaFAD have been established as close isosteric replacements for FMN and FAD in several systems (Jorns & Hersh, 1975; Hersh & Jorns, 1975; Fisher et al., 1976). These studies also showed that 5-deazaflavins can be reduced by numerous enzyme substrates but are then virtually inert to oxidation by molecular oxygen. An additional index that dihydro-5-deazaflavins are kinetically inert to some one-electron oxidations is also shown by recent studies with ferritin. Dihydroriboflavin efficiently reduces and then releases iron (as Fe^{2+}) from this protein (Sirivech et al., 1974) but dihydro-5-deazariboflavin is incapable of such reductive release.[8] Since many common biological oxidants of dihydroflavins are one-electron acceptors (oxygen, iron-containing coenzymes), it is not surprising that 5-deazariboflavin is a very potent anticoccidial agent (Graham et al., 1977).

Unique among the analogues in its reactivity is the riboflavin 5-oxide (**20**). On two-electron reduction (enzymatic or nonenzymatic), this analogue rapidly eliminates water to yield, quantitatively, oxidized riboflavin (eq 3 and Yoneda et al., 1976):[9]



20 4

For this reason and its extreme photolability, flavin 5-oxides may prove of interest in enzymatic mechanistic studies but of little value in vivo.

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References

Ashton, W. T., Graham, D. W., Brown, R. D., & Rogers, E. F. (1977) *Tetrahedron Lett.*, 2551–2554.

Becvar, J. (1973) Ph.D. Dissertation, University of Michigan.

[8] T. Jones & C. Walsh, unpublished observations.

[9] The V_{max} for riboflavin 5-oxide in Table I is, of necessity, an observed initial rate. With riboflavin 5-oxide in vast excess over enzyme in these assays, NADH oxidation accelerates smoothly from an initial relative rate of 0.21 to a steady-state rate of 1.0 as the riboflavin 5-oxide is catalytically converted to riboflavin.

Beinert, H. (1960) *Enzymes, 2nd Ed. 2,* 339–416.
Berezovskii, V., & Rodionova, E. (1958) *Zh. Obshch. Khim. 28,* 1046–1049.
Blankenhorn, G. (1975) *Biochemistry 14,* 3172–3176.
Blankenhorn, G. (1976) *Eur. J. Biochem. 67,* 67–80.
Blankenhorn, G. (1978) *Eur. J. Biochem.* (in press).
Bruice, T. (1976) *Prog. Bioorg. Chem. 4,* 1–87.
Bruice, T., & Taulane, J. (1976) *J. Am. Chem. Soc. 98,* 7769–7776.
Brustlein, M., & Bruice, T. (1972) *J. Am. Chem. Soc. 94,* 6548–6549.
Chan, T., & Bruice, T. (1977) *J. Am. Chem. Soc. 99,* 2387–2389.
Chu, C., & Bardos, T. (1977) *J. Med. Chem. 20,* 312–314.
Cresswell, R. M., & Wood, H. C. S. (1960) *J. Chem. Soc.,* 4768–4775.
Dittmer, D., Lombardo, A., Batzold, F., & Greene, C. (1976) *J. Org. Chem. 41,* 2976–2980.
Edmondson, D., & Singer, T. (1973) *J. Biol. Chem. 248,* 8144–8149.
Fisher, J. (1976) Ph.D. Dissertation, Massachusetts Institute of Technology.
Fisher, J., Spencer, R., & Walsh, C. (1976) *Biochemistry 15,* 1054–1064.
Foery, W., MacKenzie, R. E., & McCormick, D. B. (1968) *J. Heterocycl. Chem. 5,* 625–630.
Gerlo, E., & Charlier, J. (1975) *Eur. J. Biochem. 57,* 461–467.
Ghisla, S., & Mayhew, S. (1976) *Eur. J. Biochem. 63,* 373–390.
Graham, D. W., Brown, J. E., Ashton, W. T., Brown, R. D., & Rogers, E. F. (1977) *Experientia 33,* 1274–1276.
Haley, E. E., & Lambooy, J. P. (1954) *J. Am. Chem. Soc. 76,* 5093–5096.
Hemmerich, P. (1976) in *Fortschritte der Chemie Organischer Naturstoffe* (Grisebach, H., Ed.) pp 451–526, Springer-Verlag, New York, N.Y.
Hersh, L., & Jorns, M. (1975) *J. Biol. Chem. 250,* 8728–8734.
Jablonski, E., & DeLuca, M. (1977) *Biochemistry 16,* 2932–2936.
Jencks, W., & Regenstein, J. (1968) in *Handbook of Biochemistry* (Sober, H., Ed.) pp J150–J189, The Chemical Rubber Co., Cleveland, Ohio.
Jorns, M., & Hersh, L. (1975) *J. Biol. Chem. 250,* 3620–3628.
Kasai, S., Miura, R., & Matsui, K. (1975) *Bull. Soc. Chem. Jpn. 48,* 2877–2880.
Kazarinoff, M., & McCormick, D. (1975) *J. Biol. Chem. 250,* 3436–3442.
Kearney, E. (1952) *J. Biol. Chem. 194,* 747–754.
Kemal, C., Chan, T., & Bruice, T. (1977) *J. Am. Chem. Soc. 99,* 7272–7286.
Kill, R., & Widdowson, D. (1976) *Chem. Commun.,* 755–756.
Lambooy, J. P. (1975) in *Riboflavin* (Rivlin, R., Ed.) pp 303–367, Plenum Press, New York, N.Y.
Lamooy, J. P., & Shaffner, C. (1977) *J. Nutr. 107,* 245–250.
Lattes, A., & Verdier, A. (1965) *Bull. Soc. Chim. Fr., 1965,* 2037–2043.
Loach, P. (1968) *Handbook of Biochemistry* (Sober, H., Ed.) pp J27–J34, The Chemical Rubber Co., Cleveland, Ohio.
Massey, V., & Hemmerich, P. (1977) *J. Biol. Chem. 252,* 5612–5614.
Massey, V., Palmer, G., & Ballou, D. (1973) *Oxidases Relat. Redox Syst. 1,* 25–43.
Mayhew, S., & Ludwig, M. (1975) *Enzymes, 3rd Ed. 12B,* 57–118.
McCormick, D. (1962) *J. Biol. Chem. 237,* 959–962.
McCormick, D. (1964) *Biochem. Biophys. Res. Commun. 14,* 493–497.
Michaliszyn, G., Wing, S., & Meighen, E. (1977) *J. Biol. Chem. 252,* 7495–7499.
Mitchell, G., & Hastings, J. (1969) *J. Biol. Chem. 244,* 2572–2576.
O'Brien, D., Weinstock, L., & Cheng, C. (1970) *J. Heterocycl. Chem. 7,* 99–105.
Oestreicher, G., Edmondson, D., & Singer, T. (1976) in *Flavins and Flavoproteins* (Singer, T., Ed.) pp 447–452, Elsevier, Amsterdam.
Otani, S. (1976) in *Flavins and Flavoproteins* (Singer, T., Ed.) pp 323–327, Elsevier, Amsterdam.
Rivlin, R. (1975) *Riboflavin,* Plenum Press, New York, N.Y.
Shunk, C., Koniuszy, F., & Folkers, K. (1952) *J. Am. Chem. Soc. 74,* 4251–4253.
Shunk, C., Lavigne, J., & Folkers, K. (1955) *J. Am. Chem. Soc. 77,* 2210–2212.
Singer, T. P., Ed. (1976) *Flavins and Flavoproteins,* Elsevier, Amsterdam.
Sirivech, S., Frieden, E., & Osaki, S. (1974) *Biochem. J. 143,* 311–315.
Spencer, R., Fisher, J., & Walsh, C. (1976) *Biochemistry 15,* 1043–1053.
Spencer, R., Fisher, J., & Walsh, C. (1977a) *Biochemistry 16,* 3586–3594.
Spencer, R., Fisher, J., & Walsh, C. (1977b) *Biochemistry 16,* 3594–3602.
Tuazon, P., & Johnson, S. (1977) *Biochemistry 16,* 1183–1188.
Williams, R., & Bruice, T. (1976) *J. Am. Chem. Soc. 98,* 7752–7768.
Wong, S., & Frey, P. (1976) *J. Am. Chem. Soc. 98,* 7886–7887.
Yagi, K. (1971) *Methods Enzymol. 8B,* 465–467.
Yoneda, F., Sakuma, Y., Ichiba, M., & Shinomura, K. (1976) *J. Am. Chem. Soc. 98,* 830–835.