idea was further strengthened by the observation that α-L-fucosylpalmitoylethanolamine showed a weaker reaction in complement fixation as compared to the synthetic fucosylceramide, although this analogue definitely reacts with anti-fucosylceramide antibodies.

The titer of antibody directed to synthetic α-L-fucosylceramide was higher than naturally occurring α-L-fucosylceramide. Therefore, the structure of the ceramide moiety also affects immunogenicity of the liposome. It is possible that ceramide without a double bond and with a longer fatty acid chain may form a more stable liposome than that with an unsaturated sphingosine (sphingenine) and with a shorter fatty acid chain. Antibodies directed to synthetic α-L-fucosylceramide cross-react with galactosylceramide, ceramide, and liposome alone; this may be because some antibodies may also be directed to the ceramide moiety and a part of the liposome structure as well, since the cross reactivities were removed readily by absorption with galactosylceramide liposomes. Anti-galactosylceramide did not react to the liposomes containing synthetic fucosylceramide but did react to those with natural fucosylceramide (Figure 4a, solid squares with dotted line). This indicates that galactosylceramide is present as a contaminant in the natural fucosylceramide isolated from adenocarcinoma.

Recently, monoclonal antibodies directed to glycolipids have been prepared (Young et al., 1979; Nowinski et al., 1980), and the technique for targeting of chemotherapeutic drugs to tumor cells through anti-glycolipid antibodies has been developed (Urdal & Hakomori, 1980). Logically, anti-α-L-fucosylceramide antibodies are useful reagents for targeting drugs in human cancer. Such efforts are under serious consideration.

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Analogues of 8-Hydroxy-5-deazaflavin Cofactor: Relative Activity as Substrates for 8-Hydroxy-5-deazaflavin-Dependent $NADP^+$ Reductase from *Methanococcus vannielii*†

Shigeko Yamazaki,* L. Tsai, and Thressa C. Stadtman

ABSTRACT: The 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase from *Methanococcus vannielii* was examined for its ability to catalyze the reduction of a number of 5-deazaflavin analogues of the natural cofactor. Comparison of the kinetic constants revealed certain substrate structure–reactivity relationships for the enzyme. The basic heterocyclic system of the natural cofactor 2,4-dioxopyrimido[4,5-*b*]quinoline was shown to be the minimum structural requirement since neither riboflavin nor 1,5-dideazariboflavin was reduced by the enzyme. The N-10 side chain of the natural cofactor was shown not to be essential since the enzyme could reduce 8-hydroxy-2,4-dioxopyrimido[4,5-*b*]quinoline. The study also indicated that there are some steric constraints at C-8 and C-7 with respect to interaction of the cofactor with the enzyme. Specifically, (a) the 8-methoxy derivative, in contrast to the 8-hydroxy compound, was not reduced and (b) the introduction of a substituent at C-7 resulted in a marked decrease in the rate of reduction. The importance of C-5 as the site for the electron entry was suggested by the finding that 5-methyldeazariboflavin was not reduced. The latter inhibited the reduction of 5-deazariboflavin.

A novel 8-hydroxy-5-deazaflavin cofactor (8-OH-5dFl)[1] that is abundant in methane-producing bacteria (Eirich et al., 1978; Ashton et al., 1979; Pol et al., 1980) serves as electron carrier in a formate–$NADP^+$ oxidoreductase system (Tzeng et al., 1975; Jones & Stadtman, 1980). In *Methanococcus vannielii* the 8-OH-5dFl, which is reduced by formate dehydrogenase and formate, serves as a cofactor for an enzyme (5-deazaflavin–$NADP^+$ reductase) that reduces $NADP^+$ to NADPH. The *M. vannielii* $NADP^+$ reductase specifically requires

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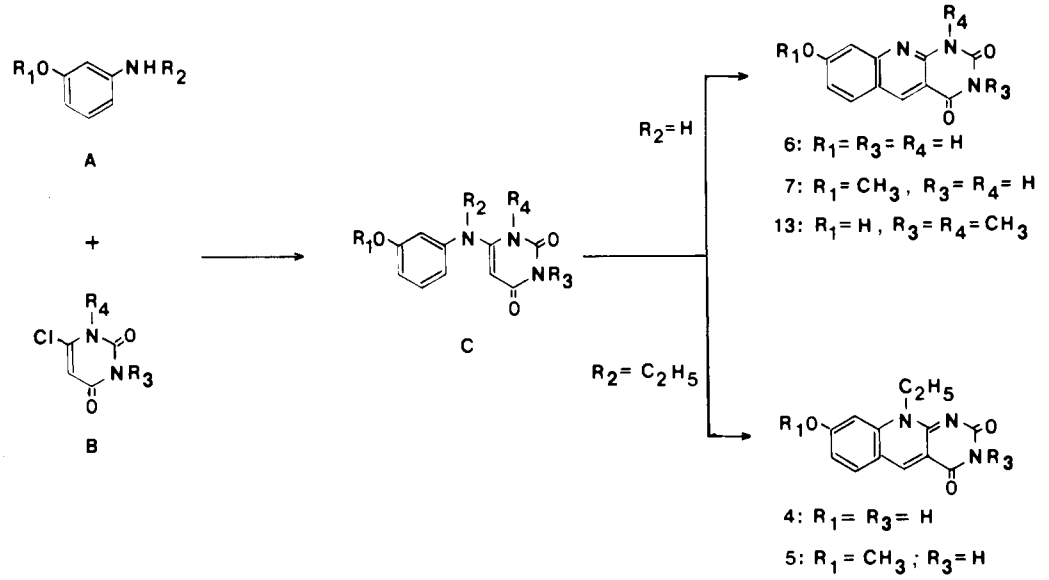
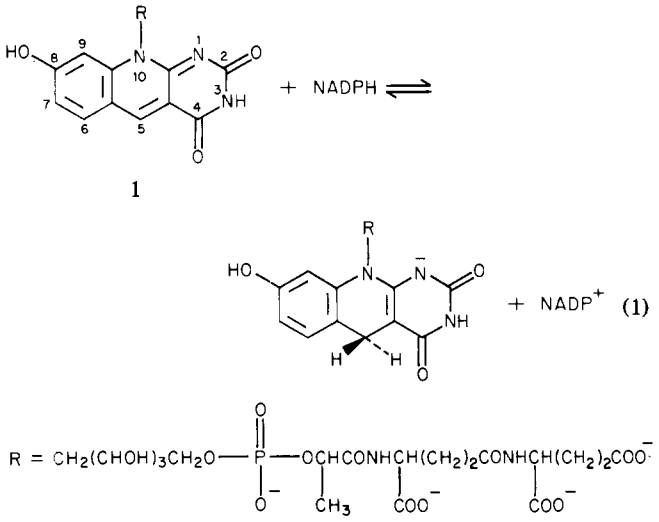
[1] Abbreviations: 8-OH-5dFl, 8-hydroxy-5-deazaflavin cofactor or coenzyme F_{420}; 5-deazaflavin–$NADP^+$ reductase, 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase; FMN, riboflavin 5′-phosphate; FAD, flavin adenine dinucleotide; NMR, nuclear magnetic resonance; ^1H NMR, proton nuclear magnetic resonance.

Scheme I



a 5-deazaflavin as cofactor whereas the formate dehydrogenases from this organism can reduce FMN and FAD in addition to 8-OH-5dFl (Jones & Stadtman, 1980; Yamazaki & Tsai, 1980). The reaction (eq 1) which is catalyzed



by homogeneous preparations of the reductase (Yamazaki & Tsai, 1980) involves a reversible hydride transfer from the C-4 position of NADPH to the C-5 of the 5-deazaflavin chromophore of 8-OH-5dFl (Yamazaki et al., 1980). Although this is similar to the familiar flavin–pyridine nucleotide oxidation–reduction system, it is the first example of the obligatory participation of a natural 5-deazaflavin instead of a flavin in such a reaction. In addition to the natural cofactor, preliminary experiments showed that the enzyme catalyzes the reduction of 8-hydroxy-2,4-dioxopyrimido[4,5-*b*]quinoline (**6**), an analogue lacking the N-10 side chain of 8-OH-5dFl. Thus, we decided to prepare other analogues of 8-OH-5dFl, and in order to probe the substrate structure–reactivity relationships for the 5-deazaflavin–$NADP^+$ reductase, we have examined the enzymic reduction of these analogues. The syntheses of some of these analogues and comparisons of their kinetic constants for the enzyme are described in this report.

Experimental Procedures

Materials. The natural cofactor 8-OH-5dFl (**1**) and 5-deazaflavin–$NADP^+$ reductase were isolated from *M. vannielii* (Yamazaki & Tsai, 1980). 7,8-Didemethyl-8-hydroxy-5-deazariboflavin (**2**) and 8-demethyl-8-hydroxy-5-deazariboflavin (**3**) (Ashton et al., 1979) were generous gifts of Dr. C. Walsh. 5-Methyl-5-deazariboflavin (**10**) and 1,5-dideazariboflavin (**12**) (Ashton et al., 1977) were generous gifts of Dr. W. T. Ashton. 6-Chlorouracil was prepared from 2,4,6-trichloropyrimidine (Cresswell & Wood, 1960). 3-(Ethylamino)phenol and 3-(ethylamino)anisole were prepared from 3-acetamidophenol and *N*-acetyl-*m*-anisole, respectively, by reduction with sodium acetoxyborohydride (Umino et al., 1976).

Table I: 6-Aminouracils

A		B			C	
R_1	R_2	R_3	R_4	reaction time (h)	mp (°C)	yield (%)
H	C_2H_5	H	H	18	317-320[a]	88
CH_3	C_2H_5	H	H	21	221-223[b]	84
H	H	H	H	2	316-318[c]	91
CH_3	H	H	H	3	312-313[d]	90
H	H	CH_3	CH_3	1.5	256-258[d]	61

[a] Sublimation at 230 °C (0.05 mm). [b] Sublimation at 170 °C. (0.2 mm). [c] Recrystallization from formic acid. [d] Recrystallization from methanol.

The synthetic approach used for the synthesis of the 8-hydroxy-2,4-dioxopyrimido[4,5-*b*]quinolines is outlined in Scheme I. This was based on the works of Yoneda et al. (1976), Janda & Hemmerich (1976), and Ashton et al. (1978). The presence of the hydroxy or alkoxy function in the aromatic ring facilitated the cyclization of the 6-(arylamino)uracils, which thus could be accomplished under much milder conditions than those used by the previous workers.

The 6-(arylamino)uracils listed in Table I were prepared from the appropriate amine and 6-chlorouracil according to the procedure of Ashton et al. (1978). For the reaction between *m*-aminophenol and 1,3-dimethyl-6-chlorouracil, dimethylformamide was used as solvent.

For the preparation of 2,4-dioxopyrimido[4,5-*b*]quinolines the following procedure was employed. To an ice-cooled suspension of 6-(arylamino)uracil in dimethylformamide (10% w/v) under argon was added slowly under stirring 2 molar equiv of phosphorus oxychloride. After the mixture was allowed to stand at room temperature for 60 min, it was diluted with 4 volumes of water, and the precipitate was collected on a filter and dried at 100 °C in vacuo for 4 h. Each product was purified either by recrystallization or by vacuum subli-

Table II: Spectral Properties of 2,4-Dioxopyrimido[4,5-*b*]quinolines

compd	condition	vis-UV λ (ε × 10^{-4})[a]	^{1}H NMR (δ)[b] solvent	H_5	H_6	H_7	H_9	others
4	0.10 N KOH	417 (4.18), 293 (1.07), 267 (2.48), 245 (3.80)	Me_2SO-d_6[c]	8.84 (s)	8.03 (d)	7.04 (dd)	7.15 (d)	4.62 (q, CH_2), 1.31 (t, CH_3)
	pH 5	394 (2.31), 382i (2.23), 265 (2.43), 249 (2.31), 232 (3.80)	D_2O–5% Na_2CO_3	7.31 (s)	6.86 (d)	6.34 (dd)	5.99	3.84 (q, CH_2), 1.00 (t, CH_3)
	1 N HCl	372 (2.88), 268i (1.90), 250 (2.24), 228 (3.92)						
5	0.10 N KOH	403i (2.41), 389 (2.56), 339 (1.17), 254 (5.22), 230 (2.96)	Me_2SO-d_6	8.92 (s)	8.13 (d)	7.21 (dd)	7.27 (d)	4.72 (q, CH_2), 1.32 (t, CH_3), 4.03 (s, OCH_3)
	pH 5	393 (2.92), 381 (2.89), 336i (0.79), 264 (3.98), 248 (3.01), 231 (5.22)						
	1 N HCl	371 (3.88), 266 (2.30), 248 (2.94), 227 (5.05)						
6	0.10 N KOH	388 (2.30), 282 (2.48), 268i (2.26), 239 (4.33)	Me_2SO-d_6	8.88 (s)	8.03 (d)	7.13 (dd)	7.10 (s)	
	pH 5	355 (1.81), 346i (1.74), 250 (4.00), 226 (3.70)						
	1 N HCl	370 (3.05), 264i (2.13), 250 (2.54), 225 (4.29)						
7	0.10 N KOH	360 (1.55), 347 (1.67), 260i (3.87), 243 (4.91), 232 (4.42)	Me_2SO-d_6	8.90 (s)	8.03 (d)	7.19 (dd)	7.17 (s)	3.94 (s, OCH_3)
	pH 5	356–344 (1.92), 251 (4.53), 226 (3.89)						
	1 N HCl	370 (3.58), 252 (3.10), 226 (5.05)						
13	0.10 N KOH	388 (2.31), 287 (2.54), 256i (3.39), 246 (3.80), 235i (3.61)	Me_2SO-d_6	8.92 (s)	8.03 (d)	7.12 (dd)	7.14	3.32 (s, NCH_3), 3.63 (s, NCH_3)
	pH 5	368i (1.60), 349 (1.68), 266i (2.48), 256 (3.73), 239 (4.13), 232i (4.00)						
	1 N HCl	374 (2.94), 266i (1.87), 243i (2.87), 228 (3.94)						

[a] λ in nm; ε in $M^{-1}\cdot cm^{-1}$; i, inflection. [b] Chemical shifts downfield from tetramethylsilane; s, singlet; d, doublet ($J_{6,7}$ = 9 Hz); dd, doublet of doublet ($J_{9,7}$ = 2 Hz, $J_{6,7}$ = 9 Hz); t, triplet; q, quartet. [c] Me_2SO-d_6, dimethyl sulfoxide.

mation. The results were as follows: **4**, mp >350 °C, yield 94%; **5**, mp 345–350 °C, yield 83%; **6**, mp >350 °C, yield 98%; **7**, mp >350 °C, yield 71%; **13**, mp 325–330 °C, yield 90%. The spectroscopic properties are shown in Table II.

Adequate C, H, and N analyses were obtained with all the new compounds except the following: compound C (R_1 = R_2 = R_3 = R_4 = H) (Anal. Calcd for $C_{10}H_9N_3O_3$: C, 54.79; H, 4.14; N, 19.17. Found: C, 54.23; H, 4.09; N, 18.87) and compound **4** (Anal. Calcd for $C_{13}H_{11}N_3O_3$: C, 60.69; H, 4.31; N, 16.34. Found: C, 60.19; H, 4.09; N, 16.18).

7-Hydroxy-10-methyl-2,4-dioxopyrimido[4,5-b]quinoline (**8**) was synthesized from *p*-(methylamino)phenol and 6-chlorouracil by the procedure described above except that the cyclization step required heating at 80 °C for 30 min. The electronic absorption and NMR spectra of this compound were in good agreement with those reported by Pol et al. (1980).

Preparation of the Reduced Form of Compounds **4** *and* **2**. Fifty milligrams of **4** in 5 mL of 5% aqueous Na_2CO_3 was stirred with $NaBH_4$ (100 mg) under argon at room temperature until the yellow color of the solution was completely bleached (60 min) and a white precipitate appeared. After acidification with a few drops of acetic acid the precipitate was collected and dried at 100 °C in vacuo for 1 h: ^{1}H NMR (D_2O–Na_2CO_3) δ 6.89, 6.37, and 6.35 (aromatics), 3.45 (H_5), 3.89 ($N_{10}CH_2$), 1.14 (C–CH_3); UV $\lambda_{max}^{pH\ 3}$ 324 nm (ε = 2.26 × 10^4); MS *m/e* 259 (M^+). Reduced compound **4** slowly reverted to the oxidized form upon exposure to air, and absorbancy measurements at 416 nm indicated the presence of 80% or more of the oxidized form. This is in contrast to the observations of Chan & Bruice (1978) on other 5-deazaflavin compounds. The yield and degree of purity of the reduced compound were difficult to ascertain since attempted purification always led to partial oxidation. However, the ^{1}H NMR of the isolated compound did not show signals other than those assigned to the dihydro-**4**. The reduced form of compound **2** was prepared by $NaBH_4$ reduction of compound **2** and purified as described earlier (Yamazaki & Tsai, 1980).

Methods. The pK_a values of the various deazaflavin analogues were determined by absorption spectral changes, measured in a mixed buffer of sodium citrate, potassium phosphate, and sodium pyrophosphate (50 mM each). The pH was adjusted by the addition of HCl or KOH. Protein concentration was determined by the Coomassie blue binding assay with bovine serum albumin as the standard (Bradford, 1976).

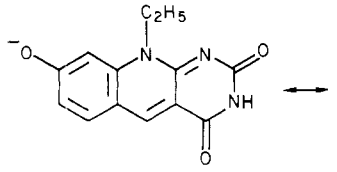
The activities of 5-deazaflavin analogues as substrates for the enzyme were measured by either fluorometric (Yamazaki & Tsai, 1980) or spectrophotometric assays of 5-deazaflavin–$NADP^+$ reductase in an argon atmosphere. Both assays were performed under the same conditions, and the reactions were initiated by the addition of enzyme. In the fluorometric assay, the excitation and emission wavelengths used were as follows: **2**, λ_{ex} 425 nm, λ_{em} 475 nm; **3**, λ_{ex} 430 nm, λ_{em} 465 nm; **4**, λ_{ex} 420 nm, λ_{em} 470 nm. In the spectrophotometric assay, the decrease in absorbancy due to reduction of the compounds was measured as follows: **4** at 415 nm; **6** at 390 nm; **8** at 418 nm; **9** at 397 nm; **11** at 420 nm. The K_m and k_{cat} values were obtained from Lineweaver–Burk plots (Lineweaver & Burk, 1934). The plots had more than eight different inverse substrate concentrations. The concentrations of the analogues used for kinetic measurements were 0.2–8 μM for **1**–**4** and 3–45 μM for the others.

Electronic absorption spectra and spectrophotometric assays were recorded on a Perkin-Elmer Model 559 spectrophotometer. A Hitachi MPF-2S fluorescence spectrophotometer equipped with Hewlett-Packard 7004B X-Y recorder was used for the fluorometric assays.

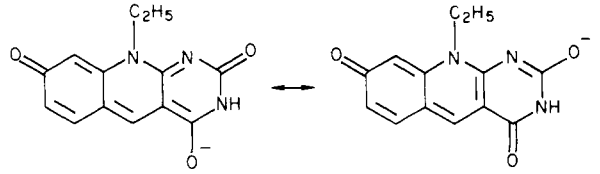
Melting points were taken on a Kolfer hot-stage apparatus and are uncorrected. Microanalyses were performed by the Section on Microanalytical Services and Instrumentation, Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases. ^{1}H NMR spectra were determined on a Varian HR 220-MHz or Nicolet 360-MHz spectrometer.

Results and Discussion

Chemical Properties of the Deazaflavin Analogues. The structures assigned to the compounds synthesized as shown in Scheme I are consistent with their spectroscopic properties (Table II). 10-Ethyl-8-hydroxy-2,4-dioxopyrimido[4,5-*b*]-quinoline (**4**), a compound having the basic heterocylic system of the natural cofactor (**1**), exhibits pH-dependent electronic absorption and NMR spectral shifts similar to those of **1**. The 8-hydroxy function in **4** is unusually acidic (pK_a = 6.1), indicating the stabilization of its anion (**4a**) by the extended conjugation as shown in **4a′** and **4a″**. On the other hand,

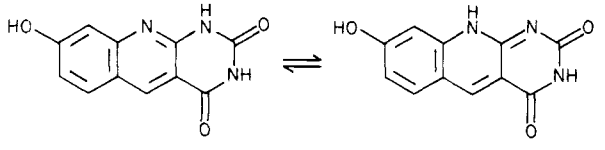


4a



4a′ 4a″

structure **4** is the predominant tautomeric state for the neutral molecule in solution, as indicated by the resemblance of its electronic absorption spectrum at pH 5 to that of the 8-methoxy derivative **5**. Among the several possible tautomeric forms for 8-hydroxy-2,4-dioxopyrimido[4,5-*b*]quinoline (**6**), the form **6′** deserves special attention because it is derived from



6 6′

the replacement of the 10-alkyl group in **4** with a hydrogen and is expected to show properties similar to those of **4**. However, both the pK_a value (Table III) of the 8-hydroxy function and the electronic absorption spectrum of **6** (Table II) indicate significant chemical differences between **6** and **4**. Furthermore, at neutral pH the spectrum of **6** resembles that of the 1,3-dimethyl derivative **13** (Table II), indicating that the predominant tautomeric state is **6** and that of the contribution of structure **6′** must be negligible.

Reduction of **4** by $NaBH_4$ or by $NaBH_3CN$ yielded a 1,5-dihydro derivative whose structure was indicated by the loss of the low-field proton signal at δ 7.31 (H_5 in **4**) in the NMR spectrum (Table II) and the appearance of a singlet at δ 3.45 attributable to the C-5 methylene protons. Reduction also resulted in the loss of absorption at 415 nm and the appearance of an absorption at 320 nm. Spencer et al. (1976) reported similar changes in spectral properties upon reduction of 5-deazariboflavin.

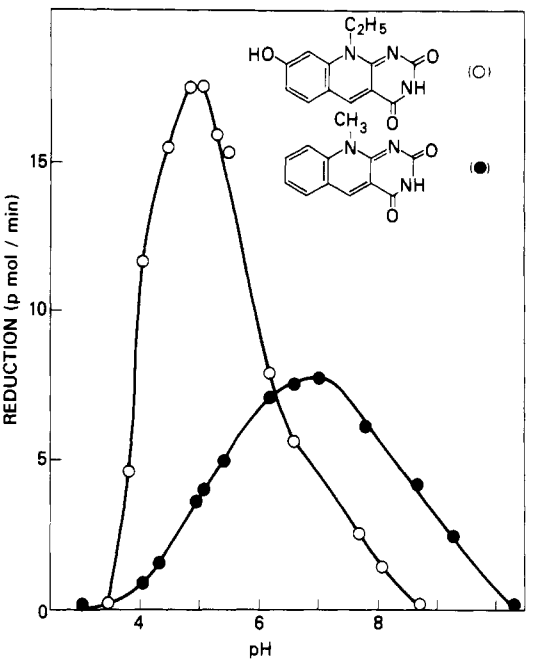


FIGURE 1: Effect of pH on the reduction of 8-hydroxy-10-ethyl-2,4-dioxopyrimido[4,5-*b*]quinoline (**4**, ○) and 10-methyl-2,4-dioxopyrimido[4,5-*b*]quinoline (**11**, ●). The rate of reduction of 5-deazaflavin (1.5 nmol) by NADPH (75 nmol) and purified 5-deazaflavin–NADP$^+$ reductase was measured by the fluorometric assay (Yamazaki & Tsai, 1980). The amount of enzyme used was 27 nanounits with analogue **11** and 13.5 nanounits with analogue **4**.

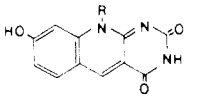
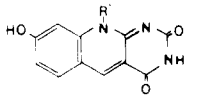
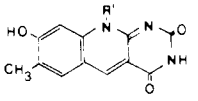
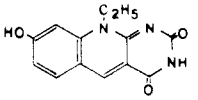
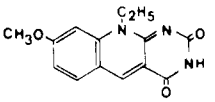
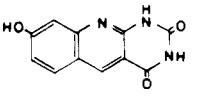
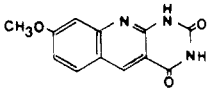
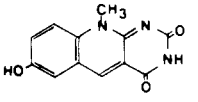
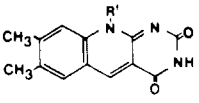
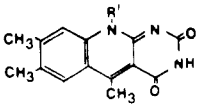
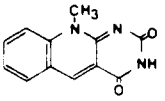
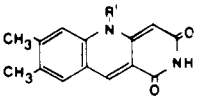
The pseudo-first-order rate constants of the chemical reduction of some of the deazaflavin analogues are given in Table III. The hydroxy compound **4** is reduced much slower by $NaBH_4$ at pH 12 than the methoxy compound **5**. This suggests that the anions are more resistant to reduction than the neutral molecules.

Enzymic Reduction of Analogues. The data of Table III show that although a variety of 5-deazaflavin compounds are reduced by the 5-deazaflavin–NADP$^+$ reductase and NADPH, the 5-deazaflavin ring system is the minimum requirement for activity of the enzyme. This view is supported by the observation that a related heterocyclic system, 1,5-dideazariboflavin (**12**), is inert as a substrate.

In order to correlate the effects of structural variations of the analogues with enzymic activity, we determined the K_m and k_{cat} values for reduction of these compounds by the enzyme (Table III). For the sake of comparison, these measurements were made at pH 7 although the optimal pH for the reduction of some of these compounds may be different. Since both the K_m and k_{cat} values vary over a wide range, the k_{cat}/K_m ratios serve as a more useful indicator of the relative reactivities of these analogues.

Effects of Modification of the Chromophore. The relative effectiveness of compounds **4**, **6**, and **11** as substrates of the enzyme are of particular interest. Compound **4** possesses the essentials of the chromophoric group of the natural cofactor **1**, except that the N-10 side chain of **1** is replaced by an ethyl group, and therefore the interaction of **4** with the enzyme may be expected to be similar to that of **1**. Although **4** is reduced at a much lower rate than **1** (Table III), its pH profile (Figure 1) follows the same pattern as that observed for the natural cofactor (Yamazaki & Tsai, 1980). In contrast, **11**, which represents the parent heterocyclic system of **1**, shows a broad pH dependence with an optimal pH at 7 (Figure 1). The

Table III: Reduction of 5-Deazaflavin Analogues[a]

no.	compd	pK_a	5-deazaflavin-NADP$^+$ reductase[b] K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min$^{-1}\cdot\mu$M^{-1})	k_{obsd}[e] (min^{-1}) $NaBH_4$[c]	$NaBH_3CN$[d]
1		6.3[f]	2.8[g]	442[g]	158	nd	nd
2		6.1[h]	3.1	174	56	0.002	0.20
3		6.1	3.2	10.8	3.4	0.001	0.23
4		6.1	4.2	53	12.6	0.001	0.27
5						0.051	0.08
6		7.2	35.9	1090	30.4	<0.001	0.12
7						nd	nd
8		8.2[i]	42.5	69	1.6	0.048	1.72
9			39.9	124	3.1	0.111	0.57
10						0.002	0.005
11			27.4	3990	146	0.354	1.45
12						nd	nd

[a] R, see eq 1; R′, ribityl; nd, not determined. [b] Kinetic values were determined at pH 7.0 and 20 °C. The k_{cat} values were estimated as the number of mol of substrates reduced per min per mol of enzyme. [c] The compound (30 nmol) in 0.1 N NaOH was reduced at 20 °C by the addition of 10 μL of 10% (w/v) $NaBH_4$. [d] The compound (30 nmol) in 1% acetic acid was reduced at 20 °C by the addition of 10% (w/v) $NaBH_3CN$. [e] The pseudo-first-order rate constant. [f] Eirich et al. (1978). [g] Yamazaki & Tsai (1980). [h] Eirich et al. (1979). [i] pK_a of 7-OH (Pol et al., 1980).

absence of an acidic 8-hydroxy function in **11** changes markedly the ionization state of the molecule, and this is reflected in the pH dependence of its enzymic reduction. From the effect of pH on enzymic activity with the natural cofactor, it was concluded earlier (Yamazaki & Tsai, 1980) that the reacting species is the neutral form of **1**. This interpretation can be applied equally well to the case of **4**, whose pK_a value is almost the same as that of **1**. On the other hand, since **11** is a neutral molecule in the pH range 3.5–10, the effect of pH on the rate of its reduction must be ascribed to the ionization state of the enzyme. The ability of the enzyme to reduce **6** at an appreciable rate is somewhat surprising because this compound, having no substituent at N-10, is a different heteroaromatic system than that of **1** or **4**. The site of reduction of **1** and related compounds has been shown to be the 1,5 conjugated system, N(1)=C(10a)—C(4a)=C(5). In contrast, the established structure of **6** necessitates the reduction to occur at the 5,10 conjugation, N(10)=C(10a)—C(4a)=C(5).[2] Since the common denominator of these two conjugated systems is position 5, the ability of the enzyme to reduce

[2] This argument is valid only if the established predominent tautomeric state of **6** in aqueous solution is the same as that in the presence of the enzyme.

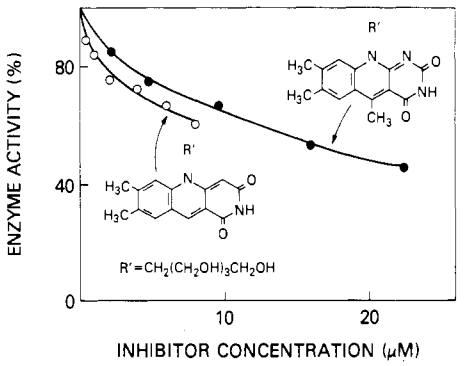


FIGURE 2: Inhibition of the reduction of 5-deazariboflavin (**9**) by 5-methyl-5-deazariboflavin (**10**, ●) and 1,5-dideazariboflavin (**12**, ○). Enzyme activity was measured by the spectrophotometric assay described under Experimental Procedures. The reaction mixture (1.0 mL) contained 0.1 M potassium phosphate, pH 7.0, 5.0 μM 5-deazariboflavin, 50 μM NADPH, purified 5-deazaflavin–$NADP^+$ reductase (1.4 microunits), and inhibitor as indicated.

both systems suggests the importance of C-5 as the site for electron entry.

From the above results it appears that the structural requirement of the chromophoric group is 2,4-dioxopyrimido-[4,5-*b*]quinoline; replacement of 5-CH by N, as in riboflavin, or N-1 by CH, as in 1,5-dideazariboflavin (**12**), renders the analogue inactive as a substrate for the enzyme.

Effects of Varying Ring Substituents. (*a*) *Position 8.* Although the natural cofactor, **1**, possesses a hydroxy function at C-8, the data in Table III show that the 8-hydroxy function is not an absolute requirement for enzymic activity. However, when the 8-hydroxy group is methylated (**5**), the resulting analogue is not reduced enzymically although it is reduced at about the same rate as **4** by $NaBH_3CN$. The effect of the methoxy group may be due to some steric restriction at this position with respect to its interaction with the enzyme. This was borne out by the fact that the addition of a 10-fold excess of **5** did not affect the rate of reduction of a half-saturating level of **4**. Similarly, addition of compound **7** did not inhibit the reduction of **6**. These results indicate that methylation of the 8-hydroxy group significantly decreases the affinity of the compound for the enzyme.

(*b*) *Position 5.* In view of the importance of position 5 as a center for the reduction, it was of particular interest to compare 5-deazariboflavin (**9**) and 5-methyl-5-deazariboflavin (**10**) as substrates. Both were reduced by either $NaBH_4$ or $NaBH_3CN$, but the rate of reduction of **10** was much slower than that of **9**. The enzyme, however, reduced only compound **9**. Compound **10** proved to be an inhibitor of the enzyme (Figure 2). The lack of enzymic activity for **10**, therefore, is not due to its lack of affinity for the enzyme but probably stems from the steric and inductive effect of the methyl group at the 5 position, which decreases the rate of reduction. Similar observations were reported by Walsh et al. (1978) on the basis of studies with NAD(P)H:flavin oxidoreductase from *Beneckea harveyi*.

(*c*) *Position 7.* Substitution at position 7 produces a profound effect on reactivity of the deazaflavins with the enzyme (Table III), as indicated by the large difference in the k_{cat} values between **11** (C-7, H) and **8** (C-7, OH) and also between **2** (C-7, H) and **3** (C-7, CH_3). However, the rates of reduction by $NaBH_3CN$ for these two pairs of compounds are comparable.

(*d*) *Inhibitors.* Two of the compounds examined in this study, 5-methyl-5-deazariboflavin (**10**) and 1,5-dideazariboflavin (**12**), were found to inhibit the enzymic reduction of 5-deazariboflavin (**9**) (Figure 2). In control experiments in the absence of enzyme, oxidation of dihydro-5-deazariboflavin by either **10** or **12** was not observed. As pointed out above the failure of the enzyme to reduce **10** may be due to steric effects of the methyl group at the 5 position. The case of **12** is particularly interesting because the only difference between **12** and **9** is the replacement of the N at position 1 by a CH group. This replacement should cause little change in the physical dimensions of the molecule, but it results in an entirely different heteroaromatic system and hence different chemical properties; for example, the E_0' of **12**, −0.370 V, is 59 mV lower than that of **9** (E_0' = −0.311 V; Walsh et al., 1978).

Oxidation of Reduced 5-Deazaflavins. In a few instances the rates of reoxidation of the reduced deazaflavin analogue by the enzyme in the presence of $NADP^+$ were studied. For example, the kinetic values for the oxidation of 1,5-dihydro-7,8-didemethyl-8-hydroxy-5-deazariboflavin (reduced **2**) by the enzyme were K_m = 11.4 μM and k_{cat} = 9870 min^{-1}. These values are similar to those obtained when the reduced natural cofactor, **1**, was used as substrate (K_m = 8.0 μM, k_{cat} = 10500 min^{-1}) (Yamazaki & Tsai, 1980). This further illustrates the relative unimportance of the lactoylglutamyl side chain of the natural deazaflavin as a determinant of cofactor activity for the $NADP^+$ reductase of *M. vannielii*.

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