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STRUCTURE AND PROPERTIES OF 5-DEAZAFLAVIN RADICALS AS COMPARED TO NATURAL FLAVOSEMIQUINONES

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Summary

In order to gain more insight into flavin radicals, on which the selection of $1e^-$- and $2e^-$-oxireduction modes in flavoproteins depends, we have investigated structure, spectral properties and decay mode of molecular species occurring in the half-reduced 5-deazaflavin 'model' system by flash photolysis and pulse radiolysis.

(1) Enforced $1e^-$-reduction of 5-deazaflavin yields the short-lived red-colored 1-HdF̊l, which is a strong reductant. In the absence of any electron acceptor, this radical decays by 1,5-prototropy (see below) and dismutation, which is rapidly reversed upon illumination. Competing with this photo-comproportionation, irreversible formation of the photo-stable σ-dimer $(HdF̊l)_2$, covalently linked via C(5), is observed, which becomes prevalent under prolonged illumination.

(2) Enforced $1e^-$-abstraction from 1,5-dihydro-5-deazaflavin yields the tautomeric 5-HdF̊l, which is a mild oxidant and is transparent at $\lambda > 480$ nm. Prototropy 5-HdF̊l $\rightleftarrows$ 1-HdF̊l can be rate-determining in 5-deazaflavin redox reactions. Hence, the radical state in the 5-deazaflavin system does not mediate double $1e^-$-oxidoreduction as do natural flavosemiquinones. Instead, 5-deazaflavin favors nucleophilic substrate addition (carbanion transfer) and formation

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of intermediate σ-adducts in (photo)reductions even over the extent observed with natural flavin. This confirms the description of 5-deazaflavin as a 'flavin-shaped nicotinamide derivative'. It explains at the same time the mechanism of 5-deazaflavin acting as a mild and yet potent photosensitizer in $1e^-$-reductions of biological redox systems.

(3) It is shown that replacement of N(5) by CH in the flavin nucleus also leads to the disappearance of the known action-pK in the photoreduction, which confirms the assignment of the latter pK in the natural flavin system to 5-protonation of the excited flavin triplet.

From these model studies the following biological conclusions can be confirmed: The tautomer equilibrium of natural flavin semiquinones is diffusion-controlled and regulated thermodynamically: 5-HF̈l ⇄ 1-HF̈l, while in flavoproteins the same equilibrium is regulated by regiospecific H-bridges from the apoprotein, which thus decides between $1e^-$- (stable 5-HF̈l) and $2e^-$-reaction (unstable 1-HF̈l) modes.

Introduction

5-Deazaflavin (dFl_{ox}) [1] is a vitamin B-2 derivative bearing a CH group in place of the central (pyridine type) nitrogen atom. Its 'bioorganic' relevance resides upon two interdependent facts.

(1) The 5-deaza-modification deletes two of the three main activities inherent in the flavin chromophore, namely electron transfer and dioxygen activation, while it retains the third, namely transhydrogenation, at least to a limited degree. In a recent review, Hemmerich et al. [1] explained this behavior by the proposal that 5-deazaflavin is a 'flavin-shaped nicotinamide', i.e. it is a functional nicotinamide derivative fitting by its steric shape into the active site of many flavoproteins. There it does not act, mechanistically, as a true flavin analog, but instead if at all, as a specific hydride transfer catalyst. By this, new perspectives in the understanding of flavin dependent biocatalysis have been opened [2].

(2) From this it follows that the 5-deaza modification removes the thermodynamic stability of the natural flavin radical [3]. Thus, under continuous illumination, the final product of dFl_{ox}-reduction is the stable, covalent dimer 1-HdFl-5,5′-dFlH-1′, in short, $(HdFl)_2$ [4]. In contrast to the case of the analogous nicotinamide radical dimer [5], the deazaflavin radical dimer formation is easily reversible in blue light under sensitization by dFl_{ox}. The steady-state concentration of deazaflavin radical monomers thus obtained represents a mild and yet potent $1e^-$-reductant [6,7], which lends itself excellently for the controlled reduction of redox enzymes.

For these two reasons it seemed desirable to investigate the structure and properties of radicals occurring in the half-reduced 5-deazaflavin system more thoroughly, and to compare the results with the known data on the natural flavin. Thus, in the present study we report on the structure and kinetics of half-reduced (monohydro) 5-deazaflavin species as revealed by parallel studies of pulse radiolysis and flash photolysis.

Materials and Methods

1. Apparatus

(a) Pulse radiolysis. The pulse radiolysis experiments were carried out using the linear accelerator of the Hebrew University of Jerusalem and the apparatus described earlier [8]. The electron pulse source was a Varian linear accelerator operated at 5 MeV, 200 mA, and pulse length of 0.1—1.0 μs, giving a dose range of 150—2000 rads per pulse. The electron pulse intensity varied within ±5%. The inductive current obtained when the electron beam passed through a coil was used to monitor the pulse intensity.

The formation and decay of the formed transients were followed spectrophotometrically in a multiple reflection silica cell (1 × 2 × 4 cm) (three passes). The electron beam was perpendicular to the light beam and was absorbed by a 1-cm thick aqueous medium. The method employed for filling the cell was based on that developed by Christensen et al. [9]. A 150 W water-cooled Xenon lamp was used as the analyzing light source. A Bausch and Lomb high intensity monochromator ($\Delta\lambda = \pm 2$ nm in the range of 250—400 nm, and ±6 nm at 400—800 nm), followed by an IP28A photomultiplier and a type 556 Tektronix double-beam oscilloscope fitted with a Polaroid camera were used for recording the transmittance changes. Light filters were used in order to avoid second-order diffraction light signals through the monochromator as well as to reduce photochemical effects. A shutter, mechanically operated by air pressure, was introduced between the irradiation cell and the light source to protect the photomultipliers and the examined solutions from excess illumination.

Deazaflavins were reduced by reaction with either hydrated electrons or CO_2^- radical ions. In the former case the solutions were freed from O_2 by extensive bubbling with Ar and 0.1 M *tert*-butanol was added to scavenge the OH radicals, whereas in the latter case the solutions were saturated with N_2O and contained 0.1 M sodium formate ($N_2O + e_{aq}^- + H^+ \rightarrow N_2 + OH$; $OH + HCOO^- \rightarrow H_2O + CO_2^-$). Dosimetry was carried out by measuring the extent of thiocyanate oxidation [10]. The initial concentration $[e_{aq}^-]_0$ used was 2.3 μM, $[CO_2^-]_0$ 5.3 or 7.6 μM. The concentrations of dFl_{ox} varied between $1 \cdot 10^{-5}$ and $1 \cdot 10^{-4}$ M. The pH was kept constant by $1 \cdot 10^{-4}$ to $1 \cdot 10^{-2}$ M potassium phosphate buffer in the weak alkaline range (pH 7—10).

(b) Flash photolysis. The flash apparatus used was that described by Vogelmann et al. [11]. The input energy of the flash was 540 J. The length of the flash profile at half of its amplitude (fwhm)* was about 6 μs. The cuvette (10 cm) was surrounded by a Kodak Wratten gelatine filter No. 1A (transmittance under 2% for wavelengths less than 380 nm) to limit the irradiation within the first absorption band of 5-deazaflavin and to avoid excitation of substrates. For the registration of the transients a cut-off filter (transparent for $\lambda > 490$ nm) or an interference filter monochromator (Oriel Optics Corp.) was mounted in the monitoring light beam in order to prevent further photochemistry.

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* fwhm, full width half-maximum.

A Zeiss DMR 10 spectrophotometer was used to record the ultraviolet-visual spectra before and after flashing.

2. Materials

Flavins and 5-deazaflavins are, by definition, alkylated at position 10 [12]. The natural residue is ribitol; for our model studies a simple methyl group is optimal in position 10. In principle, all experiments described in this paper can also be conducted with 5-deaza-vitamin B_2 = 5-deazariboflavin [13], though preparative isolation of products becomes more tedious and some photolytic damage of the polyhydroxylic side-chain in competition with reduction by photosubstrate may always occur [12].

Furthermore, in the present model studies we work for convenience with (deaza)flavins, which are methyl-substituted at position 3. Replacement of the natural proton at position 3 by methyl increases the solubility in both, aqueous as well as polar aprotic solvents. The spectral properties remain unchanged by 3-substitution and the same is true for all chemical reactions except aqueous alkaline hydrolysis, which is prevented by the natural deprotonation occurring at approx. pH 10. 3-Methyl-(deaza)flavins should thus not be handled at pH $\geqslant$ 10 [12].

Apart from the mentioned substitutions at positions 10 and 3, our experiments are conducted with two sets of deazaflavin derivatives, dFl_{ox}. (1) The 7,8-dimethylated 5-deazaflavins [4] (lumideazaflavins, dFl_{ox}). These represent the natural flavin chromophore (lumiflavin), but may give rise to side-reactions at the notoriously activated methyl group in position 8 [12]. This type of dFl_{ox} shows λ_{max} = 397 nm and ϵ_{max} = 14 300 $M^{-1} \cdot cm^{-1}$. (2) The 7,8-unsubstituted 5-deazaflavins (7,8-bis,nor-dFl). This type of dFl_{ox} exhibits a chromophore which is slightly shifted towards the blue, owing to the lack of the hyperconjugated methyl group in position 8, namely λ_{max} = 387 nm and ϵ_{max} = 12 000 $M^{-1} \cdot cm^{-1}$ [4].

For photochemical purposes a millimolar stock solution of these dFl_{ox}-compounds in acetonitrile is diluted to the desired volume in the desired solvent or buffer. For radiolytic purposes 10^{-2} M stock solution of 7,8-unsubstituted dFl_{ox} [4] in 0.1 NaOH was diluted to the desired pH with aqueous NaH_2PO_4.

3. Photosubstrates

(a) 1,4-Diazabicyclo[2,2,2]octane (DABCO). The substrate was reagent grade from Merck and purified by sublimation. For the evaluation of data obtained with this $1e^-$-transfer substrate, correction for the absorbance of the oxidized DABCO radical cation had to be made.

The spectrum of DABCO radical cation was obtained from [14], the extinction coefficient from [15]; ϵ = 2.100 $M^{-1} \cdot cm^{-1}$ at λ_{max} = 465 nm. In both references the radical cation was generated by OCl^- oxidation of DABCO in aqueous solution. More recently, the radical cation was also produced by irradiation of DABCO in frozen glassy freon solution [16]. The slightly different spectroscopic data thus obtained (λ_{max} = 470 nm with ϵ = 1.550 $M^{-1} \cdot cm^{-1}$) may be due to different solvent effects.

(b) Oxalate. In contrast to $DABCO^{\cdot+}$, the photo-products of oxalate are colorless, but oxalate is ambiguous in as far as it may react in competitive $1e^-$

and $2e^-$ pathways. Dipotassium oxalate, purity greater than 99.5%, from Merck, Darmstadt, F.R.G. was used.

(c) The reduced 5-deazaflavins H_2dFl_{red} and $(HdFl)_2$. These were used as obtained in Ref. 4, at pH $\geqslant$ 7. In the reduced forms H_2dFl_{red} as well as $(HdFl)_2$, the compounds are well soluble in water at pH $\geqslant$ 7, owing to deprotonation of the acidic function N(1)H. From these solutions, residual dFl_{ox} can be removed by extraction with $CHCl_3$. The reduced compounds can be kept under anaerobic conditions for unlimited time and aerobic solution for about 10 min, but any dFl_{ox} formed during a longer aerobic period can be reextracted with $CHCl_3$ as mentioned. Preparative isolation of the reduced compound is reported in Ref. 4. When it is required to work with reduced (deaza)flavins at pH $<$ p$K \approx$ 6, the insoluble, neutral H_2dFl_{red} or $(HdFl)_2$ has to be replaced by the water-soluble sulfonate dFl_{ox}-$(CH_2)_3$-SO_3^- [5].

3-Methyl-7,8-bis,nor-5-deaza-lumiflavin and 7,8-bis,nor-5-deaza-lumiflavin were synthesized according to Yoneda et al. [17]. Deaza-lumiflavins were obtained as reported by O'Brien et al. [18] and 5-deaza-riboflavin was synthesized according to Janda and Hemmerich [13].

3-Methyl-5-deaza-lumiflavin. For the synthesis of this compound the procedure of Yoneda et al. [17] was modified as follows, providing a 90% yield of pure product without further treatment: 2.6 g (10 mM) 3-methyl-6-(*N*-methyl-3,4-xylidino)uracil were dissolved in 20 ml dry dimethyl formamide and treated with 1 ml $POCl_3$ (11 mM). After 1 h at room temperature, the solution was heated for 15 min at 100°C, then poured on to 100 g ice and neutralized with ammonia. The precipitated product was filtered, thoroughly washed with hot water and dried at 100°C in vacuum.

Anaerobiosis was achieved by passing a stream of solvent saturated nitrogen or argon ($[O_2] < 0.1$ ppm) for 30 min through the solution.

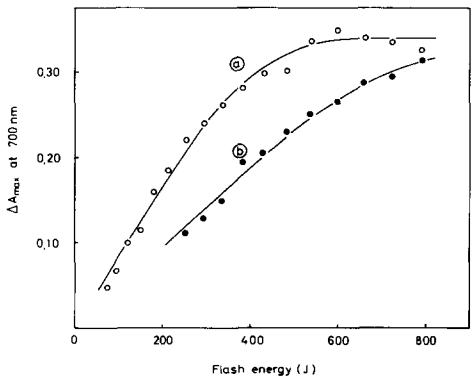
4. Calibration and correction methods used in flash photolysis

For the transient spectra, the absorbance at $t = 20$ μs was measured in 5 nm intervals at least three times. The range of noise was $\Delta A = 0.02$.

For the determination of the molar absorption coefficient of the excited 3-methyl-5-deaza-lumiflavin triplet, we tried to populate the triplet state totally in the absence of substrate by increasing the input energy of the flash. Without surrounding the cuvette by a Kodak Wratten gelatine filter No. 1A, as mentioned above, we reached at 600 J a plateau of energy-independent absorbance, indicative of the total population of the triplet state, cf. Fig. 1, curve a.

Therefore the molar absorption coefficient is calculated to be ϵ-$^3dFl^*_{ox}$ = 3.600 (±200) $M^{-1} \cdot cm^{-1}$ at 700 nm, according to $\epsilon = \Delta A(C \times d)$. With this value we determined a triplet population of 74 (±5) % with the cuvette surrounded by the Kodak Wratten filter No. 1A at the flash input energy of 540 J normally used, cf. Fig. 1, curve b. From the transient spectrum, generated by flashing $7 \cdot 10^{-6}$ M 3-methyl-5-deaza-lumiflavin in borate buffer ($5 \cdot 10^{-3}$ M), pH 9.2, the absorption spectrum of the 5-deazaflavin-excited triplet was now evaluated, Fig. 2, curve b.

Molar absorption coefficient. The molar absorption coefficient of the 5-deaza-flavosemiquinone was determined in the following way. The 5-deaza-



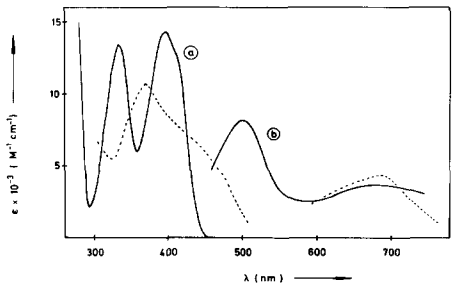


Fig. 1. Dependence of the triplet-triplet absorbance of 3-methyl-5-deaza-lumiflavin, monitored at the time of its maximum, t = 10 μs, on the flash input energy. Wavelength: 700 nm. [3-methyl-5-deaza-lumiflavin] = $9.4 \cdot 10^{-6}$ M; d = 10 cm; borate buffer, pH 9. (a) without cuvette filter; (b) with cuvette surrounded by a Kodak Wratten gelatine filter No. 1A. In case a at 600 J, a plateau of energy-independent absorbance is reached, indicative of the total population of the triplet state. From this, the extinction coefficient of the triplet state ($\epsilon_{700\ nm} = 3600\ M^{-1} \cdot cm^{-1}$) and the triplet state population in case b is evaluated.

Fig. 2. Absorption spectra of (——) 3-methyl-5-deaza-lumiflavin in water: (a) singlet-singlet absorbance, borate buffer, pH 8; (b) triplet-triplet absorbance, borate buffer, pH 9.2. In comparison: — — —, triplet-triplet absorption spectrum of lumiflavin in water, phosphate buffer, pH 7 [30].

flavin was flashed in borate buffer, pH 9, in the presence of the well-known $1e^-$-donor *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPDA) yielding 5-deaza-flavosemiquinone and Wurster's blue. (The TMPDA (98% EGA-Chemie, Steinheim, F.R.G.) was purified by sublimation prior to use and stored under argon (oxidation potential: 0.015 V vs. saturated calomel electrode, in acetonitrile [19]).) In the experimental time range of 200 μs we observed a second-order reaction for the decay of the absorbance, independent of the wavelength monitored, arising from the back donation of the electron according to the dark reaction of Eqn. 1:

$$dFl_{ox} + TMPDA \underset{-H^+}{\overset{h\nu,\ +H^+}{\rightleftharpoons}} Hd\dot{F}l + TMPDA^{\cdot+} \qquad (1)$$

At the wavelengths 610 nm and 520 nm it must be valid for the slope of a $1/(C \times \epsilon \times d)$ vs. time diagram:

$$k/((\epsilon^{Hd\dot{F}l}_{610\ nm} + \epsilon^{TMPDA^{\cdot+}}_{610\ nm}) \times d) = \text{slope at 610 nm}$$

$$k/((\epsilon^{Hd\dot{F}l}_{520\ nm} + \epsilon^{TMPDA^{\cdot+}}_{520\ nm}) \times d) = \text{slope at 520 nm}$$

From this the molar absorption coefficient of $Hd\dot{F}l$ at 520 is obtained:

$$\epsilon^{Hd\dot{F}l}_{520\ nm} = \frac{\text{slope 610 nm}}{\text{slope 520 nm}} (\epsilon^{Hd\dot{F}l}_{610\ nm} + \epsilon^{TMPDA^{\cdot+}}_{610\ nm}) - \epsilon^{TMPDA^{\cdot+}}_{520\ nm}$$

The calculation was made with $\epsilon_{610\ nm}^{TMPDA^{\cdot+}} = 13.000\ M^{-1} \cdot cm^{-1}$, recently published by Nickel et al. [20] and $\epsilon_{520\ nm}^{TMPDA^{\cdot+}} = 7.500\ M^{-1} \cdot cm^{-1}$. For $\epsilon_{610\ nm}^{HdF\dot{l}}$ we assumed that it will be negligible with respect to the absorbance of Wurster's blue at 610 nm. This is supported by the transient spectrum of the photoreaction of dFl_{ox} and DABCO, which shows a strong decrease of absorbance, beginning at 520 nm, to longer wavelengths (see below). Thus, we determined $\epsilon_{520\ nm}^{HdF\dot{l}} = 3.500\ M^{-1} \cdot cm^{-1}$ for the 3-methyl-5-deaza-lumiflavin semiquinone.

Using the 3-methyl-7,8-bis,nor-5-deaza-lumiflavin, we obtained the same molar absorption coefficient of $\epsilon_{520\ nm}^{HdF\dot{l}} = 3.500\ M^{-1} \cdot cm^{-1}$, which agrees well with the coefficient, as determined by pulse radiolysis: $\epsilon_{520\ nm}^{HdF\dot{l}} = 3.300\ M^{-1} \cdot cm^{-1}$ (see Fig. 4).

The 5-deaza-flavosemiquinone yield per flash was determined by extrapolation of the measured second-order decay reaction of the radical to the time 9 μs after flash initiation. For this we had the following reasons. Since our flash lifetime is not negligibly short with respect to the radical decay time, the end of radical production and the beginning of decay, respectively, will not be limited to a precise point of time. Therefore, we determined the time which yields the smallest error, namely 9 μs, as follows.

Eqn. 2, which was derived from the integrated rate law of a second-order reaction by multiplication with the excited triplet concentration ($[^3dFl^*_{ox}]_0$), shows that the apparent radical yield ($[HdF\dot{l}]/[^3dFl^*_{ox}]_0$), observed at a given time, depends on the initial excited triplet concentration and the time:

$$\frac{[HdF\dot{l}]}{[^3dFl^*_{ox}]_0} = 1/([^3dFl^*_{ox}]_0 \times k \times t + \frac{[^3dFl^*_{ox}]_0}{[HdF\dot{l}]_0}) \qquad (2)$$

According to this equation, the radical yield is independent of the initial excited triplet concentration at the starting point of decay, $t = 0$, and for this time the true radical yield is obtained.

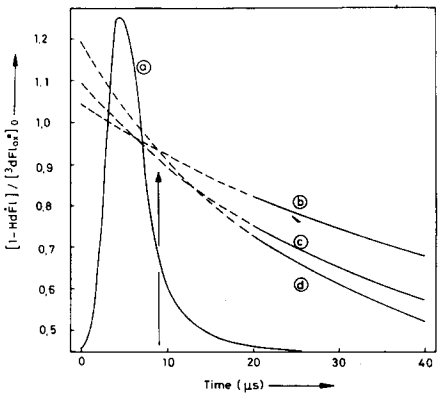
Applying this to our conditions, the most exact point of starting time will be characterized by a minimum of the differences of radical yields, obtained by reactions, which involve different concentrations of excited triplets.

In Fig. 3 the dependence of apparent radical yield upon time and flash input energy, which latter corresponds to the absolute triplet concentration, is shown under our conditions. At $t = 9\ \mu s$ the dependence of radical yields on initial triplet concentration is smallest.

Results and Discussion

(1) Pulse radiolysis

The reaction between dFl_{ox} and hydrated electron (e^-_{aq}) or $\dot{C}O_2^-$-radical was found to generate a 5-deazaflavin radical, the absorption spectrum of which (Fig. 4) is essentially independent of the reductant used at least at $\lambda > 450$ nm ($\lambda_{max} = 515$ nm, $\epsilon = 3.300\ M^{-1} \cdot s^{-1}$ at pH 7.2). An isosbestic point between the absorption of the radical and the oxidized 5-deazaflavin is observed at 425 nm ($\epsilon = 4.000$) at pH 7.2 (7,8-bis,nor-dFl). The specific rates of the radical formation monitored at 515 or 560 nm are $k_1 = 1.7 \cdot 10^{10}$ and $2.06 \cdot 10^9$



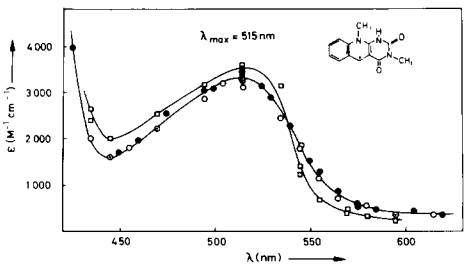
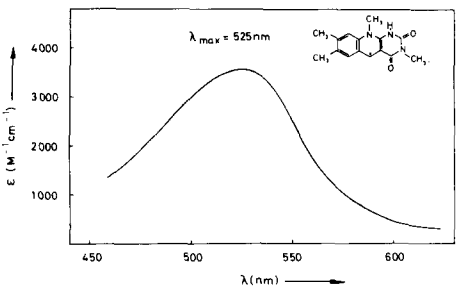


Fig. 3. Dependence of the apparent 5-deaza-flavosemiquinone yield upon time and flash input energy. The photoreaction 3-methyl-5-deaza-lumiflavin ($9.4 \cdot 10^{-6}$ M) and DABCO ($5 \cdot 10^{-3}$ M) in borate buffer, pH 9, was used. (a) flash profile (arbitrary units), (b) 294 J, (c) 434 J, (d) 542 J flash input energy. The solid curves represent the beginning of semiquinone decay, observed experimentally. The dashed curves are extrapolated from the second-order decay of the semiquinone. At 9 μs (arrow) the differences of semiquinone yields in dependence of the flash input energy (i.e., initial triplet concentration) are smallest. Therefore, according to Eqn. 2, the apparent yield of this time is the best approximation to the true yield.

Fig. 4. Absorption spectrum of the neutral 1-H tautomeric 3-methyl-7,8-bis,nor-5-deaza-lumiflavin semiquinone in aqueous buffer solution, obtained by pulse radiolysis, ○, pH 9.2; ●, pH 7.2; □, pH 5.4; ⊙, ○ + ●.

$M^{-1} \cdot s^{-1}$ (pH 7.2, cf. Table I) for the reactions with e^-_{aq} and $\dot{C}O_2^-$, respectively. The difference in the formation rate is clearly due to the much higher reactivity of the hydrated electron relative to that of the carbon dioxide radical. In the rapid reaction (with e^-_{aq}) the formation of the 5-deazaflavin radical is found to be concomitant with the decay of the hydrated electron. For the reaction between dFl_{ox} and $\dot{C}O_2^-$ the pH-dependence has been measured at $5 < pH < 10$. While there was essentially no change at $pH > 6$, we found a slight deviation, especially significant in the region around 550 nm, at pH 5.4, which at the same time is the lowest pH range permitting that the concomitant formation of the hydrogen radicals in the radiolysis be excluded. We attribute this slight alteration, as discussed below, to the protonation of the primary formed radical carboxylate $d\dot{F}l\text{-}1\text{-}COO^-$. Table I documents the pH-constancy of these rates.

When the reaction between dFl_{ox} and e^-_{aq} or $\dot{C}O_2^-$ was monitored at λ_{max} (7,8-bis,nor-dFl_{ox}) = 387 nm, a decrease in the initial absorption (arising from dFl_{ox}) was observed (Fig. 6). This decrease is composed of two distinct steps: The fast phase corresponds to the formation of the radical, which shows at this wavelength the lower extinction coefficient ($8.200\ M^{-1} \cdot cm^{-1}$, pH 7.2) compared to that of dFl_{ox} ($11.900\ M^{-1} \cdot cm^{-1}$). The slower second phase corresponds to the decay of the radical. This decay was measured both at 515 nm, where only the radical absorbs, and at 387 nm. At both wavelengths the decay kinetics were clearly of second order. Surprisingly, the decay rate was found to depend on the nature of the reductant, by which the radical was formed. When the radical was reduced by the reaction of $\dot{C}O_2^-$ the specific decay rate at pH 7.2 is $2k_2 = 9.2 \cdot 10^8\ M^{-1} \cdot s^{-1}$, while the decay of the e^-_{aq}-produced radical was nearly 10-times faster, namely $2k_2 = 1.75 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$. Thus,



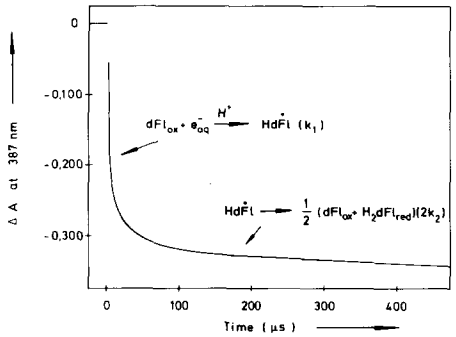


Fig. 5. Absorption spectrum of the neutral 1-H tautomeric 3-methyl-5-deaza-lumiflavin semiquinone in borate buffer, pH 9, obtained by flash photolysis. The semiquinone was generated by the photoreaction of 3-methyl-5-deaza-lumiflavin ($1 \cdot 10^{-5}$ M) with DABCO ($1 \cdot 10^{-2}$ M). The absorbance of the DABCO radical cation was eliminated and the extinction coefficient of the semiquinone was determined.

Fig. 6. Biphasic decrease of the absorbance at 387 nm in the radiolytic reduction of 3-methyl-7,8-bis,nor-5-deaza-lumiflavin. Condition: $[dFl_{ox}] = 1 \cdot 10^{-5}$ M, $[e^-_{aq}] = 2.2 \cdot 10^{-6}$ M, [t-BuOH] = 0.1 M, [phosphate buffer] = $1 \cdot 10^{-4}$ M, pH 7.2. The fast absorption decrease is concomitant with radical formation, the slow one is concomitant with radical decay, as observed at 515 nm.

TABLE I

KINETIC DATA FROM RADIOLYTIC REDUCTION OF 5-DEAZAFLAVIN

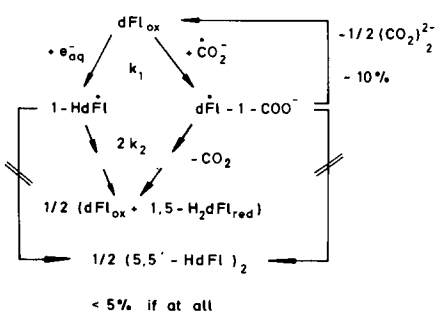
Reaction	pH[a]	Medium	Rate constant ($M^{-1} \cdot s^{-1}$)	% Bleaching
$dFl_{ox} + e^-_{aq}$	7.2	0.1 M *t*-butanol	$k_1 = (1.7 \pm 0.3) \cdot 10^{10}$ (2)[b]	51 ± 5[c] (8)[b]
$HdFl + HdFl$	7.2	0.1 M *t*-butanol	$2k_2 = (1.75 \pm 0.7) \cdot 10^{10}$ (6)	
$dFl_{ox} + \dot{C}O_2^-$	5.4	0.1 M HCOONa,	$k_1 = (2.05 \pm 0.03) \cdot 10^9$ (6)	
	7.0	N_2O satd.	$(1.96 \pm 0.1) \cdot 10^9$ (5)	38 ± 4[d] (3)
	7.2		$(2.06 \pm 0.06) \cdot 10^9$ (7)	
	9.2		$(1.92 \pm 0.03) \cdot 10^9$ (8)	
		overall average	$(2.00 \pm 0.07) \cdot 10^9$	
$dFl\text{-}1\text{-}COO^- + dFl\text{-}1\text{-}COO^-$	5.4	0.1 M HCOONa,	$2k_2 = (1.00 \pm 0.05) \cdot 10^9$ (3)	
	7.4	N_2O satd.	$(9.2 \pm 0.7) \cdot 10^8$ (2)	
	9.2		$(9.0 \pm 0.5) \cdot 10^8$ (4)	
		overall average	$(9.4 \pm 0.8) \cdot 10^8$	
$Fl_{ox} + e^-_{aq}$ [21] (lumiflavin-3-acetate)	4 to 8	0.1 M *t*-butanol	$k_1 = 3 \cdot 10^{10}$	
$HFl + HFl$ [22]	7	0.1 M *t*-butanol	$2k_2 = 2 \cdot 10^8$	

[a] 10^{-4}—10^{-2} M formate (pH = 5), phosphate (pH = 7) and borate (pH = 9) buffers.
[b] number of determinations.
[c] calculated from ϵ_{425} of dFl_{ox} (isosbestic with HdFl) (3966 M^{-1} cm^{-1}) and $[\dot{C}O_2^-]$ (see Methods).
[d] calculated from ϵ_{387} (11.900 M^{-1} cm^{-1}) and $[e^-_{aq}]_0$ (see Methods).

formation and decay of the radical are similarly dependent on the nature of the primary reductant. This suggests that the two reductants produced two spectrally identical, but structurally somewhat different, radical species (see below), namely 1-HdF̈l and dF̈l-1-COO$^-$. The unusually high decay rate of the radical formed with e^-_{aq} is noteworthy. It is probably related to the strong reducing power of the radical ($E^{\circ\prime}$ = −710 mV) [5], and it also indicates that the bimolecular decay reaction involves neutral, and not anionic radicals.

The second-order decay kinetics is consistent with the reaction being either a dismutation or dimerization. To resolve between these two possibilities, a detailed analysis of the overall decrease in dFl_{ox} absorption was carried out. In the case of dimerization, which involves 100% bleaching of dFl_{ox} at $\lambda > 350$ nm, the decrease should correspond to $\epsilon(dFl_{ox}) \times$ [Red], where [Red] refers to the effective initial concentration of e^-_{aq} or $\dot{C}O_2^-$, respectively. In the case of dismutation, however, the overall decrease should be half of that, since reconversion of dFl_{ox} is 50%. In the reaction with e^-_{aq} we measured 51 ± 5% bleaching at 387 nm, pH 7.2, in agreement with at least 90% dismutation. Even less bleaching than required for dismutation is observed in the reaction with $\dot{C}O_2^-$, namely 38 ± 4% at 425 nm. This suggests that not all radical species formed in this reaction undergo dismutation, but that there is some decay via a pathway where both radicals engaged in the bimolecular reaction give off one electron (compare below). Still, the predominant process is dismutation. This is also evident from the kinetic analysis of the second-order process monitored at 387 nm (dFl_{ox} regeneration), and 515 nm (HdF̈l decay), which give consistent rate constants only when dismutation, i.e. 50% final bleaching, is assumed. As will be discussed at length in comparison with the photochemical data in the 'Conclusions' section, we are enforced to assume, tentatively, a side reaction in the decay of the carboxylated radical by which dFl_{ox} is restored quantitatively together with formation of CO_2^- dimer, i.e. oxalate. It appears acceptable that such a reaction can compete with 1-decarboxylation or 1,5-migration of the carboxylate residue, which is required for dismutation.

Summarizing, reaction Scheme I is derived for pulse radiolysis of 5-deazaflavin:



Scheme I. Reaction pathway of the radiolytic reduction of 5-deazaflavin.

(2) Photochemical studies

General remarks. We have illuminated 10^{-4}—10^{-6} M aqueous solutions of 5-deazaflavin (dFl_{ox}) by conventional flash of 6 μs fwhm, as well as with continuous illumination. In the course of these experiments, the following parameters were monitored and compared:

(1) Formation and decay of 5-deazaflavin excited triplet ($^3dFl^*_{ox}$) at λ = 700 nm, cf. Fig. 2;

(2) Formation and decay of 5-deazaflavin radical ($Hd\dot{F}l$) at λ = 520 nm, cf. Fig. 5;

(3) The 'permanent bleaching' ($t > 1$ min) at λ = 397 nm, cf. Fig. 2.

At the same time we checked the range of $420 < \lambda < 460$ nm at $t > 20$ μs, i.e. after complete quenching of the triplet by the substrate, for an isosbestic point between starting dFl_{ox} and radical $Hd\dot{F}l$. Such a point would be indicated by a constant absorbance in the time interval $20\ \mu s < t < \infty$.

In the absence of triplet quenching substrates, we find under our standard conditions (see Materials and Methods) per flash 74% conversion of dFl_{ox} to $^3dFl^*_{ox}$, which in turn undergoes physical deactivation to the ground state. This is in marked contrast with natural flavin triplet ($^3Fl^*_{ox}$), which is deactivated up to 20% by electron transfer and back transfer via the radicals $\dot{F}l^-$ and $\dot{F}l^+$ [23,24]. With 5-deazaflavin blank, we find a negligible radical formation, around 4%, and a photodestruction as low as 2% per $^3dFl^*_{ox}$ per flash, as shown in Table II.

When we introduce triplet quenching substrates into the 5-deazaflavin photosystem, we must differentiate according to their tendency to donate either $1e^-$ (e.g., EDTA [25]) or $2e^-$-equivalents (e.g., BH_4^- [23,24]) or both. This applies in particular to organic photoreductants, RH [23,24], where in principle $1e^-$ (Eqn. 3) and $2e^-$ processes (Eqn. 4) may compete with each other. Furthermore, even if $1e^-$ donation is established by detection of 5-deazaflavin radical, distinction must be made depending on the fate of the substrate radical formed, which may yield back donation (Eqn. 3a), dismutation (Eqn. 3b) or double $1e^-$-transfer (Eqn. 3c):

$$dFl^*_{ox} + RH \xrightarrow{1e^-} Hd\dot{F}l + \dot{R} \rightarrow \begin{cases} dFl_{ox} + RH & (3a) \\ 1/2\ (dFl_{ox} + H_2dFl_{red}) + 1/2\ R_2 & (3b) \\ HdFl^-_{red} + R^+ & (3c) \end{cases}$$

$$(dFl^*_{ox}, RH)^* \xrightarrow{2e^-} HdFl_{red}R \qquad (4)$$

Furthermore, it must be noted that the pathways of 'double $1e^-$-transfer' (Eqn. 3c) and 'direct $2e^-$-transfer' (Eqn. 4) cannot always be distinguished by their final products, but only by their intermediates. The latter reaction is of non-radical nature, involving a substrate σ-addition within a flavin-substrate-

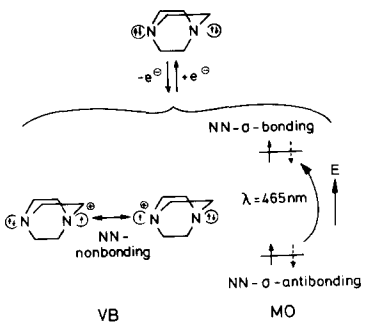
TABLE II

KINETIC DATA FROM FLASH PHOTOLYSIS OF 5-DEAZAFLAVIN

[3-Methyl-5-deaza-lumiflavin] = (1—2) · 10^{-5} M in borate buffer, pH 9.

Substrate	Concentration (M)	Quenching of		Yield of dFl-semiquinone[a,b] (%)	Final bleaching at 397 nm[a] (%)	Isosbestic point in the range 420—460 nm[c]	Bleaching at the isosbestic point[a] (%)	Decay rate of dFl-semiquinone[b] ($M^{-1} \cdot s^{-1}$) ($k \times 10^{-9}$)
		dFl_{ox}-singlet (%)	dFl_{ox}-triplet (%)					
None	—	—	—	4 (±2)	2 (±1)	—	—	—
DABCO	$5 \cdot 10^{-3}$	7	99.7	69 (±4)	0 (±1)	435 nm	0 (±1)	4.2 (±0.3)[d]
Oxalate	0.5	8	>98	90 (±4)	46 (±5)	432 nm	46 (±5)	1.0 (±0.2)[e]
H_2dFl_{red}	$1 \cdot 10^{-4}$ —$3 \cdot 10^{-4}$	<2[f]	>98	78 (±8)	25 (±4)	none	—	1.1 (±0.2)
$(HdFl)_2$	$6 \cdot 10^{-5}$	<1[f]	>97	85 (±5)	increase of absorbance: 10 (±3)%	none	—	1.4 (±0.2)

[a] the values apply for one flash and are related to the initial oxidized 5-deazaflavin concentration.
[b] measured at 520 nm and calculated with $\epsilon = 3500\ M^{-1} \cdot cm^{-1}$, see Materials and Methods.
[c] at this wavelength, there is no change of absorbance after the flash decay.
[d] correction for DABCO radical absorbance was made, see Materials and Methods.
[e] $k_{dism.} = 1/2k_{obs.}$
[f] the value was calculated using a diffusion controlled reaction rate constant and actual singlet lifetime of 4 ns (Wössner, G., Traber, R. and Kramer, H.E.A., unpublished result).



Scheme II. Valence bond and molecular orbital representation of the DABCO radical stabilization. The MO scheme describes both the ground and excited states of the radical chromophore. It should only at first sight appear disturbing, that the excited state is NN-bonding, while the ground state is NN-antibonding.

excited adduct*. The product pattern of reactions 3a-c can be safely distinguished by the extent of 'final dFl_{ox}-bleaching' (zero for 3a, 50% for 3b, 100% for 3c).

A clear-cut extreme of predominant $2e^-$-transfer (Eqn. 4) has been found for natural Fl_{ox} with borohydride as substrate [23,24]. With 5-deazaflavin, however, this reagent is not applicable, since its dark reaction with this chromophore is too fast. Natural flavin, in contrast, is not susceptible to such nucleophilic attack unless activated either by dehydrogenase apoprotein or by light excitation.

Diaza-bicyclooctane (DABCO), a specific $1e^-$-donor photosubstrate. The opposite extreme of exclusive $1e^-$-transfer, which is required as calibration in the present study, is most conveniently verified by DABCO. This amine yields a radical cation by $1e^-$-abstraction, which is stabilized by electron delocalization over the two bridge-head nitrogen centers [26] as visualized in Scheme II. Since this stable N-radical cannot assume sp^2-configuration, it cannot be neutralized by α-deprotonation and thus cannot give off a second electron. Hence, its fate is confined to back donation. This leads us to Eqn. 5 as a special case of Eqn. 3 in buffered neutral solution valid for 5-deaza- as well as for natural flavin:

$$(d)Fl_{ox} + DABCO + H^+ \underset{\text{dark}}{\overset{h\nu}{\rightleftharpoons}} H(d)\dot{F}l + DABCO^{\cdot+} \qquad (5)$$

By means of Eqn. 5 we have determined the transient spectrum for deazaflavin photoreduced by DABCO at pH 9, see Fig. 5; for necessary corrections see Materials and Methods.

The comparison of this deazaflavin radical spectrum with that obtained radiolytically (cf. Fig. 4) shows that the shapes of the spectra are practically identical but that the spectrum of 7,8-dimethylated deazaflavin radical is shifted

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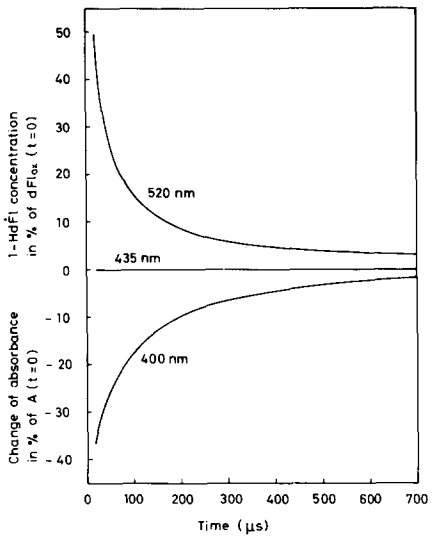
* We do not call this an exciplex, because this excited adduct is of σ-bonding type and the adduct also exists in the ground state [38].



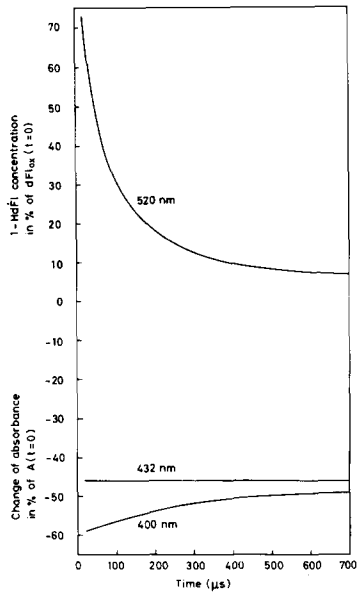


Fig. 7. Time dependence of the change of absorbance after flash decay in the photoreaction of 3-methyl-5-deaza-lumiflavin ($(1—2) \cdot 10^{-5}$ M) and DABCO ($5 \cdot 10^{-3}$ M) in borate buffer, pH 9, monitored at different wavelengths. The decay of 1-HdF$\dot{\text{l}}$ (520 nm) is concomitant with the reappearance of dFl_{ox} (400 nm). At 435 nm an isosbestic point exists between the dFl_{ox}-absorbance and the absorbance of both the 1-HdF$\dot{\text{l}}$ and $DABCO^{+\cdot}$ radicals.

Fig. 8. Time dependence of the change of absorbance after flash decay in the photoreaction of 3-methyl-5-deaza-lumiflavin ($(1—2) \cdot 10^{-5}$ M) and potassium oxalate (0.5 M) in borate buffer, pH 9, monitored at different wavelengths. During the decay of 1-HdF$\dot{\text{l}}$ (520 nm) a time-independent absorbance is observed at 432 nm after the marked bleaching at this wavelengths, generated during the flash lifetime. The initial bleaching observed at 400 nm (λ_{max} of dFl_{ox}) then decreases and approaches with time to that found at 432 nm.

about 10 nm to longer wavelengths, as is also the case in the oxidized state. The reversibility of the $1e^-$-abstraction by DABCO is shown in Fig. 7 by the quantitative reappearance of the dFl_{ox} absorbance at 400 nm concomitant with the radical decay, monitored at 520 nm. We find an isosbestic point at 435 nm as defined by the overall constancy of absorbance. Thus, at this wavelength, Eqn. 6 holds:

$$\epsilon(dFl_{ox}) = \epsilon(HdF\dot{l}) + \epsilon(DABCO^{+\cdot}) \qquad (6)$$

The photoreduction of 5-deazaflavin by oxalate. As mentioned above, 5-deazaflavin is (in contrast to natural flavin) such a good $2e^-$ acceptor, that it reacts rapidly with specific $2e^-$ donors such as borohydride in the dark. When searching for a good $2e^-$ photoreductant for 5-deazaflavin, we reconsidered our preparative results with oxalate [4]. In these long time experiments, we observed quantitative formation of the 5-deazaflavin radical dimer. By comparison with the photooxidation of authentic 1,5-dihydro-5-deazaflavin-5-carboxylate we concluded at this time that this reaction would not allow the trivial 'monophototonic' course of Eqn. 7:

$$^3dFl^*_{ox} \xrightarrow{1e^-} HdFl \longrightarrow 1/2\,(HdFl)_2 \qquad (7)$$

where the fate of the residual substrate radical ($\dot{C}O_2^-$) remains open, but instead the 'biphotonic' course of Eqns. 8a and 8b, where two triplet molecules are involved:

$$^3dFl^*_{ox} + (CO_2)_2^{2-} \xrightarrow[\text{slow}]{H^+,\,-CO_2} HdFl_{red}\text{-}5\text{-}COO^- \qquad (8a)$$

$$^3dFl^*_{ox} + HdFl_{red}\text{-}5\text{-}COO^- \xrightarrow[\text{slow}]{H^+,\,-CO_2} \begin{cases} (HdFl)_2 & (8b) \\ \text{or} & \\ 2\,Hd\dot{F}l & (8c) \end{cases}$$

Furthermore, the present photokinetic study allows one to distinguish clearly between the two pathways 7 and 8. As Fig. 8 shows, we find an isosbestic point at 432 nm. At this wavelength, we observe a rapid initial bleaching of 46% per dFl_{ox} within the decay time of the flash. Only after this time, the absorbance remains constant (in distinction to the case of DABCO, described above, where at the isosbestic point the absorbance remains constant from the beginning). At the same time, we observe at 520 nm (Fig. 8) formation of 1-Hd$\dot{F}$l radical to more than 70% for dFl_{ox}, decaying by second order. Thus, at 432 nm Eqn. 7 holds:

$$\epsilon(dFl_{ox}) = 2\,\epsilon\,(Hd\dot{F}l) \qquad (9)$$

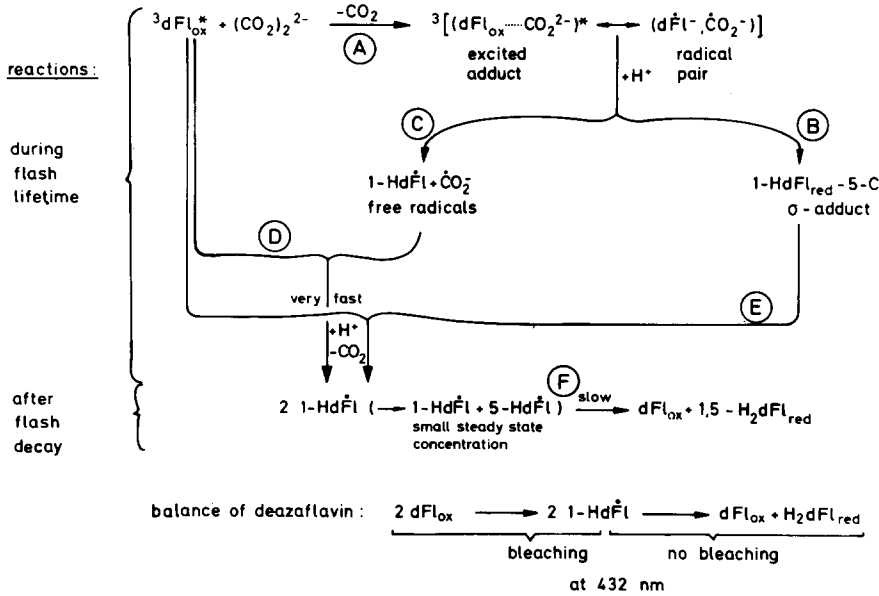
and the radical decay reaction is the pure dismutation (Eqn. 10).

$$2\,Hd\dot{F}l \rightarrow dFl_{ox} + H_2dFl_{red} \qquad (10)$$

and it follows that the formation of the dimer $(HdFl)_2$ cannot be more than a side reaction, which in long-term illumination comes to bear because of its photo-irreversibility. Eqn. 8 must thus be supplemented by Eqn. 8c: Hence, it is stringent that after 20 μs no secondary substrate radical, i.e. $^{\cdot}OOC\text{-}COO^-$ or (after decarboxylation) $\dot{C}O_2^-$, is left, which would lead to full reduction of dFl_{ox} arising from the radical dismutation, and thus would not leave a constant absorption at 432 nm. The isosbestic point we observe in the present photoreaction is evidence for the fact that the product of the initial reaction 8a and the substrate of the secondary reaction 8b, 8c is the 5-carboxylate (1-$HdFl_{red}$-5-CO_2^-) and not the unsubstituted 1,5-H_2dFl_{red} or its anion 5-$HdFl_{red}^-$. It will be shown below that in the case of the reaction with authentic 1,5-H_2dFl_{red}, in the analogous reaction no isosbestic point is found.

Furthermore, the present photoreduction confirms the radiolytic data, according to which the fate of Hd$\dot{F}$l, when left to itself, is dismutation and not dimerization. We summarize the explanation of the rapid reaction phase ($0 < t < 20$ μs). The sequence of reaction 8a, which is accelerated by the high oxalate concentration, and reaction 8c, which is fast by itself, involves the formation of one radical 1-Hd$\dot{F}$l per one starting dFl_{ox} and thus, accounts for the initial bleaching at 432 nm (cf. Eqn. 9).

The question is, however, open, as to whether the intermediate is a σ-adduct, 1-$HdFl_{red}$-5-CO_2^-, or a pair of free radicals, 1-Hd$\dot{F}$l + $\dot{C}O_2^-$. This alternative is outlined in Scheme III.



Scheme III. Mechanism of the photoreduction of 5-deazaflavin by oxalate.

In fact, we cannot distinguish experimentally between the pathways, B, E and C, D. If we neglect spin conservation, which is seemingly violated in reaction B, we must assume that both pathways are very fast. In particular, the secondary photocomproportionation E has been proved to be fast in the case of natural flavin [23,24]. In any case, if occurring, D and E will be much faster than A.

Scheme III shows furthermore that a $2e^-$-transfer pathway B can not be excluded even in the case of quantitative radical formation. Unfortunately, we can not estimate the triplet yield in the case of rapid substrate quenching. Presumably, owing to the lack of persistent triplet-triplet absorption, the yield of triplet formation per flash is enhanced from the blank value of 74% up to nearly 100%. This would explain the observed radical yield (Table II), which is calculated to 122% based on the low triplet yield of 74% in the blank reaction. If we assume 100% triplet formation, we obtain a radical yield of 90% and a permanent bleaching of 46% per triplet (= per total 5-deazaflavin). Alternatively, a radical yield of more than 100% per quenched triplet could arise from pathway C, D assuming a still rapid reaction of $\dot{C}O_2^-$ with a possible excess of ground-state dFl_{ox}. From radiolytic data above we know the rate of this reaction, namely $2 \cdot 10^9\ M^{-1} \cdot s^{-1}$, and this rate is too low to compete with the triplet quenching. Thus, pathway D, if occurring at all, must involve the excited $^3dFl^*_{ox}$ and not dFl_{ox}.

The rate of radical decay (cf. Table II and pathway F in Scheme III) by dismutation, is consistent with the dismutation rate observed radiolytically (cf. Table I).

In comparison with the σ-addition of CO_2^- found radiolytically (see above) the present data offer strong support for the assumption that oxalate reacts

with excited 5-deazaflavin by a σ-addition of the fragment CO_2^{2-}. Apart from the spin conservation 'dilemma' which is hereby encountered, we should not be surprised at this mechanism, since the structural analog, dithionite $(SO_2)_2^{2-}$, reacts with 5-deazaflavin as well as, for example, NAD^+ by σ-addition of fragment $(SO_2)^{2-}$ [27].

1,5-Dihydro-5-deazaflavin as substrate: Photocomproportionation. In order to substantiate our knowledge of the photocomproportionation reaction E in Scheme III, we continued our studies replacing oxalate as substrate by the authentic 1,5-dihydro-5-deazaflavin (H_2dFl_{red}). In any case, the question was still open as to why and how long-term illumination in the presence of oxalate would yield the radical dimer $(HdFl)_2$ instead of the end-product of Scheme III, namely H_2dFl_{red}. While we could assume that $1e^-$-abstraction from 1-$HdFl_{red}$-5-CO_2^- would lead to instantaneous loss of CO_2 and formation of the red radical 1-HdḞl, we have had to suspect that a proton would not leave C(5) in a primary radical cation 1,5-$H_2d\dot{F}l^+$ with the same ease as CO_2. This species would clearly lose at first only the mobile proton at N(1) to yield a relatively stable neutral radical tautomer 5-HdḞl. In fact, when we flash dFl_{ox} in the presence of a 10-fold excess of 1,5-H_2dFl_{red}, we observe a rapid formation of 78% 1-HdḞl per dFl_{ox} at 520 nm, but no isosbestic point in the range of $420 < \lambda < 460$ nm, i.e., at no wavelength does the absorbance reach a constant plateau after complete quenching of the triplet (Fig. 9). The possible reactions are outlined in Scheme IV in analogy to Scheme III.

The lack of an isosbestic point shows that apart from dFl_{ox} and 1-HdḞl, another chromophore must participate in the reaction, namely 5-HdḞl. Since the tricyclic resonance of 5-HdḞl in contrast to 1-HdḞl is lacking, we can con-

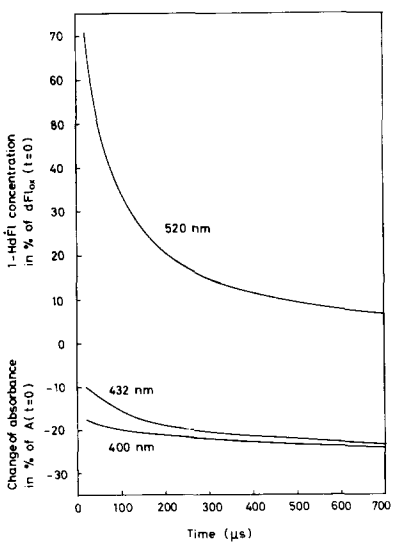
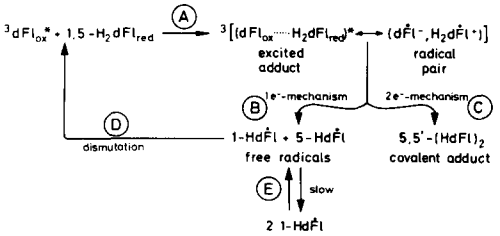


Fig. 9. Time dependence of the change of absorbance after flash decay in the photoreaction of 3-methyl-5-deaza-lumiflavin ((1—2) · 10^{-5} M) and 1,5-dihydro-3-methyl-5-deaza-lumiflavin ((1—3) · 10^{-4} M) in borate buffer, pH 9, monitored at different wavelengths. The decay of 1-HdFl (520 nm) is accompanied by an increase of bleaching, observed at 432 nm as well as at 400 nm (λ_{max} of dFl_{ox}).

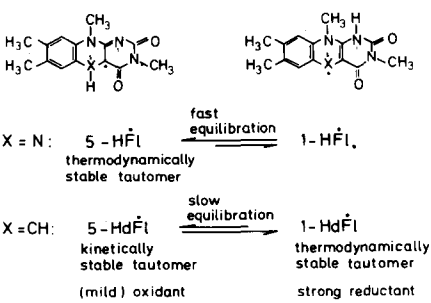


Scheme IV. Mechanism of the photoreduction of 5-deazaflavin by 1,5-dihydro-5-deazaflavin.

clude that 5-HdF̈l will not absorb at 520 nm, though it might well do so at 430 nm. While the tautomer 1-HdF̈l is known to be a strong reductant [5], 5-HdF̈l must be assumed from its structure (cf. Scheme V) to be a good oxidant. The crucial problem now is the thermodynamics and kinetics of the prototropic interconversion E (Scheme IV).

Obviously, the strongly reducing 1-HdF̈l cannot pick up a further electron and undergo dismutation without preceding partial 1,5-prototropy [5] to yield the mixture of 1-HdF̈l and 5-HdF̈l. 5-HdF̈l in turn cannot undergo 5,5′-dimerization (if at all) without preceding prototropy. In the case of natural flavin, the analogous equilibrium is established rapidly, since it involves only rupture and formation of NH-bonds. Its thermodynamics are known to be in favor of the 5-tautomer (Scheme V, X = N). With 5-deazaflavin, the radical 1-HdF̈l undergoes dismutation and not dimerization, so 1,5-prototropy must precede the dismutation (see F in Scheme III). Thus we know that 5,5′-dimerization of 1-HdF̈l must be slower than 1,5-prototropy. We must therefore conclude that the observed slow formation of the dimer $(HdFl)_2$ as the final product of 5-deazaflavin long-term photoreduction is due to the $2e^-$-transfer process C of Scheme IV.

Furthermore, the observed radical yield of 78% per dFl_{ox} plus bleaching of 25% assumed to be due to dimerization accounts well for 103% dFl_{ox} that has been turned over. If, on the other hand, 5,1-prototropy E was fast as compared to the radical decay, we have to expect more than 100% radical, i.e. 1-HdF̈l, yield (per triplet quenched) at 520 nm. This phenomenon is observed in the case of natural flavin, where prototropic equilibrium is established rapidly



Scheme V. Structure, stability and equilibration of the neutral 5-deaza-flavosemiquinone tautomers.

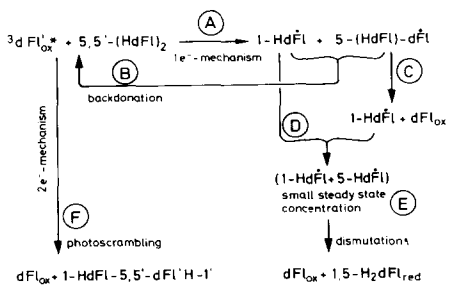
[23,24]. Its absence in the 5-deazaflavin system is independent proof for the occurrence of a second kinetically stable radical species, namely 5-HdFl.

When we compare the rates of radical decay (cf. Table II), we obtain a value of $1.0 \cdot 10^9\ M^{-1} \cdot s^{-1}$ in the oxalate reaction under the assumption that 2 mol of radical absorbing at 520 nm disappear with each other. This dismutation rate is well consistent, as mentioned, with the radiolytic value. For the case of H_2dFl_{red} as substrate, we obtain the same value under the assumption, that one radical absorbing at 520 nm reacts with another radical not absorbing at 520 nm.

Thus, in both cases, oxalate and H_2dFl_{red}, the same dismutation is the rate limiting step, for radical decay, namely reaction F in Scheme III and reaction D in Scheme IV. Hence, 1,5-prototropy is as fast as dismutation, while 5,1-prototropy is much slower. From this it follows that for deazaflavin as well as for natural flavin the radical tautomer equilibrium is in favor of the 5-protonated species (Scheme V, X = CH).

Deazaflavin radical dimer as photosubstrate: 'Photoscrambling'. In the section above we saw the occurrence of one new stable product as a result of 'photocomproportionation', namely the dimer 5,5'-$(HdFl)_2$ formed in 25% yield, while the residual part of the reaction was $1e^-$-transfer followed by dismutation, which in the case of H_2dFl_{red} as substrate equals back donation. Upon long-term illumination the dimer will be formed in 100% yield. Consequently, it was compulsive to check, in turn, this product as substrate. When we thus flashed dFl_{ox} in the presence of a 6-fold excess of $(HdFl)_2$, we observed 85% formation of 1-HdFl per dFl_{ox} at 520 nm. Since our flash with filtered light of $\lambda > 380$ nm does not cause any direct excitation of the substrate ($(HdFl)_2$ does not absorb at $\lambda > 360$ nm), the radical must be formed by dFl_{ox}-sensitization and decays preferably by back donation, as documented by the high overall stability of the substrate. Per flash, we observe now a small increase in dFl_{ox} by 10%. This is explained in Scheme VI.

Quite like in the preceding reaction with H_2dFl_{red} as substrate, we observe no isosbestic point, and thus, we must deal with a second radical, namely 5-(HdFl)-dFl, which does not absorb at 520 nm. This radical undergoes back donation (pathway B, Scheme VI) with 85—10 = 75% yield per dFl_{ox}. By a second pathway, the substrate radical is split to yield one dFl_{ox} plus one 1-HdFl, and according to what we know from above, the subsequent decay must lead to a final increase in dFl_{ox} by dismutation, and this we indeed observe. This



Scheme VI. Mechanism of the photoreaction of 5-deazaflavin by 5,5'-bis(1,1-dihydro-5-deazaflavin), $(HdFl)_2$.

pathway D, E of Scheme VI can lead to an exchange of dFl-units between the oxidized state and the dimer upon further irradiation, which we have termed 'photoscrambling' in earlier long-term illumination studies [4]. In analogy to the formation of $(HdFl)_2$ by the pathway C of Scheme IV, we must, however, admit a parallel 'direct' pathway of photoscrambling F (Scheme VI), which would not be detectable in the flash photolysis.

For each molecule of dFl_{ox} generated from $(HdFl)_2$ in this photoreaction, one molecule of H_2dFl_{red} must be present in order to preserve the redox balance. Since the latter is even more photoreactive, a photo-steady-state must be reached between dismutation (dFl_{ox}, H_2dFl_{red}) and dimerization $(HdFl)_2$, where the contribution of dismutation is relatively small due to the high photolability of H_2dFl_{red} as compared to $(HdFl)_2$. This can be proved by selective extraction of dFl_{ox} with $CHCl_3$ at pH > 7, where the two other species will be present in the anionic form. As demonstrated in Fig. 10, the content of dFl_{ox} will be restored to a constant plateau, which is dependent on the illumination energy. Photochemically, full reduction can only be reached by addition of a ternary substrate, e.g. oxalate, whereas full photoreoxidation is obtained by admission of air.

The deazaflavin triplet-pK. In the course of these studies, we became aware that in the photoreduction of 5-deazaflavin, as contrasted by natural flavin, we cannot observe a 'photo-p*K*' [28] in the pH range of 3—9. The 'photo-p*K*' was

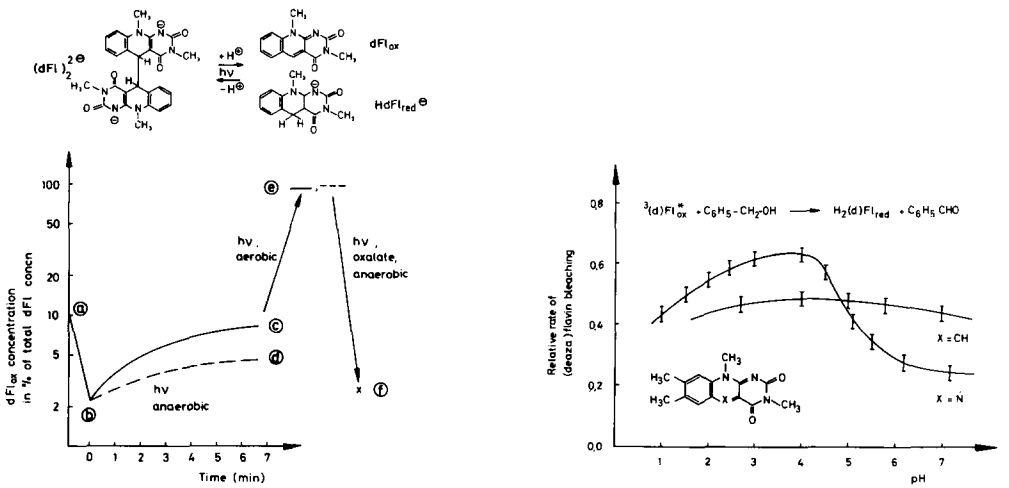


Fig. 10. The photo-steady-state of the half-reduced 5-deazaflavin. The crystalline preparation of $(HdFl)_2$ was dissolved $1.05 \cdot 10^{-4}$ M in 0.01 M borate buffer, pH 9.4 (a), and extracted three times with $CHCl_3$ (b), to remove any initial dFl_{ox} impurity. dFl_{ox} was monitored at 387 nm. The anaerobic reaction was started by illumination with light of λ = 225—350 nm (c) or λ = 380—420 nm (d), reaching a photo-steady-state after about 7 min. The steady-state yield of 5-deazaflavin was 8.7 ± 0.5% under condition c and 5.2 ± 0.5% under condition d (two experiments). The solution was now illuminated with light of λ = 390 nm in the presence of oxygen, yielding full oxidation (e). Finally, anaerobic photoreduction with 0.1 M potassium oxalate was carried out with 100% reconversion of the system to starting $(HdFl)_2$ (f).

Fig. 11. The pH-dependence of 5-deazaflavin (dFl_{ox}, X = CH) versus natural flavin (Fl_{ox}, X = N) photoreduction by benzyl alcohol. The same conditions are applied as published for natural flavin by Haas and Hemmerich [26].

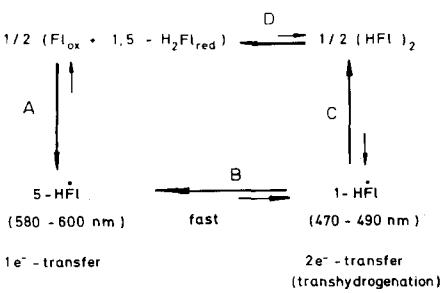
revealed by Schreiner et al. [30] to be due to the protonation of the excited triplet state of flavin. As can be expected from the replacement of N(5) by CH, the basicity located at N(1) is increased in dFl_{ox}, for which we find a pK_a of 1.8 in the ground state [29] as compared to the $pK_a \approx 0$ for the Fl_{ox} [12]. Thus, assuming that both excited triplet states of flavin and 5-deazaflavin are protonated at N(1), the 'photo-pK' ≈ 5, obtained for Fl_{ox}, should be shifted to higher values, using dFl_{ox}. In fact, this is not the case. The 'photo-pK' is found to be absent for dFl_{ox}, as shown in Fig. 11, by the comparison of the photoreductions of Fl_{ox} and dFl_{ox} with benzyl alcohol as neutral reductant.

As has been shown by Vogelmann et al. [11] the reactivity of an excited triplet state of a dye against $1e^-$-donors is strongly increased upon protonation. Therefore, we investigated the pH-dependence of the 5-deazaflavin triplet quenching constant with 1,3-dimethoxybenzene, a known $1e^-$-donor, which possesses a sufficient water solubility and does not quench the 5-deazaflavin triplet under alkaline conditions in a diffusion controlled reaction. While from pH 11 to pH 3 the triplet quenching rate constant increases only weakly, we find at pH 3 a sharp increase. Therefore, the pK of the 5-deazaflavin triplet must be lower than 3. From this and from the remarks in the previous paragraph, it follows that there are different positions of protonation in the excited triplet states of flavin and 5-deazaflavin. Since the most basic position in the dFl_{ox}-triplet molecule is the N(1), for which we find a $pK_a < 3$, the higher flavin triplet pK_a of 4.5 [30,31] must be attributed to the N(5).

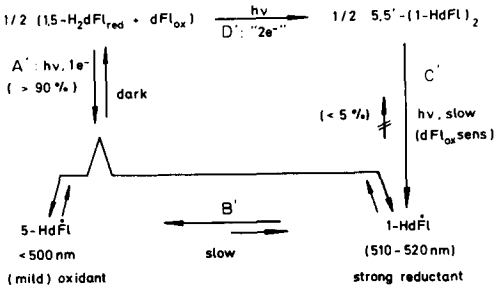
This is the new independent evidence for the assignment of flavin photo-pK to triplet protonation at N(5), which, of course, has no analogy in 5-deazaflavin.

Conclusions

For the natural flavin system, the half-reduced state is determined thermodynamically by the dismutation equilibrium A of Scheme VII. As Hemmerich and Massey [2] have shown, this equilibrium is regulated by regiospecific protonation in apoproteins. When a proton is fixed in position 5, the radical state and hence $1e^-$-oxidoreduction is favored. When, on the other hand, position 1 is kept protonated, equilibrium A is reversed via the equilibria B, C and D, which renders the radical state unstable and favors $2e^-$-transfer. Among the species listed in Scheme VII, only Fl_{ox}, H_2Fl_{red} and 5-HFl [12] are well defined in terms of chemical structure and physical properties, while the



Scheme VII. Molecular interconversion in the half-reduced natural flavin system.



Scheme VIII. Molecular interconversion of the half-reduced 5-deazaflavin model system.

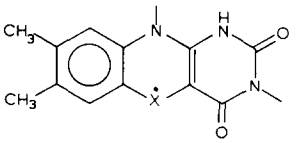
unstable radical tautomer 1-HFl is only known as artificial chemical model chromophore 1-RFl (R = alkyl [32]). Even less information is available about the dimer $(HFl)_2$. In any case, such a kinetically labile intermediate, for which we postulate a considerable biological relevance, cannot have the structure of a π-charge transfer complex ('flavoquinhydrone'). Favaudon and Lhoste [33] were first in showing that, presumably, the two flavin halves in such a labile dimer are linked in another way. This linkage has been proposed by Hemmerich to be a σ-bond in position 8 (1-HFl-8,8'-FlH-1') [34].

The present study was undertaken in order to gain more information about the thermodynamically labile as well as kinetically labile species 1-HFl and $(HFl)_2$ by a study of the more stable 5-deaza analogs. Thus, in the 5-deaza system we find a slower interconversion of radical tautomers, as shown in Scheme VIII.

The 1-monohydro isomer 1-HdFl, arising from enforced $1e^-$-reduction of the oxidized state (dFl_{ox}), absorbs at longer wavelengths due to its tricyclic electron delocalization and is a strong reductant [6,7]. On the other hand, the 5-monohydro tautomer arises upon enforced $1e^-$-oxidation of the reduced state ($1,5\text{-}H_2dFl_{red}$), it does not absorb at $\lambda > 500$ nm and is a mild oxidant. From their redox properties it follows that, preferably, the radical tautomers 1-HdFl and 5-HdFl do not react with themselves, but, rather, one with the other, which leads to dismutation. The well-known dimer $(1\text{-HdFl})_2$, which is 5,5'-linked and is the end-product of most long-term photoreductions of dFl_{ox}, must thus be formed by direct photocomproportionation, since H_2dFl_{red} is the most potent photoreductant for dFl_{ox}. A dimer of this structure can, of course, not exist in the natural flavin series, since the two N(5)-sites would repel each other. Thus, we repeat our postulate [6,7] that in the 5-deazaflavin system a second dimer exists as a short-lived intermediate, which is analogous to the σ-dimer assumed above for the natural flavin radical. This dimer, 1-HdFl-8,8'-dFlH-1', can be assumed to promote the 1,5-HdFl tautomer interconversion.

The strongly reducing radical 1-HdFl is characterized by its absorption at 510—520 nm as isoelectronic species to the flavosemiquinone cation, which was termed 'rhodoflavin' by its early discoverers, Kuhn and Stroebele [35]. The difference between 1-HdFl and rhodoflavin ($1,5\text{-}H_2Fl^+$) lies merely in the replacement of C by N^+ in position 5.

$\dot{X} = \dot{N}H^+$: $\lambda_{max} \approx 490$ nm
$\dot{X} = \dot{C}H$: $\lambda_{max} \approx 520$ nm



In the present study we have compared the formation of this radical from dFl_{ox} by three different well-defined electron donors, namely hydrated electrons, $\dot{C}O_2^-$-radical and H_2dFl_{red}. It becomes obvious from our data that electron-deficient heteroaromatic chromophores, such as (deaza)flavin, do not necessarily react (especially not with small reductant molecules) by 'direct electron abstraction' through π-orbital contact, but instead by σ-addition of substrates, followed by elimination of the substrate in its ionized state. The contact site of acceptor dye and substrate are therefore most relevant features of the redox process: Even $\dot{C}O_2^-$ does not transfer electrons, but forms with dFl_{ox} a σ-complex radical, namely $d\dot{F}l\text{-}1\text{-}CO_2^-$, which subsequently undergoes decarboxylation. Oxalate $(CO_2)^{2-}$, on the other hand, reacts with $^3dFl^*_{ox}$ to yield $[dFl_{red}\text{-}5\text{-}CO_2]^{2-}$.

With the aid of these data we can now explain the more complicated reduction of $^3dFl^*_{ox}$ by H_2dFl_{red}. Here we may obtain as first products two isomeric intermediates $(HdFl)_2$. The predominant one is 1-HdFl-8,8′-dFlH-5′, which decays rapidly, yielding the tautomeric radicals 5-Hd$\dot{F}$l and 1-Hd$\dot{F}$l. This reaction is reversed in the dark. The other dimer formed to a minor extent has the known structure 1-HdFl-5,5′-dFlH-1′. It is stable and thus accumulates in the long-term reaction.

Unexpected is the fact that 1-Hd$\dot{F}$l or $d\dot{F}l\text{-}1\text{-}CO_2^-$ formed radiolytically does not undergo direct 5,5′-dimerization to a measurable extent, but instead decays by rapid 1,5-prototropy and subsequent dismutation. This can be explained only by the fact that the steric restrictions for a 5,5′-contact are severe and that the 8,8′-contact might favor the prototropic shift. This provides further support for the assumption [34] that also the natural flavin-dependent $1e^-$-transfer operates via short-lived σ-contacts over the flavin 'edge' in position 8, which is characteristically exposed to the cytoplasm in the $1e^-$-transferring flavoprotein class of flavodoxins [36].

Summarizing, we find in the 5-deazaflavin radicals a kinetic stabilization of tautomeric forms which is analogous to the regulation of the natural flavin system by kinetically stable regiospecific hydrogen bonds between coenzyme and apoprotein. At the same time, however, we lose in the 5-deazaflavin system the thermodynamic stability of the radical which is encountered with natural 5-H$\dot{F}$l. From this it follows that 5-deazaflavin is incapable of mediating between $1e^-$- and $2e^-$-transfer, in contrast to natural flavin. It is thus corroborated, that 5-deazaflavin is a 'flavin-shaped functional derivative of nicotinamide' [1].

The whole picture shown by the 5-deazaflavin model confirms, that the decisive steps in flavin-oxidoreduction, which regulate the redox potentials of the individual oxidation state as well as the accessibility of the individual reaction sites, are proton transfers [2] to and from the N(1)/O(2α) and N(5)-region (which is C(5)H in the deazamodification). For free flavins this prototropic shift is diffusion-controlled; for bound flavins it is apoprotein-controlled, while for 5-deazaflavin it is controlled kinetically by the inter(deaza)flavin contact.

In more complex flavoproteins [37], which contain two interacting flavin units, interflavin contact and regulation by regiospecific hydrogen bridges from the apoprotein will depend on each other.

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