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Light-Induced Alkylation and Dealkylation of the Flavin Nucleus

Stable Dihydroflavins: Spectral Course and Mechanism of Formation

Wolfram H. WALKER and Peter HEMMERICH

Institut für Anorganische Chemie, Universität Basel, and Universität Konstanz, Fachbereich Biologie

Vincent MASSEY

Department of Biological Chemistry, University of Michigan, Ann Arbor

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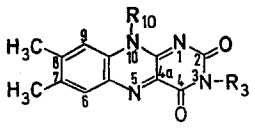
The spectral course of irreversible photoreduction of the flavin nucleus by arylacetates and related compounds has been investigated in detail. The resulting benzylated dihydroflavins and their reoxidation products have been characterized spectrophotometrically, among them 5-benzyl-flavosemiquinone radical and 5-benzylflavoquinonium cation. Reversible and irreversible flavin reduction corresponding to hydride- or group-transfer from substrate to flavin are discussed with respect to their scope and limitations and possible biological implications in the action mechanism of flavin dependent dehydrogenases.

Flavin sensitized photodecarboxylation of phenylacetate has been reported previously by our group [1,2] and was found to result in a new and irreversible type of flavin photoreduction, *i. e.* benzyl group transfer (rather than hydride or electron transfer) towards the acceptor positions 5 or 4a of the flavin nucleus (I). While "H-transfer" (the term "H-transfer" is meant formally regardless of the mechanism, *i. e.* hydride, H-atom or whatsoever), obtained for example with mandelic acid, is reversible under all conditions tested, the group-transfer reaction is



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complicated by the fact that either product, 4a-benzyl-4a,5-dihydroflavin (II, "4a-$\varphi CH_2Fl_{red}H$") and 5-benzyl-1,5-dihydroflavin (III, "5-$\varphi CH_2Fl_{red}H$"), can be isolated and has its own way of reoxidation with O_2, which is different from that of the unsubstituted $Fl_{red}H_2$ ("leucoflavin" or 1,5-dihydroflavin), the only dihydroflavin known before [3]. These pathways are illustrated in Scheme 1.

This shows that also group transfer is reversible under certain conditions. The conditions imply action of light for 4a-$\varphi CH_2Fl_{red}H$ and of mild acid for 5-$\varphi CH_2Fl_{red}H$.

It is evident that, in order to interpret the structure and function of intermediates in flavin dependent biological oxidations, the physical properties of group transfer as well as H-transfer products and all possible types of reduced flavin should be characterized thoroughly. This alone might help to understand the reasons, originating from the substrate structure or environmental conditions, which switch the course of flavin dependent "substrate" oxidation from group towards H-transfer and *vice versa*.

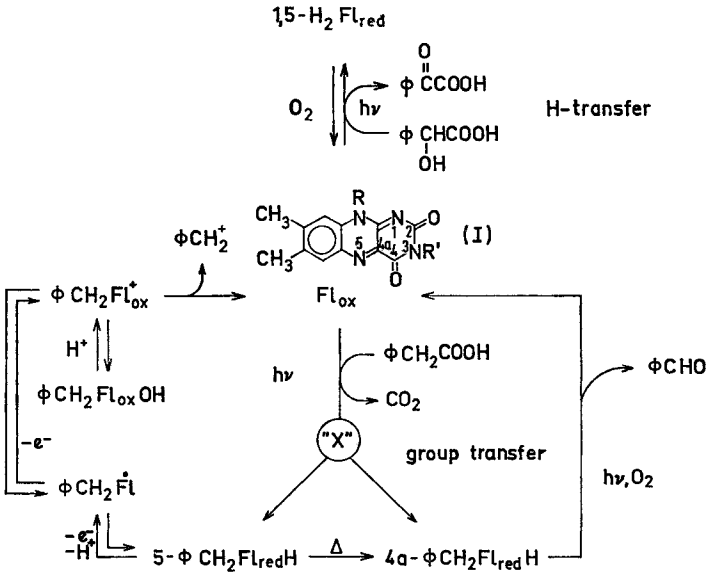
Trivial Names and Unusual Abbreviations. Flavin, 10-substituted 7,8-dimethyl-isoalloxazine; lumiflavin, 7, 8,10-trimethyl-isoalloxazine; Fl_{ox}, flavin in the neutral, oxidized state; $Fl_{red}H_2$, reduced flavin (dihydroflavin); HFl, flavosemiquinone radical; φCH_2, benzyl.

MATERIALS AND METHODS

All flavin derivatives used have been described earlier [2]. The natural flavin (I) side chain (R_{10} = ribityl) has been changed into CH_3 in order to avoid photolytic side reactions [4]. The cyclic imide group in position 3 is alkylated throughout, which affords the desired high solubility in unpolar (R_3 = benzyl) and polar ($R_3 = CH_2COO^-$) environments or at least sufficient solubility stretching out over a wide polarity range from water to toluene ($R_3 = CH_3$).

All other reagents were best grade from Fluka A. G. (Buchs, Switzerland), with the exception of tetraacetyl*iso*riboflavin, obtained by reductive alloxane condensation (and subsequent acetylation in pyridine) of 2-phenylazo-3,4-dimethyl-*N*-ribityl-aniline, which in turn was a gift from Hoffmann-La Roche.

Optical spectra were run on Cary 14 instruments. The photochemical light sources were 100–200 W tungsten lamps, the rates of photoreaction being



Scheme 1. *Reaction scheme, light-induced flavin benzylation and debenzylation*

regulated by the distance between light source and 1 cm "anaerobic" quarz cells containing the sample solution. All solutions were degassed and flushed several times with V^{2+}-deoxygenated argon or nitrogen. All measurements were taken at room temperature if not otherwise stated. The action spectra were taken with an Osram 150 W xenon arc and a Bausch and Lomb (33-86-07) grating monochromator, 3 mm slits (22 nm dispersion). The solution was irradiated for 1 min intervals, shaken to ensure uniform solution and the extent of benzylation measured by the decrease in absorbance at 450 nm over a 3 min irradiation period. The observed rates were corrected by estimating the output of the xenon arc-monochromator assembly at the wavelengths used with a IP-28 photomultiplier and correcting for the photoresponse of a typical IP-28 photomultiplier tube [5].

RESULTS AND DISCUSSION

Reversible Photoreduction

In Fig. 1 we compare spectra of free leucoflavin (obtained by hydrogenation as described earlier [6]) with the product of flavin photoreduction by mandelic acid. From this it is obvious, that mandelic acid, as well as glycine, EDTA and other α-amino acids or amines [7, 8], undergoes H-transfer towards flavin as an overall reaction.

Clearly, any substrate $\begin{matrix}R\\X\end{matrix}\!\!>\!\!CH{-}COO^-$ offers to the flavin-triplet (Fl_{ox}) the choice between dehydrogenation, *i. e.* CH-bond breakage, and oxidative decarboxylation, *i. e.* CC-bond breakage, the choice itself being governed by the nature of the residues R and X. The stoichiometry of CO_2-evolution in flavin dependent anaerobic photodehydrogenation of α-amino acids still seems controversial [7, 8]. Any CO_2 evolved from this type of system under aerobic conditions could also arise from the secondary reaction (*cf.* 2 in Eqn. 1).

$$\begin{matrix}R\\X\end{matrix}\!\!>\!\!CH{-}COO^- \xrightarrow[1]{h\nu,\ Fl_{ox},\ H_2O}$$

$$R{-}CO{-}COO^- \xrightarrow[2]{h\nu,\ Fl_{ox},\ H_2O} R{-}COO^- + CO_2\,. \quad (1)$$

This secondary reaction as such is well documented [9]. Further experiments are underway.

The spectrum of the "H-transfer product", *i. e.* 1,5-dihydroflavin (*cf.* Fig. 1 and Scheme 1), is characterized by its pronounced shoulder at 400 nm and its end absorption stretching out towards the visible range [6]. This renders concentrated "leucoflavin" solutions more deeply colored than oxidized flavins and gives rise to intense orange crystal surfaces.

Deprotonation of "1,5-$Fl_{red}H_2$" occurs with a characteristically low pK_a of 6.7 [10] in the pyrimidine subnucleus, *i. e.* not in position 5, and provokes a pronounced hypsochromic shift by restricting tricyclic delocalisation of the N(5) and N(10) lone pairs. The intensity of the transition at wavelengths above 300 nm is characteristically low (less than 4000 M^{-1} cm^{-1}). Upon admission of O_2, the Fl_{ox}-spectrum is restored quantitatively from the spectra of the reduced species.

The question of "group- *versus* H-transfer" is not easy to decide in principle as long as no decarboxy-

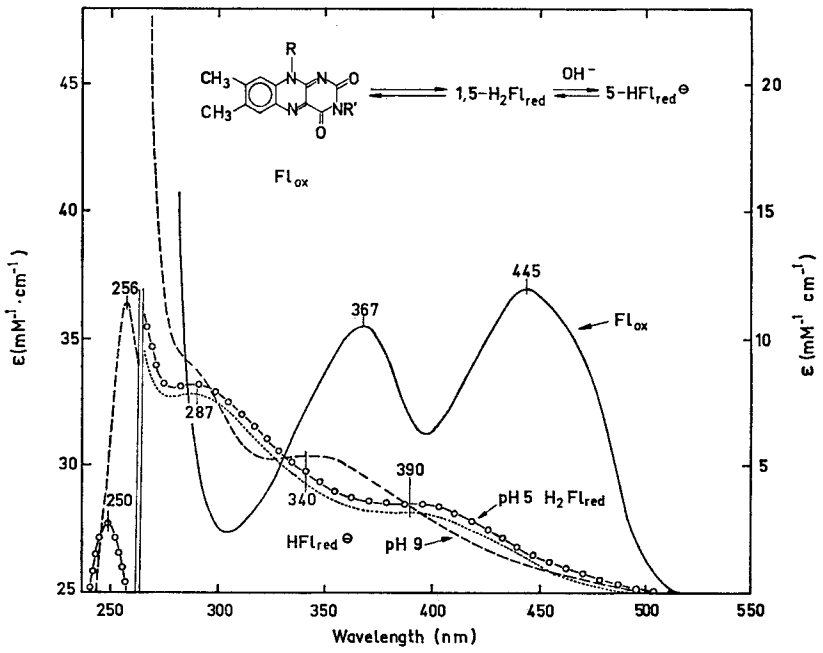


Fig. 1. *Spectral changes upon H-transfer towards flavoquinone.* Fl_{ox} = lumiflavin-3-acetic acid; ——, oxidized state (pH 7); O——O, pH 5 neutral and ---- pH 9 anionic reduced state under H_2, catalyst (Pd/SiO_2) allowed to settle down; ....., the same reduction at pH 5 done with 0.1 N mandelic acid in tungsten light. The "reduced" spectra are quantitatively reversed to the "oxidized" spectrum upon admission of air

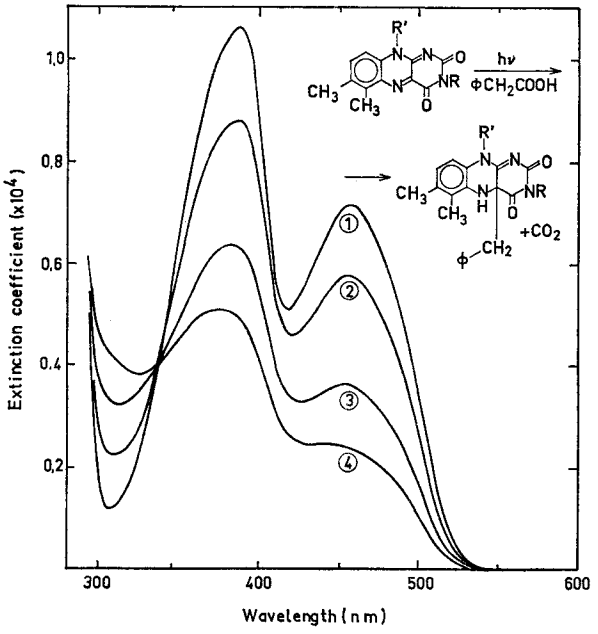


Fig. 2. "*Isosbestic*" *course of irreversible flavin photoalkylation.* Fl_{ox} = tetraacetyl*iso*riboflavin, R′ = tetraacetylribityl, R = H, in 0.25 M phenylacetate, pH 7.1: starting spectrum; 2,3,4 after about 5, 10, 15 min illumination with tungsten light under argon

lation occurs. If we were to assume that mandelic acid reacts in the same way as phenylacetic acid, the corresponding φCH(OH)-substituted dihydroflavin could be anticipated to be even more labile than the φCH_2-analogs, especially towards hydrolysis. Hence, H-transfer could be simulated by a rapid sequence of group transfer and hydrolysis, so that a real proof for H-transfer could only be given by demonstration of a deuterium effect as shown for flavin dependent dihydronicotinamide dehydrogenation by Metzler and Suelter [11].

Irreversible Photoreduction

Upon replacing mandelic acid by phenylacetic acid, the spectral course of the flavin photoreduction changes drastically and two types of reaction can be observed, isosbestic or non-isosbestic. We found the action spectrum in either case to be coincident with the flavoquinone absorption spectrum (λ_{max} 370, 450 nm in H_2O, pH 7).

The isosbestic (Fig. 2) type of reaction leads to the formation of pure 4a-benzyl-4a,5-dihydroflavin (λ_{max} 364 nm, "4a-$\varphi CH_2Fl_{red}H$", Scheme 1, Fig. 7). This is observed under a variety of conditions, *i. e.*, high temperature (>40°), low pH (<7), with sterically less accessible "substrate" (α-phenylpropionic

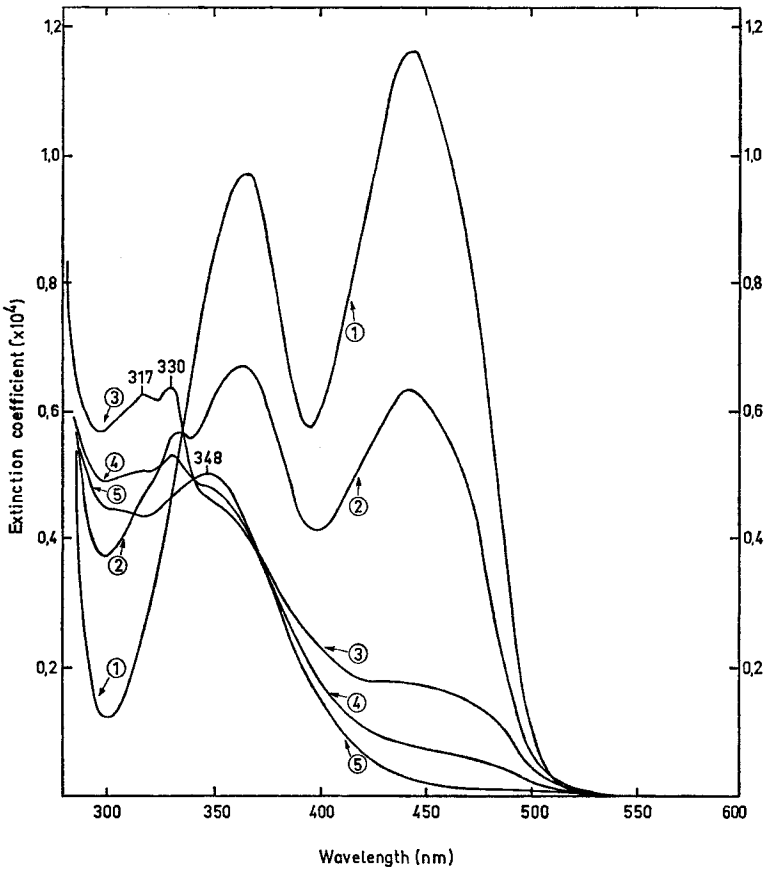


Fig. 3. *"Non-isosbestic" course of irreversible flavin photoalkylation.* Fl_{ox} = lumiflavin-3-CH_2COOH, in 0.08 M phenylacetate, pH is slowly changing from about 7 to lower values due to CO_2-liberation. Curve 1—3: Formation of intermediate "X", within about 5 min tungsten illumination under argon. Curve 3—5: Rearrangement of "X" towards a mixture of benzyldihydroflavins in the dark, within about 3 hours

acid), with flavin analogs having alkyl substituents in position 6 which renders position 5 less accessible ("isoflavins" [12]).

The non-isosbestic type of reaction is found correspondingly at temperatures below 40°, pH > 7, with 6-unsubstituted flavins and α-unsubstituted "substrates", leading to a first intermediate state shown in Fig. 3, curve 3. This state of still unknown nature marks the end of the light reaction and appears from its isosbestic mode of formation to be homogeneous (state "X", Scheme 1). What follows is a slow dark rearrangement (Fig. 3, curves 4—5, Fig. 4, curves 1—3) promoted by both heat or acid, and leading to a mixture of λ_{max} 248, 353 nm, consisting of equal parts of 4a- and 5-benzylated dihydroflavin isomers as described earlier [2]. The 4a-isomer is the same as formed in the "isosbestic" reaction (*cf.* above). The 5-isomer (λ_{max} 340 nm, "5-$\varphi CH_2Fl_{red}H$", Scheme 1, Fig. 6) undergoes a very slow, heat catalyzed alkyl migration to give the 4a-isomer (Fig. 4, curve 5).

Upon admission of air to the φ $CH_2Fl_{red}H$-isomers no reformation of Fl_{ox} (λ_{max} 445 nm) is observed in the absence of light. Hence the reaction is thermodynamically irreversible.

Reoxidation of 4a-φCH₂Fl$_{red}$H

Upon admission of air a sequence of reactions is initiated, which is very different for the two $\varphi CH_2Fl_{red}H$-isomers (*cf.* Scheme 1): While no reformation of species absorbing with λ_{max} 445 nm is observed in the dark, illumination with tungsten light restores about half the initial flavoquinone very rapidly, while the other half of the total flavin present in the mixture gives rise to the transient formation of a dark green radical and to a new final product, which in turn can be reconverted to Fl_{ox} under acid conditions, *i. e.* hydrolytically. If the isomers are separated [2], it is easily shown that the photooxidation (Fig. 7) belongs to 4a-$\varphi CH_2Fl_{red}H$. It is a light induced reversal of the "isosbestic" type of photoreduction

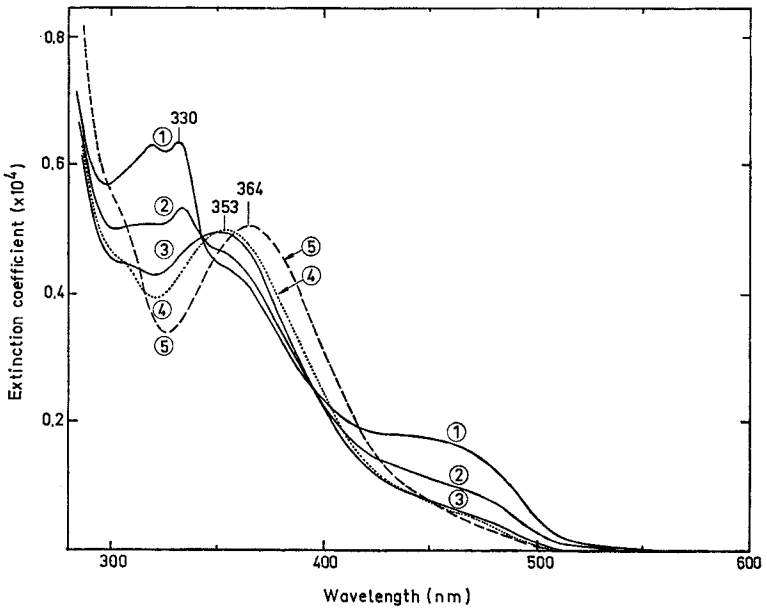


Fig. 4. *Continuation of Fig. 3.* Curves 1—3 correspond to 3—5 in Fig. 3. Curve 4: endpoint "X-rearrangement" after 20 hours. Curve 5: further very slow rearrangement (can be accelerated by heating to 40°) from 4a,5-isomer mixture to pure 4a-$\varphi CH_2Fl_{red}H$, obtained within 5 days at room temperature under argon

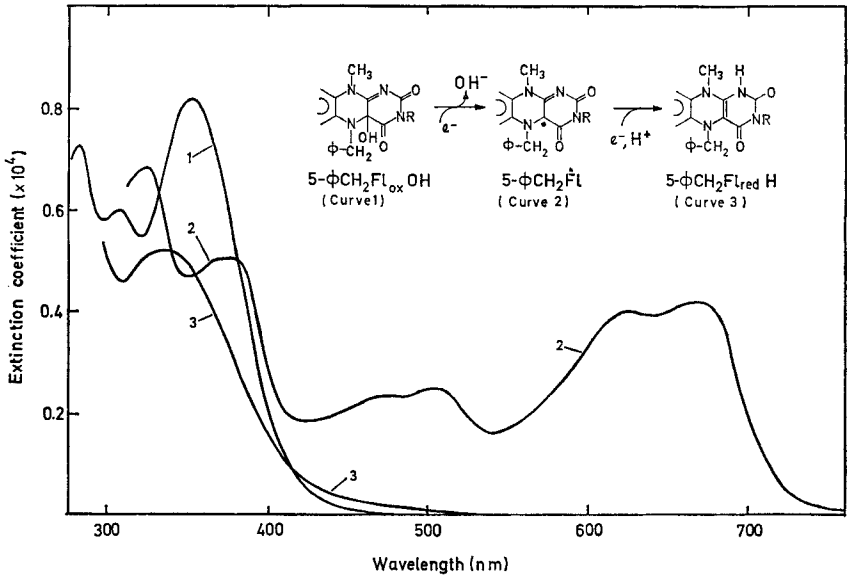


Fig. 5. *Spectral course of 5-benzyl-3-methyllumiflavosemiquinone comproportionation, in $CHCl_3$.* Curve 1: oxidized species (Ψ-base), (*cf.* Fig. 6). Curve 3: reduced species (1,5-dihydroflavin), (*cf.* Fig. 6). Curve 2: spectrum of the neutral semiquinone radical obtained by mixing equivalent amounts of oxidized and reduced species. A trace of acid (CH_3COOH) is needed to start the reaction. The spectrum 2 can also be developed by shaking the oxidized species with aqueous dithionite or mercaptans until maximal green color is developed. Full radical formation is not reached upon aeration of the reduced species in $CHCl_3$, but can be obtained by addition of a stoichiometric amount of I_2 (1 electron equivalent)

(*cf.* above, Fig. 2), giving quantitative yields of starting flavoquinone and benzaldehyde, the latter being identified by gas chromatography. (We are indebted to Dr. C. Jefcoate for the gas chromatography analysis.) The action spectrum of the reoxidation (measurements by G. Blankenhorn, more details to be given in a subsequent paper) is, not too surprisingly, very similar to that of the photoreduction, *i. e.* it is essentially identical with the flavoquinone absorption spectrum. From this it

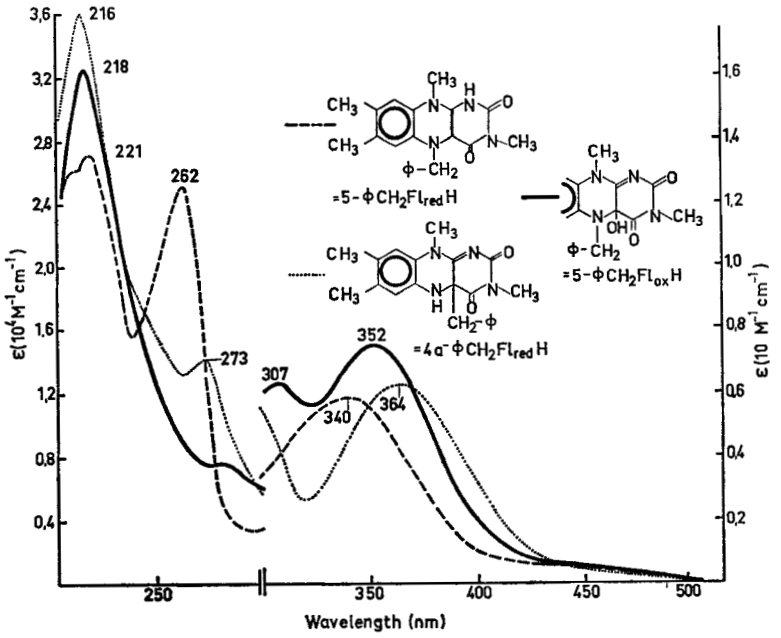


Fig. 6. *Absorption spectra of 3-methyllumiflavin photoalkylation products in CH_3OH.* For the autoxidizable 5-$\varphi CH_2Fl_{red}H$ special care is needed. The stock solution was made up 10 mM in $CHCl_3$ and shaken with aqueous $S_2O_4^{2-}$ (half saturated with NaCl in order to lower the water content of the $CHCl_3$-phase). This phase was then diluted rapidly under argon with argon-flushed MeOH

follows that the photooxidation is autocatalytic, since reoxidation starts with 4a-$\varphi CH_2Fl_{red}H$ having very low if any absorption, at 445 nm. This was indeed proven by oxidizing a very pure sample with and without addition of Fl_{ox}.

Reoxidation of 5-$\varphi CH_2Fl_{red}H$

This isomer makes up the second half of the "non-isosbestic" photoreduction products and accounts for the appearance of the relatively stable radical arising upon oxygenation of the photoreduced mixture. In contrast to the 4a-isomer mentioned above, the 5-isomer reacts with O_2 in the dark (Scheme 1). However, it is by no means certain that the reaction implies a one-electron transfer from 5-$\varphi CH_2Fl_{red}H$ towards O_2 to give 5-$\varphi CH_2\dot{F}l$; the radical can as well originate by comproportionation, as shown in Fig. 5. We obtain quantitative formation of the radical (curve 2) by mixing equivalent amounts of Ψ-base (curve 1) and reduced 5-isomer (curve 3) in the presence of catalytic amounts of acid. Since O_2 is known to be a poor one-electron acceptor [13], and since the Ψ-base 5-$\varphi CH_2Fl_{ox}OH$ is the end product of the autoxidation [2] at neutral pH, we want to point out the possibility of a reaction as given in equation 2 (the OOH-group being fixed in position 4a).

$$5\text{-}\varphi CH_2Fl_{red}H + O_2 \rightarrow 5\text{-}\varphi CH_2Fl_{ox}OOH\,. \qquad (2)$$

Subsequently comproportionation may occur [Eqn. (3)],

$$5\text{-}\varphi CH_2Fl_{ox}OOH + 5\text{-}\varphi CH_2Fl_{red}H \rightarrow 2\,(5\text{-}\varphi CH_2\dot{F}l) + H_2O_2 \qquad (3)$$

or homolytic cleavage [Eqn. (4)],

$$5\text{-}\varphi CH_2Fl_{ox}OOH \rightarrow 5\text{-}\varphi CH_2\dot{F}l + H\dot{O}_2\,. \qquad (4)$$

The Ψ-base is found to be stable in solution (Fig. 6) down to a pH of about 4, when OH^- is reversibly eliminated to give the purple flavoquinonium cation 5-$\varphi CH_2Fl^+_{ox}$ (Fig. 8, curve 1) which in turn undergoes slow benzyl transfer to the solvent with restoration of flavoquinone. In water, the benzyl group appears quantitatively as benzyl alcohol, as identified by gas chromatography.

The properties of the two benzyl isomers under acidic conditions are shown in Fig. 8. Under anaerobic conditions in acid the two $\varphi CH_2Fl_{red}H$-isomers are stable and present as the cations. Characteristically, the 4a-isomer gives a bathochromic shift, 364 → 388 nm (Fig. 8, curve 2), indicating protonation in the pyrimidine subnucleus, presumably in position 1 (*cf.* the analogous case of N(1)-protonation in Fig. 1, $Fl_{red}H^- \rightarrow Fl_{red}H_2$). The 5-isomer, however, shows a hypsochromic shift upon protonation 340 → 307 nm (Fig. 8, curve 3), indicating quaternization of N(5).

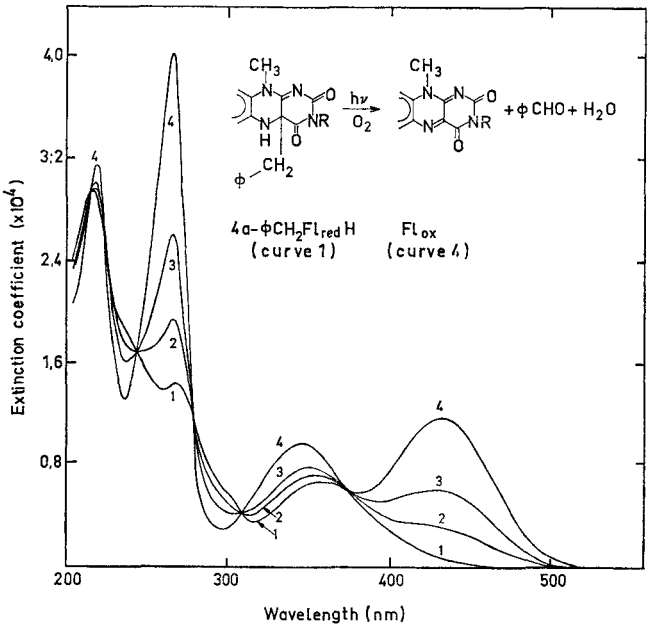


Fig. 7. *Isosbestic photooxidation of 4a-φ$CH_2Fl_{red}H$ (Fl = 3-methyllumiflavin) in CH_3OH, by air and tungsten light.* The restoration of the Fl_{ox}-spectrum (curve 4) is quantitative within about 30 min

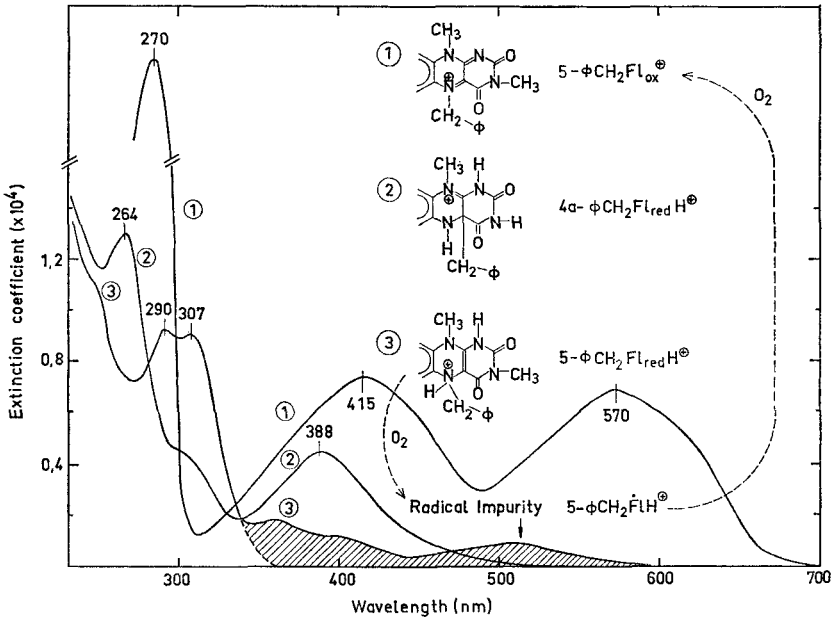


Fig. 8. *Autoxidation in 2 N $HClO_4$ of 50 μM 5-φ$CH_2Fl_{red}H_2^+$ and comparison with stable 4a-φ$CH_2Fl_{red}H_2^+$.* The long wave part of spectrum 3 is due to radical admixture, which can be avoided by addition of a trace of $TiCl_3$. Fl_{ox} = 3-methyllumiflavin

While the cation of the 4a-isomer (spectrum 2, Fig. 8) is stable in the dark against O_2, the cation of the 5-isomer (spectrum 3, Fig. 8) changes through a "rhodoflavin" [3] radical cation (5-φ$CH_2\dot{F}lH^+$, traces of which are already formed, λ_{max} 505 nm) to the purple flavoquinonium cation 5-φ$CH_2Fl^+{}_{ox}$ (Fig 8, curve 1). This extremely electron-deficient species is moderately stable in the absence of nucleophiles, *e. g.* in glacial acetic acid, but not under aqueous conditions, where it is debenzylated rapidly (*cf.* above).

From this it is proven unequivocally that, on the flavoquinone level, substitution at N(5) gives a bathochromic and at N(1) [6] a hypso-

chromic shift, while the reverse is true for flavohydroquinones.

Furthermore, stringent conclusions can be drawn as to the tautomeric structure of the neutral flavosemiquinone radical, which will be presented along with electron spin resonance evaluation in a separate paper [14]. It should be mentioned here, however, that the disproportionation equilibrium (5)

$$Fl_{ox} + Fl_{red}H_2 \overset{> 95\%}{\rightleftarrows} 2\,\dot{F}lH \qquad (5)$$

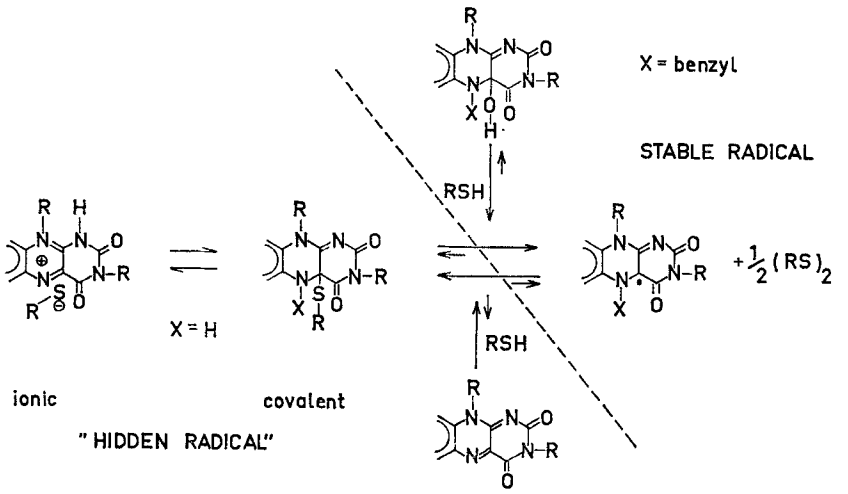
is fully displaced towards the radical side in the 5-alkylated derivatives, as follows from the results outlined above (Fig. 5), according to equation (6).

$$\begin{matrix} RFl^{+}_{ox} + OH^{-} \\ \uparrow\downarrow \\ RFl_{ox}OH \end{matrix} + RFl_{red}H \overset{\sim 100\%}{\rightleftarrows} 2\,R\dot{F}l + H_2O \qquad (6)$$

This is because the oxidized state is very high in energy, the cation due to its electron deficiency, the Ψ-base due to its non-coplanarity.

Biochemical Conclusions

We have shown that positions 4a and 5 of the flavin nucleus do have group acceptor properties, which suggests the idea of σ-transfer of redox-equivalents through these positions. The functional positions of the pyrimidine subnucleus (position 1—4), in comparison, do not seem "redox-active", as can also be deduced from the results of spin density evaluation in flavosemiquinones [14]. This is furthermore in agreement with redox-active metal ions being bound through N(5) [15] to the flavin nucleus. Now the question is to the nature of the chemical bond between position 4a or 5 and a given "substrate" group and to its manifestation in the physical, in particular spectral and magnetic properties of flavin species. Massey and coworkers [16—18] have postulated biradical complexes of substrate-flavin interaction in flavoproteins, which are on the flavosemiquinone redox-level though they do not exhibit paramagnetism and only a weak charge-transfer type long-wave absorption in the optical range. Based on the findings outlined in the present paper, Scheme 2 shows a proposal, how this behaviour could be understood in terms of chemical structure. The upper right hand side shows the chemical model, *i. e.* the Ψ-base 5-$XFl_{ox}OH$ being reduced by mercaptide to the stable radical plus disulfide as shown in Fig. 5. On an enzyme surface, a second RS$^{\cdot}$ would not normally be available at suitable distance to form a SS-bridge. With liponamide dehydrogenase the equilibrium component on the left might become stable, *i.e.* a flavoquinonium mercaptide, which might equally well dissociate homolytically as heterolytically, thus behaving like an "invisible" or "hidden" radical. The optical absorption of this species would depend on the distance S-Fl and the bond angle at C(4a), this could vary within wide limits from a true ion pair to a fully covalent bond. Kierkegaard and coworkers [19] have shown by X-ray crystallography, that in flavoquinonium iodide the I^{-} is situated next to C(4a), though the structure is perfectly ionic. This would be isoelectronic to the ionic state in Scheme 2, which thus should exhibit essentially flavoquinone plus charge-transfer absorption. The covalent state, on the other hand, should not absorb at wavelengths above 400 nm, as apparent from comparison to its isosteric analog $XFl_{ox}OH$. The apoprotein preferring either a flat or a folded flavin would finally decide which structure would be obtained. Similar explanations apply to other diamagnetic intermediates such as those found with D- and L-amino acid oxidase [17, 18].



Scheme 2. *Hypothetical scheme for the explanation of hidden flavoprotein sulfur radicals*

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W. H. Walker's present address
Division of Molecular Biology
Veterans Administration Hospital
42nd Avenue and Clement Street
San Francisco, California 94121, U.S.A.

P. Hemmerich
Fachbereich Biologie der Universität
BRD-775 Konstanz, Postfach 733, Germany

V. Massey
Department of Biological Chemistry
University of Michigan
Ann Arbor, Michigan 48104, U.S.A.