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CHEMISTRY AND MOLECULAR BIOLOGY OF FLAVIN IN THE "FULLY REDUCED" STATE

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In the history of flavin (bio)chemistry the "fully reduced state" (Fl_{red}) has drawn the least attention, as compared to the oxidized state ("flavoquinone," Fl_{ox}) and the radical state ("flavosemiquinone," $\dot{F}l$). The reason for this is to be found not so much in the realm of science, but in the psychology of scientists: Fl_{red} does not exhibit an optical spectrum as distinctive as Fl_{ox}, and even worse, it appears colorless to the eye in dilute solutions. Furthermore, unlike Fl_{ox}, it does not fluoresce. Thirdly, it is extremely unstable towards O_2. Fourthly, it is diamagnetic, unlike $\dot{F}l$. Hence, it does not seem to lend itself to "elegant" work.

Quite in contrast to this superficial evaluation, the following becomes obvious upon somewhat more detailed inspection:

(1) The absorption spectra in the visible and near-UV range of Fl_{red} are quite peculiar and call for structural interpretation.

(2) The fact that Fl_{red} has the properties of an "O_2-activator" renders it even more peculiar in view of its diamagnetism: Generally, the ground-state triplet species O_2, chemically inert because of its highly symmetrical electron distribution, needs paramagnetic species for activation—i.e. heavy metal complexes, in order to facilitate intersystem crossing.

The first researchers to assign a chemical structure to Fl_{red} were Kuhn and Weygand[1]: They proposed 1,5-dihydroflavin as shown below. Since the reduction of Fl_{ox} is thermodynamically reversible (cf. polarography[2]), we term 1,5-H_2Fl_{red} "flavohydroquinone." Kuhn's assignment of the 1,5-H_2Fl_{red} structure to "fully reduced flavin," though correct, seems more intuitive than experimentally founded, whereas the assignment of the $\dot{F}lH$-structure as "1-monohydroflavin" proposed at the same time,[1,3] proved to be incorrect.

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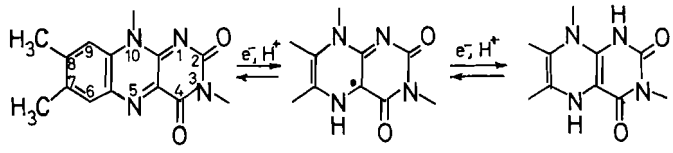
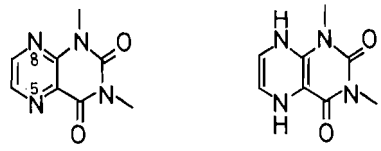
As Müller *et al.*[4] will show in the present symposium, neutral Ḟl has the structure of a 5-monohydroflavin (5-HḞl):



Fl_{ox} 5-HḞl $1,5\text{-}H_2Fl_{red}$



Pt_{ox} $5,8\text{-}H_2Pt_{red}$

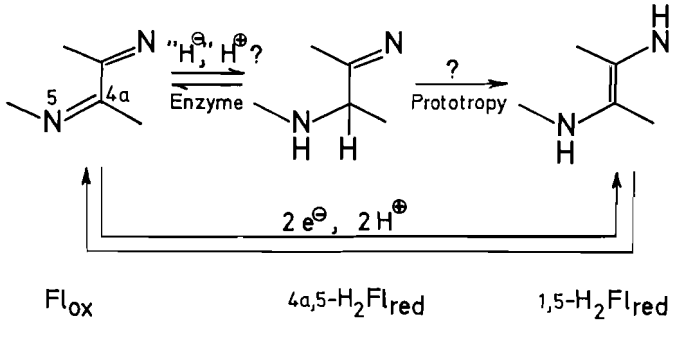
The structure assigned by Kuhn and coworkers to Fl_{red} is not as obvious as it might seem, although it was accepted from the very beginning without criticism: It is well known that the "pyrimidine part" of the flavin nucleus, or better: the N-CO-NH-CO "sub-chromophore" of positions 1–4, is electronically isolated from the residual ("quinoxaline"-)part of the tricyclic system. This is most convincingly borne out by the evaluation of the spin distribution of Ḟl-species.[4] Therefore, the highest occupied molecular orbital of $1,5\text{-}H_2Fl_{red}$ or flavohydroquinone is reminiscent of a 1,4-dihydropyrazine system, a prototype of "*anti*aromaticity"[5]: It is indeed a peculiarity of the flavin system, that the "antiaromatic" dihydro-isomer is the most stable among the possible isomers. It would be more surprising yet, if it were the only isomer of biological importance. In fact, the biologically occurring heteroaromatic compound most closely related to flavin—i.e. lumazine or pteridine-2,4-dione (Pt_{ox}) (see scheme above) does not by any means form a 1,5- or $5,8\text{-}H_2Pt_{red}$ analog. The "flavin-like" dihydropteridine is the only one of four possible dihydroisomers which is still unknown.[6]

If one looks into the pyrazine part of the flavin nucleus for potential acceptor positions, it appears obvious that the bridge carbons 5a and 9a between pyrazine and benzene rings can be neglected because these two rings are strictly in resonance. The same is true for position 10a, which appears to be a part of a cyclic amidine. In contrast, addition to position 4a would block an "azomethine" subfunction in Fl_{ox}: Cyclic azomethines are well known to undergo nucleophilic addition in electron-deficient heteroaromatic species, as was elegantly pointed out in the work of Albert and his school.[7] Therefore on theoretical grounds $4a,5\text{-}H_2Fl_{red}$ could be expected to be an Fl_{red}-species

potentially competitive with flavohydroquinone. Furthermore, since "4a-reduction" of flavin means formation of a bond to carbon (and not to nitrogen, as in "5-reduction"), 4a,5-H_2Fl_{red}-formation would involve an *irreversible* reduction in contrast to 1,5-H_2Fl_{red}-(or "flavohydroquinone") formation.

A number of results obtained in biochemical work support the notion that "irreversible flavin reduction" does indeed occur. First, Gawron[8] has postulated "4a-addition"—i.e., "hydride" addition to C(4a), in order to explain the stereochemistry of flavin-dependent dehydrogenation of succinic acid. An indication for similar phenomena is provided by Strittmatter's work[9] on NADH-cytochrome-b_5 reductase where the exchange of hydrogen isotopes at the flavin site appears to be slow. This may, of course, be due to restricted access of water to the active site, but "4a-reduction" appears to explain this result more plausibly, as NADH-cytochrome b_5 reductase exhibits a "blue semiquinone,"[10] which generally indicates accessibility of water to the active site.[11]

One should bear in mind that "5-addition" involves a strictly "in-plane" attack at the coplanar Fl_{ox} moiety, whereas "4a-addition" requires "out-of-plane" approach. Consequently, 4a-addition poses a stereochemical problem, whereas 5-addition does not necessarily do so. Furthermore, one has to differentiate between 4a-addition or removal of a "hydride equivalent," on the one hand (whatever this might be, it is unlike a proton), and 4a-addition or removal of a proton, on the other hand. In fact, prototropy from C(4a) to N(1) may occur rather rapidly, but probably not fast enough to compete with flavoenzyme catalysis:



MODES OF FLAVOQUINONE REDUCTION

In the past few years, methods have been developed which afford an experimental approach to test the validity of the ideas outlined in the preceding chapter. We found that there are several quite different modes of flavoquinone reduction, depending on the reductant.

Catalytic hydrogenation

We observed in many experiments that catalytic hydrogenation of flavin leads to uptake of more than one equivalent of molecular hydrogen (Fig. 1). This was not understood until we tried CF_3COOH as solvent, when we suddenly obtained quantitative "overreduction" with a total consumption of eight redox equivalents. The resulting compound was shown to be a 1,5,5a,

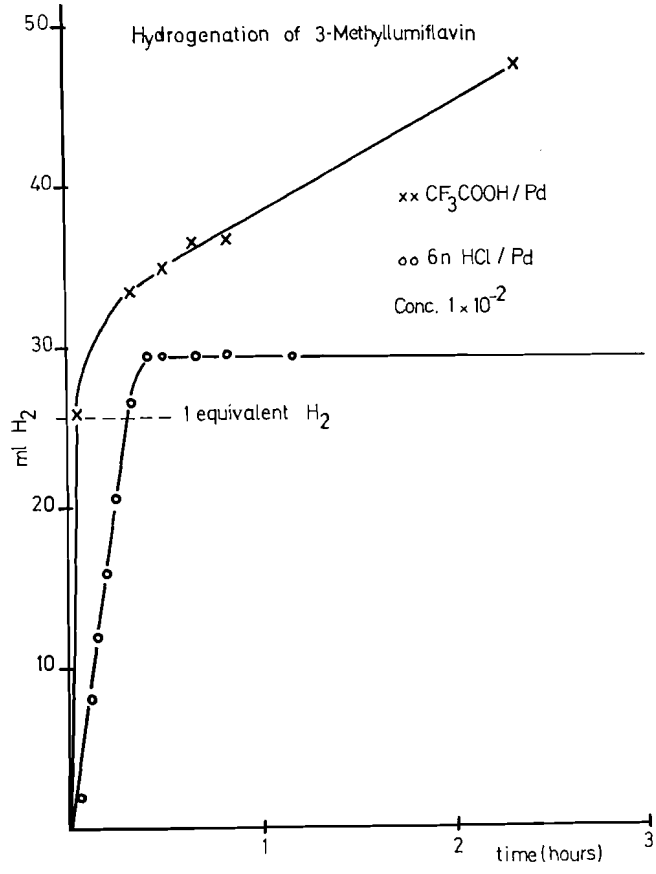
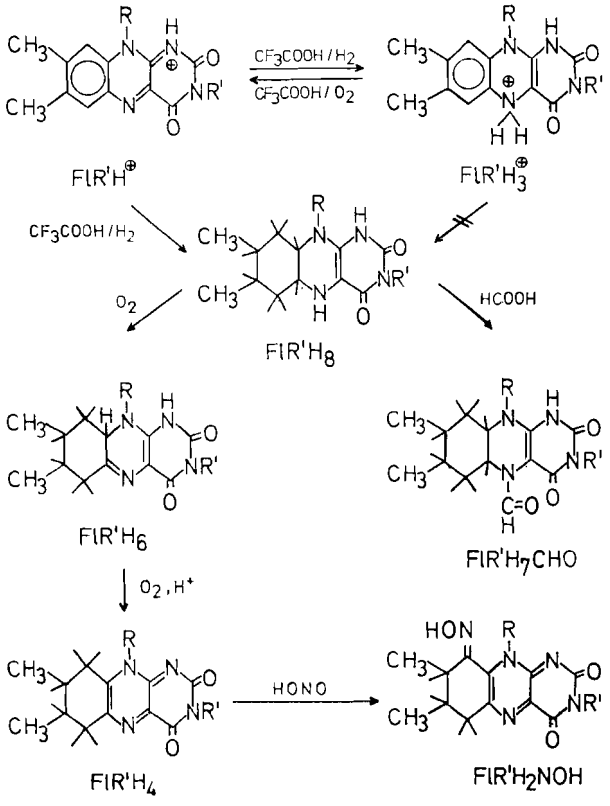


Fig. 1. Course of catalytic hydrogenation of flavoquinone.

6,7,8,9,9a,10-octahydroflavin or cyclohexanotetrahydropteridine (see Fig. 2). This compound, upon reoxidation, behaved like a normal tetrahydropteridine. This new and unique path from the flavin into the pteridine series has been fully elucidated.[12] The feature most interesting with respect to flavin chemistry is that the irreversible "overhydrogenation" of flavoquinone does

not occur via 1,5-dihydroflavin. In contrast, the overreduction is much slower than the formation of 1,5-H_2Fl_{red} and operates only under conditions of hydrogenation, where the "dihydrogenation" equilibrium is not fully displaced towards Fl_{red}. This is true with CF_3COOH as solvent, where reversible hydrogenation does not appear to proceed further beyond the radical state. Therefore, H_2-uptake in the benzenoid nucleus must be assumed to occur with $Fl_{ox}H^+$, competing with the fast but incomplete and reversible formation of



R = -H, -CH_3, ribityl, ribityl - P

R' = -H, -CH_3

Fig. 2. Irreversible catalytic hydrogenation of, and pteridine synthesis from, flavins.

$1,5\text{-}H_2Fl_{red}$. The possibility that the radical cation $\dot{F}lH_2^+$ might be responsible for further reduction can be excluded by the observation that 5-alkyl-1,5-flavohydroquinones ($5\text{-}RFl_{red}H$) do not enter this reaction, although they do form normal radical cations, $5\text{-}R\dot{F}lH^+$. They cannot, however, form normal flavoquinone cations, since normal $Fl_{ox}H^+$ is identical with $1\text{-}HFl^+{}_{ox}$ —i.e., Fl_{ox} protonated at N(1) and not at N(5).[13]

Reduction by borohydride

BH_4^- reacts only slowly with flavins, so that under aerobic conditions no reduction is observed at all, because reoxidation is faster than reduction. Light accelerates the reductive step and the "fast" product of this reduction is the normal flavohydroquinone (Fig. 3). If this reaction is continuously reversed by an excess of O_2, a second, irreversible path of reduction becomes dominant, which leads to "3,4-dihydroflavin." This reaction has been elucidated and its products characterized.[14] Clearly, the reduction of the 4-carbonyl group in Fl_{ox} by BH_4^- is abnormal and biologically insignificant. But the

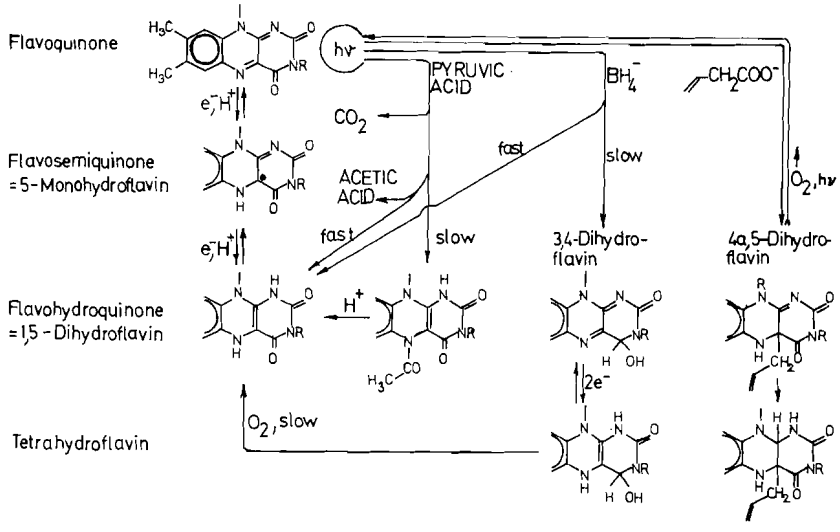


Fig. 3. Modes of reversible and irreversible reduction of flavins.

biologically interesting result emerges from the fact that 4-CO-reduction can be brought about in amino acid oxidases,[35] leading to *biologically active* 3,4-dihydroflavoproteins. Therefore, position 4 cannot play a role at least in these flavin-catalyzed dehydrogenations. This again gives support to the assumption that the "active site of the flavin nucleus" is the 4a,5-C=N-bond.

Reduction by electrons (Fig. 3)

Flavoquinone is reduced by Fe^{2+} at pH>7 as well as by Ti^{3+} and Sn^{2+} at pH<1, and by metals, yielding flavohydroquinone. Dithionite reacts similarly, although there are many indications for a SO_2^{2-}-unit–i.e., two electrons at a time, being transferred through an unstable sigma-bonded intermediate.[16] The structure of flavohydroquinone is non-coplanar (see below).

(Photo)reduction by "hydrogen donors"

Flavins are reduced by amines and light. In this process the "substrates" undergo breakage of a CH-bond in the position α to the functional group.[17] The first reduction product to be seen in most of these reactions is again flavohydroquinone. The same reaction is observed with other activated methylene centers, as in the reduced nicotinamide nucleotides,[18] with keto acids and (di)sulfides, but not alcohols and acylated amines.[19] This suggests that "hydrogen transfer" (Fig. 4) is the primary reaction. A hydrogen transfer could be simulated, however, by a group transfer, if only the group R^-, transferred along with an electron pair to make up a σ-bond to the flavin nucleus, is able to dissociate without activation (or with low activation) from the nucleus in the form of R^+ (see Fig. 4). Clearly, this prerequisite is fulfilled for the "substrate" residues mentioned above, which are of the type R-=>CX-, X being a donating group like NH_2, NR_2, SR, or a vinylogue of these (e.g., in NADH).

Nucleophilic (photo)alkylation of Fl_{ox}

We have demonstrated a chemical example of such a group transfer, where the R-Fl intermediates could be isolated (R=benzyl or allyl (Fig. 3),[20] although these intermediates are surprisingly reactive and allow a catalytic cycle of flavin oxido-reduction to be established under suitable conditions. Similarly, photoacylation (Fig. 3) was discovered.[21]

Simultaneously it was shown that alkyl transfer toward the flavin nucleus may occur at positions 4a as well as 5.[20] Studies which aim at differentiating precisely between H-and R-transfer in the case of more reactive R groups are under way in our laboratory. The direct photochemical synthesis of these alkyl-dihydroflavins is, however, limited by the fact that only "active" akyl residues can be introduced, like allyl and benzyl—i.e., residues containing a frontier orbital of largely π-character.

Electrophilic alkylation of Fl_{red}

To understand flavin reactivity in terms of Klopman's concept of frontier and charge-controlled substitution,[22] it was desirable to check the electrophilic alkylation of Fl_{red}. The only reactions of this type that were previously known used "stable" flavohydroquinone derivatives as starting materials—

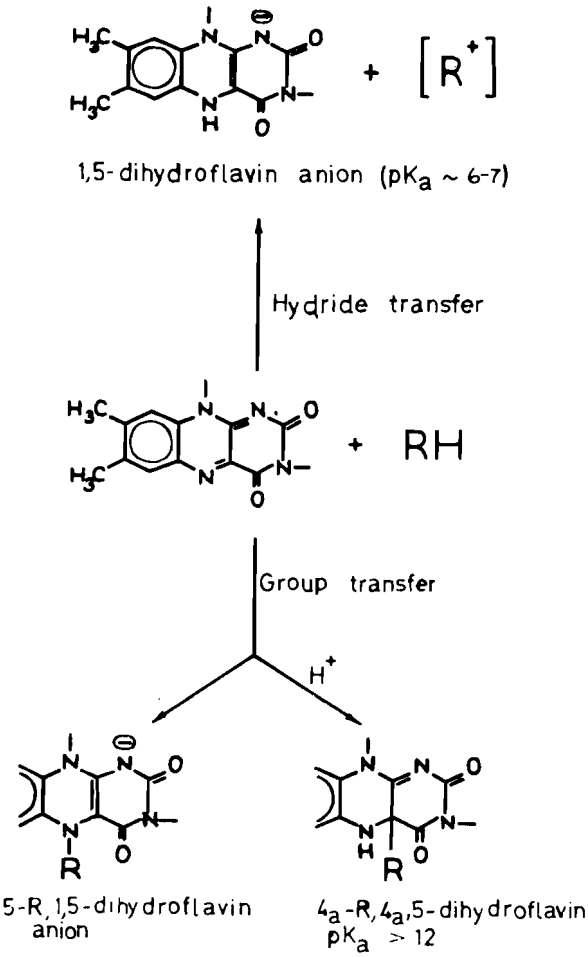


Fig. 4. Schematic outline of group versus hydride transfer.

e.g., 5-acetyl Fl_{red} H, compounds which are not attacked by O_2.[23] These compounds are alkylated at the oxygens of positions 2 and 4, or at O(2) and N(3), but never at N(1) (Fig. 5). This is apparently due to overcrowding effects between N(1) and N(10), which prevent peri-disubstitution.[23]

If, on the other hand, 1,3-dialkylated lumichrome (Fig. 6) is the starting material, O-alkylation does not occur, and positions N(10) and C(4a) compete for the substitution.[24] Surprisingly, unprotected Fl_{red} behaved entirely differently from 5-acetylated Fl_{red} in the alkylation (Fig. 7).[25] Although there should be a much higher charge density at O(2,4) of the reacting anion

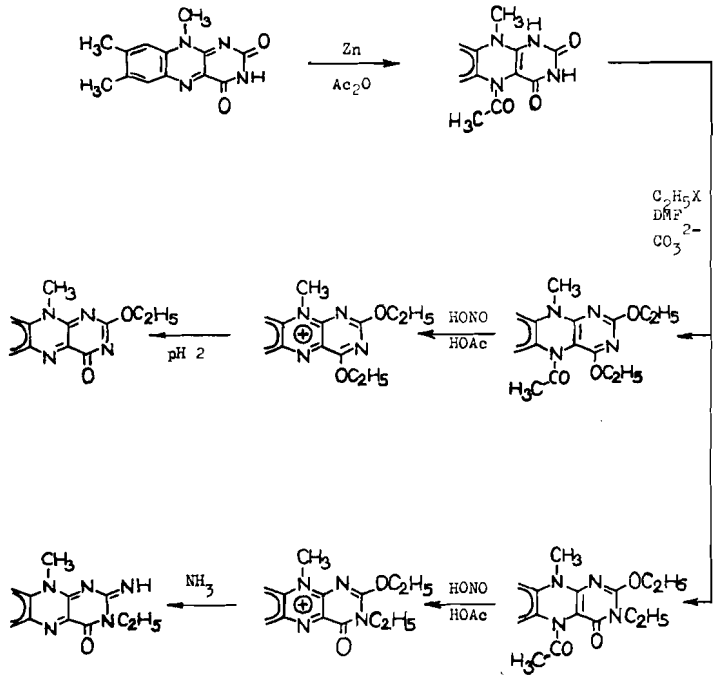


Fig. 5. Alkylation of 5-protected dihydroflavin.

$Fl_{red}H^-$ (=$5HFl^-_{red}$!), the reaction with saturated alkylating agents occurs preferably at N(5), yielding mainly the 5,5-dialkyl-1,5-dihydroflavins and, to a minor extent, also 4a-substitution products.

The proof of twofold N(5)-substitution is given by NMR: 5,5-dimethyl-1,5-dihydroflavin shows a sharp signal (6 protons) at $\delta = 4.13$ ppm ($CDCl_3$).[25] This signal lies at relatively low field and can therefore only be assigned to methyl groups attached to oxygen- or electron-deficient nitrogen. The corresponding ethyl analog shows a well-resolved ABX spectrum (δH_A-H_B = 1.83 ppm (=111 cps); J_{AB} = 12 cps), as shown in Fig. 8. These results can only be explained if one assumes diastereoisotopic* disubstitution at a highly polar asymmetric center. 5,5-disubstituted flavins are mesoionic in neutral solution (Fig. 6). They are protonated at N(1) with a pK_a of 3.7.[25]

The ease with which N(5) is substituted and even doubly substituted is surprising in view of the fact that this nitrogen is neither basic ($pK^{H^+}_{N(5)H_2^+}$

* For scope and limitation of the term "diastereoisotopism," see: K. Mislow and M. R. Raban, *Topics in Stereochemistry*, Interscience (Wiley and Sons), 1967, Vol. 19 ff.; and M. Gorkam and G. E. Hall, *Quart. Rev. London*, *22*, (1968) 14.

<O) nor acidic ($pK^{H^+}_{N(5)H}$>14). In spite of this, N(5) is not a true "pyrrole"—i.e., it has no coplanar (sp^2, pz) nitrogen. Thus the geometry of the nitrogen centers of 1,5-$Fl_{red}H_2$ becomes most important.

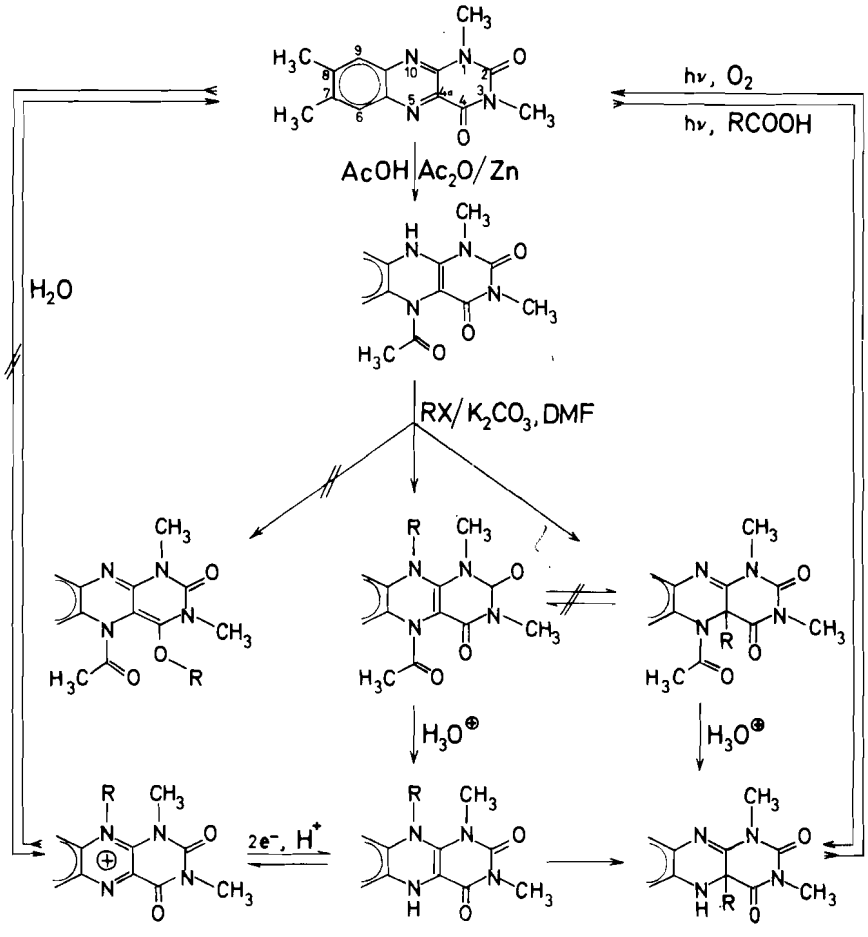


Fig. 6. Alkylation of 5-protected dihydrolumichrome.

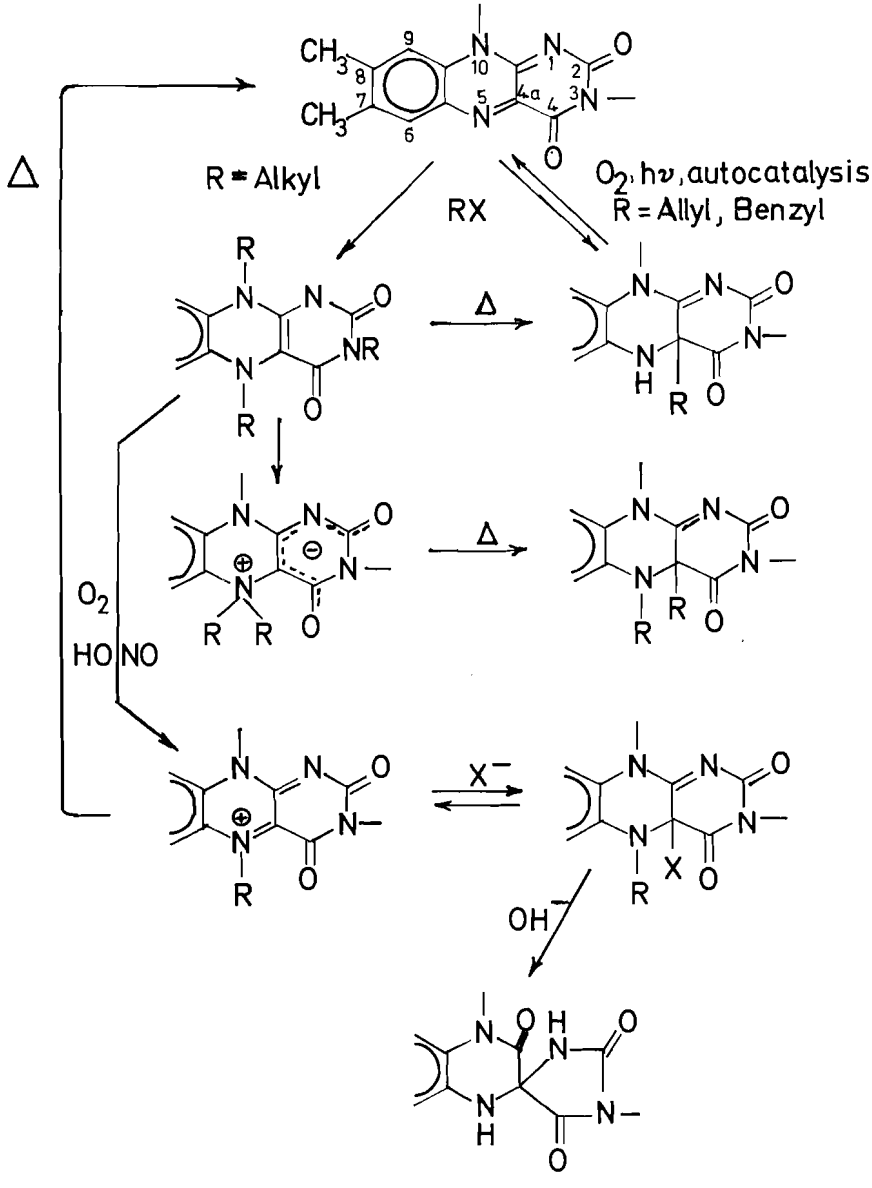


Fig. 7. Direct alkylation of flavohydroquinone. Reaction conditions are either DMF/K_2CO_3 or $CHCl_3/(C_2H_3)_3N$ at 20–50° C for ½–5 h., with simultaneous addition of excess alkylating agent. Flavoquinone was prereduced by adding conc. $S_2O_4^{2-}$ aq. to the DMF solution or shaking the $CHCl_3$ solution with aq. $S_2O_4^{2-}$.

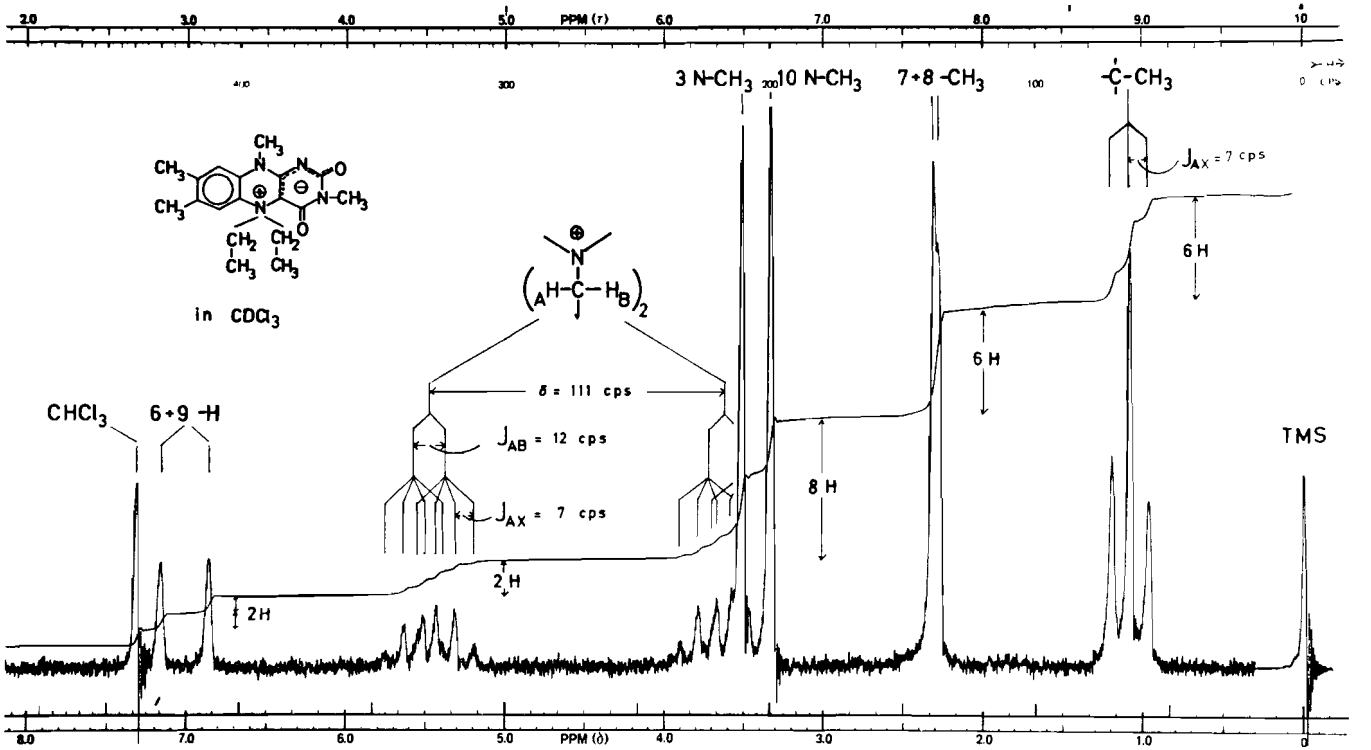


Fig. 8. NMR of 5,5-diethyl-substituted mesoionic flavohydroquinone.

THE STRUCTURE OF FLAVOHYDROQUINONE

In 1964 we pointed out[13] that 1,5-H_2Fl_{red} must be assumed to deviate considerably from coplanarity because of its optical properties: Fig. 9 shows once more the spectrum of 1,5-H_2Fl_{red} with the very characteristic shoulder at 390–400 nm of $\varepsilon = 4000\ M^{-1}\ cm^{-1}$. If the flavohydroquinone is folded along the N(5) N(10) axis, then it is easily understandable that this transition will reflect the degree of folding: In the long-wave "tail" of the Fl_{red}-absorption a n-π^* transition is hidden, which becomes more allowed (or more π-π^*) as the molecule tends to flatten. Hence, this transition reflects "tricyclic" electron delocalization in the Fl_{red}-nucleus. Again, from the UV-spectrum[13] one must conclude that the anion HFl^-_{red} is more strongly folded than the neutral H_2Fl_{red}, and this is easily understood: Since the whole Fl_{red} nucleus is extremely electron-rich, tricyclic delocalization should be decreased by the formation of a full negative charge in the pyrimidine nucleus—i.e., through increased folding.

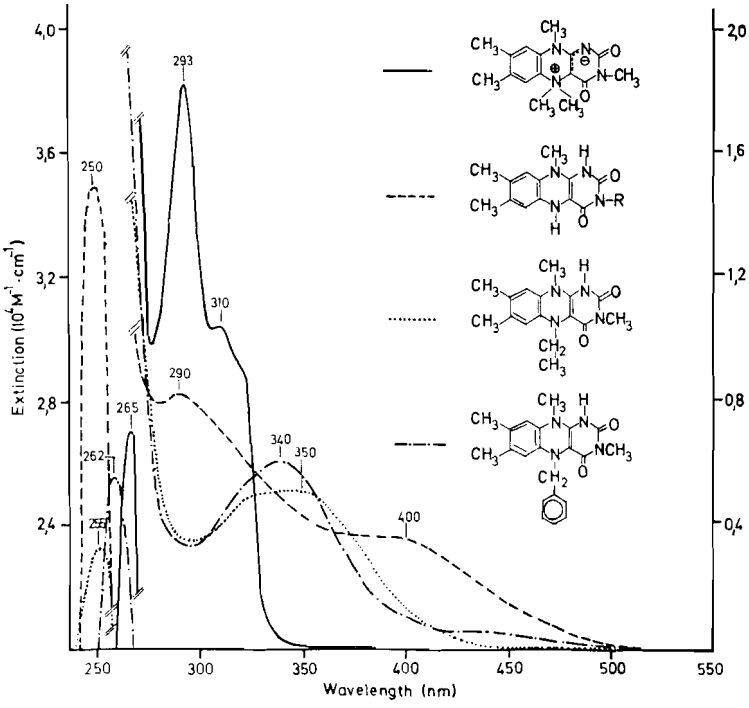


Fig. 9. UV-spectra of neutral flavohydroquinones, and influence of substituents.

A similar conclusion can be drawn from the bathochromic shift observed in $FADH_2$ as compared to $FMNH_2$ (Fig. 10): The adenine-flavin interaction in a "hairpin" configuration clearly tends to flatten the Fl_{red}-nucleus somewhat over the extent found in $FMNH_2$. The spectral difference shown in Fig. 10 is absent at pH 3, when protonation of the adenine moiety prevents the "hairpin" configuration.

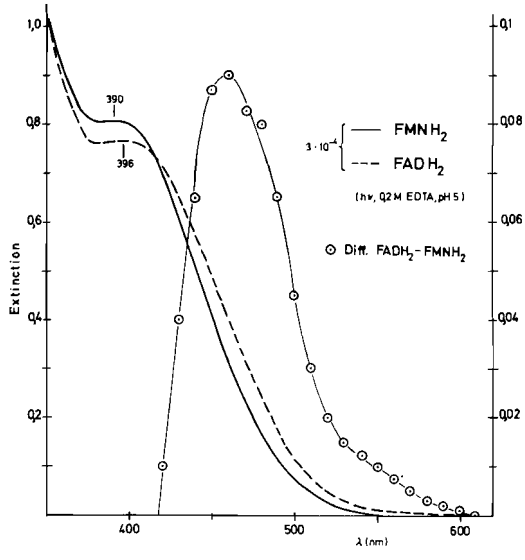


Fig. 10. Influence of FAD-stacking on the long-wave spectrum of $Fl_{red}H_2$.

Substitution at the center nitrogens causes further bending of the molecuel through electronic as well as steric effects. This is clearly reflected in the spectra (Fig. 9). In 5,5-disubstituted compounds, no absorption at >330 nm remains. Consequently, the cations do not reflect steric or electronic influence of substituents at N(5) in their UV spectra (Fig. 11), but only the symmetry of substitution. Therefore 5,5-dialkyl and 5,5-diprotonated species have similar light absorption (315 and 318 nm), whereas 5-monoalkyl, 5-monoprotonated species are different (305 nm). Kierkegaard *et al.*[26] provided final proof of the structure, as shown in Fig. 12.

It is clearly seen from this that a stable "butterfly wing" configuration generates centers of chirality at N(5) and N(10). From this it follows that hydride or group transfer towards N(5) involves a stereochemical problem, in a way quite similar to that of hydride transfer from alcohols towards nicotinamide

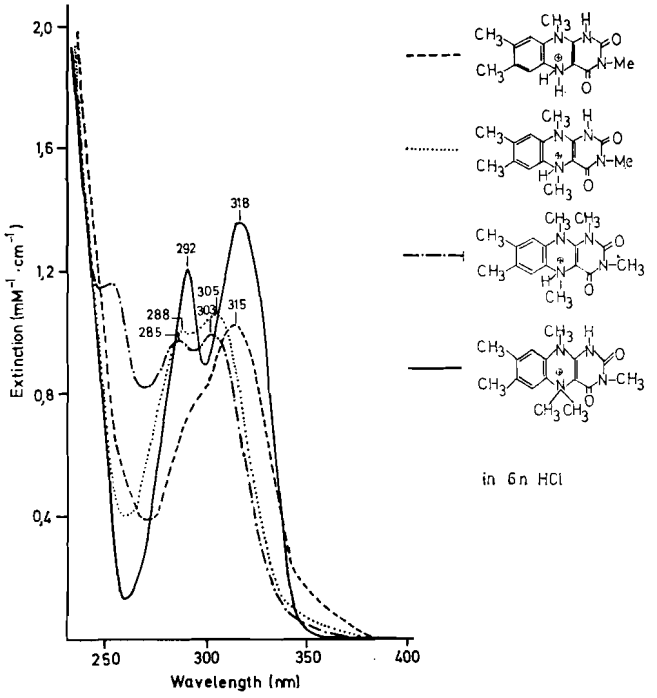


Fig. 11. UV-spectra of flavohydroquinonium cations, and influence of substituents.

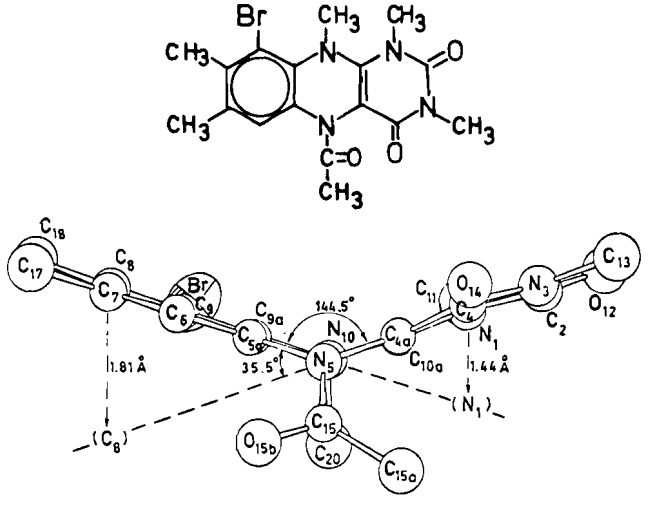


Fig. 12. Crystal structure of 5-acetylflavohydroquinone (Courtesy of Dr. Peder Kierkegaard).

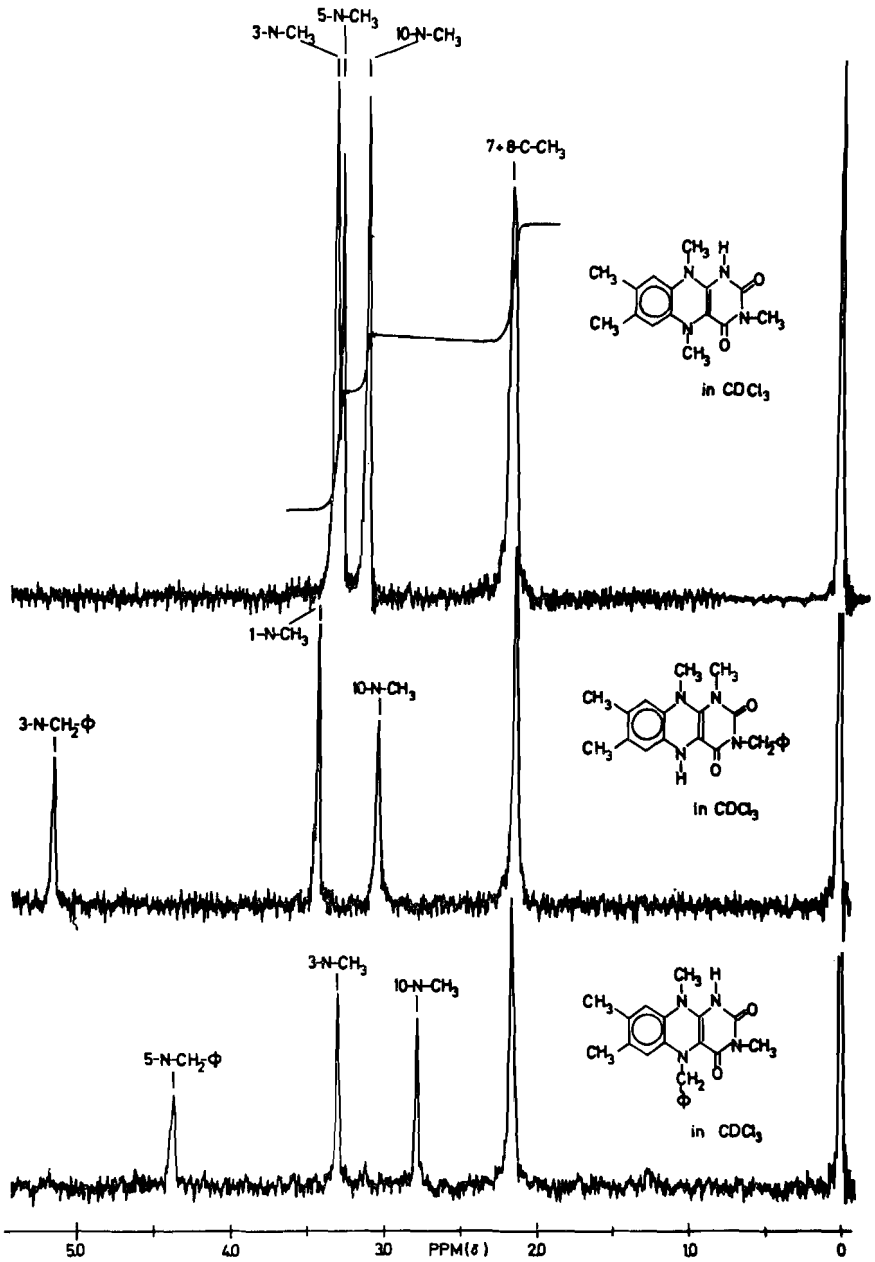


Fig. 13. Influence of substituent overcrowding on N(10)CH_3 chemical shift in flavohydroquinones.

nucleotides. In the latter case, however, the chiral center, once formed, remains stable, while the critical parameter in the flavin case must be the inversion time of the nitrogen center as compared to the rate of flavin catalysis. It can be assumed that flavohydroquinone inversion at the active site of enzymes is slow, or even impossible—in other words, the apoprotein determines which diastereomeric state of Fl_{red} is stabilized upon reduction.

Clearly, it remains very important to know the quantitative inversion characteristics of the free Fl_{red} species. We hope to resolve this question by NMR. Preliminary data show that substituent overcrowding in positions 1, 10 and 4,5 influences the bending. We conclude this from the position of the N(10) CH_3 signal, which is increasingly shifted toward higher field throughout the series of model compounds listed in Fig. 13. This reflects the increasing sp^3-character of N(10).[25]

PROPERTIES OF ALKYL-DIHYDROFLAVINS

The most important feature of alkyl-dihydroflavins is the ease of addition and removal of substituents. In general, 4a-and 5-monosubstituted derivatives can be recognized not only by their different courses of reoxidation, but also by differences in protonation.

4a-alkyl-dihydroflavins are protonated at N(1), yielding a bathochromic shift~360→390 nm (Table 1), while N(5)-isomers will be protonated at N(5) in acid, yielding a hypsochromic shift~340→305 nm. Through this shift, 5,5-dialkyl quaternary salts are easily distinguished from 4a,5-dialkyl isomers. The ease of removal of substituents from alkyl-dihydroflavin is summarized in Table 2.

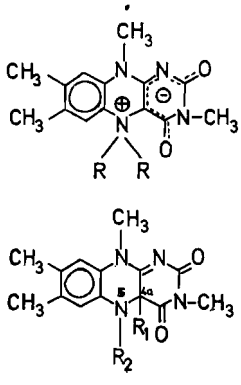


TABLE 1

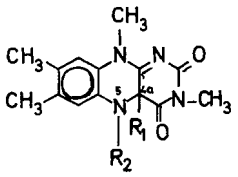
PROTONATION-INDUCED SPECTRAL SHIFTS IN 4A- AND 5-SUBSTITUTED DIHYDROFLAVINS

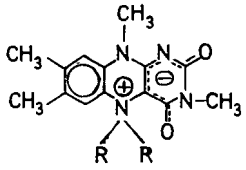
Compound	*pH 7* λ_{max} (*nm*) $\varepsilon(x10^4)$	*6N HCl* λ_{max} (*nm*) $\varepsilon(x10^4)$	*Shift*
4a-Methyl	360 0.88	385 0.37	+25
4a-Allyl	362 0.56	399 0.23	+37
4a-Benzyl	365 0.70	395 0.42	+30
4a, 5-Dibenzyl	345 0.65	365S 0.35	+20
4a-Allyl-5-methyl	330 1.16	315 0.94	−15
4a, 5-Diethyl	327 0.97	322 1.01	− 5
5-Methyl	355 0.55	305 1.06	−45
5-Ethyl	330 0.60	305 1.06	−25
5-Benzyl	340 0.58	307 0.88	−33
5,5-Dimethyl	310 1.04	318 1.36	+ 8
5,5-Diaethyl	310 0.93	318 1.21	+ 8

TABLE 2

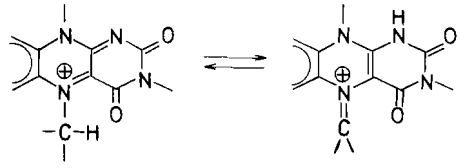
CONDITIONS OF 4a- AND 5-DEALKYLATION OF DIHYDROFLAVINS. APPROXIMATE YIELDS OF FLAVOQUINONE (%), MEASURED AT 450 NM, AS OBTAINED AFTER MAX. 60 MIN. UNDER THE GIVEN CONDITIONS. NO QUANTIFICATION MEANS LESS THAN 10 PERCENT YIELD. IN PARENTHESES, FATE OF SUBSTITUENT, IF ANALYZED (GAS CHROMATOGRAPHY)

			50% *AcOH*			6*N HCl*
Compound	O_2	O_2, $h\nu$	*HONO*, 20°	*HONO*, $h\nu$	*HONO*, 90°	*HONO*, 90°
4a-Methyl	—	—	—	—	—	—
4a-Allyl	—	98				
4a-Benzyl	—	98(φCHO)				
4a, 5-Dibenzyl	—	—	—	—	34	99 (φCOOH)
4a-Allyl-5-methyl	—	—	—	—	96	
4a,5-Diaethyl	—	—	—	—	—	—
5-Methyl	—	83	100(CH_2O)			
5-Aethyl	—	—	—	93(AcOH)	31	
5-Benzyl	100(BzOH)					
5,5-Dimethyl	—	—	—	—	—	92(HCOOH)
5,5-Diaethyl	—	—	—	—	—	—





Saturated alkyl in position 4a is the only substituent which cannot be removed at all without destruction of the heteroaromatic nucleus. Even the most stable residues, like methyl and ethyl, are easily split from N(5) by oxidation. These observations support the idea that alkyl-dihydroflavins may act as intermediates in the catalysis of flavin enzymes. The important species in this context, however, are not the dihydroflavins themselves, but their oxidation products, 5-alkyl-flavoquinonium salts and the corresponding ψ-bases (Fig. 6). In this state electron withdrawal from the 5α-CH bonds towards the extremely electron-deficient nucleus may help dehydrogenate the N(5)-substituent by the prototropic shift from a 5-alkyl flavoquinonium salt towards a 5-alkylidene flavohydroquinonium salt, which in turn is easily hydrolyzed to yield "free" $Fl_{red}H_2$ and aldehyde, as shown below.



The course of spectral changes during the 5-RFl^{+}_{ox}→5-$RFl_{ox}OH$ interconversion is shown in Fig. 14. More detailed work is in progress.

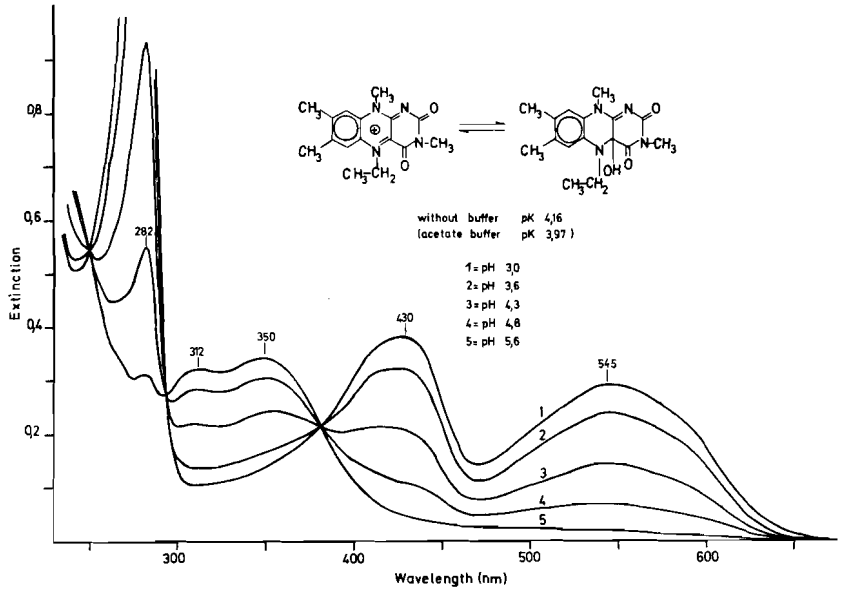


Fig. 14. 5-ethylflavoquinonium ion and spectral changes during neutralization.

It is our hope that these studies may encourage biochemists to take an active interest in the properties of flavins in the fully reduced state, as they have done so successfully in the recent past with regard to the oxidized and semiquinoid states of these compounds. We would predict that such endeavors will produce new insights into the mechanism of flavoprotein catalysis.

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DISCUSSION

CHANCE: I would like to ask about your mechanism for pyridine nucleotide-flavoprotein interaction. Were the products fully oxidized pyridine nucleotide or radical?

HEMMERICH: The products would be oxidized pyridine nucleotide and reduced flavin, as shown at the bottom of Fig. 1, below. Figure 2, p. 104, suggests a chemical mechanism for hydrogen ion transfer in a pyridine nucleotide-flavin complex. The radical would be the product of artificial homolytic splitting of the complex, as shown on the right-hand side of Fig. 1. This experiment could be most easily done by shining light on the very sensitive intermediate.

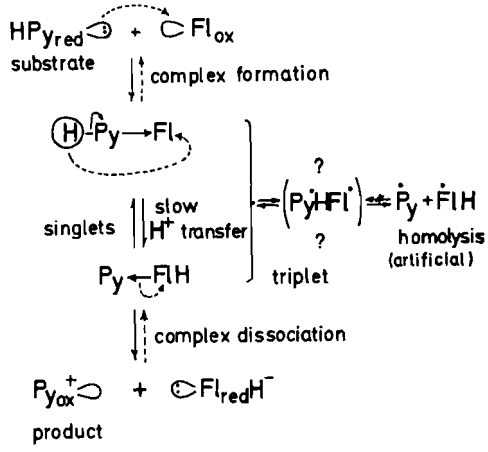

Fig. 1. Flavin substrate complexes.

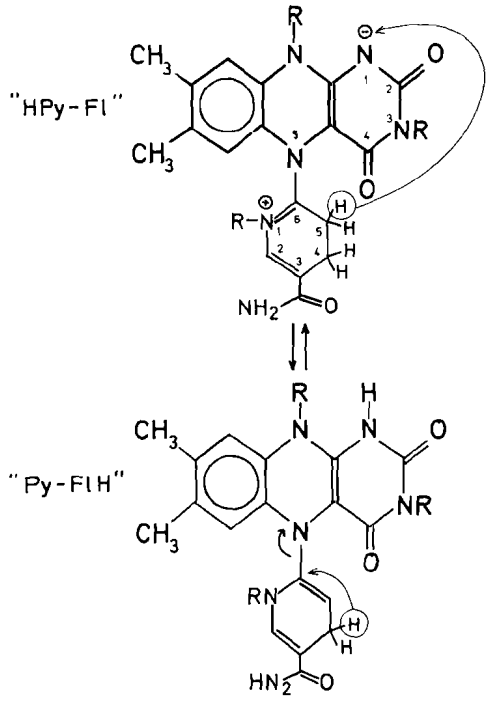

Fig. 2. A possible mechanism for hydrogen ion transfer in a pyridine nucleotide-flavin complex.

ORME-JOHNSON: You were dealing with nicotinamide, is that correct?

HEMMERICH: Yes.

SONG: The pK of the triplet estimated from a log $t_{1/2}$ vs. pH plot is subject to severe spectroscopic complications because pH will not only affect the triplet population and rate of photoreduction, but also other processes, such as fluorescence, radiationless transition, and intersystem crossing. Therefore, the apparent pK of the triplet which you estimated is the result of combinations of many factors, rather than just the $pK_a(^3Fl^*_{ox}H^+)$ involved in the rate-determining step.

HEMMERICH: All I can conclude from Dr. Song's question is that he neglects the definition of the term pK_a: A pK_a is detected by the observation of a pH-dependent change in a physical property of a given system, if the change fits the law of mass action. From this, it follows that the pH-dependent change of reaction rate which we observed must be due to an acidic dissociation. I

have explained that we do not know to which species this observed pK_a belongs, and that we propose to assign it to the $(Fl^*_{ox}H^+)$-pK_a since we cannot imagine any other species being rate-limiting and having a pK_a in the observed range. We may be wrong with this assignment, but we cannot be wrong, I believe, in assuming that this effect reflects a true pK_a.

MASSEY: I think a point of clarification is required because there is a lot of putting carts before horses here today. Dr. Hemmerich has shown, in his last illustration, a hypothetical mechanism, and some of you people are thinking that this is something which he has actually done or proved. This is a hypothetical mechanism for some new spectral intermediates with four different flavoproteins, which we reported on in Konstanz a couple of weeks ago and which I think some of the people from Ann Arbor will comment on later. I like your idea, Dr. Hemmerich, as I like most of your ideas. It would just be nice to have some real experimental evidence.

HEMMERICH: This is always very nice, if one has a lot of results one cannot explain. One tells anybody who wants to explain something of these results that he lacks experimental evidence!