# The Reduction of Flavins by Borohydride: 3,4-Dihydroflavin

## Structure, Absorption and Luminescence

F. Müller, V. Massey, C. Heizmann, P. Hemmerich, J.-M. Lhoste, and D. C. Gould

The University of Michigan, Department of Biological Chemistry, Ann Arbor, Michigan
Fachbereich Biologie der Universität Konstanz
and Harvard Medical School, Department of Biological Chemistry, Boston, Massachusetts

The reduction of flavins by $BH_4^-$ is slow, but strongly catalyzed by light. The main product of the reaction is 1,5-dihydroflavin, i. e. normal leucoflavin, which is rapidly reoxidized upon admission of air. Under continuous aeration, however, a second reaction, irreversible by oxygen in the dark, becomes dominant yielding a tetrahydroflavin which, upon exhaustive reaction of $BH_4^-$, is autoxidized to give a new dihydroflavin of structure I. This compound is stable toward further oxidation in the crystalline state and in solution with organic solvents. Compound I has to be considered as a formal 3,4-dihydroflavin. It is photooxidizable to give starting flavin, reacts very slowly with $S_2O_4^-$ compared to flavoquinone (II), but is easily reduced by EDTA in the presence of light. The compound was also synthesized by an unequivocal route and found identical with the product obtained by $BH_4^-$-reduction of lumiflavin. The light absorption spectrum of I shows absorption maxima at about 405, 320, 270, and 220 nm and is practically identical with that of the $BH_4^-$-reduction product of the flavoproteins D- and L-amino acid oxidase. The $BH_4^-$-reduction products of the amino acid oxidases are catalytically active. From this it follows that position 4 of the flavocoenzyme is not involved in enzymatic dehydrogenation.

The first photo-excited triplet state of I has been investigated by optical and electron paramagnetic resonance spectroscopy. The structure of I was elucidated by means of infrared and nuclear magnetic resonance spectroscopy.

Although flavins are well known to be ubiquitous redoxcoenzymes, much remains to be investigated in the chemistry of flavin oxido-reduction. It was recently shown by Massey et al. [1] that the flavoproteins D- and L-amino acid oxidase are readily reduced by $BH_4^-$ to give a product absorbing at 410 nm. The products thus obtained showed enzymatic activity. Hence the natural redox function of the active group was left undisturbed by the action of $BH_4^-$. Therefore, establishment of the chemical background of this reaction is of great enzymological interest. Obviously, action of $BH_4^-$ must result primarily in a hydride-transfer, i. e. two-electron transfer, to the flavin nucleus. A survey of the known features of flavin $2e^-$-reduction shows the following:

The normal product of flavin reduction (cf. Scheme 1, II), is the same as that of two consecutive one-electron reductions, yielding the well known 1,5-dihydroflavin III as anticipated by Kuhn et al. [2]. Compound III is termed "flavohydroquinone", since its formation is thermodynamically reversible, which implies rapid reoxidation of III also in the presence of small amounts of oxygen. Other "formal" two-electron transfers are exerted on flavins by photodecarboxylation of phenylacetate [3] and by the formation of the flavin sulfite adduct [4]. Apart from N(5)-substituted 1,5-dihydroflavins one observes hereby the formation of 4a-substituted 4a,5-dihydroflavins (IV). This leads to the question, whether or not flavin-dependent dehydrogenation of substrates might always imply a rapid 4a-addition, whereas the 1,5-dihydroflavin might arise from 4a,5-dihydroflavin by a secondary proton shift from position 4a to position 1 (IV → III, Scheme 1). This possibility was first suggested by Gawron [5].

1,5- and 4a,5-dihydrogenation delete the characteristic flavoquinone spectrum. The tricyclic electron delocalization in 1,5-dihydroflavin retains some color, which gives a brown-orange appearance to even very pure 1,5-dihydroflavin crystals and a reddish color to its concentrated solutions [6]. 4a,5-Dihydroflavin, on the other hand, shows a pale yellow color in a concentrated solution and in crystalline state. The $BH_4^-$-reduction of amino acid oxidases as observed by Massey et al. [1] with products absorbing maximally at 410 nm must therefore imply a different chemistry, which is elucidated in the present paper.

## EXPERIMENTAL PROCEDURE

### MATERIALS AND METHODS

Melting points were determined with a heating block (capillary) and are uncorrected. The infrared spectra were recorded with a Perkin-Elmer 234 spectrophotometer; all samples were pressed in KBr discs consisting of 100 mg KBr and 0.3—0.4 mg flavin. The numbers given in parentheses with the infrared spectra refer to the position of the functional group in the flavin molecule (cf. Scheme 1, formula I). The phosphorescence spectra of the triplet state of the flavins were recorded on an Aminco-Bowman spectrophosphorimeter. Fluorescence spectra were measured with the ratio recording fluorimeter previously described [7]. Light absorption spectra were recorded on a Cary model 14 spectrophotometer, 1 cm cells were used. Specially constructed anaerobic cells were used for flavin reduction experiments as described previously [8]. Nuclear magnetic resonance spectra were taken on Varian A 60, DP 60, and HA 100 NMR spectrometers using tetramethylsilane as an internal standard. Chemical shift values are expressed as $\delta$ in ppm. Electron paramagnetic resonance spectra were recorded on a X-band Varian spectrometer. Plates of MN-polygram SIL S-HR (binder:starch) from Macherey-Nagel and Co. (Düren, Germany) were used for thin layer chromatography. The abbreviations stand for the following solvent systems: (A) chloroform—ethanol (47:3), (B) benzene—isopropylether—ethanol (2:2:1), (C) $n$-butanol—ethanol—water (7:2:1), (D) chloroform—ethanol (7:3) saturated with water. Methanol of analytical reagent purity was a Baker Chemical Co. product. Lithium borohydride (99%) was an Alfa Inorganics Inc. product. Ether was a product from Mallinckrodt. $^2H_2O$ (99.7 atom %) was a Merck Sharp and Dohme of Canada Ltd. product. $NaB^3H_4$ was purchased from Nuclear-Chicago (13.2 mC/mg). The radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer. [5-$^2$H]Pyridine was purchased from Merck Sharp and Dohme of Canada Ltd.

The experiments were conducted at room temperature (22—25°) when not otherwise stated.

### SYNTHESES

#### N(3)-Methyl-3,4-dihydrolumiflavin
#### (I, $R_1 = R_2 = CH_3$, $R_3 = H$)

In a 1000 ml Erlenmeyer flask were placed 300 ml anhydrous ether, cooled in an ice bath to 3—6° and 350 mg $LiBH_4$ added. The mixture was illuminated by two 100 W tungsten lamps placed about 30 cm from the reaction vessel. To the practically clear illuminated ethereal $LiBH_4$ solution was slowly added during the course of 1 h 100 mg $N$(3)-methyllumi-flavin [9] (II, $R_1 = R_2 = CH_3$) dissolved in 300 ml absolute methanol. The reaction mixture was illumi-

nated throughout the procedure and air admitted frequently until no further change of the light absorption spectrum was observed, i. e. loss of the 445 nm absorption peak to about 90%. If at this state a colorless precipitate was formed, due to the formation of boric acid or other boron compounds, 50—100 ml methanol were added in order to obtain a clear solution (the precipitate caused a decrease in the yield of the product). The ice bath was removed and the reaction mixture illuminated further for 8 h with gentle stirring at room temperature. The ether was removed from the reaction mixture under reduced pressure at room temperature. To the residual methanolic solution were added 400 ml $H_2O$ and the mixture extracted 6 times with 150 ml volumes of chloroform. The chloroform phase was washed 3 times with 150 ml volumes of 0.3 M phosphate buffer of pH 7.0, the organic phase then dried with anhydrous $Na_2SO_4$ and evaporated to dryness under reduced pressure at room temperature. The yellow-brown residue thus obtained was suspended in 10 ml chloroform, the suspension warmed to 50° for a few minutes and allowed to stand for 30 min at room temperature. The bright yellow micro crystals weighed 40 mg (57%, based on the quantity of II used). 40 mg starting material was recovered from the filtrate by addition of ether.

The product crystallized from methanol or chloroform was spectrophotometrically and chromatographically pure (solvent mixtures A, B, C, D), but gave no consistent elementary analysis due presumably to boron impurities. Suitable purification was obtained by dissolving I ($R_1 = R_2 = CH_3$, $R_3 = H$) in 0.1 N perchloric acid to form the cation and reprecipitation by addition of aqueous sodium bicarbonate, whereupon bright yellow crystals, which decomposed at about 280° were obtained nearly quantitatively. (Found: C, 61.47; H, 5.91; N, 20.43. $C_{14}H_{16}O_2N_4$ requires C, 61.75; H, 5.92; N, 20.58.)

*Light absorption spectra:* in methanol: $\lambda_{max}$ 400 nm, $\varepsilon = 13900$; $\lambda_{min}$ 345 nm, $\varepsilon = 4280$; $\lambda_{max}$ 315 nm, $\varepsilon = 5780$; $\lambda_{min}$ 295 nm, $\varepsilon = 3220$; $\lambda_{max}$ 269 nm, $\varepsilon = 28600$; $\lambda_{min}$ 233 nm, $\varepsilon = 6200$; $\lambda_{max}$ 217 nm, $\varepsilon = 36600$; in 0.3 M phosphate buffer, pH 7.8: $\lambda_{max}$ 405 nm, $\varepsilon = 13900$; $\lambda_{min}$ 355 nm, $\varepsilon = 5500$; $\lambda_{max}$ 331 nm, $\varepsilon = 6100$; $\lambda_{min}$ 292 nm, $\varepsilon = 2500$; $\lambda_{max}$ 269, $\varepsilon = 23900$; $\lambda_{min}$ 227 nm, $\varepsilon = 5000$; $\lambda_{max}$ 215 nm, $\varepsilon = 30000$; in HCOOH: $\lambda_{max}$ 373 nm, $\varepsilon = 18100$; $\lambda_{min}$ 287 nm, $\varepsilon = 1250$; $\lambda_{max}$ 260 nm, $\varepsilon = 35100$; $\lambda_{min}$ 230 nm, $\varepsilon = 10150$; $\lambda_{max}$ 212 nm, $\varepsilon = 35350$; in chloroform: $\lambda_{max}$ 405 nm, $\varepsilon = 13900$; $\lambda_{min}$ 351 nm, $\varepsilon = 4160$; $\lambda_{max}$ 316 nm, $\varepsilon = 6450$.

*Infrared spectrum:* $\nu$ OH(4) 3130 cm$^{-1}$ (3.19 μ), 2880 cm$^{-1}$ (3.48 μ); $\nu$ CO(2) 1625 cm$^{-1}$ (6.16 μ).

*Nuclear Magnetic Resonance Spectrum:* ([5-$^2$H]-pyridine solvent): 7.75 $\delta$ (1 H, singlet, [C(6)-H]);

6.17 $\delta$ (1 H, doublet, [C(4)-H]); 4.82 $\delta$ (1 H, exchangeable, broad (approx.0.20 $\delta$), singlet [C(4)-OH]); 3.79 $\delta$ (3 H, singlet, [N(10)-CH$_3$]); 3.59 $\delta$ (3 H, singlet, [N(3)-CH$_3$]); 2.28 $\delta$ (3 H, singlet, [C(8)-CH$_3$]); 2.22 $\delta$ (3 H, singlet, [C(7)-CH$_3$]). The aromatic proton on C(9) is hidden under the pyridine absorption at 7.58 $\delta$.

If in the procedure described above $N$(3)-methyllumiflavin was replaced by lumiflavin (II, $R_1 = CH_3$, $R_2 = H$), the yield of the 3,4-dihydrolumiflavin was decreased to about 15% due to the solubility properties of the starting material and the smaller stability of the product towards oxygen. However, the isolated product was identical with that of the total synthesis (see below) as was judged by comparison of the light and infrared absorption spectra as well as thin layer chromatography.

A small amount of radioactive labeled I ($R_1 = R_2$ = $CH_3$, $R_3$ = H) was prepared in the way outlined above using 80 ml ether, 80 mg LiBH$_4$, 2.5 mg NaB$^3$H$_4$, 30 mg II and 80 ml methanol.

### Total Synthesis of 3,4-Dihydrolumiflavin
### (I, $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_2CH_3$)

100 mg $N$,3,4-trimethyl-6-($p$-carboxyphenylazo)-aniline [10] was dissolved in 150 ml glacial acetic acid and hydrogenated with palladium on carbon as catalyst at room temperature and atmospheric pressure yielding VI. The suspension was filtered to remove the catalyst and 150 mg isodialuric acid (V) [10] and 150 ml absolute methanol added to the filtrate. The reaction mixture was stirred mechanically at room temperature for 30 min and then allowed to stand at 6° for 6 h. To the brown-red reaction mixture were added 700 ml H$_2$O and the mixture extracted 2 times with 300 ml chloroform. The combined organic phase was washed 3 times with equal volumes of 0.3 M phosphate buffer, pH 7.0 in order to remove the acetic acid. The organic phase was dried with anhydrous Na$_2$SO$_4$ and evaporated to dryness under reduced pressure at room temperature. The residue was dissolved in 7 ml chloroform, filtered and allowed to stand at −6° for 10 h during which time yellow crystals formed. The collected crystals were dried with ether giving 30 mg of I. A further 10 mg of I was obtained by concentration of the filtrate to about 3 ml and allowing it to stand at −6° for 10 h. The total yield was 42% of 3,4-dihydrolumiflavin (I) which decomposed at about 150°. The product was several times recrystallized from small volumes of chloroform until only one spot was observed on the thin layer chromatogram (solvent systems as described above). (Found: C, 61.19; H, 6.24; N, 18.99. C$_{15}$H$_{18}$O$_2$N$_4$ requires C, 61.0; H, 6.40; N, 18.90.)

Chloroform contains ethanol as stabilizer, hence the ethanol content of I ($R_1$ = $CH_3$, $R_2$ = H, $R_3$ = $CH_2CH_3$). This compound has enhanced solubility in all organic solvents compared to I ($R_1 = R_2$ = $CH_3$, $R_3$ = H). This indicates that the 4-OH group is replaced by −OH$_2$CH$_3$ (see nuclear magnetic resonance spectrum below). 3,4-Dihydrolumiflavin can also be synthesized by BH$_4^-$-reduction of lumiflavin by the procedure given above for I ($R_1 = R_2$ = $CH_3$, $R_3$ = H). The product thus obtained is somewhat difficult to purify.

*Light absorption spectra:* In chloroform: $\lambda_{max}$ 400 nm, $\varepsilon$ = 13900; $\lambda_{min}$ 347 nm, $\varepsilon$ = 4100; $\lambda_{max}$ 320 nm, $\varepsilon$ = 5000; $\lambda_{min}$ 292 nm, $\varepsilon$ = 1100; $\lambda_{max}$ 267 nm, $\varepsilon$ = 22500; in methanol: $\lambda_{max}$ 395 nm, $\varepsilon$ = 13800; $\lambda_{min}$ 345 nm, $\varepsilon$ = 5200; $\lambda_{max}$ 317 nm, $\varepsilon$ = 6000; $\lambda_{min}$ 290 nm, $\varepsilon$ = 3650; $\lambda_{max}$ 265 nm, $\varepsilon$ = 26800; $\lambda_{min}$ 233 nm, $\varepsilon$ = 8100; $\lambda_{max}$ 215 nm, $\vartheta$ = 37500; in 0.3 M phosphate buffer, pH 7.0: $\lambda_{max}$ 400 nm, $\varepsilon$ = 14000; $\lambda_{max}$ 330 nm (shoulder), $\varepsilon$ = 6100; $\lambda_{min}$ 295 nm, $\varepsilon$ = 2800; $\lambda_{max}$ 265 nm, $\varepsilon$ = 25500; $\lambda_{min}$ 233 nm, $\varepsilon$ = 8000; $\lambda_{max}$ 215 nm, $\varepsilon$ = 36500; in HCOOH: $\lambda_{max}$ 373 nm, $\varepsilon$ = 18100.

*Infrared spectrum:* $\nu$ NH(3) 3180 cm$^{-1}$ (3.19 $\mu$), 3030 cm$^{-1}$ (3.25 $\mu$), $\nu$ CO(2) 1655 cm$^{-1}$ (6.03 $\mu$).

*Nuclear magnetic resonance spectrum:* ([5-$^2$H] pyridine solvent): 7.75 $\delta$ (1 H, singlet, [C(6)-H]); 6.14 $\delta$ (1 H, doublet, [C(4)-H]); 4.82 $\delta$ (1 H, exchangeable, broad singlet, [N(3)-H]; 3.78 $\delta$ (3 H, singlet, [N(10)-CH$_3$]); 2.28 $\delta$ (3 H, singlet, [C(8)-CH$_3$]); 2.22 $\delta$ (3 H, singlet, [C(7)-CH$_3$]); 3.6 $\delta$ (2 H, quartet) and 1.0 $\delta$ (3 H, triplet) assigned to [C(4)-OCH$_2$CH$_3$]. The C(9) proton is not seen for the reasons given above.

Integration of the peaks at 3.6 and 1.0 $\delta$, respectively, showed that there was about 1 OCH$_2$CH$_3$ per flavin present as indicated by the elemental analysis.

### RESULTS

As a model flavin for the synthesis of 3,4-dihydroflavin, $N$(3)-methyllumiflavin [9] (Scheme 1, II, $R_1 = R_2 = CH_3$) was found most suitable for the study described, because of its photostability and adequate solubility. LiBH$_4$ was preferred over NaBH$_4$ because of its better solubility under the reaction conditions. Methanol as solvent was used because the BH$_4^-$-product is much more stable towards autoxidation under this condition than in aqueous solution.

Anaerobic reduction of flavins (II) by BH$_4^-$ is slow in the absence of light but is strongly catalyzed by light. The main products thus obtained are 1,5-dihydroflavins (III) (Scheme I) as judged by spectral comparison with the product obtained by catalytic hydrogenation of II. 1,5-Dihydroflavin is instantaneously and quantitatively reoxidized upon admission of air (Scheme 1). Furthermore, 1,5-dihydroflavin (III) is unaffected by even prolonged treatment with BH$_4^-$. However, an increasing loss of absorbance of oxidized flavin at 450 nm is observed with progressive cycles of photocatalyzed reduction of II by BH$_4^-$ and air reoxidation and a gradual

increase of absorbance at about 400 nm due to the formation of a new flavin, namely, 3,4-dihydroflavin (I) (Scheme I). The photocatalyzed reduction of II by $BH_4^-$ is fast for the formation of III and rather slow for the production of I (Scheme I), hence the formation of I requires many cycles of reduction and
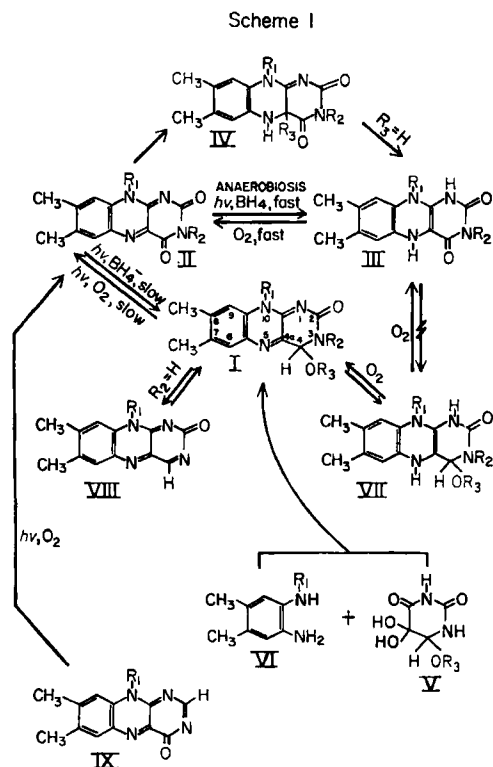
Scheme I



reoxidation. This also indicates that the formation of I does not occur via 1,5-dihydroflavin (III) but only via oxidized flavin (II) (Scheme I). The attempt to synthesize I in an aqueous medium resulted in very low yields. However, it is obtained readily in methanolic solution as described in the Methods section.

The total synthesis (Scheme I) was achieved in a way known in principle from earlier work [10]. If alloxan is replaced by isodialuric acid (V) in a Kuhn condensation [11], the product to be expected is 3,4-dihydrolumiflavin (I, $R_1 = CH_3$, $R_2 = H$), but it could not be isolated as such in a pure state due to its sensitivity towards oxygen under the conditions employed. However, if equal volumes of methanol and acetic acid were used in the condensation of isodialuric acid (V) and prehydrogenated $N$,3,4-trimethyl-6-($p$-carboxyphenylazo)-aniline (VI) and the condensation conducted at lower temperature the product could be isolated in a relatively high yield. Isolated in the crystalline state, the product is as stable as its $N$(3)-methyl analogue obtained by

$BH_4^-$-reduction. The total synthesis product shows the same light and infrared absorption as the product obtained by $BH_4^-$-treatment of lumiflavin (II, $R_1 = CH_3$, $R_2 = H$) and is also identical in fluorescence and thin layer chromatography. The instability of this product under acidic conditions is apparently due to splitting off alcohol from compound I ($R_1 = CH_3$, $R_2 = H$, $R_3 = CH_2CH_3$), and the formation of the dehydrated 3,4-dihydroflavin VIII, ($R_1 = CH_3$) ("4-deoxylumiflavin"), which may undergo further reactions of self-condensation as a true azaquinoid compound. This conclusion is strongly supported by the fact that when a concentrated acid solution of I ($R_2 = H$, $R_3 = CH_2CH_3$) was prepared a green intermediate was observed which upon aeration turned red-brown exhibiting a light absorption maximum of about 480 nm. This behaviour is similar to that previously described [12] for the self condensation of flavinium salts.

Although the Kuhn-condensation in dilute acetic acid or neutral solution (rather than glacial acetic acid) would usually be expected to yield 1-alkyl-2-chinoxalone-3-carbonic acid ureides [13], the procedure given above for the total synthesis of I did not yield anything but flavins (I, II).

$N$(3)-Methyl-3,4-dihydrolumiflavin (I, $R_1 = R_2 = CH_3$, $R_3 = H$), the $BH_4^-$-reduction product of II ($R_1 = R_2 = CH_3$), shows a light absorption spectrum similar to that of the starting material except that the visible absorption bands are shifted about 40 nm to shorter wavelengths (Fig.1) whereas the ultraviolet absorption remains practically unchanged. In contrast to II, organic solvents have little effect on the resolution of the 405 nm band of I but influence the near ultraviolet absorption band at about 320 nm to a much greater extent than the corresponding absorption of II. The $BH_4^-$-reduction product of lumiflavin (I, $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_2CH_3$) and the "total synthesis" product show identical light absorption spectra which are also practically identical with that of I, where $R_1 = R_2 = CH_3$ and $R_3 = H$, apart from a small hypsochromic shift of the long wavelength absorption maximum. Furthermore, the molar extinction coefficients of the first absorption maximum of 3,4-dihydroflavins are increased about $11\%$ whereas the molar extinction coefficient of the second absorption maximum is decreased about $40\%$ compared to those of their parent flavins.

3,4-Dihydroflavins were found to be protonated with a $pK_a$ of 2.5 compared to a $pK_a$ of 0.18 [14] for the parent flavins. Reduction of the 4-carbonyl group in II renders the flavin nucleus more basic at N(1), as to be expected. Protonation of I displaces the 400 nm band about 30 nm to shorter wavelength and the 320 nm band to longer wavelength resulting in a one band absorption spectrum in the visible region (Fig.1) with an absorption maximum at 373 nm.
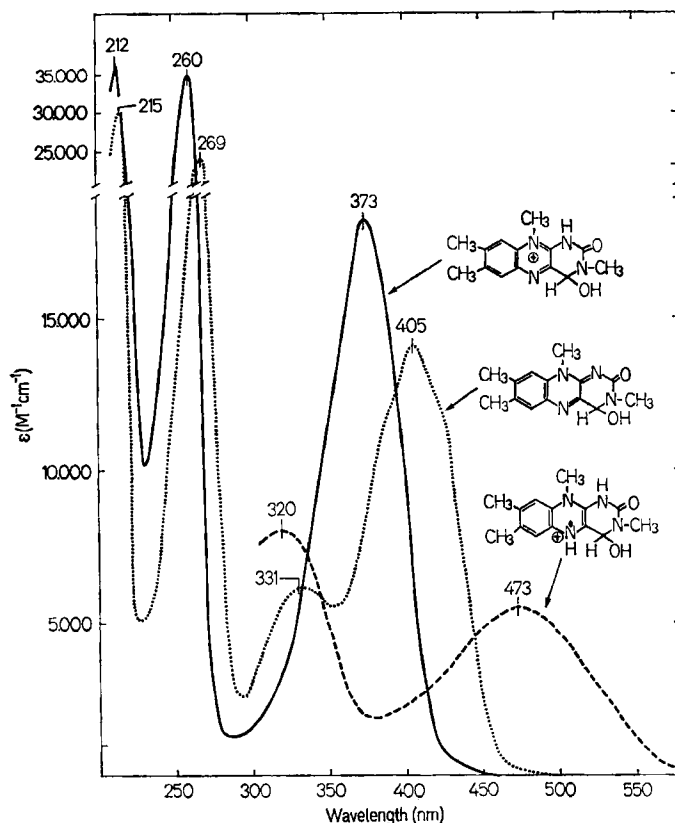
Fig. 1. *Comparison of the light absorption spectra of different species of* N(3)-methyl-3,4-dihydrolumiflavin: Neutral form in 0.3 M phosphate buffer, pH 7.0 (· · · ·), cationic form in HCOOH (———), and radical cation in HCOOH (- - - -), obtained as described in the text (note different scales)

Upon reduction of I in absolute HCOOH by Zn dust under anaerobic conditions, a red intermediate is formed showing absorption maxima at 473 and 320 nm (Fig. 1). Upon admission of air to the sample thus reduced the original light absorption spectrum is quantitatively restored (Fig. 1, cationic form of I). This red intermediate exhibits an electron paramagnetic resonance signal similar to that obtained for II under the same conditions [15] and represents therefore the cationic form of the 3,4-dihydroflavin-radical. However, when reduction was allowed to proceed beyond 50% the original light absorption spectrum upon reoxidation was never completely restored, probably due to the breakdown of the molecule under these conditions.

3,4-Dihydroflavins I are very slowly photo-oxidized in aqueous solution in the presence of oxygen to give starting material II. The spectrophotometric course of this reaction is demonstrated in Fig. 2. Three isosbestic points were observed at 337, 375, and 425 nm. The conversion of I into II is much more rapid in strong acid solutions than at neutral pH. Furthermore, 3,4-dihydroflavins are indefinitely stable towards oxygen in the absence of light in methanolic solution.

Compound I is very slowly reduced by $S_2O_4^-$ to give 1,3,4,5-tetrahydroflavin VII in marked contrast to the rapid rate of reduction of flavoquinones II under identical conditions. However, I is rapidly reduced by EDTA in the presence of light [16] as demonstrated in Fig. 3. Two isosbestic points were observed at 347 and 445 nm during the reduction. The final light absorption spectrum thus obtained shows absorption maxima at 412 and 380 nm. The color of the sample changes from pale yellow to deep yellow during the course of reduction whereas the reduction of flavoquinone yielding 1,5-dihydroflavin (III) under identical conditions leads to a practical colorless solution. From the fact, that the molar extinction coefficient of VII at 450 nm is increased about three-fold compared to that of III, we conclude that VII is more planar than III according to results published earlier [6]. However, air-oxidation of VII restores the original light absorption spectrum only to about 50%. Two additional cycles of reduction and reoxidation of the same sample yield finally II indicating the reaction sequence I ⇌ VII → III ⇌ II (cf. Scheme 1). Replacement of EDTA by phenyl-acetate in the experiment described above led to a final spectrum practically identical to that shown in
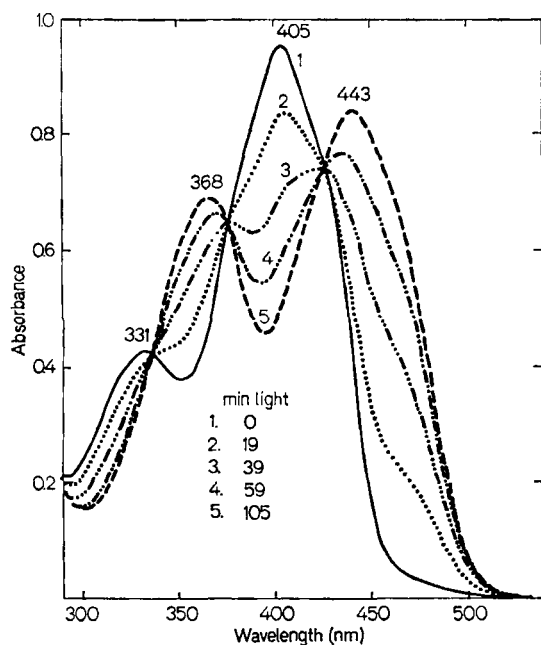
Fig. 2. *Spectral course of the conversion of* N(3)-methyl-3,4-dihydrolumiflavin *into its parent compound* [N(3)-methyl-lumiflavin]. 68.2 µM flavin in 0.3 M phosphate buffer, pH 7.0, illuminated with a 100-W tungsten light bulb under aerobic conditions at room temperature for the time indicated on the individual curves
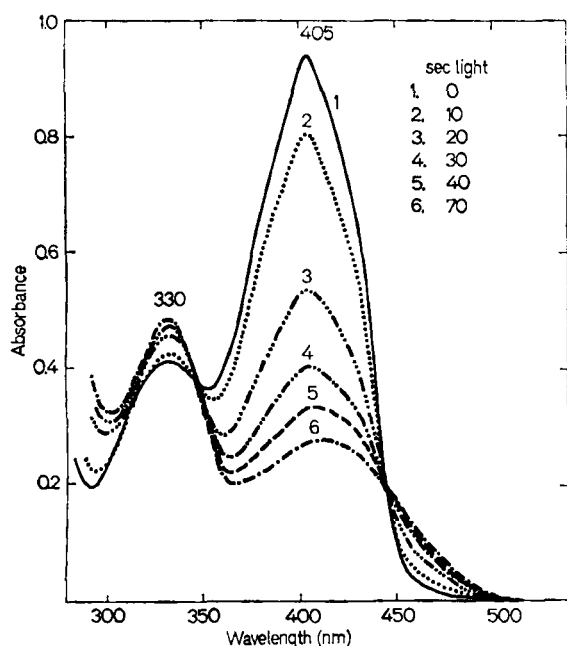


Fig. 3. *Spectral course of the anaerobic reduction of* N(3)-methyl-3,4-dihydrolumiflavin. 67.7 µM flavin in 0.3 M phosphate buffer, pH 7.0, in the presence of 6.0 mM EDTA illuminated at 6° with a 100-W tungsten light bulb [14] for the time shown on the individual curves

Fig. 3 indicating that neither N(5) nor 4a-substitution of I occurred by this procedure [3]. Furthermore, prolonged illumination of a sample of VII in the presence of phenylacetate did not alter the light absorption spectrum but prolonged illumination of a sample of VII in the presence of EDTA caused a secondary very slow and irreversible reaction leading to the loss of the visible light absorption.

The light absorption spectra of 3,4-dihydroflavins I are practically identical with those obtained by $BH_4^-$-reduction of the flavoproteins amino acid oxidases [1] apart from a small bathochromic shift and a somewhat better resolution of the long wavelength absorption band of the $BH_4^-$-reduced enzymes. These differences, however, become insignificant upon release of the coenzyme from the apoprotein [1].

The fluorescence activation spectra of I ($R_1=CH_3$, $R_2 = H$, $R_3 = CH_2CH_3$), obtained by total synthesis and by $BH_4^-$-reduction of lumiflavin (I, $R_1 = R_2 = CH_3$, $R_3 = H$), are identical in all details (Fig.4). The released coenzyme of $BH_4^-$-treated amino acid oxidases [1] shows the same fluorescence spectrum as obtained for I, except for lower quantum yields (Fig.4), indicating that the adenine moiety is still in close contact with the flavin moiety of the modified FAD released from protein. However, treatment of the 3,4-dihydro-FAD thus obtained with snake venom phosphodiesterase [1] yielding the FMN-analog leads to an absolutely identical fluorescence spectrum to that obtained for I ($R_1 = CH_3$, $R_2 = H$, $R_3 = CH_2CH_3$) (Fig.4). The $BH_4^-$-reduction product of methyllumiflavin (I, $R_1 = R_2 = CH_3$, $R_3 = H$) shows a similar fluorescence spectrum as illustrated in Fig.4 except that the fluorescence emission band is shifted about 10 nm to longer wavelength. Furthermore, a quantitative comparison of the fluorescence intensity of I ($R_1 = R_2CH_3$) with that of its parent compound II showed a ratio of 1.66.

In order to complete the spectroscopic characterization of 3,4-dihydroflavins (I, $R_1 = R_2 = CH_3$, $R_3 = H$), its first photo-excited triplet state has been investigated by optical and electron paramagnetic resonance spectroscopy in rigid ethanolic solution at 77° K.

The phosphorescence of the triplet state has a maximum intensity at 550 nm and a life time of 0.32 sec. Its integrated intensity is about two orders of magnitude lower than that of the fluorescence emission under the same conditions of solvent and temperature, as observed for flavoquinone compounds II. After correction for the spectral output of the xenon excitation lamp the excitation spectra of both luminescences correspond to the absorption spectra of the molecules I and II, thus eliminating the possibility of emission contamination from parent compounds.

The electron paramagnetic resonance of the photo-excited triplet state of I has also been recorded, in-
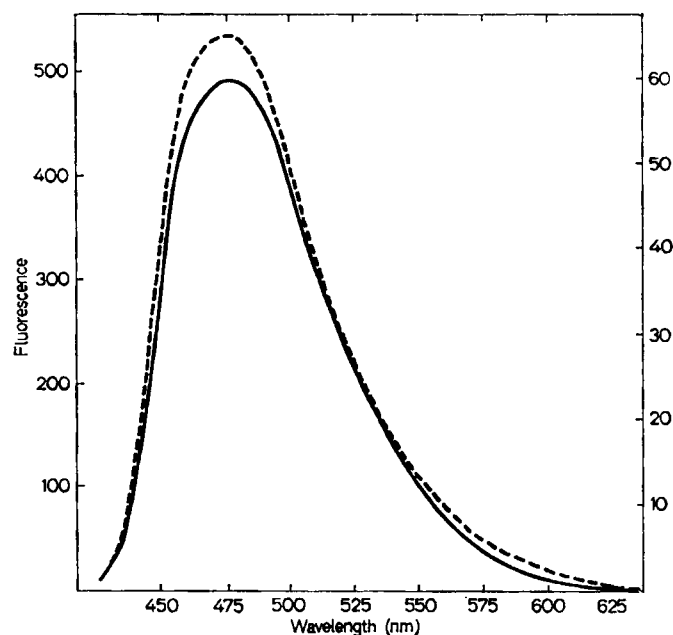
Fig. 4. *Comparison of fluorescence emission spectra of 3,4-dihydrolumiflavin and the borohydride-modified flavin released from L-amino acid oxidase.* All spectra were recorded in 0.03 M phosphate, pH 7.0 at 20°, at a concentration corresponding to an absorbancy at 400—410 nm of 0.05, using an exciting wavelength of 405 nm. L-Amino acid oxidase was reacted with borohydride [1], the protein precipitated with 5% (w/v) trichloroacetic acid and centrifuged. The supernatant solution was extracted with ether to remove trichloroacetic acid. Solid line (fluorescence intensity scale on right), the fluorescence emission spectrum of the modified FAD released. Dashed line (scale on left), after conversion to FMN form by *Naja naja* venom. The identical emission spectrum (- - - -) was given by 3,4-dihydrolumiflavin

cluding the $m = 1$ transitions, using the procedure previously reported for the investigation of other flavin compounds [17]. The zero field splittings of the magnetic sub-levels of the triplet state measured from the spectrum are $D/hc = 0.0674\ cm^{-1}$ and $E/hc = 0.0173\ cm^{-1}$. For the triplet state of II we measured corresponding values of 0.0553 and 0.0150 $cm^{-1}$, respectively.

Evidence for the structure of I was obtained by the unequivocal route of the synthesis of ($R_1 = CH_3$, $R_2 = H$, $R_3 = CH_2CH_3$). The product thus obtained showed the same chemical and physical properties as those obtained by $BH_4^-$-reduction of $N(3)$-methyl-lumiflavin and lumiflavin as outlined above. Furthermore, the infrared spectra of I (Fig. 5) show lack of the first carbonyl-stretching compared to those of their parent compounds (II). This carbonyl-streching at about 5.85 $\mu$ represents the carbonyl group in position 4 of the isoalloxazine ring [18]. The remaining carbonyl stretching at about 6 $\mu$ representing the carbonyl group in position 2 of the flavin nucleus, is shifted to longer wavelength and less intense in the case of $N(3)$-methyl-3,4-dihydrolumiflavin whereas the analogous absorption of 3,4-dihydrolumiflavin remains practically unchanged compared to their parent compounds. This difference is explained by the fact that $N(3)$-alkylation of lumiflavin causes a decrease of the intensity and a small bathochromic

shift of the first carbonyl absorption as shown in Fig. 5. These effects are somewhat larger for the 3,4-dihydroflavin derivatives (Fig. 5). The infrared spectrum of I ($R_1 = R_2 = CH_3$, $R_3 = H$) shows in the 3.2—3.5 $\mu$ region two additional absorptions as compared to its parent compound II (Fig. 5). The intensity of these bands, which we assign to OH-stretching absorption, depends on the procedure employed for the purification of I ($R_1 = R_2 = CH_3$, $R_3 = H$). Thus, the product crystallized from organic solvents, *i. e.* $CHCl_3$ or methanol, showed a weaker intensity of these bands than that crystallized from 0.1 N $HClO_4$. This indicates that compound I is primarily formed as the boric ester or methyl acetal, *i. e.* $OB(OCH_3)_2$ or $OCH_3$ replacing OH in position 4 of the flavin nucleus. Furthermore, the observed decrease of absorption at 3.2—3.5 $\mu$ and the expected formation of a new band at 4.3 $\mu$ (Fig. 5) on the infrared spectrum of I after recrystallization from 0.1 N $^2HCl$ supports the correctness of our assignment of the bands at 3.2—3.5 $\mu$ to OH-stretching absorption. However, these bands are much weaker for 3,4-dihydrolumiflavin ($R_1 = CH_3$, $R_2 = H$, $R_3 = CH_2CH_3$) as expected.

The nuclear magnetic resonance spectrum could only be obtained in [5-$^2H$]pyridine, since $(C^2H_3)_2SO$ did not dissolve compound I at the desired concentration and $CF_3COOH$ destroyed the compound

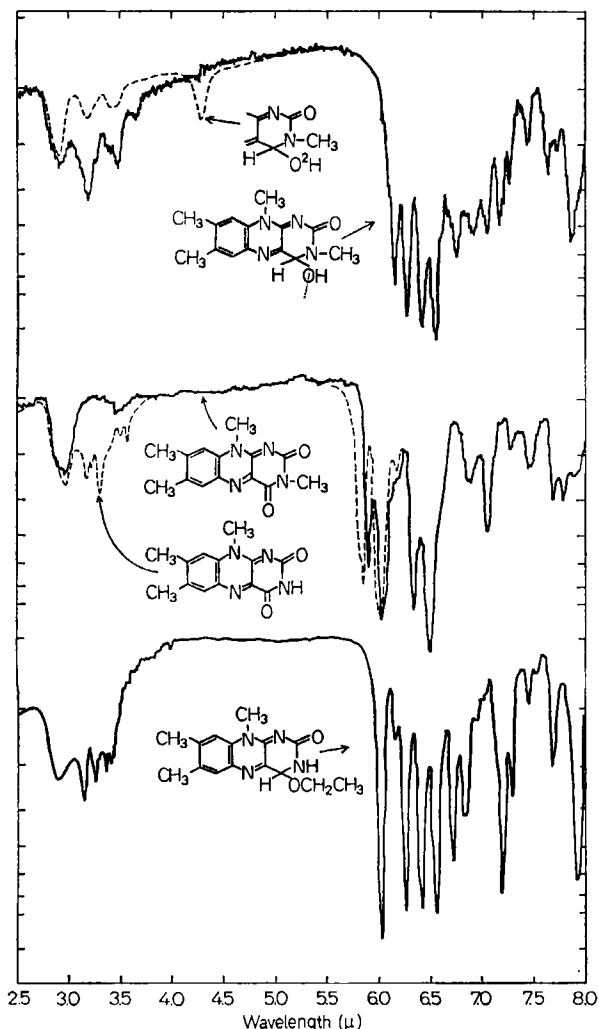Fig. 5. *Comparison of the infrared spectra of 3,4-dihydroflavins with those of their parent compounds in the region of 2.5—8.0 μ obtained as described in the text.* Upper curves: solid line, *N*(3)-methyl-3,4-dihydrolumiflavin; dashed line, after recrystallization from 0.1 N ²HCl; middle curves: *N*(3)-methyllumiflavin (———) and lumiflavin (- - - -); lower curve: 3,4-dihydrolumiflavin from "total synthesis" and BH₄⁻-reduction of lumiflavin

presumably due to trifluoroacetylation of the 4-OH group in I and subsequent removal of $CF_3COO^-$ along with disproportionation. However, it was possible to obtain a nuclear magnetic resonance spectrum of I in aqueous (deuterated) acid, but the relatively fast oxidation of I and concomitant formation of a flavin radical made it more difficult to obtain a pure spectrum. The nuclear magnetic resonance spectrum of I ($R_1 = R_2 = CH_3$, $R_3 = H$) in [5-²H]pyridine thus obtained showed absorptions at 7.75, 6.17, 4.82, 3.79, 3.59, 2.28 and 2.22 $\delta$, respectively, which are assigned to C—H(6) [C—H(9) is overlapped by the strong pyridine absorption at about 7.58 $\delta$], C—H(4),

O—H(4), $NCH_3(10)$, $NCH_3(3)$, $CCH_3(8)$ and $CCH_3(7)$, respectively. Addition of a small volume of ²H₂O to the sample caused the disappearance of the broad absorption at 4.82 $\delta$, as expected (Fig. 6) and the conversion of the doublet at 6.17 $\delta$ into a singlet as expected (the change from the doublet to the singlet did not alter the integrated area of these peaks). Illumination of the same sample with time caused gradually a decrease of the intensity of the peaks at 7.75, 6.17, 3.79, 3.59, 2.28 and 2.22 $\delta$, respectively, and concomitantly the formation of new peaks at 7.96, 7.46, 4.01, 3.60, 2.34 and 2.14 $\delta$, respectively. An intermediate spectrum thus obtained after 132 h of illumination is also shown in Fig. 6. Illumination over a period of 320 h led to the complete loss of the absorptions of I ($R_1 = R_2 = CH_3$, $R_3 = H$) and the formation of the spectrum of II (Fig. 6). Furthermore, spectra taken and integrated at about 24 h intervals during the illumination period showed that the conversion of the $NCH_3$ and $CCH_3$ peaks of I into those of II and the loss of the single proton at 6.17 $\delta$ occurred at the same rate. Furthermore, one of the peaks of the two single aromatic protons shifted gradually to lower field and the other shifted to higher field during the illumination period.

Finally, the synthesis of a small quantity of I ($R_1 = R_2 = CH_3$, $R_3 = H$) using $NaB^3H_4$ and the radioactivity measurement of solutions of I thus obtained showed that one tritium atom per flavin was incorporated into the flavin nucleus. No radioactivity was found after an illumination experiment of the type shown in Fig. 2 and extraction of II thus formed with chloroform.

## DISCUSSION

The hypsochromic shift of absorption, fluorescence and phosphorescence spectra of about $2000\,\mathrm{cm}^{-1}$ relative to the flavoquinone spectra clearly indicates a lower conjugation of the $\pi$-electron cloud in the 3,4-dihydroflavins. This lower delocalization is further evidenced for the two unpaired $\pi$-electrons in the first triplet state from the increase in their magnetic dipole interaction energy responsible for the zero field splittings of the state. However, the effect of 4-keto-reduction upon $\pi$-electron delocalization is relatively small and comparable to the isoalloxazine effects [17]. This suggests, as well as the long life-time of the triplet state, that the conjugated part of the molecule remains planar upon reduction of the 4-CO group in contrast to 4a reduced compounds IV for which we observed a fluorescence emission only in the glassy solutions at low temperature and could not detect any phosphorescence emission.

The spectral properties of the new dihydroflavins I are consistent with those of protein-linked $BH_4^-$-reduced flavocoenzymes as previously described [1].

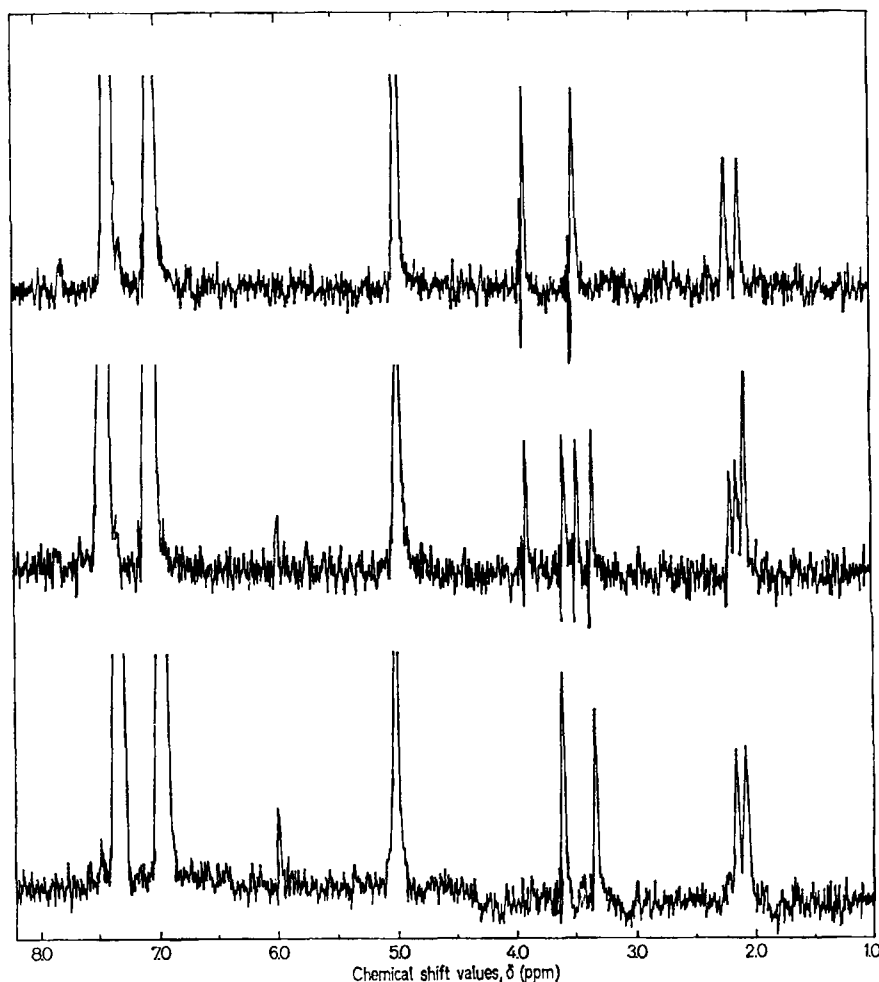Fig. 6. *Nuclear magnetic resonance spectrum recorded in [5-²H]-pyridine at 31° in the presence of 10 μl ²H₂O (total volume 0.3 ml).* Lower curve: N(3)-methyl-3,4-dihydrolumiflavin; middle curve: the same as lower curve but 132 h illumination; upper curve: after 300 h illumination yielding N(3)-methyllumiflavin. The scale that is indicated is only approximately correct due to a non-linear sweep of the magnetic field. The correct values are given in the text

It is conceivable that reduction of the 4-CO group does not alter very much the flavoquinone light absorption spectrum, but shifts it to shorter wavelengths. The most characteristic behavior is the enhanced basicity of the new flavin, which it has in common with the analogous 2,3-dihydroflavin (or "2-deoxyflavin", IX, Scheme I) described earlier [18]. Compound I and IX also share the photooxidizability to give II. The oxido-reductive system in the flavin nucleus remains essentially undisturbed by reduction of the 4-CO chromophore since substrate reduction of BH₄⁻-reacted D- and L-amino acid oxidases has been demonstrated [1]. This indicates that flavin-dependant substrate dehydrogenation does not involve position 4 of the flavin nucleus. The proposal of Gawron [5] of reduction at

positions 5 and 4a followed by internal proton transfer to position 1, however, remains a possibility.

REFERENCES

1. Massey, V., Curti, B., Müller, F., and Mayhew, S. G., J. Biol. Chem. 243 (1968) 1339.
2. Kuhn, R., and Ströbele, R., Chem. Ber. 70 (1937) 753.

3. Walker, W., Hemmerich, P., and Massey, V., *Helv. Chim. Acta,* 50 (1967) 2269.
4. Müller, F., and Massey, V., *J. Biol. Chem.,* in press.
5. Gawron, O., *Nature,* 194 (1963) 1270.
6. Dudley, K. H., Ehrenberg, A., Hemmerich, P., and Müller, F., *Helv. Chim. Acta,* 47 (1964) 1354.
7. Casola, L., Brumbry, P. E., and Massey, V., *J. Biol. Chem.* 241 (1966) 4977.
8. Massey, V., Gibson, Q. H., and Veeger, C., *Biochem. J.* 77 (1960) 341.
9. Hemmerich, P., *Helv. Chim. Acta,* 47 (1964) 464.
10. Bamberg, P., Hemmerich, P., and Erlenmeyer, H., *Helv. Chim. Acta,* 43 (1960) 395.
11. Kuhn, R., and Weygand, F., *Chem. Ber.* 67 (1934) 1409.
12. Ehrenberg, A., Müller, F., and Hemmerich, P., *European J. Biochem.* 2 (1967) 286.
13. Dudley, K. H., and Hemmerich, P., *J. Org. Chem.* 32 (1967) 3049.
14. Suelter, C. H., and Metzler, D. E., *Biochim. Biophys. Acta,* 44 (1960) 23.
15. Hemmerich, P., Müller, F., and Ehrenberg, A., unpublished observations.
16. Massey, V., and Palmer, G., *Biochemistry,* 5 (1966) 3181.
17. Lhoste, J.-M., Haug, A., and Hemmerich, P., *Biochemistry,* 5 (1966) 3290.
18. Müller, F., Walker, W., and Hemmerich, P., *Helv. Chim. Acta,* 49 (1966) 2365.

F. Müller and V. Massey
Department of Biological Chemistry
University of Michigan Medical School
Ann Arbor, Michigan 48104, U.S.A.

C. Heizmann and P. Hemmerich
Fachbereich Biologie, Universität Konstanz
BRD-775 Konstanz, Postfach 733, Germany

J.-M. Lhoste's permanent adress:
Laboratoire de Biophysique
Musée d'Histoire Naturelle, F-75 Paris-5, France

D. C. Gould
Department of Biological Chemistry, Harvard Medical School
Boston, Massachusetts 02115, U.S.A.