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Studies on Succinate Dehydrogenase

Site of Attachment of the Covalently-Bound Flavin to the Peptide Chain

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Improved methods have been devised for the isolation in μmole quantities of a pure flavin pentapeptide and its acid-hydrolysis product (SD-flavin) from inner-membrane preparations of heart mitochondria and from soluble, purified succinate dehydrogenase. SD-flavin differs from riboflavin in still having an amino acid covalently linked to the isoalloxazine ring system. SD-flavin may be compared with riboflavin and with various 8α-substituted synthetic flavins by optical spectrophotometry in the neutral and cationic states and by ESR and ENDOR spectrometry in the cationic radical state. On the basis of these experiments is was concluded that the FAD prosthetic group of mitochondrial succinate dehydrogenase is covalently linked through the 8α-position to the peptide backbone of the protein. This conclusion is in accord with the acid stability of the natural product and its tendency to yield riboflavin under reductive conditions. The unusual pH-fluorescence spectrum of the flavin strongly suggests that the 8α-methylene group is linked to an amino acid through a tertiary nitrogen group.

Early in 1950 the suggestion arose that, in addition to riboflavin, FMN and FAD, a fourth form of flavin exists in nature, one which is not extracted from tissues by conventional denaturation methods but requires proteolytic digestion for extraction [1,2]. Unmistakable evidence that this new form of riboflavin, which subsequently became known as "bound flavin" or "covalently bound flavin" originates from succinate dehydrogenase had to await the first isolation of the enzyme in soluble, purified form [3]. Shortly thereafter Kearney and Singer [4,5] presented evidence that succinate dehydrogenase contained covalently-linked FAD and that on digestion with trypsin and chymotrypsin a flavin peptide with markedly-different properties from known flavins could be isolated. These conclusions were confirmed and extended by Wang *et al.* [6].

Subsequent studies [7,8] disclosed that SD-flavin differs from previously-known flavins in showing a hypsochromic shift of its 375-nm absorption band to 345–350 nm in the neutral oxidized state, inactivity in the D-amino acid oxidase test at the FAD level, greater water solubility, failure to yield free authentic lumiflavin on alkaline irradiation, and a characteristic pH-fluorescence curve which shows a maximum at pH 3.2 to 3.4 with a pK of 4.5 ± 0.1, and no significant fluorescence at neutral pH. Kearney [8] also showed that hydrolysis at 95 °C in 6-N HCl yields a flavin which was different from all previous-known flavins, particularly ribo-

Abbreviations and Definitions. ESR, electron-spin resonance; ENDOR, electron nuclear double resonance; NMR, nuclear magnetic resonance; SD-flavin, acid-hydrolysis product of a flavin pentapeptide isolated from inner membrane preparations of heart mitochondria; non-phosphorylating preparation (ETP in other publications), a non-phosphorylating inner-membrane preparation from beef-heart mitochondria; phosphorylating preparation (ETP_H in other publications), a phosphorylating inner-membrane preparation from beef-heart mitochondria.

Enzymes. Succinate dehydrogenase, or succinate : acceptor oxidoreductase (EC 1.3.99.1); trypsin (EC 3.4.4.4); chymotrypsin (EC 3.4.4.5).

flavin. By a series of chromatographic methods she also isolated small quantities of a flavin peptide, believed to be a hexapeptide, containing 1 mole each of serine, threonine, alanine, valine, and glutamic acid and a second, N-terminal serine [8].

The studies of Kearney [7,8] left little doubt that the peptide backbone of succinate dehydrogenase is attached to the isoalloxazine ring system and not to the ribityl side chain of FAD linked to the 10-position, since alkaline photolysis yields a lumiflavin derivative which is not identical with lumiflavin. Later Wang *et al.* [10] eliminated the 1-, 2- and 3-positions (Fig. 1) as the site of attachment since hydrolysis in mild alkali yields urea, not a ureido peptide. The exact site of attachment of the peptide to the flavin, the nature of this bond, the identity of the amino acid adjacent to riboflavin, and the peptide sequence at the active center remained undetermined partly because of the lack of a method for the isolation of SD-flavin in quantities sufficient for chemical studies, partly because of the lack of unambiguous and sensitive physical and chemical methods required for the resolution of these remaining questions.

A collaboration, initiated in 1965 between the laboratories in Konstanz, San Francisco and Stockholm has resulted in the unambiguous demonstration that, in contrast to alternative speculations [10], the peptide chain is linked to the 8α-methylene on the benzene ring of riboflavin [11,13]. It was demonstrated [14] that histidine is the adjacent amino acid, linked through an imidazole ring nitrogen. Histidyl-8α-riboflavin was synthesized [15] and the peptide sequence at the active center was elucidated [16,17].

The present paper is a detailed description of the synthetic, preparative, and analytical methods developed in conjunction with this program. The next paper [17] deals with the identification of histidine as the amino acid immediately linked to riboflavin and the synthesis of histidyl-8α-riboflavin.

MATERIALS AND METHODS

Chromatographic Procedures

Chromatographic procedures for flavin peptides on Dowex-50 (NH_4^+ or pyridinium forms), DEAE-cellulose (acetate form) and on Florisil were as described by Kearney [8].

In all *column chromatography* the elution profiles of flavins were obtained by monitoring absorbance at 445 or 450 nm and, occasionally, also at 350, 280, and 220 nm. In addition, the fluorescence ratio at pH 3.4/pH 7.0 was determined. In routine work the distribution of ninhydrin-positive material in effluents was followed by spotting aliquots on filter paper and spraying with 0.3% (w/v) ninhydrin in *n*-butanol, containing 10% (w/v) zinc acetate, and heating for 5 min at 110 °C.

Thin-layer chromatography was carried out on either silica gel G, containing 13% (w/w) $CaSO_4$ as binder (Merck A.G., Darmstadt) or crystalline cellulose, particle size 19 (Brinkmann Co., New York, N.Y.) as supporting phase. The best separation of flavin peptides from each other and from amino acids was obtained with *n*-butanol—acetic acid—H_2O solvent systems in the ratios (by vol.) of 4:2:2 (Solvent A) or 4:2:4 (Solvent B) for silica gel and 5:2:3 (Solvent C) for cellulose, respectively. For preparative thin-layer chromatography silicic acids (Supelcosil 12A and 42A, Supelco, Inc., Bellefonte, Pa.) were used. The silicic acid was washed 3 times with water, twice with 10% (v/v) pyridine, twice with water, twice with 10% (v/v) acetic acid, and 5 times with water.

Paper chromatography (descending) was done on Whatman No. 1 or 3 paper with Solvent A (above) as the moving phase for 15 h. Occasionally in qualitative paper chromatography the following additional solvents were used: *n*-butanol—acetic acid—H_2O (4:1:5, v/v/v, upper phase); phenol—H_2O (75:25, w/w); 5% (w/v) Na_2HPO_4; and ethanol —34% (w/v) NH_4OH (67:33, v/v).

Spectrometry

Flavin concentration was calculated from the molar absorption coefficient of FAD (ε_{450} = 11300) for dinucleotides and from that of riboflavin and FMN (ε_{450} = 12200) for mononucleotides and dephosphorylated SD-flavin samples. ENDOR spectra were recorded with a Varian E-700 system. The flavin radical concentration was about 1 mM, the total sample 0.5 to 2 μmol, and the sample tubes 5-mm internal diameter unless otherwise mentioned. Other details of the ENDOR experiments have already been published [18].

Flavin radical cations for ESR spectrometry were prepared in 6-N HCl anaerobically by reduction with $TiCl_3$. The latter was added from a dilute solution in 6-N HCl by a microsyringe under spectrophotometric control to maximum absorbance at 490 nm. ESR spectra of these solutions were recorded under argon with a Varian 4045 spectrometer in 1-mm quartz tubes or a Varian E-3 spectrometer in flat quartz cells at 1.0 to 0.1 mM flavin concentrations, room temperature, non-saturating microwave power condition and modulation amplitude of 0.5—1.5 gauss (reduction of modulation would not improve hyperfine resolution in the given system). In the case of hydrolyzed preparations fluorescence was measured at about 10 nM sample concentration in 0.1-M phosphate-citrate buffers at pH 3.4 and 7.0 [13] with a Farrand model A-2 fluorometer. Total P was determined by the method

of Bartlett [19] and total amino acids by the ninhydrin method [20], following hydrolysis in 6-N HCl [8]. Crystallized trypsin and chymotrypsin were obtained from the Worthington Biochemical Corp. Non-phosphorylating inner-membrane preparation was prepared from beef-heart mitochondria as in previous work [21] and phosphorylating inner-membrane preparation by the method of Hansen and Smith [22]. Soluble succinate dehydrogenase was isolated by the method of Bernath and Singer [23], except that an acetone powder of the non-phosphorylating inner-membrane preparation, rather than of mitochondria, was the starting material and dilute acetic acid was used in the neutralization of the glycine extract.

Isolation of SD-Flavin Peptide

Procedure A. For the early ESR studies [11] pooled samples of a phosphorylating preparation were worked up in an adaptation of procedures previously published for the extraction of flavin peptides [13]. The treatment consisted of acetone-HCl extraction for removal of cytochromes followed by treatment with trichloroacetic acid, for removal of loosely-bound flavin, and predigestion with trypsin and chymotrypsin (50 mg of crystalline enzymes per g of phosphorylating preparation protein used). Incubation was done for 4 h at 38 °C and pH 8.0; the sample was protected from light and stirred continuously. Proteolysis was stopped by adding trichloroacetic acid. The acidified mixture was incubated an additional 15 h at 38 °C in order to hydrolyze the flavin peptides from the FAD to the FMN level.

This solution was chromatographed on a Florisil column. After washing, first with 5% (w/v) acetic acid, then water and finally 0.5% (w/v) pyridine, in each case until A_{280} was less than 0.05 in the effluent, the flavin was finally eluted with 5% (v/v) aqueous pyridine. The pyridine was removed by extraction with chloroform. The flavin in the water phase showed a pH 7 versus pH 3.4 fluorescence ratio of 0.2 to 0.4, indicating partial breakdown of SD-flavin to "normal" flavin during manipulation. The extent of this breakdown was highly variable.

This material was hydrolyzed in 6-N HCl at 95 °C for 12 h under argon. Hydrolysis was accompanied by considerable melanin formation. Most of the latter was removed by careful treatment with a small amount of charcoal. The filtrate was evaporated *in vacuo*, dissolved in water and applied to a Sephadex G-10 column. The elution patterns have been published [11,12] and show up to 3 flavin bands in varying amounts. The third band was always identical in chromatography, electron-spin resonance, and absorption spectra with riboflavin, whereas the first two partially overlapping bands were due to SD-flavin and a second, apparently still 8α-substituted, decay product of the latter. ESR spectra of these two fractions before and after photolysis have been published [11].

Since both the destruction of flavin and reduction of SD-flavin to riboflavin are functions of the peptide impurities present which particularly affect the acid hydrolysis step, it was decided to purify SD-flavin as much as possible by the following procedure (B) before subjecting it to strong acid conditions.

Procedure B. 50 g of a non-phosphorylating preparation (protein basis) in 965 ml of 0.25-M sucrose at 0 °C were blended with 3 l acetone (−15 °C) in 300 ml batches and the acetone powder was prepared [23]. The latter (71 g) was washed by extraction with 2.5 l of 0.1-M phosphate, pH 7.5, and succinate dehydrogenase was then extracted with 2.5 l of 0.06-M glycine, pH 10.3. The supernatant solution was adjusted to pH 8.2 with 2-N acetic acid and precipitated with 361 g of $(NH_4)_2SO_4$ per l of solution, stirred for 30 min at 0 °C, and the precipitated enzyme was collected by centrifugation for 15 min at 11000 × *g*. After redissolving in 100 ml of 5-mM phosphate, pH 7.5, the enzyme was dialyzed overnight against the same buffer. The resulting solution (168 ml, containing 2 g of protein) was precipitated at 0 °C with 0.1 volume of 55% (w/v) trichloroacetic acid and centrifuged. The residue was washed once with 50 ml of 5% (w/v) and then with 1% (w/v) trichloroacetic acid until the supernatant solution gave no fluorescence. The pellet was then homogenized in 150 ml of Tris base, brought to 38 °C, the pH adjusted to 8.0, and 0.2 g each of crystalline trypsin and chymotrypsin were added. After 4 h at 38 °C at pH 8.0, 15 ml of 55% (w/v) trichloroacetic acid were added and suspension was maintained for 15 h at 38 °C to hydrolyze the flavin dinucleotides. The solution was precipitated with 5% (w/v) trichloroacetic acid at 0 °C; the precipitate was washed with 20 ml of 5% (w/v) trichloroacetic acid and the supernatant solutions were combined. The solution (about 3 μmol of SD-flavin) was placed on a Florisol column (3 × 50 cm), washed with 2 l of 5% (v/v) acetic acid, 0.5 l of H_2O, and eluted in a narrow band with 5% (v/v) pyridine. The pyridine eluate (2.5 μmol of SD-flavin), after concentration to about 5 ml, usually contained a precipitate which was removed by centrifugation; the precipitate was washed free from adhering flavin with water. The combined solutions were again concentrated to about 2 ml volume and centrifuged if turbidity was present. The procedure was repeated until on concentration to 2 ml no turbidity developed.

The flavin was chromatographed on Sephadex G-25 (3 × 150 cm column, equilibrated with H_2O); of the two fluorescent bands appearing the leading

one (B1) contained 65% of the flavin placed on the column (optical ratio $A_{345}/A_{445} = 0.82$) and the second one (B2) 17% (optical ratio = 1.1). Neither fraction gave any evidence of free (*i.e.*, non-SD) flavin from pH 7/pH 3.4 fluorescence ratios. About 90% of the ninhydrin-positive material was eluted ahead of these two flavin bands. The flavin in the main or leading band was used for purification of flavin peptides or for acid hydrolysis.

The flavin peptide used for ENDOR studies was obtained by chromatography of fraction B1 (2 μmol) on DEAE-cellulose (acetate cycle), equilibrated with a 12:7 (v/v) mixture of 95% (v/v) ethanol—H_2O [8]. The flavin was eluted with 7:3 (v/v) mixture of 95% (v/v) ethanol—1-N acetic acid. The main band, obtained in 75% yield, was lyophilized (fraction B3).

For the preparation of the pure flavin pentapeptide 2.5 μmol of fraction B1 (0.5 ml) were applied to a Whatman 3 paper (46 × 57 cm), prewashed with the butanol-acetic solvent system A. The flavin was chromatographed (descending mode) with Solvent A for 26 h. Several flavin bands developed, the principal one of which had 63% of the mobility of FMN. This was eluted with water and yielded 1.47 μmol of flavin and 26 mol of amino acid/mol of flavin. The sample was concentrated to a small volume, applied to a preparative thin-layer-chromatography plate (Supelcosil 42A) and chromatographed in solvent system A for 7 h. Aside from the main band (R_F value = 0.28) only a minute trace of an additional flavin band was detected. (This system is excellent for separating non-flavin peptides which move with a much higher R_F value.) The flavin peptide was eluted and found to be homogeneous in all the neutral and basic solvents listed in Methods on paper chromatography. It contained 0.01 mol P/mol of flavin and, on acid hydrolysis, 1 mol each of serine, threonine, alanine, valine, and histidine [16].

Isolation of Acid-Hydrolyzed SD-Flavin (Histidyl-Riboflavin)

Fraction B1 and B2 obtained were combined (3.9 μmol of flavin), concentrated to 2 ml, precipitated impurities removed by filtration, concentrated to dryness, and sublimed in high vacuum for 1 h at 50 °C. The residue was dissolved in 1 ml of 6-N HCl, filtered, and the sublimation was repeated but at 20 °C. The residue was then hydrolyzed in 1 ml of 6-N HCl in a pyrex tube at 95 °C in high vacuum for 15 h. A small amount of dark precipitate formed during this procedure and the fluorescence ratio pH 7/pH 3.4 rose by about 15%, indicating some breakdown of SD-flavin. The suspension was filtered and the filtrate lyophilized. The flavin, dissolved in 0.2 ml of H_2O, was chromatographed on 2 thin-layer-chromatography plates (Supelcosil 42A) in Solvent B for 9 h. A brown-black impurity and several blue fluorescent bands moved ahead of the flavin. The flavin shows an R_F value of 0.40 under these conditions. Elution with 5% (v/v) pyridine yielded 1.95 μmol of acid-hydrolyzed SD-flavin containing 25 mol of amino acids/mol of flavin. The preparative thin-layer-chromatography step was repeated, using one plate, yielding 1.65 μmol of flavin (1.5 mol amino acids/mol of flavin). A second small flavin band with twice the R_F value of histidyl riboflavin and a more orange-yellow rather than greenish-yellow fluorescence was also eluted. Its optical spectrum showed maxima at 445 and 345 nm but the pH 7/pH 3.4 fluorescence ratio was 1.0. This is apparently a breakdown product of histidyl riboflavin.

The main SD-flavin band was eluted, concentrated, and chromatographed a third time by thin-layer chromatography in solvent system B, yielding material which contained only 1 amino acid (histidine) per mole of flavin.

Syntheses

8α-Oxo-lumiflavin (IIa). 5 g (19.5 mmol) lumiflavin (Ia) were dissolved in 20 ml conc. sulfuric acid and 3 ml (58.5 mmol) bromine were added. The reaction mixture was allowed to stand at 50 °C for 5 days; the course of the reaction was followed by thin-layer chromatography (System A). Tetrahydrofuran (20 ml) was added and the deep-red solution added dropwise, with stirring to 500 ml of ice-water. The suspension was allowed to stand at 60 °C for 4 h in order to hydrolyse the 8α-dibromo compound which formed as an intermediate. The precipitate was filtered off, washed with water, ethanol and ether and dried *in vacuo* at 80 °C overnight to yield 4.5 g (85%) of the crude IIa. Analysis after repeated recrystallization from acetic acid/water: F > 325 °C (dec.). Found: C 57.35, H 3.73, N 20.55%, $C_{13}H_{10}O_3N_4$ (M_r 270.24) requires C 57.77, H 3.73, N 20.73%. λ_{max} (ε) at pH 7: 452 (9000), 348 (8500), 260 (24000) and 220 nm (21200). λ_{max} (ε) in 6-N HCl: 410 sh (6500), 370 (12500), 270 (20000), 236 (15500) and 220 nm (18000). IR (KBr): 3420 (ν_{N-H}), 1730, 1720 and 1710 cm^{-1} ($\nu_{C=O}$).

3-Methyl-8α-oxo-lumiflavin (IIb) was synthesized in a manner analogous to IIa starting from Ib. It showed physical properties corresponding to those of IIa. Infrared bands ($CHCl_3$): 1710, 1697 and 1665 cm^{-1} ($\nu_{C=O}$).

8α-Hydroxy-lumiflavin (V). 0.4 g lithium borohydride were added to a suspension of 0.5 g (1.8 mmol) IIa in 50 ml methanol in several portions with constant stirring for 30 min. The color of the reaction mixture turned green and finally yellow brown indicating formation of dihydroflavin. Thirty ml 2-N acetic acid were added, the precipitate

formed was filtered, washed with water, methanol and ether and dried to yield 420 mg of the crude V (84%). This compound was dissolved in hot formic acid, treated with charcoal and Celite, and the hot solution allowed to crystallize at 10 °C. The pure V (380 mg, 65%) crystallizes as mono-formate, F = 330 to 340 °C (dec.). Found: C 52.43, H 4.11, N 17.96%; $C_{14}H_{14}O_5N_4$ (M_r 318.29) requires C 52.83, H 4.43, N 17.60%. λ_{max} (ε) at pH 7: 442 (11800) and 358 nm (8300). λ_{max} (ε) in 6-N HCl: 380 nm (16000). IR (KBr): 3470 (Broad, ν_{OH}) 1705, 1700 cm^{-1} ($\nu_{C=O}$). NMR (CF_3COOH): δ = 8.67 (6-H), 8.41 (9-H), 5.35 (8-CH_2), 4.70 (10-CH_3) and 2.62 ppm (7-CH_3).

8α-Acetamido-lumiflavin (IIIa). 1 g (3.6 mmol) of the crude IIa were dissolved in a minimum of acetic acid—water (4:1, v/v) and 0.5 ml phenylhydrazine were added to the hot solution. After 15 min standing at room temperature the solvent was removed *in vacuo* and the solid residue, a black powder, washed on a filter with water, methanol, chloroform and ether, and dried to yield 0.8 g of the phenylhydrazone of the aldehyde IIa. λ_{max} (chloroform): 526, 325 and 275 nm. The phenylhydrazone (200 mg) was dissolved in 3 ml of hot acetic acid—acetic anhydride (1:1, v/v) and 300 mg zinc dust were added over 20 min at refluxing temperature. This solution was filtered and the solvent removed *in vacuo*. The solid residue was treated with hot 2-N acetic acid, filtered from insoluble material and allowed to crystallize at 10 °C. The crude product was recrystallized from dilute formic acid to yield 80 mg (47% from the phenylhydrazone) of the pure IIIa, F > 350 °C (dec.). Found: C 56.97, H 5.10, O 16.60%; $C_{15}H_{15}N_5O_3$ (M_r 313.3) requires C 57.50, H 4.83, O 15.32%. λ_{max} (ε) at pH 7: 442 (11100), 358 (8000) and 269 nm (29600). λ_{max} (ε) in 6-N HCl: 375 (14700) and 365 nm (29600). NMR (CF_3COOH): δ = 8.45 (6-H), 8.32 (9-H), 8.22 (broad, amide NH) 5.05 and 4.95 (doublet, 8-CH_2; after exchange of amide NH and ^2H_2O, singlet at 5.00), 4.67 (10-CH_3), 2.78 (7-CH_3) and 2.47 ppm (CO—CH_3).

8α-Succinamido-lumiflavin (IIIb) was synthesized from the phenyl hydrazone of IIa with succinic anhydride/succinic acid in ethylene glycol dimethyl ether as solvent in the manner described for IIIa. The reaction mixture was distributed between chloroform/water at pH 7, the water extract adjusted to pH 3 with HCl and extracted again with $CHCl_3$. The $CHCl_3$ extracts were dried over magnesium sulfate and then evaporated to yield IIIb. This compound showed absorption spectra analogous to those of IIa: F > 350 °C.

8α-Amino-lumiflavin (IV). 10 mg of the pure IIIa were dissolved in 2 ml 6-N HCl, the solution was refluxed, the reaction course was followed by thin-layer chromatography (System A). After 12 h the acid was distilled off *in vacuo*. The residue was homogeneous by thin-layer chromatography and contained a free NH_2-group according to anaerobic pH-titration. Because the free base is very sensitive to air, and the salts are difficult to isolate, the aqueous solution was used for measurement of optical spectra and fluorescence directly.

3-Methyl-8α-(N)-morpholino-lumiflavin (VIa). 0.5 g (1.85 mmol) of the crude IIb were dissolved in 2 ml morpholine and this solution kept for 2 min at 100 °C. The deep-red solution was cooled to room temperature and 200 mg sodium borohydride were added in several portions over a period of 20 min. To this solution 20 ml of water were added, the aqueous phase was extracted three times with $CHCl_3$, and the $CHCl_3$ solution dried over magnesium sulfate and reduced to an oil *in vacuo*. This residue was dissolved in 20 ml 1-N acetic acid, treated with charcoal, filtered, the filtrate adjusted to pH 8 with sodium carbonate and extracted four times with $CHCl_3$. The dried organic phase was evaporated to yield 230 mg of the crude VIa (29%). This compound was homogeneous by thin-layer chromatography (System A). Recrystallization from chloroform/isopropylether yielded orange needles, F 218 to 222 °C. The compound shows pK_a values of −0.5 (flavin nucleus) and of 5.2 (morpholino residue). Found: C 60.72, H 5.86, N 19.99%; $C_{18}H_{21}N_5O_3$ (M_r 355.39) requires: C 60.83, H 5.96, N 19.71%). λ_{max} (ε) at pH 9: 445 (11800), 357 (9600), 271 (40600) and 222 nm (31400). λ_{max} (ε) at pH 3: 445 (9500), 338 nm (9200). λ_{max} (ε) (H_0 = −1): 400 sh (8600), 365 nm (14300). NMR (C^2HCl_3): δ = 8.21 (6-H), 7.75 (9-H), 4.14 (10-CH_3), 3.69 (8-CH_2—), 3.54 (3-CH_3) and 2.54 ppm (7-CH_3). NMR (CF_3COOH): δ = 8.75 (6-H), 8.60 (9-H), 5.02 (8-CH_2—), 4.68 (10-CH_3), 3.78 (3-CH_3) and 2.90 ppm (7-CH_3).

The following compounds have been synthesized analogously.

3-Methyl-8α-(N)-imidazolyl-lumiflavin (VIb). λ_{max} (pH 9): 442, 342 and 268 nm. λ_{max} (pH 3): 442, 335 and 268 nm. λ_{max} (6-N HCl): 400 sh, 355 and 265 nm.

3-Methyl-8α-(p-methoxycarbonylanilino)-Lumiflavin (VIc). F = 350 °C (dec.). λ_{max} (ε) at pH 7: 443 (11900), 358 (8800), 305 (21400), 272 (43000) and 225 nm (33000). λ_{max} (ε) in 6-N HCl: 405 sh (9200), 368 (14000), 268 (32000) and 225 nm (34000). NMR (CF_3COOH): δ = 8.43 (6-H), 8.38 (9-H), 8.28, 8.13, 7.48 and 7.33 (AB-quadruplet of the benzene protons), 5.03 (8-CH_2—), 4.43 (O—CH_3), 4.08 (10-CH_3) and 2.60 ppm (7-CH_3).

3-Methyl-8α-anilino-lumiflavin (VId). This compound shows absorption spectra analogous to those of VIc.

RESULTS AND DISCUSSION

As mentioned in the introduction, positions 1, 2, 3, and 10 in the isoalloxazine nucleus may be

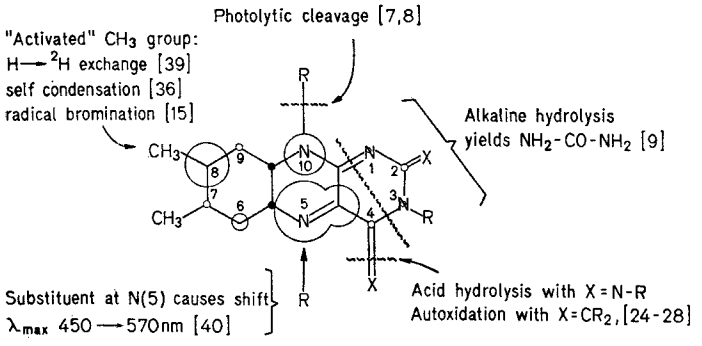


Fig. 1. *Map of flavin submolecular structure and reactivity.* The size of the open circles indicates relative spin densities. Full dots indicate sites of unknown spin density

eliminated as the site of attachment of the peptide [7—9] (Fig. 1). Positions 4 and 5 are also excluded on the basis of extensive studies on the stability of model compounds substituted in these positions [24—28]. Thus, OR, NR_2, and SR substituents are easily split from the 4-position by both acid and alkaline hydrolysis, yielding normal flavin, while H-, alkyl, and aryl substituents are also removed from C(4) by photo-oxidation, yielding again normal flavin. Alkyl substituents on N(5) on the other hand, are easily hydrolyzed in acid media. No transformation of SD-flavin into known normal flavin occurs under similar hydrolytic or oxidative conditions. Of the remaining positions, 6 to 9, ESR activity in flavin radicals is connected only with position 6 and 8, while the spin density is low in positions 7 and 9 (Fig. 1) [29—31]. In radical cations spin density is also small in position 6 [31].

ESR Spectra

Fig. 2 compares the ESR spectra of flavosemiquinone cations for riboflavin (A, B) and SD-flavin (C). Although the preparation used to obtain C was not completely free from extraneous amino acids, the spectrum is as well resolved as that of pure synthetic SD-flavin prepared later on [17]. Higher resolution would require less polar solvent [32] in which SD-flavin is unfortunately not soluble. The total width of spectrum C, 46 G, is reduced as compared with A and B, 52 G, with an amount corresponding closely to twice the main line spacing. A similar reduction is found on comparing the ESR spectra of radical cations of lumiflavin (I, Fig. 2E) and 8α-hydroxy-lumiflavin (V, Fig. 2D). This reduction is typical for substitution of a hydrogen of a methyl group with proton couplings about equal to the main spacing.

The individual hyperfine structure and resolution of the signals in case of riboflavin are different in 6-N and conc. HCl, whereas the total width remains constant. This is consistent with the view that when a comparatively bulky substituent is introduced for one methyl hydrogen, which previously had a total isotropic hyperfine coupling of $3a$ (three equivalent protons), then the total coupling of the methylene group will be a, if the rotation is hindered, so that the substituent is situated on one side of the isoalloxazine plane and the two methylene hydrogens on the other. Depending on the effective equilibrium position of these hydrogens the coupling a may come mainly from one of them or it could be distributed more evenly between them, thus giving different hyperfine patterns. We have observed that in many instances a position seems to be preferred which gives one of the methylene hydrogens a dominant coupling. This is what we have sometimes called "loss" of two ESR-active protons when a methyl hydrogen is substituted. If more than one equilibrium position is possible the spectrum will be further complicated and depend on the frequency of oscillation. It might also be important to consider changes of the relaxation time, e.g. because of short radical lifetime in a dynamic equilibrium, which could affect ESR-line width and resolution. In all these situations, however, the contribution to the total width of the ESR spectrum will be a. The exact behavior of the ribityl side chain is thus different in conc. HCl and 6-N HCl. No similar effect has been detected with the SD-flavin, presumably because of restriction of possible ribityl chain conformation introduced by H-bonding towards the 8α-substituent.

As shown earlier [1], alkaline photolysis of SD-flavin derivatives results in an increase of total spectral width due to change of ribityl in SD-riboflavin to methyl in SD-lumiflavin. The alkaline photolyzate, however, is not homogeneous in the flavin component: in contrast to riboflavin, which yields 100% lumiflavin upon alkaline photolysis, photolytic breakdown of SD-flavin invariably results in a mixture of lumiflavin and lumichrome. Hence, with the small amounts of natural SD-flavin at hand, further study of photolysis was not feasible.

Nevertheless, ESR spectrum C provides final evidence for a link between peptide backbone and a hyperfine coupled methyl group of riboflavin. Since the only strongly coupled methyl group of the riboflavin nucleus is in position 8α, the site of attachment of the covalently bound flavin to the peptide is fixed by this result unequivocally.

Optical and Ultraviolet Spectra

It is known from Kearney *et al.* [7,8] that SD-flavin peptide in the oxidized state exhibits a shift of the second band from 370 towards 340—350 nm under aqueous, neutral conditions as compared with riboflavin, while the first band at 445 nm remains the same. This second band of SD-flavin peptide also reflects the pK of fluorescence quenching at 4.5 by a small but reproducible shift between

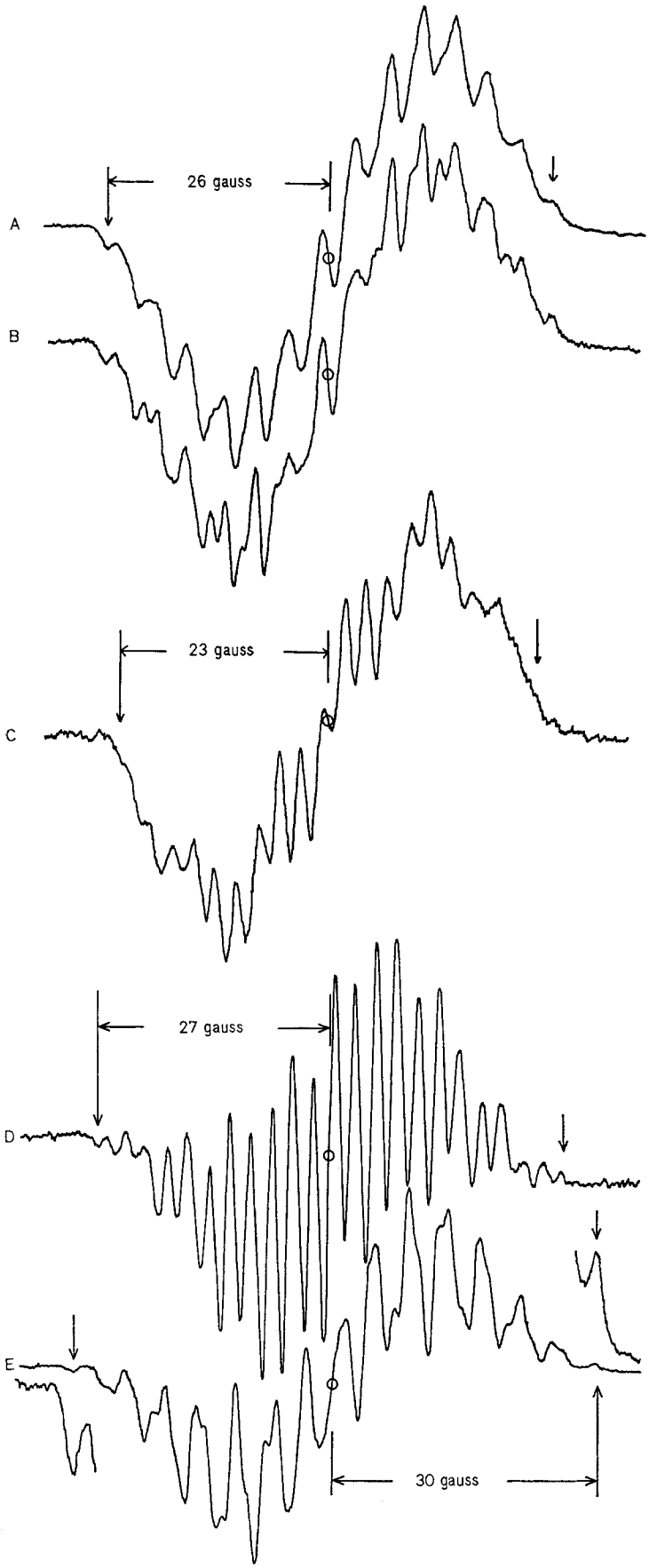


Fig. 2. *Comparison of ESR spectra of flavosemiquinone cations.* (A) Riboflavin in conc. HCl. (B) Riboflavin in 6-N HCl. (C) SD-flavin in 6-N HCl. (D) 8α-Hydroxy-lumiflavin (V) in 6-N HCl. (E) Lumiflavin (Ia) in 6-N HCl. For precise conditions of sample preparation *cf.* Methods and Materials. "Half total widths of signal" are measured from the top of the outermost ESR-line to the signal center. No further wing lines are revealed by increasing the gain 10-fold

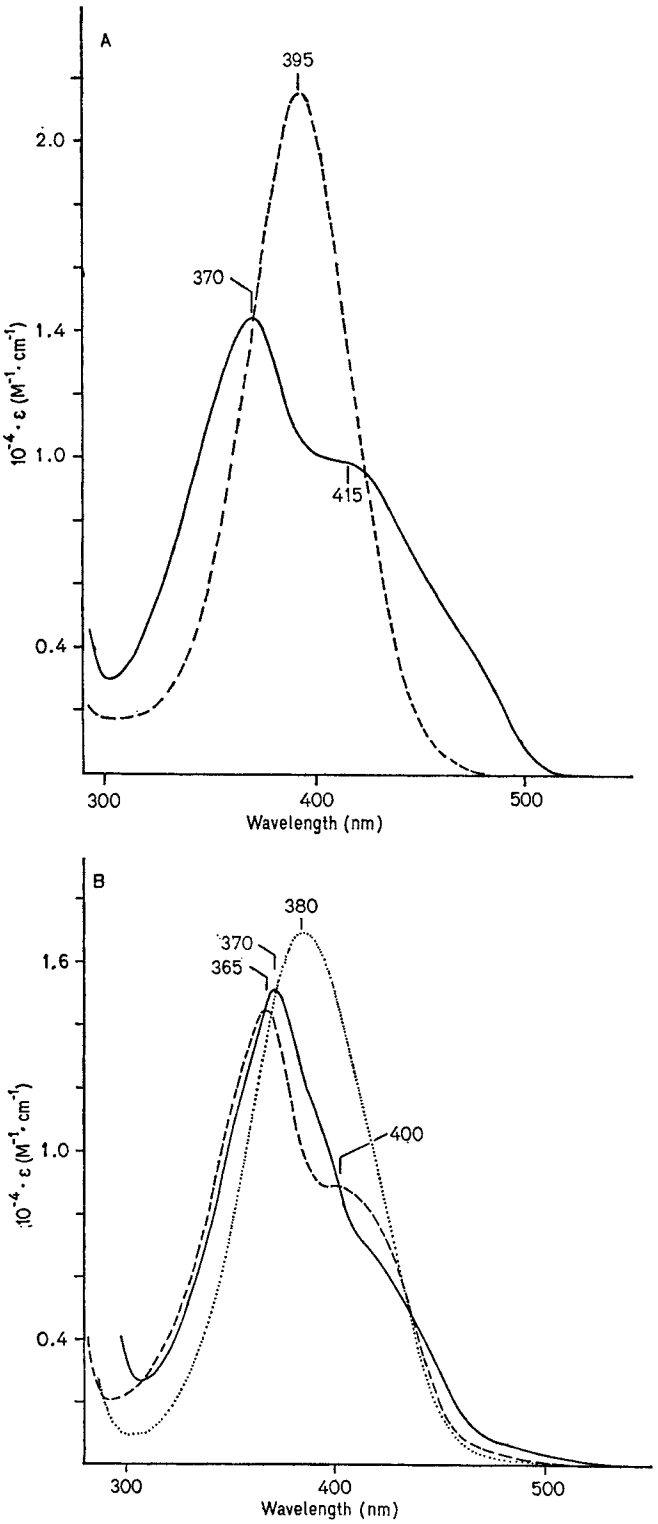


Fig. 3. (A) *Comparison of electronic spectra of SD-flavin trication (——) and riboflavin cation (— — —)* both in 6-N HCl. (B) *Spectra in 6-N HCl of "SD-flavin models"*, 8α-hydroxy-lumiflavin (V) (·····), 8α-oxo-lumiflavin (IIa) (— — —) and 8α-(*N*-morpholino)-3-methyl-lumiflavin (VIa) (——)

340—350 nm [18]. Exactly the same spectral behavior is observed for acid-hydrolyzed SD-flavin. Hence, we define, with support from electrophoretic data [17], SD-flavin between pH 9 and 4.5 as "neutral", between pH 4.5 and 3 as "monocation" and, since it contains an intact α-amino acid group as seen from its ninhydrin reaction, we may expect a "dication" to exist between pH 3 and 0 and a "trication" at pH 0. The first protonation is reflected only slightly in the second band of the absorption spectrum and may, therefore, be concerned with the 8α-function. The second protonation involving the COO^--group is not reflected spectrally, while the third protonation, occurring at N(1) as in riboflavin, causes a drastic shift of absorption. Fig. 3 A compares the SD-flavin trication with riboflavin cation, both in 6-N HCl. The first two absorption bands of neutral riboflavin at 370 and 445 nm telescope into a single one at 395 nm of double intensity. It has been found a characteristic feature of SD-flavin that N(1) protonation causes a less pronounced effect than in riboflavin, resulting in a main band at 373 and a shoulder at about 410 nm. The phenomenon is characteristic of flavins which are functionally substituted at C(8α), as shown in Fig. 3 B. Fig. 4 B shows the spectrum of SD-flavin pentapeptide in 6-N HCl oxidized (curve 1), and successively reduced to the radical cation (curves 2, 3). It is important to note that in the SD-radical cation there is also an hypsochromic shift of about 25 nm as compared to riboflavin radical cation [33]. The peak of 350 nm (Curve 2) represents the second band of the radical cation while the peak at 315 (Curve 3) represents the first band of the fully-reduced cation, in agreement with known data on normal flavins [33].

ENDOR Studies

Additional strong support for 8α-substitution in SD-flavin came from ENDOR studies, performed in collaboration with Dr J. S. Hyde of Varian Associates [18]. In order to permit unambiguous assignment of the different ENDOR signals to individual functional groups in the flavin ring system, the ENDOR spectra of radicals of systematically selected model compounds were recorded and analyzed and comparison was made with the previously obtained ESR data [34].

Fig. 5 B shows the ENDOR spectrum of 3-methyl-lumiflavin radical in 6-N HCl. The intense signal at 13.25 MHz is the matrix-ENDOR and is due to dipole coupled protons, mainly solvent protons in the vicinity of the free radical. The signals centered at about 18 and 20 MHz, which are 4.75 and 6.75 MHz from the free proton frequency, are due to the 8-CH_3 and 10-CH_3 groups, respectively, with couplings of 3.4 G and 4.8 G. The CH_3 groups at the 3- and 7-positions have very small hyperfine couplings and thus contribute only to the

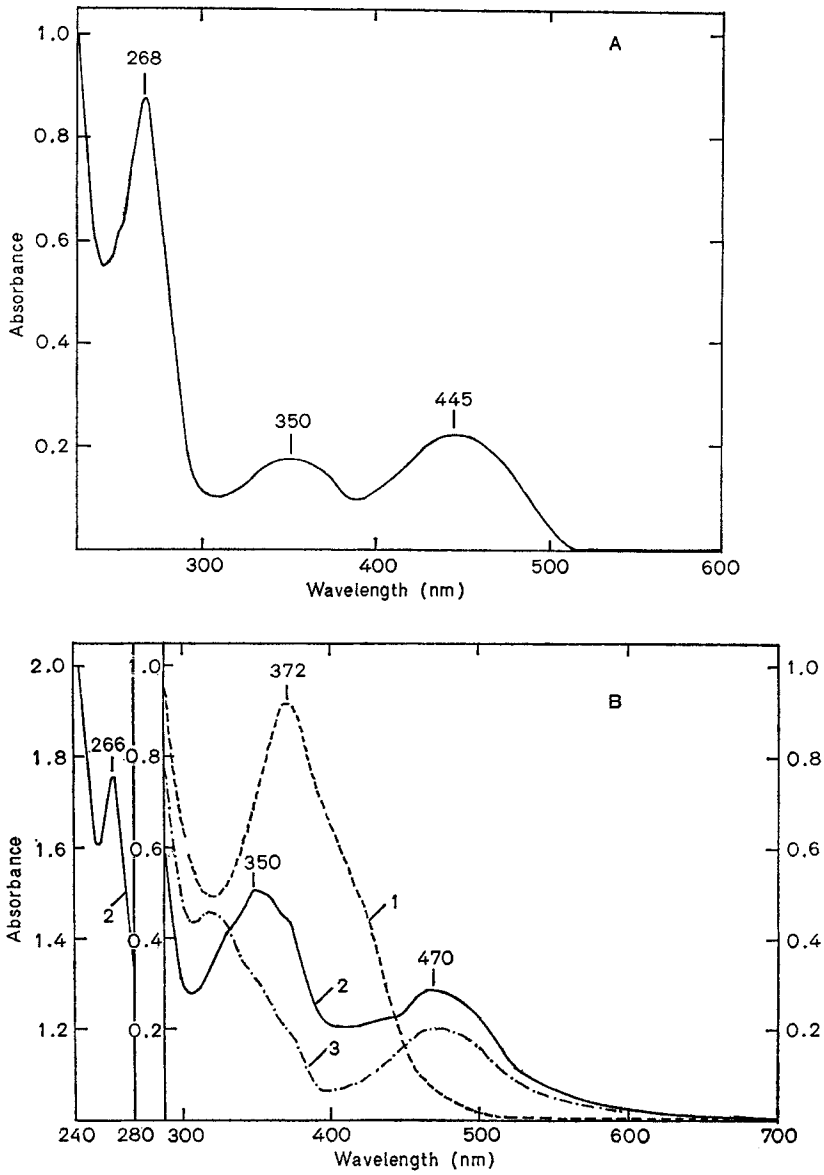


Fig. 4. *Optical spectra of flavin peptide in various states.* The material used in these experiments was fraction B3 (as described in Materials and Methods). (A) Neutral, oxidized form, monophosphate level. (B) Protonated form at various stages of reduction. Curve 1: Oxidized (in 6-N HCl). Curve 2: After addition of slightly less than 1 equivalent of Ti^{+3} (radical cation not yet completely formed). Curve 3: After further addition of Ti^{+3} (radical cation overreduced)

shoulder of the matrix-ENDOR. Riboflavin (Fig. 5A) shows only the intense signal at 4.75 MHz from the free pronto frequency, which must be due to the 8-CH_3 group, since the 10-CH_3 group is absent here and the methylene protons in the 10 position do not give a strong ENDOR signal for reasons discussed below. The strong ENDOR signal due to the 8-CH_3 group has been previously identified in the radical of lumiflavin [34] and in the FMN radical of photoreduced old yellow enzyme [35].

Fig. 6A, 6B, and 7B reproduce the ENDOR spectra of cation radicals of 8α-morpholino-3-methyl-lumiflavin, 8-nor-8-chloro-lumiflavin, and 10-methyl-isoalloxazine, respectively. These compounds show only the intense signal due to the 10-CH_3 group at around 20 MHz with the shoulder at the high frequency side (about 21 MHz), which is considered to be anisotropic contribution of the same CH_3 group and is seen in all compounds (Fig. 5B, 6A, 6B, and 7B) containing this group. It is quite clear, therefore, that the signal at around 18 MHz is due to the 8-CH_3 group, since all compounds in which this is absent lack the corresponding signal. Comparison of these ENDOR spectra

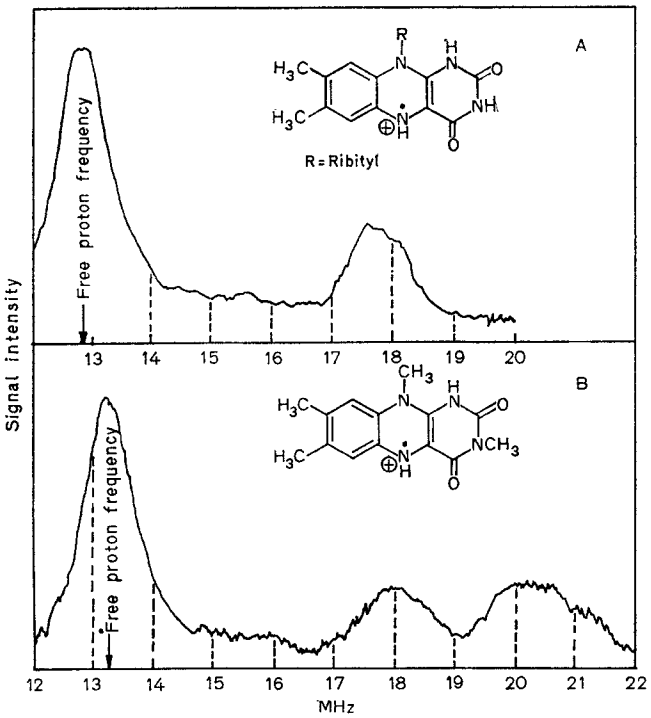


Fig. 5. *ENDOR spectra of radicals of riboflavin (A) and 3-methyl-lumiflavin (B)* in 6-N HCl

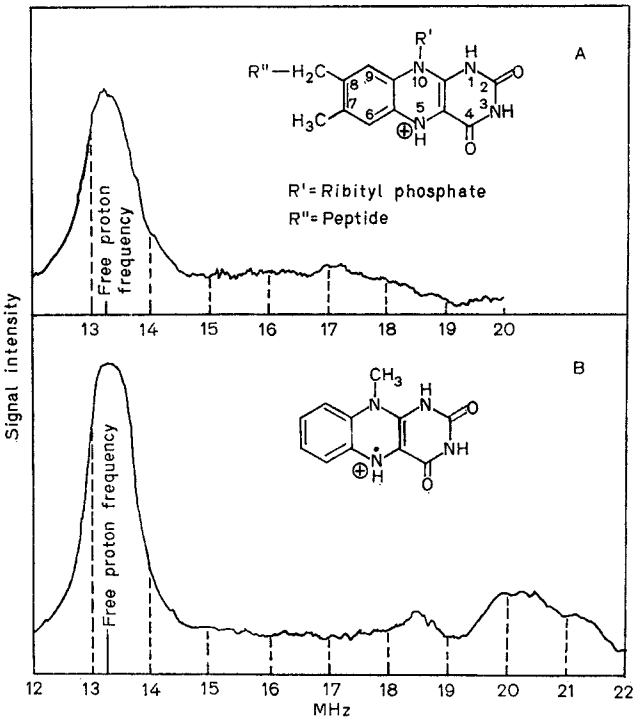


Fig. 7. *ENDOR spectra of radicals of SD-flavin peptide (A) and of 10-methylisoalloxazine (B)* in 6-N HCl. The peptide preparation was the same as in Fig. 4

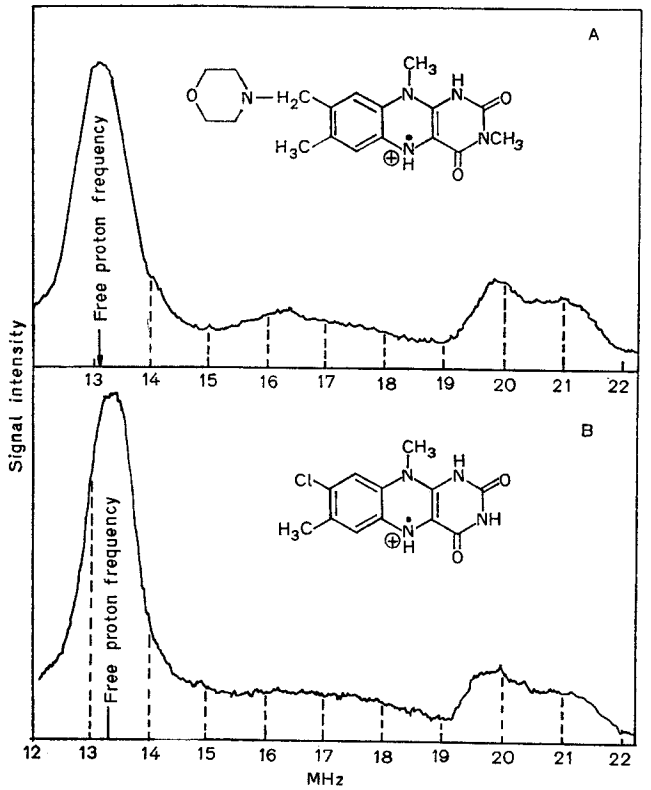


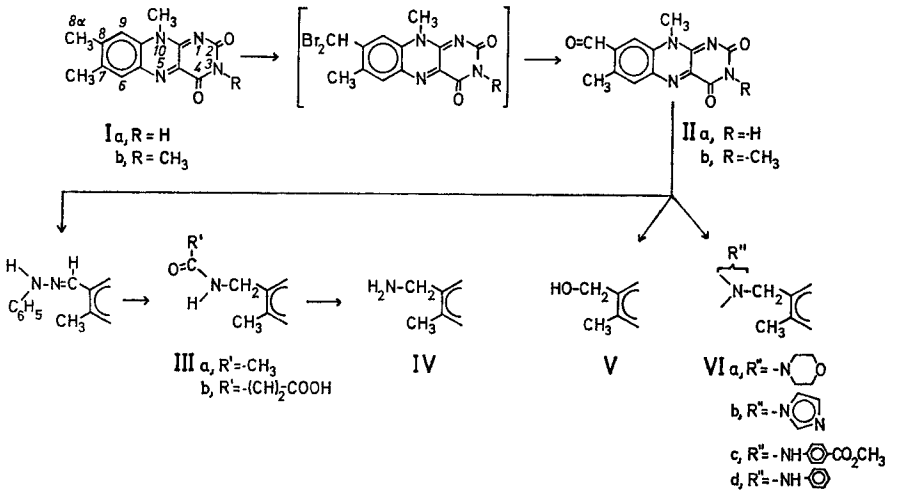
Fig. 6. *ENDOR spectra of radicals of 8α-morpholino-3-lumiflavin (A) and 8-nor-8-chloro-lumiflavin (B)* in 6-N HCl

show that the 8- or 10-methylene protons, in contrast with methyl protons, do not give a strong ENDOR signal. The hyperfine coupling to the methylene protons is highly dependent on their orientation with respect to the molecular framework; if this orientation is not well-defined, the resulting ENDOR signal broadens and is usually not discernible at low temperature. Comparison of the ENDOR spectra of riboflavin and 8α-morpholino-3-methyl-lumiflavin with that of 8-nor-8-chloro-lumiflavin nevertheless shows that the 8-methylene protons contribute to the broad shoulder in the region 3 to 5 MHz from the matrix-ENDOR.

In the light of these facts the ENDOR spectrum of SD-flavin peptide (Fig. 7 A) may be readily interpreted. The signal at 6.75 MHz from the free proton frequency (around 20 MHz) is missing because the 10 position carries a methylene, not a methyl group. The signal at about 18 MHz (4.75 MHz from the free proton frequency) is also missing, which confirms the conclusion that the 8α-methyl group is substituted. The broad signals between 16 and 18 MHz might in part be due to the methylene protons in the 8α and 10α positions.

Synthesis of SD-Flavin Model Compounds and Variation of Fluorescence with pH

It was stated earlier that the most obvious difference between SD-flavin and "normal" flavin

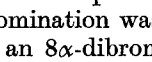
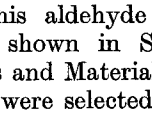
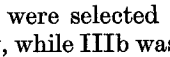
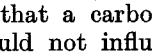


Scheme 1. *Synthesis of SD-flavin-models*

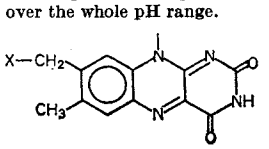
was in the absorption of the protonated flavin nucleus (Fig. 3A). In model compounds exhibiting similar spectra this effect is characteristic of flavins bearing functional substituents in position 8α. Since it was desirable to eliminate the sensitive 10-ribityl side chain in favor of a stable methyl group, a whole series of such model compounds was prepared starting from lumiflavin. This modification could be anticipated to have no influence on the flavin chromophore. Since it was known that the methyl group in position 8 of the flavin nucleus would be susceptible to electrophilic substitution in conc. H_2SO_4 [36], bromination was attempted under these conditions and an 8α-dibromide was obtained. This compound was easily hydrolyzed to yield the aldehyde, 8α-oxo-lumiflavin (II). The above mentioned series of 8α-monofunctional derivatives was prepared from this aldehyde by different reductive pathways, as shown in Scheme 1 and outlined under Methods and Materials. Compounds IIIa, IV, V, VIa—VId, were selected according to increasing proton affinity, while IIIb was meant to be a model of a SD-flavin-glutamyl-peptide. From this it was demonstrated that a carboxylate group in such a side chain would not influence flavin fluorescence by any kind of excit one transfer or "hairpin complexation". However, all the "homoconjugated" basic 8α-functions have been found to quench flavin fluorescence.

Localization of the peptide chain at the 8α position explains all the characteristic properties of SD-flavin except one: the quenching of fluorescence with a pK of 4.6 ± 0.1. Since this property was considered to be a clue to the nature of the substituent in the 8α position, the pH-fluorescence curve of SD-flavin was compared with that of flavin model compounds substituted in this position (Table 1). From this table it can be seen that

Table 1. pK_a-*Values of flavocoenzymes and 8α-substituted models*
As determined fluorometrically from the "pH of half quenching"

Substituent X	pK_a of XH[a]	pK_a of 8α-X-Fl_{ox}	Maximum 8α-induced quenching[b]
— H (FMN)	—	10.0[c]	None
— OH (V)	—	10.2[c]	None
— $NHCO(CH)_2COOH$ (IIIb)	≈ 3	10.0[c]	None
— NH_3^+ (IV)	9.2	8.4	>50%
—  (VIa)	8.2	5.3[d]	>95%
SD-flavin	?	4.6	>95%
FAD	(3.3)[e]	(3.7)[e]	80%[e]
—  (VIb)	6.9	3.8	>75%
—  (VId)	4.6	≈ 2	>95%
—  (VIc)	2.4	<2[f]	>90%

[a] The pK-values of the free bases are taken from [37].
[b] High quenching percentages cannot be determined precisely because of residual fluorescence from background and impurities. In the case of IV, 8α- and 3-deprotonation are partially overlapping.
[c] "Alkaline" quenching due to flavin-N(3) deprotonation.
[d] The same pK was determined spectrophotometrically and by pH-titration.
[e] Quenching and pK due to adenine-N(1) deprotonation [38].
[f] pK_a of 8α-function lower than pKa of flavoquinone-excited singlet state: fluorescence quenched over the whole pH range.

8α-X-Fl_{ox} = 

"SD-flavin" is intermediate between 8α-(morpholyl) (VIa) and 8α(*N*-imidazolyl) flavin (VIb). Electrophoresis demonstrates that an additional positive charge arises upon acidification of SD-flavin with a *pK* of 4.5. The fact that this *pK* is slightly reflected by the absorption spectra and strongly by the fluorescence together with the simulation of this phenomenon by model compounds, does not leave much choice for the nature of the peptide-flavin linkage: C(8α) of the flavin must be linked directly to a tertiary nitrogen function of *pK* 4.5, preferably a heteroaromatic nitrogen. Histidine, therefore, seemed a good possibility for the 8α-substituent.

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