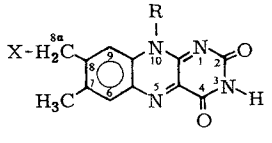
The Synthesis of Succinate-Dehydrogenase Riboflavin

By *Sandro Ghisla*, *Ursula Hartmann*, and *Peter Hemmerich* [*]

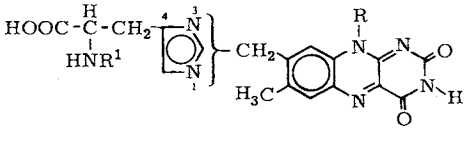
Singer and *Kearney* [1], in their early studies, recognized that the flavocoenzyme of mitochondrial succinate dehydrogenase ("SD-flavin") [**] was a derivative of flavin-adenine-dinucleotide which was covalently bound to the protein. In cooperation with Dr. *Singer*'s team [***], we have attempted to elucidate the structure of this coenzyme. In the first preliminary communication [2], we concluded from the hyperfine structure of electron spin resonance (ESR) spectra of partially purified SD-riboflavin (obtained from SD-FAD-peptide mixtures by total hydrolysis in 6 N HCl) that

a) SD-riboflavin is irreversibly converted into riboflavin *(1a)* by reduction and

b) the bond between the leaving group "X" [cf. *(1)*] and the heteroaromatic flavin nucleus is connected through position 8α.



(1a), X = H, R = Ribityl
(1b), X = Morpholino, R = CH_3
(1c), X = Br, R = Tetraacetyl-ribityl



(2a), R = Tetraacetyl-ribityl, R^1 = C_6H_5CO
(2b), R = Ribityl; R^1 = H

The light absorption of neutral SD-riboflavin is hardly different from that of riboflavin *(1a)* (λ_{max} 445 nm), but the near UV spectrum of the oxidized cation differs more drastically (Fig. 1, λ_{max} shifted from 395 [2] to 373 nm). Most characteristic is the pH-dependence of the fluorescence obtained with SD-riboflavin (Fig. 2) and, as mentioned above, the ESR hyperfine structure of the red radical cation (Fig. 3). Since these ESR results could be interpreted unequivocally [2] in terms of 8α-substitution of the flavin nucleus, we have synthesized 8α-substituted flavin derivatives [3], *e.g. (1b)*, which mimic the light absorption as well as the fluorescence properties of SD-riboflavin closely. This work enabled us to predict that the characteristic quenching of SD-riboflavin fluorescence with p*K* 4.4 (Fig. 2) originates from a secondary or tertiary nitrogen function in position 8α. Subsequently, *Walker* and *Singer* [4] succeeded in identifying the X-fragment obtained from reductive cleavage of the flavin-X-bond as *histidine*.

We have now synthesized SD-riboflavin *(2b)* as follows: Riboflavin *(1a)* is peracetylated in pyridine-acetic anhydride and then monobrominated in position 8α with an 80 % yield by refluxing 40 min with an 1.3-fold excess of Br_2 and catalytic amounts of dibenzoyl peroxide in dioxane. The yield is estimated from the decrease of the C(8)CH_3-peak in the NMR spectrum (δ = 2.45 ppm).

In addition to the C(8)CH_2Br-signal (δ = 4.7 ppm), the product of bromination *(1c)* shows only a trace of C(8)$CHBr_2$-signal (δ = 7.0 ppm [6]), indicating the presence of dibrominated analog [6]. The dibrominated product is obtained quantitatively upon heating with a larger excess of bromine in

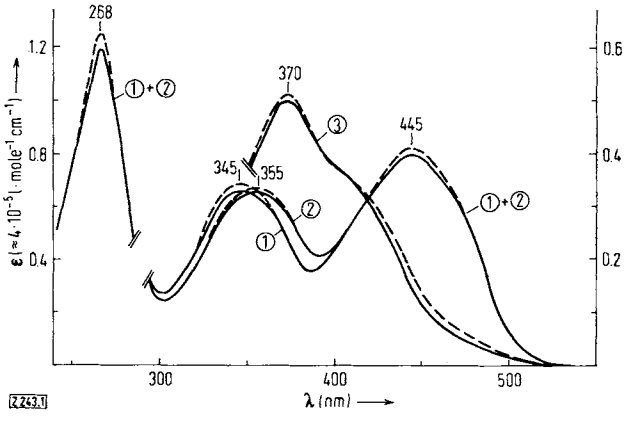


Fig. 1. Electronic spectra of native (---) and synthetic (—) SD-riboflavin.
① Neutral SD-riboflavin, pH 7, λ_{max} = 445, 355, 268, 221 nm.
② SD-riboflavin monocation protonated in the imidazole ring, pH 3, λ_{max} = 445, 345, 268, 221 nm.
③ Dication, 6 N HCl, λ_{max} = 405 (S), 373, 265, 221 nm.

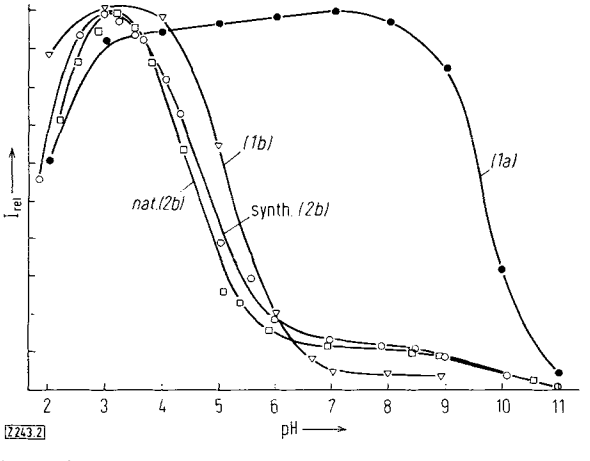


Fig. 2. Dependence of the relative intensity of fluorescence I_{rel} on the pH of unbuffered solutions of native and synthetic SD-riboflavin *(2b)*, riboflavin *(1a)*, and the model compound *(1b)*.

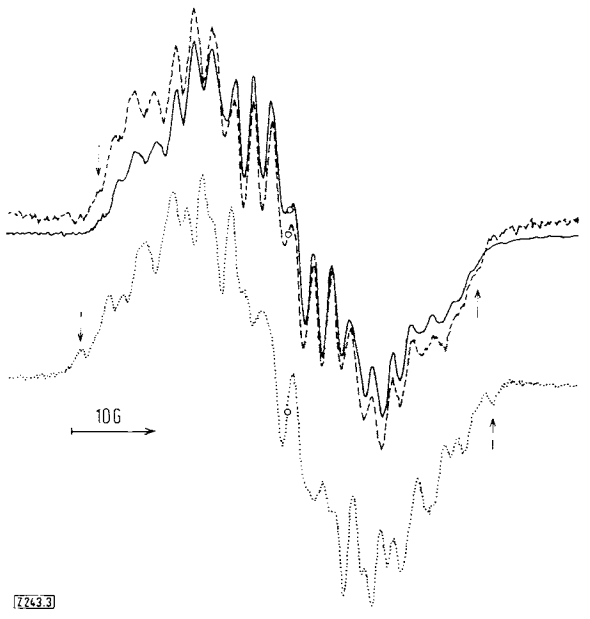


Fig. 3. Hyperfine structure of the ESR of SD-riboflavosemiquinone cation in 6 N HCl obtained by reduction with $TiCl_3$. The arrows indicate the outermost lines of the spectrum. Riboflavin *(1a)* shown for comparison, exhibits 2 more ESR active 8α-protons than SD-riboflavin *(2b)* since in contrast to the 8-methyl group in riboflavin *(1a)*, the bulky histidyl-CH_2 group in *(2b)* is not free to rotate. (An analogous situation, for instance, exists with 5-methyl- and 5-benzylflavosemiquinone [5].) All ESR spectra have been recorded at a modulation amplitude of 0.5 gauss and 25 mW power in an anaerobic flat cell with a Varian E3 spectrometer. ———: native SD-riboflavin, —: synthetic SD-riboflavin, ····: riboflavin.

presence of pyridine[6]. The monobromide *(1c)* was heated for 15 hours at 90 °C in absolute dimethylformamide (without addition of a basic catalyst) with a 2.5-fold excess of N_α-benzoylhistidine. The resulting flavin mixture was shaken in aqueous buffer of pH 7. The aqueous phase contains the 8α-histidyl flavin *(2a)* which can be separated from the unreacted histidine by extraction with chloroform at pH 3 after saturation of the aqueous phase with ammonium sulfate. Finally, *(2a)* is hydrolyzed to *(2b)* by refluxing in 6 N HCl for 2 hours (overall yield 35 %).

The ESR and light absorption spectra of the resulting 8α-(*N*-histidyl)riboflavin are identical with those of authentic native material[4] (Figs. 1, 3). The pH derived from quenching of fluorescence (Fig. 2) of the synthetic product is slightly less clear cut than that of the native material. We conclude from this that the synthesis leads to a mixture of isomers because the imidazole nitrogens are chemically not equivalent. The native and synthetic products appear homogeneous and identical in thin layer chromatography on silica gel. The chromatographic separation of the presumed isomers and their structural assignment is presently under investigation.

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[**] The general term "flavin" denotes all species having formula *(1)* with any groups R and X, but not R = H.

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[1] *T. P. Singer*, *J. Hauber*, and *E. B. Kearney*, Biochem. Biophysic. Res. Commun. *9*, 146 (1962); *E. B. Kearney*, J. biol. Chemistry *253*, 865 (1960).

[2] *P. Hemmerich*, *A. Ehrenberg*, *W. H. Walker*, *L. E. G. Eriksson*, *J. Salach*, *P. Bader*, and *T. P. Singer*, FEBS-Letters *3*, 37 (1969).

[3] *S. Ghisla* and *P. Hemmerich*, unpublished.

[4] *W. H. Walker* and *T. P. Singer*, J. biol. Chemistry, in press.

[5] *F. Müller*, *P. Hemmerich*, *A. Ehrenberg*, *G. Palmer*, and *V. Massey*, Europ. J. Biochem. *14*, 185 (1970).

[6] *D. B. McCormick*, J. heterocyclic Chem. *7*, 447 (1970).