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Evidence from electron-spin-resonance spectra for metal chelation shifting flavin-leucoflavin equilibria towards radical state

It has been shown as early as 1938 by MICHAELIS AND SCHWARZENBACH[1] that the redox system of flavin coenzymes appears to be more than 90% disproportionated at neutral pH, the radical state contributing only slightly to the total flavin in the half-reduced state:

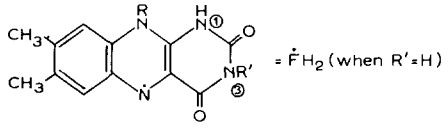
$$\begin{matrix} FH_3 + FH \\ \Updownarrow \\ (FH_2)_2 \end{matrix} \rightleftharpoons 2\,\dot{F}H_2$$

$(FH_2)_2$ behaves essentially as a mixture of FH_3 and FH, as stated by one of us[2] and confirmed by GIBSON *et al.*[3], though it can arise from dimerisation of flavin radical. $(FH_2)_2$ exists to measurable extent only in concentrated ($c > 10^{-3}$ M) polar solution and thus has presumably no biological significance. Furthermore it is not paramagnetic, as found in the course of the present study.

The pH dependance of the above-mentioned equilibrium obeys a function which runs through a minimum of radical concentration just in the physiological pH region[4], a behaviour which is confirmed by the ESR data of EHRENBERG[5]. Furthermore, one of us[6] has given evidence, by studying visible absorption changes and proton release in half-reduced flavin on addition of metal ion, that the semiquinone radical anion FH^- is the only flavin species exhibiting measurable affinity for normal non-reducing d-metal ions in aqueous solution. Thus, by adding such cations to the half-reduced flavin system, the above-mentioned equilibrium is displaced in favour of a radical chelate at physiological pH:

$$FH_3 + FH + 2\,Me^{2+} \rightleftharpoons 2[\dot{F}HMe]^+ + 2\,H^+$$

This proton release occurs at position 1, not at position 3, of the isoalloxazine nucleus



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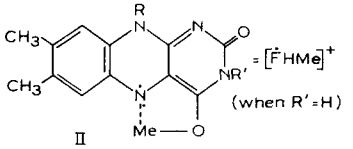

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since flavins alkylated in position 3 appear to form even stronger radical chelates than the unsubstituted analogues.

These assignments of visible spectra and proton release phenomena have been confirmed in the present study, where the radical character of these chelates was established by measurement of the ESR spectrum. Measurements were made on two types of solutions:

(a) Polar type: $5 \cdot 10^{-3}$ M lumiflavin-3-acetic acid (see ref. 4) (I, R = CH_3, R' = CH_2COO^-) in $5 \cdot 10^{-2}$ M Veronal buffer (pH 7.5), 0.1 N in $NaClO_4$, hydrogenated with

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Abbreviations: FH, riboflavin, FMN or FAD in the oxidized state = "flavoquinone"; FH_3, reduced state = "leucoflavin"; $\dot{F}H_2$, flavin radical = "flavosemiquinone"; $(FH_2)_2$, charge-transfer complex between FH_3 and FH = "radical dimer" or "flavoquinhydrone"; ESR, electron-spin resonance; Me^{2+}, divalent metal ion.

palladium on silica as catalyst, filtered under N_2, and about 50% reoxidized by air, with and without 10^{-2} M $MeSO_4$ (Me = Ni, Zn, Fe).

(b) Non-polar type: 10^{-2} M tetraacetyl-riboflavin (I, R = peracetylribityl, R' = H) (see ref. 4) in $CHCl_3$, reduced by shaking with 1.0 M aq. $Na_2S_2O_4$ saturated with NaCl, 50% reoxidized by controlled addition of 0.1 M I_2 in $CHCl_3$.

In Case b the solution was mixed under nitrogen with a 4-fold excess of 0.1 M triethylamine in $CHCl_3$ to neutralize all protons released, and then with an equivalent quantity of 0.01 M metal in CH_3CN, either as the benzyltrimethylammonium-chloride–tetrachloroferrate complex, $[C_6H_5CH_2N(CH_3)_3]_2Fe^{II}Cl_4$, or as $Zn(CH_3COO)_2$.

All solutions were kept thoroughly anaerobic.

In each case the 50%-reduced yellow flavin solutions turned brown-red on addition of metal, thus indicating the formation of a flavin–metal complex. This colour is practically independent of the nature of the metal. Blank solutions of metal without flavin show negligible visible absorption under the same conditions of dilution and neutrality.

The metal-free blanks show only a very weak ESR signal at $g = 2.00$, the hyperfine structure of which could not be resolved. But on addition of Zn^{2+} there was, along with the colour change, a 20-fold increase in signal intensity, from which a neat hyperfine resolution could be obtained (Fig. 1). In contrast, the addition of

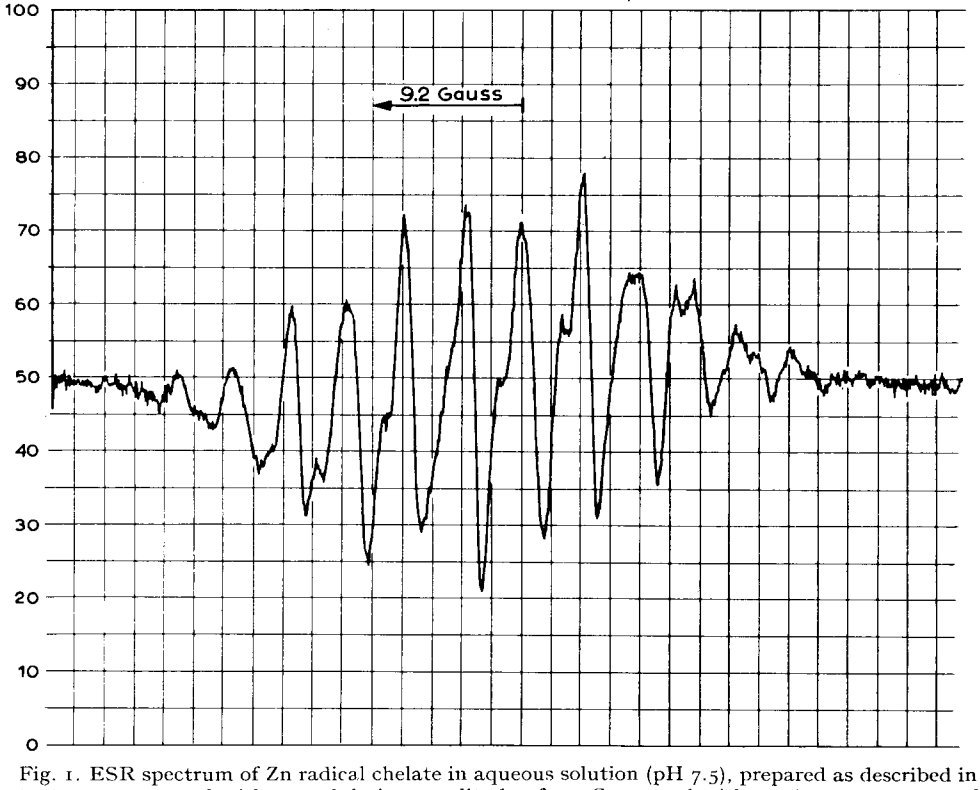

Fig. 1. ESR spectrum of Zn radical chelate in aqueous solution (pH 7.5), prepared as described in text, was measured with a modulation amplitude of 0.2 Gauss and with a microwave power of 1 mW in a Varian aqueous solution cell. The measurements were performed in a Varian V 4510 X-band spectrometer.

the paramagnetic ions Fe^{2+} and Ni^{2+}, though leading to the same colour change, did not alter significantly the weak signal obtained with the metal-free solutions, thus demonstrating spin–spin interaction in the case of chelates that are built up from radical ligand and paramagnetic metal ion.

When there was added enough I_2 or O_2 to convert all the iron into the trivalent state, a new signal could be observed at $g = 4.00$ at $-194°$, but not at room temperature. The metal blank showed no such absorption.

The same results were obtained in polar (type a) as well as in non-polar (type b) solutions.

We wish to make the following statements concerning the biological significance of these findings:

One of us has demonstrated the striking similarity of the visible spectra of the flavin radical chelates and the native mitochondrial metalloflavoproteins[6–8]. The ESR spectra of such enzymes were measured by BEINERT *et al.*[9] as well as by us[10]. Four kinds of signals were obtained: at $g = 1.94$, 2.00, 2.01, 4.00, respectively, at $-194°$; only the $g = 2.00$ signal was found at room temperature. The $g = 1.94$ and 2.01 signals were tentatively assigned to a Fe^{2+} compound by BEINERT[9], the $g = 4.00$ signal to Fe^{3+} and the $g = 2.00$ signal to the free flavin radical.

We can confirm by the model studies the last two assignments, but the first two remain in question. Our ESR studies of flavo-coenzyme model compounds support the Fe^{3+}-flavosemiquinone chelate structure at the active site of the oxidized enzyme, and furthermore a possible role of non-heme iron acting as stabilizer of the flavin radical. The spin coupling shows that the metal chelates may be mesomeric entities.

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