Detailed ESR Spectra of the Free Radicals of FMN and FAD

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In the last years considerable attention has been paid to the properties of flavin radicals because of their possible role in enzymatic activity. Spectrophotometry [1-3] and electron spin resonance, esr, [4,5] have been used for the study of the free and protein bound flavin radicals. In the latter investigations it was not possible to detect any hyperfine structure of the esr spectra. Commoner *et al.*[6] claimed recently that there is a hyperfine splitting that consists of thirteen lines in the flavin radicals. By improved sensitivity and technique it has now been possible to study these spectra in more detail.

FAD and FMN were half reduced by $Na_2S_2O_4$ or Pd-H_2 and studied at room temperature in an X-band spectrometer equipped with a 100 kc field modulation.

The two coenzyme radicals gave identical spectra but the shape of the hyperfine splitting varied with pH as can be seen from Fig. 1. At both pH 0 and 7 the hyperfine spectrum consists of at least 32 symmetrically arranged lines. The spacing between consecutive resolved lines is not constant and varies at pH 0 from 1.3 to 2.1 gauss and at pH 7 from 1.2 to 1.7 gauss. The total splitting between the uttermost observable hyperfine lines increases with the acidity, being 46 gauss at pH 7 and 50 gauss at pH 0. The accompanying colour change from yellow-green to reddishbrown indicates that a proton dissociation is operable with a pK between these values. The attachment of the proton hence seems to influence the distribution of the odd electron in a way so that its absolute spin density is somewhat increased near the nuclei giving rise to the hyperfine splitting. The theoretical calculations available [7,8] suggest that all the four nitrogens in the *iso*alloxazine nucleus has to be accounted for in this interaction, possibly also the two unsubstituted hydrogens of the benzene part [7]. No direct influence can at present be ascribed to the nuclear magnetic moment of the dissociable proton.

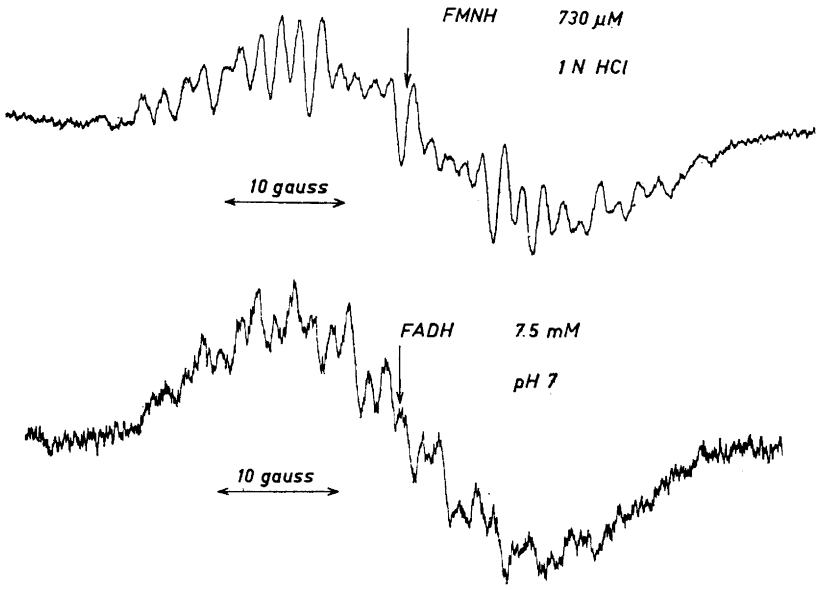

Fig. 1. Derivative records of the esr absorption of the free radicals of FMN at pH 0 (above) and of FAD at pH 7 (below).

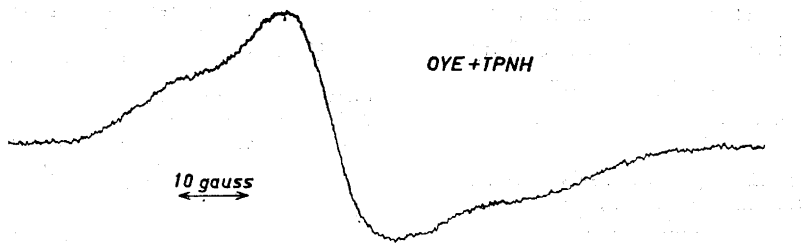

Fig. 2. Derivative record of the esr absorption of the free radicals formed in OYE upon reaction with TPNH.

The hyperfine spectra of Fig. 1 are superposed on broader lines with a width of about 21 gauss between the inflexion points Neither solution contained only radical molecules but were mixtures also containing radical dimers, oxidized and reduced molecules and possibly further complexes of these molecular species. An explanation for the broad underlying absorption might be that the lifetime of the radicals is shortened enough by the dynamic equilibrium established between the solute constituents, in analogy with the explanation forwarded by Ward and Weissman for the equilibrium between naphthalene and its negative ion [9]. The possibility to determine the mean lifetime of the radicals as well as the rate constant of the electron or hydrogen transfer process is now being investigated.

In the previous investigation on old yellow enzyme, OYE, Ehrenberg and Ludwig [4] could not detect any difference between the esr spectra of the FMN free radical and the radical formed in the reaction between OYE and TPNH. In order to achieve enough sensitivity for the detection of the radicals in the latter reaction it was at that time necessary to use a very wide field modulation, that made any search for hyperfine structure impossible, and only the g-values and mean widths of the signals could be compared. With the present technique there is still no hyperfine structure to be seen in the esr absorption of these radicals; see Fig. 2. The spectrum is unsymmetrical and shows only a weak structure consisting of a relatively sharp central peak with broader wings. This observation confirms that the free radicals formed in the reaction between OYE and TPNH really are bound to the protein. The rotational motion of the big protein molecule will be too slow to make the averaging of the anisotropic terms of the interaction between the electron and the magnetic nuclei effective, as it is for the more rapidly tumbling smaller molecules like FMN and FAD. As would be expected the coenzyme radicals give under certain conditions in frozen solutions esr spectra that are rather similar to that of the enzyme radical.

A more detailed report will be published later in this journal.

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1. Beinert, H. *J. Am. Chem. Soc.* **78** (1956) 5323.
2. Beinert, H. *Biochim. et Biophys. Acta* **20** (1956) 588.
3. Beinert, H. *J. Biol. Chem.* **225** (1957) 465.
4. Ehrenberg, A. *Acta Chem. Scand.* **11** (1957) 205.
5. Ehrenberg, A. and Ludwig, G. D. *Science* **127** (1958) 1177.
6. Commoner, B. and Lippincott, B. B. *Proc. Natl. Acad. Sci. u.s.* **44** (1958) 1110.
7. Pullman, B. and Pullman, A. *Proc. Natl. Acad. Sci. u.s.* **45** (1959) 136.
8. Grabe, B. *Arkiv Fysik* **17** (1960) 97.
9. Ward, R. L. and Weissman, S. I. *J. Am. Chem. Soc.* **76** (1954) 3612.

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