*SPIN RESONANCE STUDY OF SEROTONIN-FMN INTERACTION**

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A variety of indoles form complexes with riboflavin.[1, 2] These complexes have been interpreted as ones involving electron transfer, the indoles acting as an electron donor and the riboflavin as an acceptor. Theoretical studies[3] have shown that the positions of the energy levels of these molecules are consistent with this interpretation. The indoles have moderately high occupied energy levels, thus making them fairly good donors, while riboflavin has a low unoccupied energy level, thus making it a good electron acceptor.

This paper will report a study of serotonin and flavin mononucleotide (FMN) by the method of electron spin resonance (ESR). Tryptophan and FMN were also studied, but, for reasons stated below, not as extensively. To help identify the spectra obtained, the semiquinone of FMN at acid pH was prepared by reduction with zinc, by reduction with dithionite and photoreduction.

If an electron donor complexes with an electron acceptor, the donor giving an electron to the acceptor, the complex will not necessarily yield a signal in SR. In such a complex the donated electron will not necessarily be completely uncoupled from its partner and the two electrons will probably form a weak covalent bond

and hence the complex will yield no signal. Furthermore, even if the electrons were completely uncoupled, two free radicals in such close proximity would broaden each other's signal to a considerable extent by dipole-dipole broadening,[4, 5] and the resultant signal might be so broad as to completely avoid detection. Consequently, the technique of ESR, at least under the conditions reported here, cannot examine the complex directly. It does shed light on the complex, however, since, as will be shown, it indicates that at sufficiently acid pH the complex may dissociate into two free radicals. Ordinary charge transfer complexes, of the type extensively studied by Mulliken and his school, cannot do this. Consequently, it appears that the complexes of indoles and FMN must be classified differently. As suggested previously,[1] the complex appears to be one in which the indole gives a "complete" electron to riboflavin,† resulting in an ionic structure. It might be appropriate to call the ordinary charge transfer complexes cases of "weak charge transfer," reserving the name "strong charge transfer" for complex resulting in an ionic structure. Miller and Wynne-Jones,[6] and Bijl, Kainer, and Rose-Innes,[7] have examined complexes that also appear to be cases of strong charge transfer.

Technique.—The ESR apparatus was a Varian 4500-Spectrometer, equipped with a 12-inch magnet and a Varian 4560 100 kc modulation unit. Many features of the hyperfine structure, such as symmetry properties, are more evident when the second derivative of the absorption is obtained rather than the first. To achieve this, a dual modulation method, patterned after that of Smaller and Yasaitis,[8] was adapted for use with Varian equipment. One side of the 100 kc signal was fed to the Varian 1270A low frequency phase detector. The modulation of both sweeps, but more especially, that of the low frequency sweep, was kept very low so that essentially second derivative spectra were obtained.

Solutions were examined in flat quartz cells of internal dimensions 0.1 mm × 7 mm × 52 mm. The cells were oriented in the microwave cavity so as to place the liquid in a minimum electric field.

The FMN was Nutritional Biochemical Corporation's riboflavin 5′ phosphate Sodium. The serotonin was the California Corporation for Biochemical Research Company's serotonin-creatine H_2SO_4 complex,‡ and the tryptophan was the H. M. Chemical Company's L-tryptophan. The acid used was HCl double distilled in glass. For spectra run at acid pH identical results were obtained over a wide range of acid concentration. Thus, while the results reported below were obtained mainly with undiluted constant boiling point HCl, solutions made using a few per cent HCl gave identical spectra.

The red acid semiquinone of FMN was prepared by dropping zinc into an acid FMN solution and waiting until the color of the solution became dark red. The solution was then separated from the zinc and poured into the flat cell. Reduction by dithionite instead of zinc, as well as photoreduction, gave identical results. Photoreduction was achieved by the method of Commoner and Lippincott.[9] The solution was deoxygenated and placed for 5 hr at about 10 inches from a 100-watt desk lamp. Photoreduction at neutral pH yielded a different spectrum from the photoreduction at pH < 1.

Results and Discussion.—Figure 1 shows the first derivative of the absorption for the red riboflavin free radical, while Figure 2 shows the second derivative.

Figure 2 shows mirror image symmetry with respect to the midpoint of the

FIG. 1.—First derivative ESR spectrum of 10^{-2} *M* FMN in acid solution reduced by zinc. Marker indicates one gauss. Arrow indicates free electron resonance position.

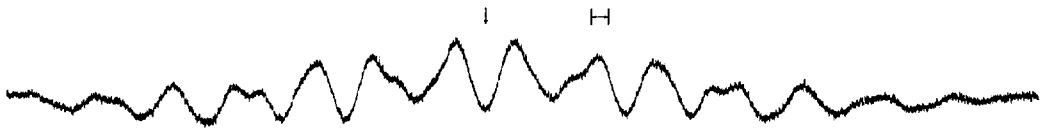

FIG. 2.—Second derivative ESR spectrum of 10^{-2} *M* FMN in acid solution reduced by zinc. Marker indicates one gauss. Arrow indicates free electron resonance position.

absorption. This is what is to be expected from a single free radical species. For two free radicals to show such marked symmetry, their g values must coincide. Since this is most unlikely, it seems reasonable to assume that Figures 1 and 2 are the spectra of one species of free radical and that this free radical is the riboflavin semiquinone at pH < 1.[10, 11]

Figure 1 demonstrates that the hyperfine structure shows considerable overlap. Attempts to obtain a better resolution of these lines, either by lowering the sweep modulations or by lowering the concentration of FMN, met with no success. At small modulation amplitude the lines do appear to be made up of narrower lines but the sensitivity of the instrument becomes poor under these conditions so that a complete resolution of the spectrum is lacking. Actually, a result such as this is not unexpected. In a molecule such as FMN the spin density will be large at a few places on the molecule. This will give rise to the spectrum shown. The remaining atoms will have a small but finite spin density. The nitrogen and hydrogen atoms not giving rise to the large splitting will broaden the lines causing them to overlap. The overlap thus occurs because of unresolved hyperfine structure.

Serotonin in acid gave no ESR signal and neither did FMN. Serotonin and FMN, taken together, did yield a signal.

Figure 3 shows a second derivative spectrum of serotonin and FMN. It will be seen that this spectrum is similar but not identical to the riboflavin semiquinone spectrum. It is markedly asymmetrical, indicating that there are at least two free radicals present. From the similarity to the riboflavin spectrum it may be concluded that one of these is riboflavin. The other may be serotonin. However, since the serotonin free radical spectrum is not known, it may also be a secondary free radical rather than the serotonin free radical itself. The data are therefore consistent with the following scheme.

$$\begin{array}{ccccccc} S + R & \underset{k_1}{\rightleftharpoons} & S^+ R^- & \underset{}{\overset{k_2}{\rightleftharpoons}} & \dot{S} + \dot{R} \\ & & & & \Vert \quad \Vert \\ & & & & P_2 \rightleftharpoons \dot{P}_1 \quad P_3 \end{array} \qquad (1)$$

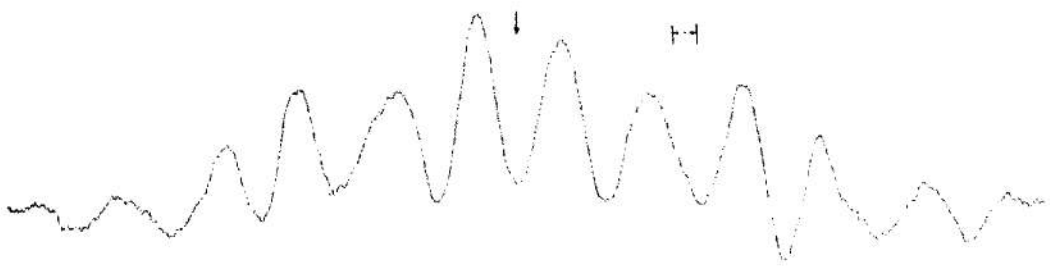

FIG. 3.—Second derivative ESR spectrum of serotonin and FMN in acid. Solution was 0.16 *M* with respect to FMN and 0.59 *M* with respect to serotonin. Marker indicates one gauss. Arrow indicates free electron resonance position.

P_1 may not exist. It is included for the reasons just mentioned. P_2 and P_3 may be dimers or other products of the free radicals. The symbol S^+R^- is to be interpreted as indicating that the complex is primarily ionic and is not meant to indicate that the total charge on the complex is zero. The complex may pick up protons from the solution and hence have a net positive charge.

The above scheme may be supported by the following experiment: A solution of 10^{-2} *M* FMN in acid was placed in a quartz capillary and tested in the spin resonance apparatus. No signal was obtained. A small amount of dry serotonin was added to the sample and stirred with a fine platinum wire. This yielded a signal. Successive small increments were added and the signal became larger. Finally, however, the signal saturated and further addition of serotonin gave no further change in signal in spite of the fact that the additional serotonin went into solution. At this point, the amount of signal corresponded to one free radical per 250 FMN molecules. The interpretation may be made that the serotonin drove all of the riboflavin into the complexed form. However, the amount of free radical present is determined by the dissociation constant k_2 and this dissociation, and hence the amount of free radical in the experiment just mentioned, is not under the control of the investigator.

Tryptophan and FMN in acid also yielded a signal in ESR. However, the signal was much weaker than with serotonin, so much weaker that the hyperfine structure could not be well resolved. This finding is consistent with the observation[1] that serotonin is a stronger electron donor than tryptophan.

The existence of strong charge transfer complexes permits a unified theoretical framework in the scheme of charge transfer. At the one extreme, for weak donors and/or acceptors, there exist charge transfer complexes of the type benzene-iodine in which the ground state is overwhelmingly nonionic. For example, it is estimated that only about 3 per cent of an electronic charge is transferred from benzene to iodine.[12] At the other extreme are the well-known cases of semiquinone formation in which an electron is transferred from the reducing agent to the oxidizing agent, no stable complex being formed. As one considers donors successively stronger than benzene, one passes from weak charge transfer to cases of stronger charge transfer, finally arriving at a predominantly ionic structure. Whether this ionic structure dissociates into free radicals or not depends on whether the free energy of the free radicals, as such, is lower than that of the complex. This, in turn depends, in part, on the nature of the medium, dissociation being favored by ionizing media such as water.

No ESR signal has been observed when serotonin and FMN were mixed at neutral pH. This is evidence that the complex does not dissociate into free radicals at neutral pH. At acid pH the complex does dissociate, probably because

the riboflavin free radical is more stable in a protonated condition than in an unprotonated state. It may also be, however, that in the complex, at acid pH, the FMN picks up protons from the medium and becomes a somewhat better acceptor. There may be, therefore, a somewhat stronger charge transfer complex in strong acid.

In spite of the power of ESR techniques, a point of caution should be noted. Consider a solution of serotonin and FMN in acid. This is brown at room temperature. If the solution is frozen it becomes black, indicating, evidently, a greater degree of charge transfer complexing in the frozen state. In spite of this, such a frozen sample, when tested, yielded no ESR signal. It should not be concluded from this, however, that no free radical is present. Even if a large number of unpaired electrons were present, ESR techniques might not detect them. This may easily be shown also by freezing a 10^{-3} M solution of $MnCl_2$. While at room temperature a characteristic 6-line manganese spectrum is obtained, in the frozen state this signal disappears. One sees, therefore, that electron spin resonance, while a powerful tool, cannot always by itself be relied upon for determining the charge transfer state of a given system.

Summary.—Even though the technique of electron spin resonance does not measure a property of the complex directly, it does shed light on the nature of the complex. It seems most unlikely that a weak charge transfer complex could dissociate into free radicals. In the case of strong charge transfer, however, under proper environmental conditions, such dissociation seems reasonable. Spin resonance techniques yield two overlapping spectra, one of which is that of the riboflavin free radical. This is evidence that the serotonin-riboflavin complex is of the strong charge transfer or predominantly ionic type and is consistent with data previously obtained using optical absorption methods.

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† The term "complete" is used to indicate that there is a transfer of one, or almost one, electronic charge from indole to riboflavin.

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