

Studies on Flavin Semiquinone

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The nature of reddish compound arising in oxido-reduction of flavins was investigated by measuring its spectrophotometric properties and electron spin resonance, and it was proven to be a semiquinone. Descriptions were made of the procedures for determining the absorption spectrum of the semiquinone in the presence of other oxidoreductive forms of flavins and for evaluating the equilibrium constants between these various forms. Using the values of the equilibrium constant, the spectra of the intermediate semiquinones, both of RF and FMN, were determined in the range from 230 to 580 $m\mu$.

The reddish intermediate occurring in the course of oxido-reduction of flavins in strongly acidic solutions has been thought to be the semiquinone form of flavins¹⁻⁶. In their previous work the present authors⁷ have determined, by analysing graphically the spectra of equilibrium mixtures of the oxidized and reduced forms of FMN*, the absorption spectrum and the extinction coefficients in the visible region of the intermediate semiquinone. In the present work, the measurement was extended to the ultraviolet region using not only FMN but also RF. With both forms of flavins the semiquinone nature of the red-colored intermediates was definitely proven by measuring their ESR.

EXPERIMENTAL

The samples of FMN and RF used were those commercially obtained, which have been confirmed to be chromatographically pure. The concentrations of both flavins in their test solutions were determined spectrophotometrically by taking their molecular extinction coefficient as $12.2 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 450 $m\mu$ and pH 7.0⁸. Spectrophotometric measurements were performed using a Hitachi photoelectric spectrophotometer model EPU-2A. The pH's of test solutions were measured after each experiment with a glass electrode pH-meter model HM-5A of the Toa Electronic Co. The ESR spectrometer used was a model JES-3B X-band instrument of Japan Electron Optics Laboratory Co. which was combined with a 30 cm magnet and a proton resonance fluxmeter. The microwave frequency was measured by a microwave frequency meter model NJM-133B of the Nihon Musen Co.

The procedure of treating the test solutions in the spectrophotometric measurements was

* Abbreviations used are: FMN, flavin mononucleotide; RF, riboflavin; ESR, electron spin resonance.

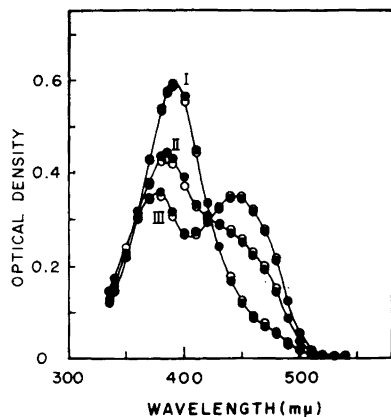


Fig. 1. Absorption spectra of oxidized RF in different concentrations of hydrochloric acid and in presence and absence of zinc chloride. RF concentration was 3.0×10^{-5} M. Curves I, II and III were obtained at the hydrochloric acid concentrations of 2.12, 0.69 and 0.19 N, with pH values of < 0 , 0.2 and 0.7, respectively. The values shown by solid and open circles are those obtained in presence and absence, respectively, of 0.2 M ZnCl_2 .

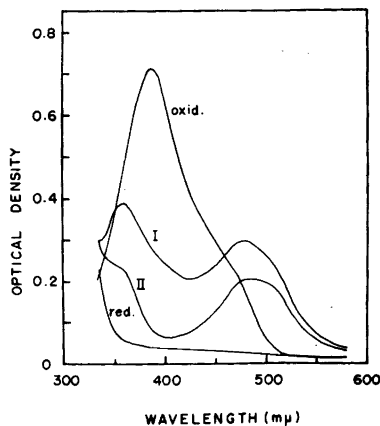


Fig. 2. Absorption spectra in the visible region of oxidized, reduced and intermediary oxidation levels of RF. RF concentration, 4.5×10^{-5} M; pH, -0.1 . In curve I, 58.9% of oxidized and 41.1% of reduced forms were mixed in test solution, and in curve II, 25.5% of oxidized and 74.5% of reduced forms were mixed.

the same as that previously reported; namely the oxidized flavin was titrated under the anaerobic condition with the reduced form keeping the concentrations of acid and total flavin concentrations constant. In the ESR measurements, the test solution was introduced into a quartz capillary, 0.5 mm in inner diameter and 150 mm in length, by using an injection syringe, and the capillary, after being sealed with vaseline, was inserted into the microwave cavity. Diffusion of molecular oxygen into the test solution was found to be negligible even after one hour. The concentration of free spins in the test solution was calculated by double integration of the derivative spectra, and by comparison with the value found for standard solution which contained 1 mM CuSO_4 . The g-value of the ESR spectrum was determined by measuring the field strength and microwave frequency at the center of the resonance spectrum. All the measurements were made at room temperature.

RESULTS

Spectrophotometric measurements. The absorption spectrum of the oxidized FMN, as it changes with pH, has been measured in detail in our previous paper⁷. The corresponding measurement was made with RF in the pH range which was of interest in the present study. The measurements were made in the presence and absence of zinc ion to see whether or not the absorption spectrum of the oxidized form of RF is affected by the presence of zinc ion, since zinc powder was used in preparation of the reduced RF. The results obtained are presented in Fig. 1. As may be seen, the absorption spectrum of RF changed markedly with pH, and was not affected by the addition of zinc ion. Isosbestic points were found at 355 and 423 $m\mu$, and the extinction coefficient at the latter wavelength was estimated to be $9.8 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. The results coincides with those obtained earlier with FMN⁷. In Fig. 2 are shown the absorption spectra of RF

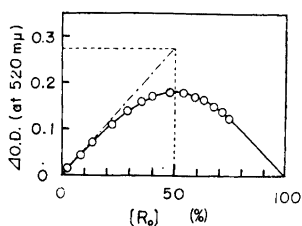


Fig. 3. Plot of $\Delta O.D._{520}$ against relative values of $[R_0]$. The maximum amount of semiquinone was found to be 66% of the total flavin. Straight line represents tangent of the curve at $[R_0] = 0$. RF concentration used was 4.5×10^{-5} M; pH, -0.1. Solid curve represents the theoretical values calculated by applying the experimental value ($K = 15$) obtained for the equilibrium constant of effective semiquinone formation.

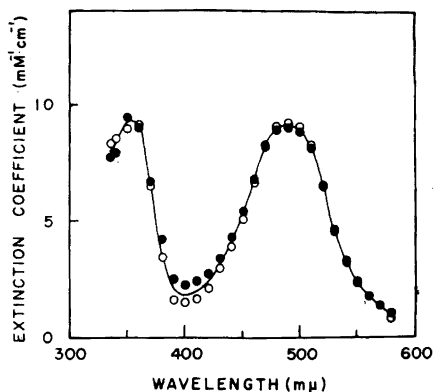


Fig. 4. Spectrum of semiquinone of RF at pH -0.1. The curve was obtained by calculation using the K value obtained in Fig. 3. The values shown by solid and open circles are those calculated from curve I and curve II, respectively, of Fig. 2.

in its oxidized, reduced and two intermediate oxidation levels at pH -0.1*. Worth noticing is the existence at 520 $m\mu$ of an isosbestic point of the oxidized and reduced RF. At this wavelength, the increment of optical density ($\Delta O.D._{520}$) may be assumed to be proportional to the amount of semiquinone formed; namely,

$$\Delta O.D._{520} = (\epsilon_S - \epsilon_T)[S]$$

where ϵ_S and ϵ_T are, respectively, molecular extinction coefficients of semiquinone and the oxidized form of RF at 520 $m\mu$, and $[S]$ the concentration of the semiquinone. In Fig. 3, $\Delta O.D._{520}$ is plotted against the relative value of the initial concentration of the reduced form $[R_0]$. Since the semiquinone is formed by the reversible reaction Oxidized + Reduced \rightleftharpoons 2Semiquinone, and in our experiments the total concentration of RF has been kept constant, we may deduce⁷ that the tangent of the curve in Fig. 3 at $[R_0] = 0$ should be given by the expression:

$$\Delta O.D._{520} = 2(\epsilon_S - \epsilon_T)[R_0]$$

The value of $(\epsilon_S - \epsilon_T)$ and therefore the value of ϵ_S can thus be obtained graphically. Consequently, the values of $[S]$ at different R_0 values can also be calculated. Experimental results showed that at pH -0.1 the amount of semiquinone was maximal at $[R_0] = 50\%$, being 66% of the total RF concentration. The equilibrium constant of effective formation of semiquinone⁹, *i. e.* $K = [\text{Semiquinone}]^2 / [\text{Oxidized}] \times [\text{Reduced}]$, was calculated to be 15. The solid curve in Fig. 3 is that drawn theoretically by using the K -value mentioned above which

* Under the said condition, the exact value of hydrogen ion concentration could not be determined titrimetrically owing to the presence of zinc ion in the mixture. This may be checked by referring to the experimental data presented in Fig. 1. The hydrochloric acid concentration in the experiment shown in Fig. 2 was estimated to be 1.1 N.

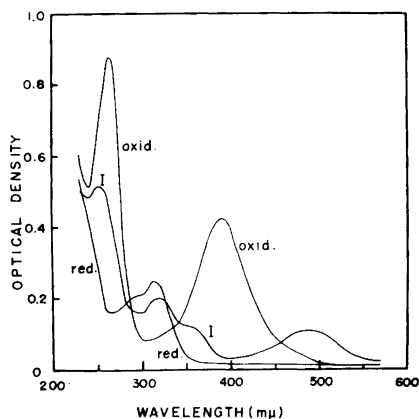


Fig. 5. Absorption spectra of oxidized, reduced and intermediary oxidation levels of RF in the ultraviolet and visible region. RF concentration, 2.2×10^{-5} M; pH, -0.4 . The curve for intermediary level was obtained with a mixture containing 25.7 % of oxidized and 74.5 % of reduced forms.

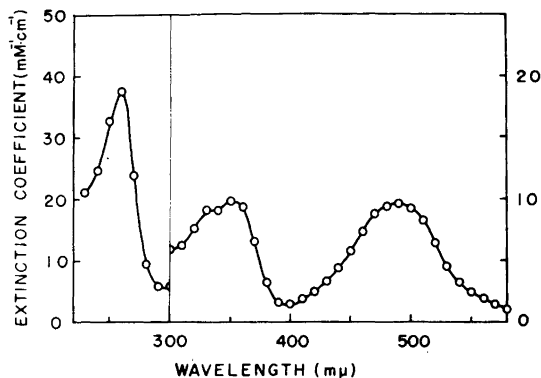


Fig. 6. Spectrum of semiquinone of RF at pH -0.4 .

is in good agreement with the experimental results. Calculations made, on the basis of these considerations, with the data presented in Fig. 2, led to the conclusion that the spectrum of the semiquinone in question would be such as given in Fig. 4. The curves computed for $[R_o] = 41.1\%$ and 74.5% coincided with each other almost perfectly, showing two absorption maxima at 350 and 490 $m\mu$ with the extinction coefficients of 9.2 and $9.1 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$, respectively. Determination of the absorption spectrum of RF semiquinone was made at various pH's, extending, in some cases, the measurement to the ultraviolet region. In Fig. 5 are presented the spectra thus obtained at pH -0.4 for the oxidized, reduced and intermediate states of RF. The whole spectrum of RF semiquinone at pH -0.4 is shown in Fig. 6. Almost the same spectrum was obtained by the similar measurements performed with FMN at pH -0.4 . It may be noticed that also in this case the semiquinone spectra has peaks at 260, 350 and 490 $m\mu$ and a shoul-

Table 1. Spectrophotometric characteristics of RF and FMN semiquinones and equilibrium constants for their effective formation at different pH values.

Semiquinone	pH	[S] _{max} %	K	λ _{max} mμ	Extinction coefficient 10 ³ M ⁻¹ cm ⁻¹	Total flavin concentration 10 ⁻⁵ M	
RF	-0.4	69	20	260	37	2.2	
				(330)			
				350			
	-0.1*	66	15	490	9.8	4.5	
				350	9.6		
				490	9.2		
0.3*	51	4.3	350	9.1	5.9		
			490	8.9			
			490	9.1			
FMN	-0.4	70	22	260	45	2.6	
				(330)			
				350			10
				490			9.5

* Measurements were made only in the spectral range from 340 to 580 mμ.

der at 330 mμ. The K-value calculated for FMN in this case was 22 in comparison with 20 for RF obtained from the data presented in Fig. 5. All these results are summarized in Table 1.

ESR measurements. The ESR measurements with RF and FMN were performed at pH -0.4. As expected, no significant signals were recorded with the oxidized and reduced forms of both flavins. When, however, the oxidized and reduced forms were mixed, there appeared a distinct signal indicating the formation of an intermediate being a free radical. Relevant data are shown in Fig. 7. The g-values of the signals obtained from the intermediates of RF and FMN were identical, being in both cases 2.003. The relationship between the concentration of free spin and [R₀] in the case of FMN is presented in Fig. 8, which shows a satisfactory agreement with the corresponding curve obtained for semiquinone determined by spectrophotometry.

DISCUSSION

According to Beinert⁴, there are two forms of flavin semiquinone; one which is reddish in color arises in strongly acidic solution, and the other, greenish in color, is observed at neutral pH when the flavin concentration is rather high. The latter was characterized by absorption bands at 565 and 880 mμ, which were thought to be due to the existence of the monomer and dimer of the semiquinone, respectively. However, Gibson *et al.*¹⁰ and Massey *et al.*¹¹ have recently suggested that the compound observed in the intermediary mixture showing a broad absorption band in the infrared region is a charge transfer complex of the oxidized and reduced forms, and that the compound showing an absorption at 570 mμ is a complex of free radical and the reduced form of flavin. The flavin concentrations used in the present study were of the order of 1/100 to 1/1000 of those used by Gibson *et al.* so that the possibility of formation of a complex molecule, if any, seems to be slim. Since the semiquinone spectra obtained by using various total concentrations of flavin were identical, the formation of a dimer form

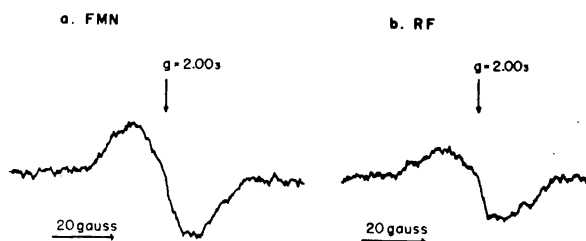


Fig. 7. ESR spectra of FMN and RF semiquinones at pH -0.4 . Spectrum *a* was obtained with 3.8×10^{-5} M FMN solution, in which 57% of oxidized and 43% of reduced forms were mixed. The estimated concentration of semiquinone was 2.7×10^{-5} M. Spectrum *b* was obtained with 3.8×10^{-5} M RF solution, in which 13% of oxidized and 87% of reduced forms were mixed. The estimated concentration of semiquinone in this case was 2×10^{-5} M. The field strength decreases from left to right and the arrows indicate $g = 2.003$. Measurements of spectra *a* and *b* were performed using different capillaries.

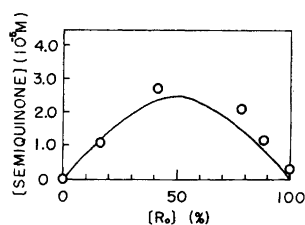


Fig. 8. Plot of FMN semiquinone concentration against relative values of $[R_0]$. Open circles show the concentrations estimated by ESR measurements, and the curve represents the concentration measured by spectrophotometry. FMN concentrations was 3.8×10^{-5} M; pH, -0.4 .

is unlikely in our experiments. It may, therefore, be justifiable to assume that under our experimental conditions the equilibrium mixture contained only three molecular forms; namely, oxidized, reduced and semiquinone forms. The g -values of ESR signals obtained with FMN and RF agreed well with that reported elsewhere¹², and they are no doubt ascribable to free radicals. That the red intermediate compound here studied was a semiquinone is evidenced by the fact that the free spin concentration obtained by the ESR measurement and the concentration of semiquinone determined by spectrophotometry coincided with each other quantitatively.

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