Kinetics and Equilibria in Partially Reduced Flavine Solutions[†]

Bruce G. Barman[‡] and Gordon Tollin*

ABSTRACT: Equilibrium constants have been determined at pH 4.5 and 9.0 for the reactions occurring in solutions ($<10^{-4}$ M) of FMN which have been partially reduced. For the equilibria

$$F + FH_2 \overset{K_{12}}{\rightleftharpoons} F\text{–}FH_2 \overset{K_{34}}{\rightleftharpoons} 2FH\cdot$$

K_{12} is 1.9×10^2 M^{-1} at pH 4.5 and 1.6×10^2 M^{-1} at pH 9.0 and K_{34} is 8.0×10^{-5} M at pH 4.5 and 1.2×10^{-5} M at pH 9.0. At higher concentrations, radical dimerization is shown to occur, and a paramagnetic solid which separates out of solution under these conditions has been characterized as a mixture of F–FH$_2$ and (FH$\cdot$)$_2$. The kinetics of these various equilibria have been investigated at pH 4.5 using the temperature-jump method and rate constants have been evaluated for several of the individual steps. The equilibrium constant for the dissociation of the radical dimer has been estimated to be 5×10^{-7} M at pH 4.5.

Gibson *et al.* (1962) have shown that the principal species present in a dilute ($<10^{-3}$ M) partially reduced FMN solution are oxidized flavine (F), fully reduced flavine (FH$_2$), half-reduced semiquinone (FH$\cdot$), and a complex between oxidized and reduced flavines (F–FH$_2$). The following scheme was suggested to be operative:

$$F + FH_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} F\text{–}FH_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} 2FH\cdot$$

where the complex F–FH$_2$ is a precursor to the semiquinone. Swinehart (1965), using the temperature-jump method, calculated the rate constants for such a scheme. However, his estimate of k_4 (6×10^6 M^{-1} sec^{-1}) is almost three orders of magnitude less than that obtained using flash photolysis (3×10^9 M^{-1} sec^{-1}; Vaish and Tollin, 1971) or pulse radiolysis (1.14×10^9 M^{-1} sec^{-1}; Land and Swallow, 1969) to study the disproportionation of flavine radicals. In order to resolve this discrepancy, we have carried out a series of spectrophotometric, temperature-jump and flash photolysis experiments on partially reduced FMN solutions at pH 4.5 and 9.0.

Experimental Section

Materials

FMN was obtained from Calbiochem, Los Angeles, Calif., and was purified by DEAE-cellulose column chromatography at pH 7.0. Concentrations were determined spectrophotometrically using published values for the molar extinctions. EDTA was obtained from Allied Chemical, Morristown, N. J., in reagent grade.

Methods

Partially Reduced Flavine Solutions. An anaerobic reduction apparatus was used for the preparation of partially reduced flavine samples for electron spin resonance and temperature-jump experiments. The apparatus was double armed with a 1-mm path-length cell on one arm and a 10-mm path-length cell on the other. The two path-length cells permitted the determination of absorption spectra of concentrated solutions of oxidized flavine using the 1-mm cell and also the weak absorption spectrum of semiquinone flavine and F–FH$_2$ using the 10-mm cell. Reduction was accomplished by irradiating the flavine sample, which contained a 50-fold molar excess of EDTA, with a 650-W, heat-filtered, Sylvania Sun Gun lamp. Prior to irradiation, the sample was deoxygenated by alternate evacuation and nitrogen purging and left under a slight positive nitrogen pressure. To effect half-reduction, equal volumes of the sample were divided between the two arms. Only one arm was irradiated while the other was kept dark. Irradiation was continued until the sample showed no fluorescence under uv light, thus indicating complete reduction. The samples from the two arms were then combined, yielding a half-reduced mixture.

For temperature-jump studies (Barman and Tollin, 1972), the partially reduced solution was transferred anaerobically to the temperature-jump cell within a nitrogen-filled glove bag. The cell was kept under a positive nitrogen pressure with constant purging. Total flavine concentrations ranged from 5×10^{-4} to 5×10^{-3} M. All kinetics were determined at 10° unless otherwise stated.

For electron spin resonance (esr) measurements, the top of the reduction apparatus was fitted with an aqueous solution esr cell which could be made anaerobic. The half-reduced sample was forced into the esr cell by inverting the entire apparatus within a glove bag. By momentarily opening the bottom stockcock, the positive nitrogen pressure could be made to fill the cell. Flavin concentrations were 10^{-3} M (total flavine). Spectra were obtained using a Varian V-4501 spectrometer equipped with 100-kc modulation and a Fieldial control unit.

All reductions were carried out at either pH 4.5 or 9.0, generating upon mixing the neutral or anionic form of the semiquinone, respectively. Acetate buffer (0.05 M) was used at pH 4.5, while glycine buffer (0.05 M) was used at pH 9.0. All samples contained 0.1 M KNO$_3$ whether they were used for temperature-jump or esr experiments.

Isolation of a Paramagnetic Precipitate from Concentrated Half-Reduced Flavine Solutions. For samples in which the total flavine concentration was above 5×10^{-3} M, a flocculent

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precipitate formed upon mixing oxidized and reduced flavine. This same behavior has been observed previously (*cf.* Beinert, 1960). The precipitate was green at pH 4.5 and red at pH 9.0. It was possible to collect this species as a paste by anaerobic centrifugation at 20,000*g*. The paste could be freeze-dried anaerobically by vacuum sublimation and stored indefinitely without decomposition. Even under air, decomposition was relatively slow (many hours).

Determination of Mull Absorption Spectra. It was of interest to characterize the freeze-dried precipitate isolated from the concentrated partially reduced FMN samples. The determination of its absorption spectrum was thus of importance. Since the sample underwent disproportionation in aqueous solutions and was found to be insoluble in organic solvents, the spectrum of a solid sample had to be determined. Inclusion of the material in potassium bromide pellets did not yield reproducibility. However, mull spectra in mineral oil proved to be quite reproducible. The freeze-dried precipitate was ground to homogeneity into deoxygenated mineral oil using a mullite mortar and pestle. This was done within a nitrogen glove bag to prevent oxidation. Spectra were determined using anaerobic Thunberg cuvets with the Cary 14R spectrophotometer. Opal glass was used in both the monitoring and reference beams to compensate for scattering due to the sample particles. Mineral oil was used as a reference.

Determination of Relative Spin Concentrations of Solutions of Flavine Semiquinone. For aqueous solutions of flavine containing semiquinone, relative spin concentrations were determined as a function of pH. Samples which were 1×10^{-3} M in total flavine were half-reduced at pH 4.5 and 9.0. The relative radical concentrations were approximated by comparing the product of the maximum peak to peak height and the square of the width of the esr absorption curves at the two pH's. Instrument parameters were held constant. The sample was overmodulated such that only a broad, unstructured resonance was observed.

Determination of Absolute Spin Concentration per Molecule of FMN in the Paramagnetic Precipitate. Electron spin resonance spectra were determined for the solid paramagnetic precipitate obtained from reduction of concentrated flavine solutions. Samples were ground and sealed in 2-mm cylindrical quartz tubes under a nitrogen atmosphere. Esr signals were compared to those from known spin concentration standards made by grinding diphenylpicrylhydrazyl into dry KBr. The hydrazyl concentrations were determined spectrophotometrically by dissolving the standard in a measured volume of chloroform and using an extinction of 37 g^{-1} cm^{-1} at 529 nm. It was assumed that there was one spin per hydrazyl molecule. Total flavine concentrations were determined by dissolving the solid sample in a measured quantity of oxygenated water and determining the absorbance at 445 nm.

Because the resonances of hydrazyl and flavine semiquinone overlap, simultaneous determination of spectra could not be performed. Therefore extreme care was taken to keep all instrument parameters constant for both hydrazyl and flavine samples.

The actual integration of the absorption curves was done by computer using a double summation method. The number of spins per molecule of flavine was calculated by dividing the number of spins corresponding to the integrated area, as determined from the hydrazyl standard, by the total flavine concentration.

Determination of Equilibrium Constants for the Principal Species Present in a Mixture of Oxidized and Reduced FMN. The principal species present in a dilute ($\sim 10^{-4}$ M) solution of partially reduced FMN are (Gibson *et al.*, 1962) FMN, $FMNH_2$, FMN–$FMNH_2$, and FMNH·. Neglecting any minor contributions due to radical dimerization, $FMNH_2$ dimerization, etc., it is possible to ascertain the equilibrium concentrations of these species spectrophotometrically by making absorbance measurements at 360, 445, and 1100 nm. A molar extinction of 4700 M^{-1} cm^{-1} for FMNH· was used (Land and Swallow, 1969). From these concentrations, the two equilibrium constants can be calculated.

TABLE I: Equilibrium Constants for Partially Reduced FMN Solutions.

pH	K_{12} ($\times 10^2$) (M^{-1})	K_{34} ($\times 10^{-5}$) (M)
4.5	1.9 ± 0.2	8.0 ± 0.2
9.0	1.6 ± 0.2	1.2 ± 0.2

Results and Discussion

Equilibrium Constants in Dilute Solution. The two equilibrium constants ($K_{12} = k_1/k_2$ and $K_{34} = k_3/k_4$) for the reaction mechanism given in the introduction were determined spectrophotometrically at both pH 9.0 and 4.5 and are presented in Table I. In all determinations, the concentrations of oxidized and fully reduced flavine were kept equal. This minimized dimerization of the fully reduced flavine, which Gibson *et al.* (1962) consider to be negligible under these conditions.

At a given pH, the concentration of the semiquinone as a function of the concentration of oxidized and fully reduced flavine is as shown in eq 1. Therefore, the ratio of semiquinone

$$[FH\cdot]_{pH} = \sqrt{K_{12}K_{34}[F][FH_2]_{pH}} \tag{1}$$

concentration at pH 4.5 to that at pH 9.0, when F and FH_2 are equal at both pH's, is given by eq 2. Using the equilibrium

$$\frac{[FH\cdot]_{4.5}}{[FH\cdot]_{9.0}} = \frac{\sqrt{K_{12}K_{34}}\ \text{pH 4.5}}{\sqrt{K_{12}K_{34}}\ \text{pH 9.0}} \tag{2}$$

constants given in Table I, the ratio of $[FH\cdot]_{4.5}/[FH\cdot]_{9.0}$ is 2.8 (±0.1). In order to test this, solutions of equal concentrations of F and FH_2 at the two pH's were prepared and their electron spin resonance (esr) spectra recorded. Inasmuch as the spectral line shape is approximately independent of pH, the spin concentrations can be taken as proportional to signal height times width squared. The ratio of the spin concentrations was measured to be 3.2 ± 0.1. Therefore the esr signal ratio compares well to that calculated using the equilibrium constants.

The equilibrium data show that the neutral flavine complex F–FH_2 is much less stable than is the anion complex F–FH^-. This same pattern was found for the radical species by measurements of the rates of disproportionation in flash photolysis experiments at pH 5.0 and 9.0 (Vaish and Tollin, 1971). This may have biological significance inasmuch as FH^- is probably the principal form of fully reduced flavine at physiological pH. It should be noted that the equilibrium constants determined at pH 4.5 are in fair agreement with the constants determined by Michaelis (1938) for riboflavine at pH 5.0 ($K_{12} = 6.0 \times 10^2$ M^{-1}, $K_{34} = 10^{-4}$ M) but differ by an order of

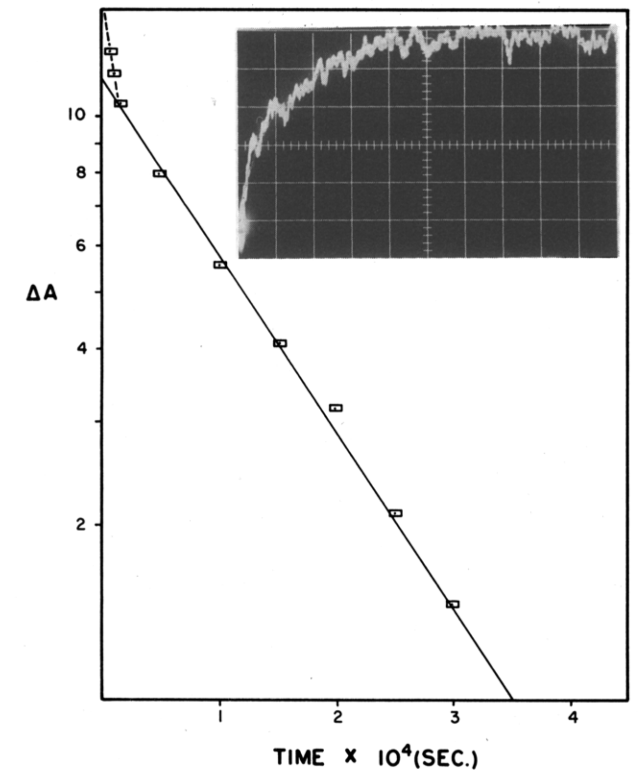

FIGURE 1: Plot of log ΔA *vs.* time for the determination of the relaxation time for a half-reduced FMN solution (experimental trace superimposed; 100 μsec/division). Transient was recorded at 580 nm; pH 4.5, 0.05 M acetate buffer, 10°.

magnitude from those estimated by Gibson *et al.* (1962) ($K_{12} = 2.0 \times 10^3\ \mathrm{M}^{-1}$, $K_{34} = 10^{-6}\ \mathrm{M}$) for FMN at pH 6.5. The equilibrium constants at pH 9.0 are the first such determined involving the anion form of the flavine semiquinone.

Temperature-Jump Kinetics. For the reaction scheme shown above, three possible situations can occur: the first equilibrium (K_{12}) is established rapidly compared to the second (K_{34}), the second is established more rapidly than the first, or both are established in the same time range. The last possibility can be eliminated by studying the temperature-jump relaxation behavior at pH 4.5 as a function of wavelength. In the region 480–620 nm, there is a fast[1] (<10 μsec) optical density increase followed by a slower optical density increase (>100 μsec) (Figure 1). At wavelengths beyond 680 nm (where only the F–FH_2 complex absorbs), one can only observe a fast (<10 μsec) transient corresponding to an optical density decrease (the sensitivity of the apparatus at this wavelength is much less than at the lower wavelengths). The direction of the optical density changes are the same as has been observed by studying the temperature dependence of the spectrum of partially reduced FMN solutions (Beinert, 1956). From these observations it is evident that one of the above two equilibria must be established more rapidly than the other.

A possible source of the above-mentioned discrepancy between the values for k_4 determined by temperature jump and by flash photolysis is that in the former experiments dithionite reduction was used to obtain a half-reduced flavine solution. In view of this, we decided to repeat the experiments of Swinehart (1965) using photochemical reduction with EDTA.[2]

An analysis of the two remaining possibilities with respect to the relative rates of attainment of equilibria leads to two sets of relaxation equations with different concentration dependencies. For the case in which the first equilibrium (K_{12}) is established rapidly compared to the second, eq 3 and 4 characterize the two relaxation times. If the reverse is the case, eq 5 and 6 hold. By considering the concentrations used in the experiments and the magnitude of the equilibrium constants (K_{12} and K_{34}), the above expressions for $1/\tau_2$ can be simplified as follows:

$$\frac{1}{\tau_1} = k_1([\overline{\mathrm{F}}] + [\overline{\mathrm{FH}_2}]) + k_2 \qquad \text{fast} \quad (3)$$

$$\frac{1}{\tau_2} = \frac{k_3K_{12}([\overline{\mathrm{F}}] + [\overline{\mathrm{FH}_2}])}{1 + K_{12}([\overline{\mathrm{F}}] + [\overline{\mathrm{FH}_2}])} + 4k_4[\overline{\mathrm{FH}\cdot}] \qquad \text{slow} \quad (4)$$

$$\frac{1}{\tau_1} = k_3 + 4k_4[\overline{\mathrm{FH}\cdot}] \qquad \text{fast} \quad (5)$$

$$\frac{1}{\tau_2} = \frac{4k_2[\overline{\mathrm{FH}\cdot}]}{K_{34} + 4[\overline{\mathrm{FH}\cdot}]} + k_1([\overline{\mathrm{F}}] + [\overline{\mathrm{FH}_2}]) \qquad \text{slow} \quad (6)$$

$$\frac{1}{\tau_2} = 4k_4[\overline{\mathrm{FH}\cdot}] + k_3 \quad (7)$$

$$\frac{1}{\tau_2} = k_1([\overline{\mathrm{F}}] + [\overline{\mathrm{FH}_2}]) + k_2 \quad (8)$$

Therefore a plot of $1/\tau_2$ *vs.* either $4[\overline{\mathrm{FH}\cdot}]$ or $[\overline{\mathrm{F}}] + [\overline{\mathrm{FH}_2}]$ should be linear depending upon which mechanism is correct. The concentration of [FH·] can be evaluated at 580 nm while the sum of concentrations (F and FH_2) must be calculated from the equilibrium constants using the initial concentrations. It was found that a plot of $1/\tau_2$ *vs.* $4 \times A_{580}$ was linear (Figure 2), as was also observed by Swinehart (1965). However, due to the small concentration range that is experimentally accessible, a plot of $1/\tau_2$ *vs.* [F] + [FH_2] also appears linear. Therefore, in order to calculate the kinetic constants, an assumption must be made as to which of the two reactions (either complex formation or radical formation) is faster than the other. Because the rate of flavin complexation is fast (Barman and Tollin, 1972) and complexation does not involve a chemical change whereas radical formation does, it is probably reasonable to assume that the slower step is the production of radical. This assumption is further supported by the fact that radical formation would involve the generation of a thermodynamically unstable species, which could tend to slow down the rate of this reaction. On the basis of this assumption, the rate constant k_4 was calculated from the slope of the plot in Figure 2, using a molar extinction of 4700 M^{-1} cm^{-1} (Land and Swallow, 1969). From the intercept of this plot, k_3 can be determined. We can also obtain lower limits for the rate constants k_1 and k_2 from the heating time of the instrument and the previously calculated equilibrium constant (K_{12}), since $1/\tau_1 = k_1([\overline{\mathrm{F}}] + [\overline{\mathrm{FH}_2}]) + k_2$ and $K_{12} = k_1/k_2$. The calculated constants are given in Table II together with the values obtained by Swinehart (1965). The agreement between the two sets of experiments is seen to be good. The agreement is even better if Swinehart's data are recalculated using a larger value for the extinction of the flavine semiquinone [4700 M^{-1} cm^{-1} (Land and Swallow, 1969) rather than

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[1] This is within the heating time of the instrument.

[2] We also did a temperature-jump experiment using a dithionite-reduced sample and obtained the same results as did Swinehart.

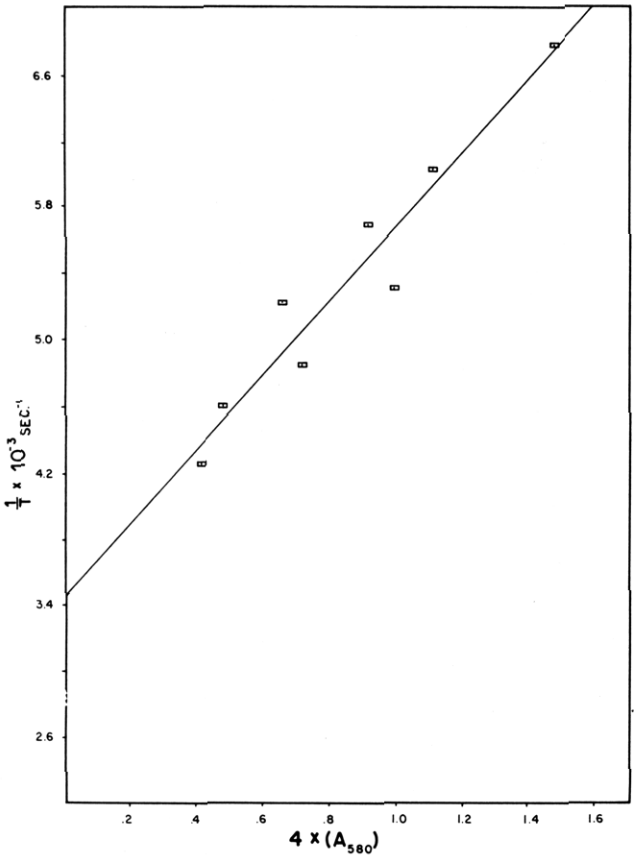

FIGURE 2: Plot of $1/\tau_2$ *vs.* 4(A_{580}] for the calculation of the kinetic constants for half-reduced FMN solutions at pH 4.5.

3050 M^{-1} cm^{-1} as he used]. This yields values of $k_4 = 9.4 \times 10^6$ M^{-1} sec^{-1} and $k_3 = 1.8 \times 10^3$ sec^{-1} which are closer to the constants calculated in the present study. Thus it appears that the method of reduction does not affect the kinetic results and the source of the discrepancy between flash and temperature-jump experiments must lie elsewhere.

From these data, it appears that the major factor controlling rates of establishment of the two equilibria are the magnitudes of k_2 and k_3; *i.e.*, the complex dissociates into F and FH_2 much more rapidly than it comproportionates into radicals. It was not possible to determine the kinetics at pH 9.0 since the anion semiquinone absorption is obscured by the oxidized flavine absorption.

It should be noted that the above mechanisms assume that the complex $F\text{–}FH_2$ is the precursor of the semiquinone. However, there is another mechanism that also leads to a linear dependence of $1/\tau_2$ on [FH·]

$$F + FH_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} F\text{–}FH_2 \qquad (9)$$

$$F + FH_2 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} 2FH\cdot \qquad (10)$$

From our equilibrium data it is possible to calculate the equilibrium constant K_{56} to be 1.5×10^{-2}. The rate constant k_6 as calculated from the plot of $1/\tau_2$ *vs.* 4[FH·] is identical with k_4 (1.0×10^7 M^{-1} sec^{-1}). From the value of K_{56}, k_5 is found to be 1.5×10^5 M^{-1} sec^{-1}. The rate constants k_1 and k_2 are the same as before.

The question of the actual precursor of the semiquinone, the complex $F-FH_2$ or the free flavine forms (F and FH_2), cannot be resolved kinetically. However, the results of Gibson *et al.* (1962) and of Fox and Tollin (1966) indicate that the complex is a precursor to the semiquinone and that the first mechanism proposed is probably correct.

TABLE II: Rate Constants for the Equilibria in Partially Reduced FMN Solution (pH 4.5) at 10°.

Rate Constant	This Study	Swinehart (1965)
k_4 (M^{-1} sec^{-1})	1.0×10^7	6.0×10^6
k_3 (sec^{-1})	3.5×10^3	5.0×10^3
k_2 (sec^{-1})	$>10^6$	$>10^6$
k_1 (M^{-1} sec^{-1})	$>1 \times 10^8$	$>10^9$

Flash Photolysis Experiments. In flash photolysis experiments the total flavine concentration is generally about 5×10^{-6} M, and all the flavine is initially in the oxidized state. In the temperature-jump studies, the total flavine concentration was $>10^{-3}$ M and equal quantities of oxidized and reduced flavine were present. Therefore, flash photolysis experiments were performed on flavine solutions that more closely paralleled those used for the temperature-jump experiments. That is, samples were made as concentrated in flavine as possible (5×10^{-4} M), the buffer and EDTA concentrations were identical with those used in the temperature-jump studies, and the sample was half-reduced initially. Higher flavin concentrations could not be used because of the high absorbance of the solutions. Figure 3 shows a plot of $1/\Delta A$ *vs.* time ob-

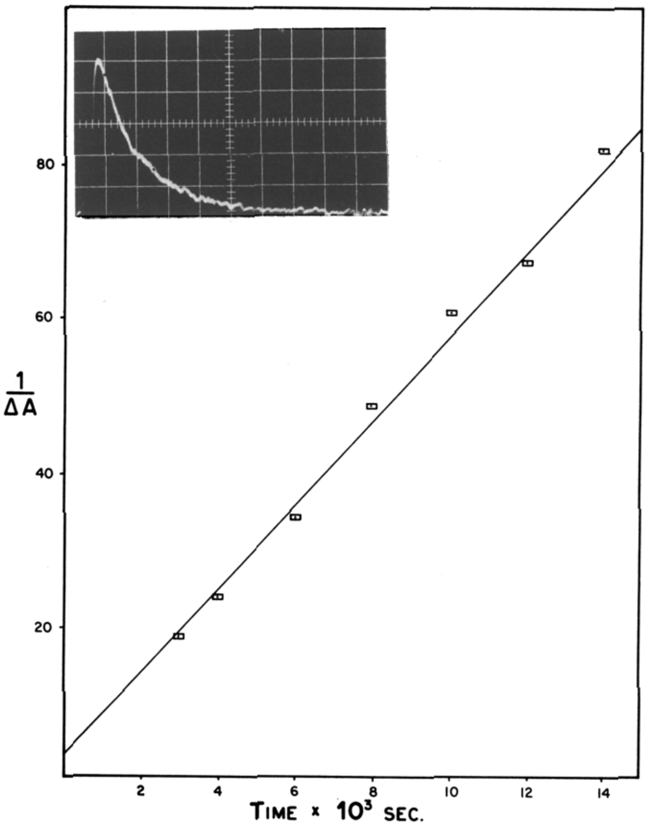

FIGURE 3: Plot of $1/\Delta A$ *vs.* time for the determination of the rate of radical disproportionation by means of flash photolysis at pH 4.5. Total FMN (5×10^{-4} M); total $FMNH_2$ (5×10^{-4} M); acetate buffer (0.1 M).

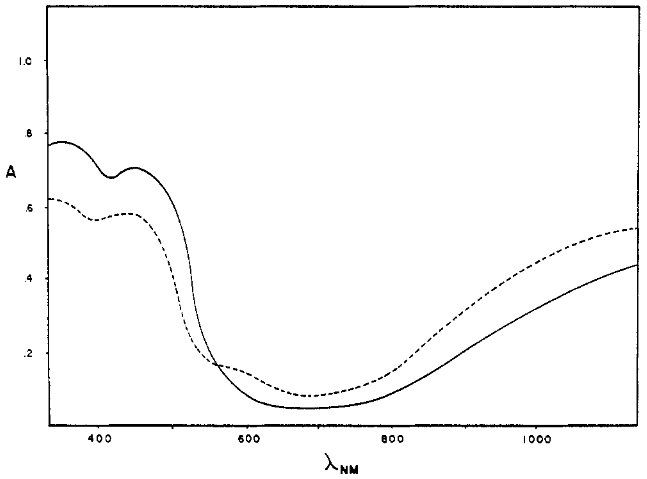

FIGURE 4: Mull absorption spectra of paramagnetic solids isolated from half-reduced FMN solutions (~10^{-2} M). pH 4.5 (----) and pH 9.0 (——).

tained under these conditions from which the rate constant for the disappearanec of semiquinone can be calculated. Again k_4 was found to be 3×10^9 M^{-1} sec^{-1} and good second order behavior was followed. It should be noted, however, that the sample concentration used was still only 10% or less of that used in the temperature-jump experiments.

Flavine Radical Dimerization in Concentrated Solutions. It was noted that when the concentration of a half-reduced FMN solution approached 10^{-2} M, a flocculent precipitate was formed. This precipitate was green at pH 4.5 and red at pH 9.0, suggesting that it might be a form of the flavine semiquinone (Beinert, 1956). The precipitate was collected and characterized as described in the Experimental Section with the following results. The mull spectra of samples obtained at pH 4.5 and 9.0 are shown in Figure 4. It is instructive to compare these spectra with those obtained for partially reduced flavine solutions (Figure 5). Examination of Figure 4 shows that at both pH's there is a broad long-wavelength absorption that is similar to that seen in solutions (Figure 5) of partially reduced flavines which is attributed to either a charge-transfer complex of oxidized and reduced flavin (Gibson *et al.*, 1962) or to a radical dimer of the semiquinone (Beinert, 1960). In addition, at pH 4.5 there is a shoulder around 600 nm that is absent at pH 9.0. This is probably due to the absorption of the neutral semiquinone which absorbs strongly at 580 nm in solution at pH 4.5 but has no 580-nm absorption in basic solutions. There are also peaks around 450 and 380 nm which have peak ratios characteristic of a mixture of both oxidized and fully reduced flavine.

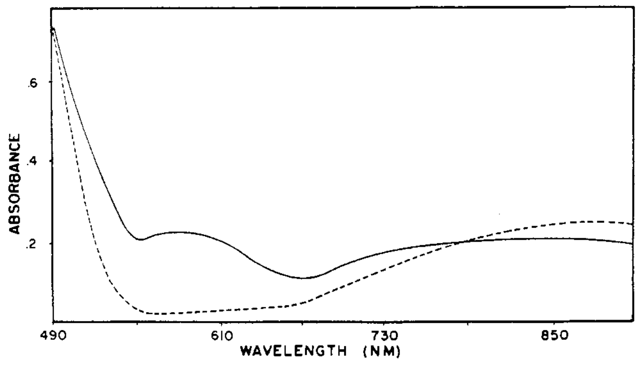

FIGURE 5: Absorption spectra of half-reduced FMN solutions (10^{-3} M). pH 4.5 (----) and pH 9.0 (——).

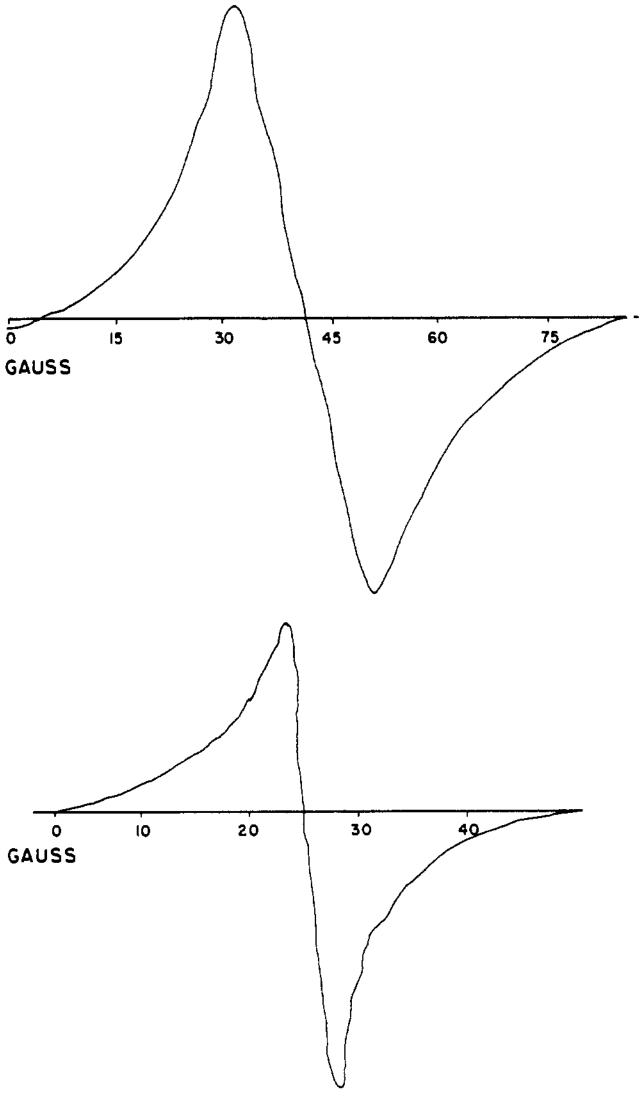

FIGURE 6: First derivative esr absorption spectra of the FMN paramagnetic solids. Upper trace: pH 4.5; lower trace: pH 9.0.

The precipitate was found to be paramagnetic[3] and the per cent spin per molecule of FMN was calculated as previously described. The esr spectra are shown in Figure 6. It was found that at pH 4.5 and pH 9.0 the precipitate averaged 0.51 and 0.33 (four determinations each) spins per mol of FMN, respectively. Note that, as in solution (see above), the low pH system has the higher spin concentration.

The above experiments lead to the conclusion that the precipitate is most likely a complex of oxidized and reduced FMN that is in equilibrium with a semiquinone pair. Thus, it may be represented by the following equation

$$\text{F-FH}_2 \rightleftharpoons \text{FH}\cdot\text{-FH}\cdot$$

It is noteworthy that there is narrowing of the peak to peak line width (ΔH) in going from the pH 4.5 complex to the pH

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[3] The possibility exists that this species has triplet character in the ground state. However, a search for an esr signal at half-field was unsuccessful.

9.0 complex. Such a line narrowing may result from a faster electron exchange rate between the two flavines in the pH 9.0 complex. At pH 9.0 the fully reduced and radical forms of FMN are largely in the anion state due to the loss of a proton. These would be expected to pick up a metal cation (K^+) upon precipitation. This could act to mediate electron exchange and thus account for the narrowing of the esr signal. Substitution of a metal cation for a proton might also change the spin distribution with a possible narrowing of ΔH. Furthermore, it is reasonable to expect that the spin dipole–dipole interaction would be different in complexes of different states of protonation.

To further document this model, the stoichiometry of the solids was determined by anaerobically dissolving a measured weight of the complex in degassed buffer and recording the spectra. In sufficiently dilute solution ($<10^{-4}$ M) the above species should dissociate to give an equimolar mixture of oxidized and reduced FMN. Other complexes that can be imagined as being present in partially reduced flavin solution (*e.g.*, F–2FH$_2$, F–FH·, etc.) would yield odd ratios of oxidized to reduced flavin upon dilution. In five separate determination at pH 4.5, a one-to-one correspondence between oxidized and reduced FMN was found. This result is thus consistent with the conclusions arrived at from the esr and mull spectra experiments. It seems reasonable to conclude that the species absorbing at 900 nm, which is called a charge-transfer complex by some and a radical dimer by others, is probably best characterized as a mixture of these two types.

On the basis of these experiments, it is possible to account for the difference in radical disproportionation kinetics determined by flash and temperature-jump experiments. The temperature-jump experiments were performed in a concentration range that permits appreciable complex formation and dimerization of the flavin semiquinone. Therefore, an additional step must be included in the mechanism of semiquinone formation to account for the dimerization process (eq 11). If

$$\mathrm{F} + \mathrm{FH_2} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{F{-}FH_2} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} [\mathrm{FH\cdot}]_2 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} 2\mathrm{FH\cdot} \qquad (11)$$

we assume that the first two steps are fast (complexation and dimerization) compared to the third step (radical formation),[4] this mechanism can be simplified to

$$[\mathrm{FH\cdot}]_2 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} 2\mathrm{FH\cdot} \qquad (12)$$

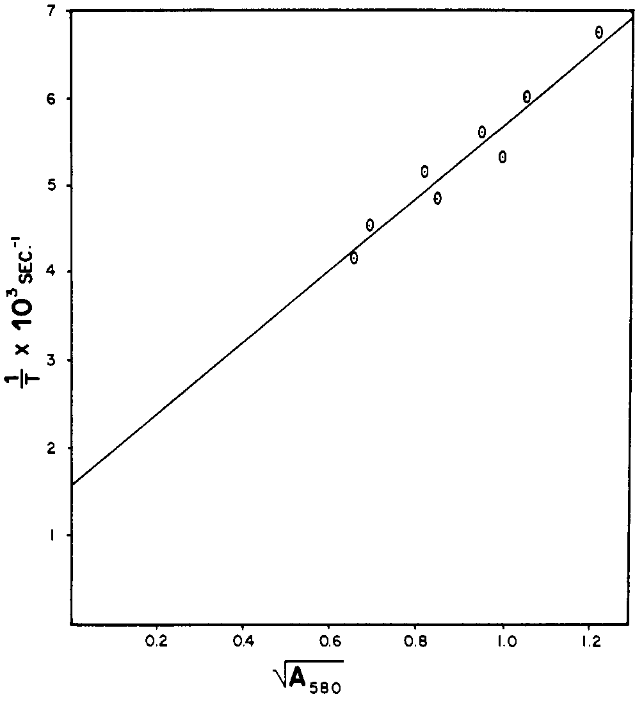

FIGURE 7: Plot of $1/\tau$ *vs.* $(A_{580})^{1/2}$ for the determination of the kinetics of FMN semiquinone dimerization (pH 4.5).

and the expression for $1/\tau_2$ would be identical with eq 7, except that k_3 and k_4 have now to be replaced by k_5 and k_6. A plot of $1/\tau_2$ *vs.* 4[FH·] should now yield the rate constants k_5 and k_6. However, this cannot be accomplished by plotting $1/\tau_2$ *vs.* 4[A_{580}] (as in Figure 2), inasmuch as both the dimer and monomer semiquinone absorb at 580 nm. Indeed, because of the apparent slowness of radical disproportionation as measured by temperature jump, most of the radical must be dimerized at these concentrations. Therefore, the greater part of the absorbance at 580 nm is due to the dimer. We can make use of the equilibrium relationship between dimer and monomer and solve for the concentration of monomer in terms of A_{580} as shown

$$[\mathrm{FH\cdot}] = \sqrt{\frac{K_{56}^{-1}}{2\epsilon_{\mathrm{FH}}}}\sqrt{A_{580}}$$

Therefore a plot of $1/\tau_2$ *vs.* $(A_{580})^{1/2}$ should be linear with a slope proportional to k_6 and intercept equal to k_5 (Figure 7). The exact concentration of [FH·] cannot be determined and therefore k_6 cannot be calculated. However, the intercept does yield k_5 (1.5×10^3 sec^{-1}) and if this is combined with k_6 (3×10^9 M^{-1} sec^{-1} as determined by flash photolysis), the equilibrium constant between monomer and dimer ($K_{56} = k_5/k_6$) may be estimated to be 5×10^{-7} M. Thus, radical dimerization is apparently an energetically quite favorable process.

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[4] An alternative assumption is that the establishment of the second equilibrium is rate determining. In this case, the relaxation time should be independent of concentration, which is contrary to observation.