solution. From this result one would predict a calorimetric observation of two peaks, or at least a shoulder, in the e.m.f. trace of Figure 1. The lack of such a feature in Figure 1 suggests that the resolution accomplished by Scott and Scheraga might not be completely correct. In making the resolution it was assumed that the heat of each step was independent of temperature. We have noted a small but definite heat capacity change for the transition, which would be contrary to this assumption. We have found that the calorimetric fraction temperature dependence in Figure 2 can be explained by either a two-step simultaneous process or a two-step successive process for various combinations of heat of reaction per step and transition temperature. It is also possible to obtain correspondence with the curve in Figure 2 by helix–coil transition theory. For example, if the transition reaction consisted of successive steps which involve equal amounts of heat, then the fraction as determined by the heat measurements provides a significant parameter for determining the extent of the reaction. There is no special reason to believe that this situation holds in ribonuclease in contrast to simple synthetic polypeptides; indeed, some of the evidence points to the contrary. However, if one does go ahead and make this questionable assumption, then it is possible to apply the results of helix–coil transition theory[16] to the experimental dependence of θ with temperature. When this is done then one finds various combinations of parameters, σ and n, which can give theoretical curves that fit the experimental curve reasonably well. It is of interest in this connection to note that when one takes a σ-value of 2×10^{-4}, which has been found to apply to polypeptide transitions, that a value of n of 70, which corresponds to the estimated number of carbonyl–amide hydrogen bonds in ribonuclease,[17] does not give a theoretical curve that fits the experimental data. For this value of σ a much larger value of n would be needed for a reasonably fitting transition curve.

In conclusion the calorimetric results serve to confirm the notion that the ribonuclease transition is not a simple helix–coil polypeptide transition, nor is it a transition which consists of a single step or several independent steps with significantly different transition temperatures. The enthalpy measurements indicate the transition reaction consists of more than one closely affiliated or similar step.

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Kinetics of the Flavin Mononucleotide System

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The kinetics of the equilibria present in a partially reduced aqueous solution of flavin mononucleotide are examined by the temperature-jump–relaxation method. The equilibria considered are the formation of a dimer from the oxidized and reduced forms of flavin mononucleotide (eq. i) and the formation of free radicals from the dimer (eq. ii). (i) is established rapidly compared to (ii). In the pH range 3.9–5.2 at 11° and ionic strength 0.1 the rate constants for (ii) are $k_{32} = 4 \times 10^7\ M^{-1}\ sec^{-1}$ and $k_{23} = 0.8\ sec.^{-1}$. For (i) k_{21} and k_{12} are greater than $2 \times 10^5\ sec.^{-1}$ and $4 \times 10^8\ M^{-1}\ sec.^{-1}$, respectively.

Introduction

The species and equilibria present in partially reduced aqueous flavin solutions have long been a subject of interest. Kuhn and Wagner-Jauregg[2] reduced flavin dyes in the presence of HCl and noted a transition from the original intense yellow color to red and then to pale yellow, the color of reduced flavins. The red species was assumed to be a semiquinoid radical. Michaelis and his co-workers[3,4] made a detailed quantitative study of a number of flavin systems by analysis of potentiometric titration curves. At low concentrations of the flavin and over a wide pH range it was concluded that the intermediate form in the reduction is represented by a free radical. At higher concentrations of the flavin there is a partial dimerization of the free radical. Lowe and Clark[5] made potentiometric titration studies of several flavins over a wide pH range. They concluded that for flavin mononucleotide the amount of flavin as free radical and dimer was essentially constant over the entire pH range at a total concentration of 10^{-4} M in flavin.

Changes in the color of flavin solutions upon reduction were noted by early workers.[2-4] However, Beinert was the first to assign species to the absorption bands which appeared during the reduction of flavins.[6,7] Bands at about 570 and 900 mμ were assigned to the free radical and dimeric forms, respectively. A strong temperature and dilution dependence was noted for the longer wave length band. It was observed that a plot of the optical density of the 570 mμ band *vs.* the optical density of the 900 mμ band did not follow

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the same line when flavin mononucleotide in the oxidized (FMN) and reduced ($FMNH_2$) forms were in excess. This difference was attributed to the slow formation of dimer ($FMN \cdot FMNH_2$) from the free radical ($FMNH\cdot$). Massey and Palmer[8] studied the characteristics of the 570 and 900 mμ bands at various stages in the reduction of FMN. They concluded that the species corresponding to the 900 and 570 mμ bands were not related by dimerization as previous investigators had postulated, but came from $FMNH_2$ and FMN. The authors postulated a charge-transfer complex for the 900 mμ band. Later work by Massey, Gibson, and Atherton[9,10] using spectrophotometry, fluorescence, and e.s.r. spectrometry indicated that the 900 mμ band is a charge-transfer complex between $FMNH_2$ and FMN ($FMN \cdot FMNH_2$) and the 570 mμ peak arises from $FMNH\cdot$ and a complex between $FMNH_2$ and $FMNH\cdot$ ($FMNH_2 \cdot FMNH\cdot$). There is a direct correspondence between the magnitude of the 570 mμ peak and the e.s.r. signal indicating that both $FMNH\cdot$ and ($FMNH_2 \cdot FMNH\cdot$) have e.s.r. signals.

The e.s.r. signal in FMN solutions was first observed by Beinert[6] when FMN was partially reduced in a 50% ethanol–50% water solution 1 *N* in HCl by Zn. Ehrenberg[11] found such a signal in partially reduced FMN solutions which were frozen at liquid nitrogen temperatures to reduce the dielectric loss caused by water. Recently several workers have studied the resolved e.s.r. spectrum of the semiquinone of FMN in an attempt to characterize this biologically important compound.[12,13]

Several kinetic studies of the equilibria involved in flavin oxidation–reduction systems have appeared in the literature. Burn and O'Brien studied the reaction between dithionite and FMN in a semiquantitative manner.[14] Addition of dithionite to a 10^{-2} *M* flavin solution increases the absorption at 900 mμ to a maximum in about 1 sec. followed by a slow decrease to zero in a few seconds.

Gibson and Hastings[15] made a complete study of the oxidation of $FMNH_2$ by molecular oxygen. They found the oxidation reaction to be autocatalytic due to the ability of a compound formed from the interaction between FMN and $FMNH_2$ to react with oxygen.

Holmström,[16] while studying the photoreduction of FMN, determined the rate constants for the equilibrium

$$2FMNH\cdot \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} FMN + FMNH_2$$

to be $k_2 = 3 \times 10^8\ M^{-1}$ sec.$^{-1}$ and $k_{-2} \simeq 10^6\ M^{-1}$ sec.$^{-1}$ at room temperature. When the semiquinone is in the anionic form the rate constants are $1 \times 10^8\ M^{-1}$ sec.$^{-1}$ and $<10^5\ M^{-1}$ sec.$^{-1}$, respectively.

This communication describes a preliminary study of the kinetics of the equilibria present in partially reduced aqueous solutions of FMN. Information is also presented on the rate constants for complex formation between flavin mononucleotide and the electron donors L-tryptophan and serotonin creatinine sulfate. The latter complexes are described by Isenberg and Szent-Györgyi.[17] All of the systems described were studied by the temperature-jump–relaxation method.[18]

Experimental

Chemicals. The flavin mononucleotide (riboflavin-5′-phosphate sodium salt, H. Schroeder, Munich, Germany) used was 93% pure, and was used without further purification. L-Tryptophan (Mann Research Laboratories), serotonin creatinine sulfate (Fluka, A.G., Zurich, Switzerland), sodium dithionite (Merck, reagent grade), and potassium nitrate (Merck, reagent grade) were used without further purification.

Preparation of Solutions. FMN solutions with L-tryptophan and serotonin creatinine sulfate were prepared in doubly distilled water. Enough KNO_3 was added to make the ionic strength 0.1.

Partially reduced FMN solutions were prepared in the following manner. Two solutions of FMN of the desired ionic strength and pH were prepared for each experiment, one to be reduced to $FMNH_2$ and the other to be added to the $FMNH_2$ solution. The concentration of each was such that upon dilution the desired initial concentrations of FMN and $FMNH_2$ were present. Both solutions, as well as a spectrophotometer cell, temperature-jump cell, and sodium dithionite solution (approximately 10^{-1} *M* with added NaOH to prevent turbidity), were outgassed with nitrogen which passed through chromous bubblers before and after the system. Sufficient sodium dithionite solution was added to the desired FMN solution to nearly reduce it. The $FMNH_2$ solution was added to the FMN solution and mixed. The mixed solution was forced under nitrogen pressure into both the spectrophotometer and the temperature-jump cells, which were sealed off with stopcocks during the experiments.

Apparatus. The pH meter was a Radiometer pH meter 22 (Radiometer, Copenhagen, Denmark). The instrument was standardized with commercially available buffers. The spectrophotometer was a Beckman DK-2A with matched 1-cm. cells. A variable temperature, nitrogen flushed, cell holder attachment cooled from a circulating ice bath was used for work at temperatures between 1 and 20°.

The theory of relaxation techniques and a description of the temperature apparatus appear elsewhere.[18] However, a brief résumé is given here. The conditions for using relaxation methods to evaluate the kinetics of a system are the following. First, the system must be in equilibrium. Second, when a perturbation (temperature, pressure, electric field, etc.) is applied to the system to change the equilibrium, the change must be small enough so that the rate equations can be linearized, that is, $\Delta G/RT << 1$. Then $dc_i/dt = -(c_i - \bar{c}_i)/\tau$, where c_i and $\bar{c}_i$ are concentrations of the *i*th species in the perturbed and the equilibrium state, and τ is the relaxation time. The relaxation time is related to the rate constants and concentrations of the species in the

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equilibrium state established after the perturbation in a unique way depending on the mechanism.

In the study described here perturbation of the equilibrium was accomplished by an increase in temperature. A 30-kv. source was discharged across an air gap with the cell containing the solution under study in series. The temperature increase and time required for the increase were controlled in part by the cell resistance. The temperature increase, which occurs in about 10^{-9} sec., was 10°. The temperature was then constant for several seconds. The initial temperature of the solution was about 1° so that effects from the change in density of the solution with temperature were minimized. The changes in concentration of the species in the equilibria were followed spectrophotometrically in the 450 to 700 mμ region. The change as a function of time was recorded on an oscilloscope. The values for τ, the relaxation time, were taken from photographs of the traces. The minimum observable relaxation time was 5 μsec. due to the circuitry of the photomultiplier system, and the maximum observable relaxation time was about 1 sec. due to convection effects from solution cooling.

Results and Discussion

Kinetics of FMN Complexes with L-*Tryptophan and Serotonin Creatine Sulfate.* The equilibrium studied is $\mathrm{FMN} + \mathrm{X} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \mathrm{FMN \cdot X}$, where X represents L-tryptophan or serotonin creatinine sulfate. The relaxation time, τ, is related to the rate constants and concentrations by[18]

$$\tau = \{k_{21} + k_{12}([\mathrm{FMN}] + [\mathrm{X}])\}^{-1}$$

In both systems a temperature perturbation of 10° (from 1 to 11°) produced an optical density change between 450 and 550 mμ. This is the spectral region in which Isenberg and Szent-Györgyi observed an absorption band for the FMN·X complex.[17] The change occurred in a time period short compared to the relaxation time of the instrument, 5 μsec. A simple mixing experiment between X and FMN shows that the rate constants involved in the process are not small. For all measurements [X] was greater than [FMN], and the smallest [X] was 5×10^{-4} M. If $K = k_{12}/k_{21} = [\mathrm{FMN \cdot X}]/([\mathrm{FMN}][\mathrm{X}])$

$$1/\tau = k_{21}\{1 + K([\mathrm{FMN}] + [\mathrm{X}])\}$$

At room temperature K's for the FMN complexes with serotonin creatinine sulfate and tryptophan are about 400 and 60 M^{-1}, respectively.[17] Thus, $1/\tau$ is a lower limit on k_{21}. k_{21} is greater than 2×10^5 sec.$^{-1}$. The rate constant for the formation of the FMN–serotonin creatinine sulfate complex, k_{12}, is greater than 8×10^7 M^{-1} sec.$^{-1}$. This result will be of interest when we analyze the results of the FMN system.

Kinetics of the Equilibria in Partially Reduced FMN Solutions. There are a number of acid–base equilibria in partially reduced FMN solutions, some of which are represented by the following pK's: FMN, −0.2[4] (riboflavin) and 9.8[13]; $\mathrm{FMNH \cdot}$, 1.2[4] (riboflavin) and 7.3[16]; and $\mathrm{FMNH_2}$, 6.8.[16] Therefore, the predominant equilibria in the pH range studied, 3.9 to 5.2, are[9,10]

$$\mathrm{FMN} + \mathrm{FMNH_2} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (\mathrm{FMN \cdot FMNH_2}) \qquad \text{(i)}$$

$$(\mathrm{FMN \cdot FMNH_2}) \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} 2\mathrm{FMNH \cdot} \qquad \text{(ii)}$$

$$\mathrm{FMNH_2} + \mathrm{FMNH \cdot} \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} (\mathrm{FMNH_2 \cdot FMNH \cdot}) \qquad \text{(iii)}$$

Since excess FMN over $\mathrm{FMNH_2}$ was used in the experiments the predominant equilibria are (i) and (ii). The possibilities exist that equilibrium (i) is established rapidly compared to (ii), (ii) is established rapidly compared to (i), or both are established in the same time range.

One of the possibilities, the latter, can be eliminated by studying the character of the optical density change at various wave lengths when a partially reduced solution of FMN is perturbed by a temperature increase. At wave lengths between 500 and 600 mμ there is an optical density increase of variable magnitude which occurs faster than 5 μsec. followed by a relaxation within the time range of the instrument corresponding to an optical density increase. Between 600 and 650 mμ the temperature perturbation has little effect on the optical density of the system. Between 650 and 700 mμ there is a rapid decrease in optical density of the system with increasing temperature. The sensitivity of the instrument in this wave length region is not good enough to say whether any relaxation follows the rapid optical density change. The direction of the change at various wave lengths corresponds exactly with those observed from temperature-dependence studies of the spectrum of partially reduced FMN solutions.[6,7] At the sensitivity at which these experiments were carried out a temperature perturbation caused no appreciable optical density change in solutions of FMN or $\mathrm{FMNH_2}$. Therefore, it seems reasonable to assume that either (i) is established rapidly compared to (ii) or (ii) is established rapidly compared to (i).

Two equilibrium constants are defined

$$K_{12} = [(\mathrm{FMN \cdot FMNH_2})]/([\mathrm{FMN}][\mathrm{FMNH_2}])$$

$$K_{23} = [\mathrm{FMNH \cdot}]^2/[(\mathrm{FMN \cdot FMNH_2})]$$

If (i) is established rapidly compared to (ii)

$$1/\tau = \frac{k_{23}K_{12}([\mathrm{FMN}] + [\mathrm{FMNH_2}])}{2\{1 + K_{12}([\mathrm{FMN}] + [\mathrm{FMNH_2}])\}} + 2k_{32}[\mathrm{FMNH \cdot}] \qquad (1)$$

and if (ii) is established rapidly compared to (i)

$$1/\tau = \frac{4k_{21}[\mathrm{FMNH \cdot}]}{4[\mathrm{FMNH \cdot}] + K_{23}} + k_{12}([\mathrm{FMNH_2}] + [\mathrm{FMN}]) \qquad (2)$$

The magnitude of the equilibrium constants K_{12} and K_{23} determined by Michaelis and his co-workers for the riboflavin system at pH 5 are approximately 600 M^{-1} and 10^{-4} M, respectively.[3,4] Gibson, *et al.*,[9] have estimated the values for the FMN system to be 2×10^3 M^{-1} and 10^{-6} M, respectively, at pH 6.3. Thus, $1/\tau$ should be a linear function of $[\mathrm{FMNH \cdot}]$ or $([\mathrm{FMNH_2}] + [\mathrm{FMN}])$ for the mechanisms represented by eq. 1 and 2, respectively. Table I records data for a series of temperature-jump experiments carried out at 550 mμ in the pH range 3.9 to 5.2. The initial temperature was 1° and final temperature 11°. Figure 1 shows a plot of $1/\tau$ as a function of twice the optical density of the corrected $\mathrm{FMNH \cdot}$

absorption at approximately 570 mμ. Run 8 in Table I corresponds to an experiment where the original concentrations of FMN and $FMNH_2$ in the mixed solution were 9.5×10^{-3} and 5×10^{-4} M, respectively. Thus $\{[FMNH_2] + [FMN]\}$ must be at least 9.0×10^{-3} M, which is approximately twice the value for run 11 and three times the value for run 16. This indicates that of the two possible mechanisms the most likely is that in which (i) is established rapidly compared to (ii). Run 8 indicates that a relaxation related to equilibrium iii is not being measured. For (iii) alone

$$1/\tau = k_{43} + k_{34}\{[FMNH_2] + [FMNH\cdot]\}$$

In runs 11 and 16 $\{[FMNH_2] + [FMNH\cdot]\}$ is of the order of 2×10^{-3} and 1×10^{-3} M, respectively, while in run 8 this sum is no greater than 5×10^{-4} M, about the same as in run 7.

Table I. Relaxation Data at 11° and Ionic Strength 0.1

Run	pH	Total concn. of flavin in soln., $M \times 10^3$	$1/\tau$, sec.$^{-1}$	2 × O.D. of FMNH· at 570 mμ
1	5.2	2.2[a]	5,300	0.36
2	5.2	1.8[a]	4,800	0.30
3	5.2	1.5[a]	4,700	0.24
4	5.2	1.0[a]	3,300	0.20
5	5.0	1.8[a]	6,300	0.36
6	5.0	1.5[a]	5,600	0.32
7	5.0	0.9[a]	5,000	0.20
8	5.0	10.0[b]	10,000	0.80
9	5.0	0.7[a]	3,700	0.15
10	5.0	1.1[a]	5,400	0.20
11	5.0	5.5[a]	10,000	0.66
12	3.9	1.5[a]	4,800	0.30
13	3.9	1.1[a]	5,600	0.20
14	3.9	0.7[a]	2,600	0.16
15	3.9	2.2[a]	8,000	0.48
16	3.9	2.9[a]	9,600	0.56
17	3.9	1.8[a]	7,600	0.40

[a] Equal volumes of $FMNH_2$ and FMN of this concentration were added together to form the solutions. [b] FMN (100 ml. of 9.6×10^{-3} M) was added to 1 ml. of 5×10^{-2} $FMNH_2$. Run was done in duplicate.

Several values for the extinction coefficient of FMNH· at about 570 mμ appear in the literature. To say the least the agreement is not good. Beinert[19] gives a value of 1000 M^{-1} cm.$^{-1}$ (λ 560 mμ, pH 6.1). Gibson, *et al.*,[9] find the value to be between 8000 and 13,600 M^{-1} cm.$^{-1}$ (λ 570 mμ, pH 6.3). Holmström[16] determined the value to be 3050 M^{-1} cm.$^{-1}$ (λ 560 mμ, pH 6) and 1300 M^{-1} cm.$^{-1}$ when the free radical is in the anionic form.

Using the value of 3050 M^{-1} cm.$^{-1}$ and eq. 1, k_{32} can be calculated from the slope of Figure 1. From such a calculation $k_{32} \approx 4 \times 10^7$ M^{-1} sec.$^{-1}$ at 11° and ionic strength 0.1. Using a value of $K_{23} \approx 10^{-6}$ M,[9] $k_{23} \approx 8 \times 10^{-1}$ sec.$^{-1}$ ($k_{23} = k_{32}K_{23}$). The

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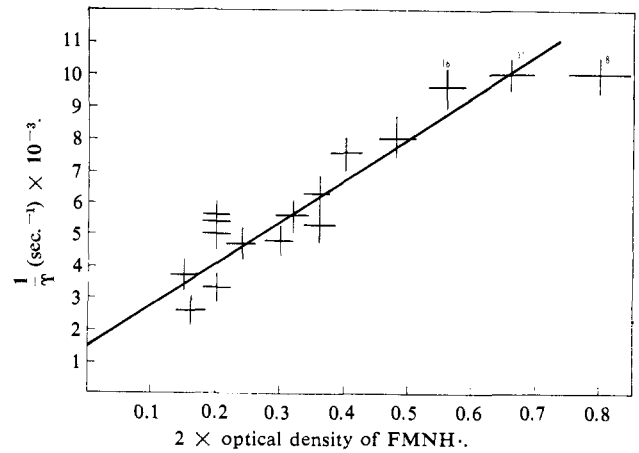

Figure 1. $1/\tau$ *vs.* 2 × optical density of FMNH· at 570 mμ; pH 3.9 to 5.2, temperature 11°, and ionic strength 0.1.

rate constants in the equilibrium process represented by (i) must be greater than the rate constants for (ii). A relaxation is not seen for (i); thus, the rate constants for this process must have lower limits of the same order of magnitude as the FMN–serotonin creatinine sulfate or tryptophan system, that is k_{21} and k_{12} are greater than 2×10^5 sec.$^{-1}$ and 4×10^8 M^{-1} sec.$^{-1}$, respectively. Since the complex between the acceptor FMN and the donor serotonin creatinine sulfate is of the charge-transfer type,[20] it seems likely that the complex (FMN·$FMNH_2$) is also a charge-transfer complex, and that the electron transfer takes place in step (ii). This is in agreement with the conclusion of Gibson, *et al.*,[9] that the species represented by the 900 mμ band is a charge-transfer complex and a precursor of the free semiquinone.

More work is being carried out to determine the rate constants for the various equilibria involved in the partially reduced FMN system and the effect of pH on these rate constants. Preliminary data indicate that the relaxation time is radically altered as the pH is increased to values greater than six, but as the data in Table I indicates, the relaxation time, and thus the mechanism associated with it, is the same in the pH range 3.9 to 5.2. This indicates that the electron transfer is intimately connected with a proton transfer and that the rate constants are thus radically altered by the presence of the unprotonated forms of $FMNH_2$ and FMNH·.

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