The Kinetics of Flavine Oxidation–Reduction. I. Dismutation in Nonaqueous Solvent†

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ABSTRACT: The dismutation reactions of flavines in dimethylformamide have been investigated using the stopped-flow technique under anaerobic conditions. The ionization constants of fully reduced and oxidized tetraacetylriboflavine were measured spectrophotometrically in buffered dimethylformamide. The dismutation equilibrium of the flavine as a function of pH in dimethylformamide was roughly comparable to that reported in water and allowed the estimation of the pK_a value of the flavosemiquinone. The dismutation kinetics of tetraacetylriboflavine in unbuffered dimethylformamide were investigated using the fully oxidized and reduced flavines in their neutral form at constant product of concentrations and varying the reduction degree. The kinetics at very low reduction ratios (<3%) were triphasic. The kinetic analysis of the initial and simultaneous formation of the anionic and neutral radicals revealed a second-order reaction. The electron transfer between the oxidized and reduced flavines was not directly coupled with proton exchange. The multiphasic time course of the reaction proceeded primarily from differences in the intrinsic rates of the direct and mixed backward dismutation reactions of the two radical species, and finally from a change in the equilibrium conditions resulting from the accumulation of anionic flavohydroquinone. An acidic–basic negative catalytic effect from the neutral flavohydroquinone appeared progressively as the reduction degree was increased. It was complete at reduction ratios higher than 30%, i.e. under conditions where the radical anion could not be observed at any reaction time. Acids with a pK_a value lower than the second one of the flavosemiquinone exhibited a similar catalytic effect. These acidic–basic catalytic effects are associated with changes in the ionic state of labile intermediate dimers formed in the forward as well as in the backward directions of the dismutation reaction. Such a transient complex revealed by the kinetic analysis could be observed directly by absorption spectroscopy in alkaline-buffered dimethylformamide. Its spectral characteristics, as well as the kinetic effects induced by substitution of the benzenoid part of the flavine, can hardly be taken into account by a quinhydrone-like structure for the intermediate dimers at any pH value. The experimental results favored a more specific interaction, possibly of covalent character, involving the benzenoid part of the isoalloxazine ring.

Among the properties associated with the existence of the three redox states of the flavines, namely the flavoquinone, the flavosemiquinone, and the flavohydroquinone, the dismutation reaction has been considered since the early investigations of Stern (1934), Michaelis (Michaelis et al., 1936; Michaelis and Schwarzenbach, 1938), and Beinert (1956) as a possible biochemical reaction. It could provide a link between the two-electron and the one-electron carriers in electron-transferring chains (Beinert and Sands, 1961; Ingraham, 1962; Hemmerich et al., 1970). This idea has now gained strong biochemical support (Siegel et al., 1971; Norris et al., 1972; Masters et al., 1975; Blandin-Capeillère et al., 1975) and has been for a long time the subject of model studies in vitro starting with equilibrium investigations which already revealed the existence of dimers, named as the flavoquinhydrone complexes, formed by the fully reduced and oxidized flavines (Beinert, 1956; Gibson et al., 1962). The kinetics of the reaction have been extensively studied in aqueous solvent using various techniques: stopped flow (Burn and O'Brien, 1959), temperature jump (Swinehart, 1965; Barman and Tollin, 1972), flash photolysis (Holmström, 1964; Vaish and Tollin, 1971), and pulse radiolysis (Land and Swallow, 1969). These investigations have underlined the importance of acidic–basic reactions associated with the electron transfer, but were unable to describe the intermediate states of the dismutation reaction, due to its very fast rate in water.

It was the aim of the present study to use a nonaqueous solvent, *N,N'*-dimethylformamide, to slow the dismutation reaction down in order to investigate the existence and the nature of such possible intermediates using conventional stopped-flow techniques. Indeed, such a solvent allows for a control of the acidic–basic reactions in an environment which is probably more comparable to that in a flavoprotein. Furthermore, it hinders the formation of stacked dimers such as the flavoquinhydrone complex stabilized by hydrophobic interactions. Finally, such a medium favors flavine–metal interactions which may interfere with the dismutation reaction and provide new pathways for oxidoreduction. Considering the widespread occurrence of metalloflavoproteins, it appeared worthwhile to investigate these phenomena altogether. They will be reported in the following paper (Favaudon and Lhoste, 1975).

Experimental Procedure

Solvent. N,N'-Dimethylformamide was used as a solvent throughout the present work. It was distilled under a low pressure of dry nitrogen in order to eliminate the major part of water as well as decomposition products such as amines and formic acid which could overcome the acidic–basic effects of the added solutes. The water content remaining

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Table I: pH Values of Equimolar Solutions (0.01 *M*) of Some Organic Acids and of Their Tetrabutylammonium Salts in Dimethylformamide at 20°C.[a]

Nature of the Acid	pH	Nature of the Acid	pH
p-Toluenesulfonic	2.2	Benzoic	11.4
Dichloroacetic	7.4	Acetic	13.4
Monochloroacetic	9.4	Propionic	13.9
2-Chlorobenzoic	10.6		

[a] G. Demange-Guerin, private communication.

after distillation (ca. $\geq 10^{-3}$ *M*) varied to some extent, which could affect the kinetics of flavine oxidation–reduction. Thus, each experimental series was carried out using the same solvent sample in order to prevent systematic errors in the relative rate measurements.

The determination of a pH scale in dimethylformamide has been carried out by measuring the potential of a glass electrode (Tacussel, type DMF B 10) dipping in solutions of 1 to 10^{-3} *M* perchloric acid in dimethylformamide, the ionic strength being kept constant ($\mu = 0.1$) using tetrabutylammonium perchlorate (Demange-Guérin, 1970). A saturated calomel electrode (Tacussel, type DMF C 10) was taken as a reference. The dependence of the electrode potential on the concentration of perchloric acid obeys the Nernst equation in this concentration range. This equation gives the origin of the pH scale used ($pK_{HClO_4} = 0$). On the other hand, the pH may be controlled in dimethylformamide by means of various buffers, as described by Juillard (1970) in the acidic pH range. We used equimolar mixtures of organic acids and of their tetrabutylammonium salts (G. Demange-Guérin, personal communication) at a concentration of 10^{-2} to 10^{-1} *M* in order to reach alkaline values of the pH. Tetrabutylammonium salts were prepared from tetrabutylammonium hydroxide (Fluka) and dehydrated in vacuo; then equimolar amounts of the corresponding acids were added. Buffers were used immediately after dissolution in dimethylformamide in order to minimize the solvent decomposition which occurs under very acidic as well as under very basic conditions. The pH of a given buffer sample may indeed differ to some extent from the expected value (Table I) due to decomposition of the solvent and/or of the tetrabutylammonium salts upon dehydration. Thus, the pH was measured immediately before any experiment.

Flavines. Tetraacetylriboflavine (Ac_4RF)[1] was synthesized following the method of McCormick (1970) and then recrystallized twice from glacial acetic acid–water mixtures and dried in vacuo.

8α-Bromo-Ac_4RF, as a starting material for the synthesis of 8α-morpholino-Ac_4RF, was prepared following the methods of Ghisla et al. (1970) and Walker et al. (1972). One millimole (0.6 g) of anhydrous crystallized 8α-bromo-Ac_4RF was dissolved in 10 ml of 0.2 *M* morpholine in chloroform. This solution was boiled for 20 sec, then cooled in ice and precipitated by anhydrous cold ether, and the precipitate was desiccated in vacuo. Purification of the crude material was carried out chromatographically on Dowex 50W-X2 resin (Baker) equilibrated with 1-butanol–acetic acid–water (2:1:1) as eluting solvent. The 8α-morpholino-Ac_4RF was adsorbed at the upper part of the column, while all the other flavines were eluted down. Desorption of the morpholino derivative by pH 6.8 phosphate buffer was followed by chloroform extraction from the aqueous medium and by precipitation with isopropyl ether. The final yield of 8α-morpholino-Ac_4RF was 20% with respect to the starting 8α-bromo-Ac_4RF. Purity controls were carried out by absorption spectroscopy in 8 *N* HCl and by nuclear magnetic resonance (NMR) spectroscopy (Salach et al., 1972).

Isotetraacetylriboflavine, 8-nor-8-chlorolumiflavine, and 3-benzyl-8-norlumiflavine were kindly provided by Professor P. Hemmerich (University of Konstanz, Germany). The flavine derivatives were reduced with hydrogen using a palladium catalyst adsorbed on silica powder (Fluka). In dimethylformamide the neutral flavohydroquinone is formed without proton release. The exposure to hydrogen of some substituted flavines must be carefully limited in order to prevent further reduction of the isoalloxazine ring (Lambooy, 1967).

Stopped-Flow Apparatus. The Durrum Instruments stopped-flow spectrophotometer was modified in order to allow working under anaerobic conditions. The air-exposed joints (between the valve block, the optical cuvette, and the drain valve block) were sealed up with Araldite. The valve packing nuts were forced into the valve block with Teflon tape. The drive syringes were forced into conical Teflon caps made in the laboratory, and the syringe holders were flushed continuously with pure nitrogen. Argon or hydrogen was bubbled through the solutions by means of stainless-steel tubings. The solutions were contained in vessels made of inactinic glass and fitted with three-way stopcocks which allowed the apparatus to be flushed with argon and the reactants to be transferred to the drive syringes. The vessel used for the reduction of the flavines with H_2/Pd was fitted with a fritted disk. Positive pressures of gases (0.5 to 1 bar) were used in order to prevent air leakage. The anaerobicity was judged by the good stability of 10^{-5} *M* solutions of flavohydroquinone after mixing with argon-degassed solvent.

All the experiments were performed at controlled temperature (20°C, unless otherwise stated). The mixing dead time of 2.5 ± 0.2 msec was kept constant for all the experiments.

Results and Discussion

Dismutation Equilibrium of Ac_4RF in Dimethylformamide. The pK_a values of the fully oxidized and reduced forms of Ac_4RF were measured spectrophotometrically (Figure 1). They appear in the pH scale defined for dimethylformamide about four units higher than the reported values for flavines in water (Lowe and Clark, 1956; Draper and Ingraham, 1968). Optical titration at 530 and 600 nm was combined with electron spin resonance (ESR) in order to estimate the pK_a values of the flavosemiquinone (Figure 1).

The equilibrium of dismutation in dimethylformamide as a function of pH appeared strictly bimolecular at all flavine concentrations and degrees of reduction. As expected from the measured pK_a values, the equilibrium of dismutation as a function of pH (Figure 1) is very similar to that measured in water (Ehrenberg et al., 1967). However, at pH values higher than 13, a true equilibrium could not be observed and the large radical formation may be due to the solvent degradation under strongly alkaline conditions.

The formation constant of neutral radical at 50% reduc-

[1] Abbreviations used are: Ac_4RF, tetraacetylriboflavine; iso-Ac_4RF, isotetraacetylriboflavine; LF, lumiflavine; $F_{red}H_3$, FH_2, and $F_{ox}H$, neutral form of flavohydroquinone, flavosemiquinone, and flavoquinone; DMF, dimethylformamide.

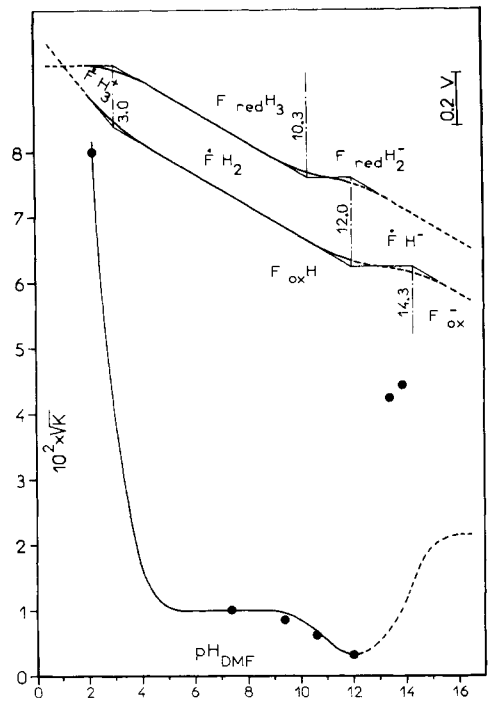


FIGURE 1: Diagram of oxidation-reduction potentials and acidic-basic equilibria of Ac_4RF in buffered dimethylformamide. The diagram was established following the Nernst equation using the equilibrium formation constant of radical measured at pH 7.4 ($K = 1.07 \times 10^{-4}$, $\Delta E = -0.24$ V) and at pH 12 ($K = 1.1 \times 10^{-5}$, $\Delta E = -0.30$ V) and the pK_a values of fully oxidized and reduced flavines measured spectrophotometrically. The pK_a of semiquinone was assumed to correspond to the pH value of minimum radical formation. The equilibrium of dismutation $K^{1/2}$ recalculated in the entire pH range (lower line) from this diagram for best fit with the experimental data (filled circles) suggests a second pK_a value of 3.0 for the semiquinone. Strong deviation with the model is observed at high pH values, possibly due to rapid solvent degradation.

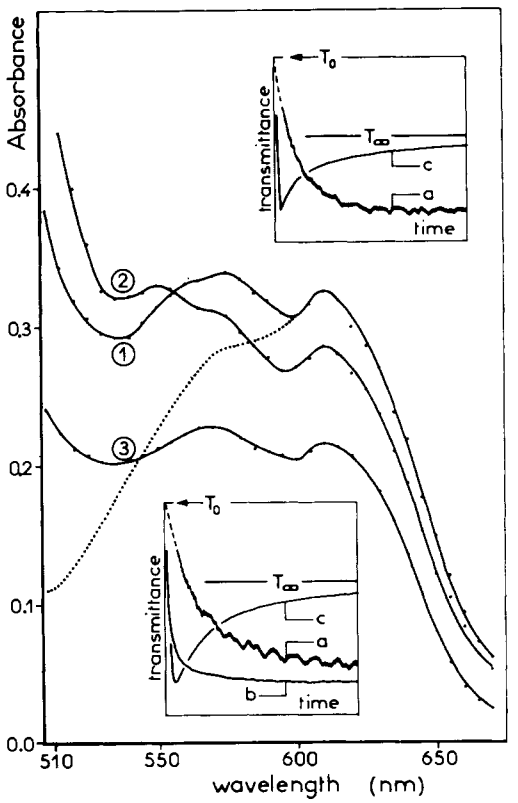


FIGURE 2: Transient absorption spectra for the dismutation reaction of Ac_4RF in pure dimethylformamide at (1) 20 msec, (2) 250 msec, and (3) infinite reaction times. The initial concentrations in fully reduced and oxidized flavines were $[F_{red}H_3]_0 = 2.5 \times 10^{-4}$ M and $[F_{ox}H]_0 = 3.6 \times 10^{-2}$ M. The large residual absorbance of the unreacted flavoquinone was subtracted from the spectra. The dotted line corresponds to the spectrum of neutral radical at the concentration obtained at 20 msec. The oscillograms obtained at 540 nm (lower) and 610 nm (upper) exhibit the triphasic character of the reaction course. The time scale calibration a, b, and c corresponds to reaction times of 25 msec, 250 msec, and 2 sec, respectively. T_0 and T_∞ denote the initial and equilibrium transmittance.

tion in unbuffered dimethylformamide was measured spectrophotometrically, using a value of 5000 ± 100 M^{-1} cm^{-1} for the molar extinction coefficient of neutral semiquinone at 610 nm (Favaudon and Lhoste, 1975). This formation constant is equal to that in dimethylformamide buffered at pH 7.4:

$$K = [\dot{F}H_2]^2/[F_{red}H_3][F_{ox}H] = (1.07 \pm 0.14) \times 10^{-4} \text{ at } 20°C \quad (1)$$

while radical formation appears endothermic. At 20°C:

$$\Delta G = 5.32 \pm 0.08 \text{ kcal/mol}$$

$$\Delta H = 3.60 \pm 0.10 \text{ kcal/mol}$$

$$\Delta S = -5.87 \pm 0.60 \text{ cal/(mol deg)} \quad (2)$$

The negative entropy change may be essentially due to acidic-basic reactions.

The formation in dimethylformamide of the quinhydrone dimer, characterized by a weak absorption in the near infrared (Beinert, 1956), is hardly observable and must be at least two orders of magnitude lower than in aqueous solution.

Kinetics of Dismutation of Ac_4RF in Unbuffered Dimethylformamide. The dismutation reaction of Ac_4RF was always initiated by mixing fully oxidized and fully reduced flavine. Radical formation, as measured spectrophotometrically in the 500–750-nm wavelength range, could not simply be related to the elementary reaction scheme (eq 3) corresponding to the equilibrium data. In particular, the kinetics appeared very dependent upon the degree of reduction.

$$F_{red} + F_{ox} \rightleftarrows 2\dot{F} \quad (3)$$

Kinetics at Half-Reduction. At half-reduction, only the neutral radical $\dot{F}H_2$ appears in a monophasic reaction. This reaction is first order with respect to the total flavine concentration, having a constant half-reaction time and an initial reaction rate V_i linearly dependent upon the total flavine concentration.

Kinetics at Variable Reduction Degree. At variable degrees of reduction maintaining the initial product $[F_{red}H_3]_0[F_{ox}H]_0$ constant, the kinetics depend upon the reduction degree and appear triphasic at the lowest reduction ratios when the observation is fixed in a spectral region where the radical anion does absorb (Figure 2). Thus, anionic semiquinone is formed initially together with the neutral radical in stoichiometric amounts and with the same initial rate (Figure 2). This rate, V_i, is now proportional to the product $[F_{red}H_3]_0[F_{ox}H]_0$, as expected for a second-order reaction.

However, the initial rate of radical formation decreases and radical anion disappears as the reduction degree is increased (Figure 3). At the highest reduction ratios (≥50%) V_i varies linearly with the reciprocal concentration in reduced flavine (eq 4), corresponding to a negative catalytic

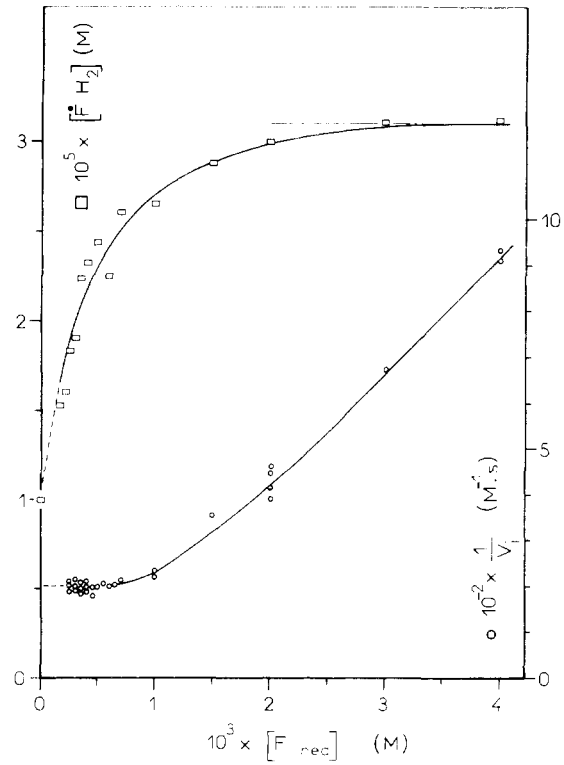


FIGURE 3: Reciprocal initial formation rate (O) and equilibrium yield (□) of the neutral semiquinone produced by dismutation of fully reduced and oxidized Ac_4RF in pure dimethylformamide at initially constant product, $[F_{red}H_3]_0[F_{ox}H]_0 = 9 \times 10^{-6}\ M^2$. All measurements were carried out at 610 nm. Radical anion is also present at the lowest reduction degrees. It should represent 50% of the total radical yield at infinite dilution in flavohydroquinone corresponding to strictly aprotic conditions. Indeed, the extrapolated radical concentration is equal to that measured at 50% reduction in dimethylformamide buffered at a pH value of 12 equal to the pK_a of flavosemiquinone.

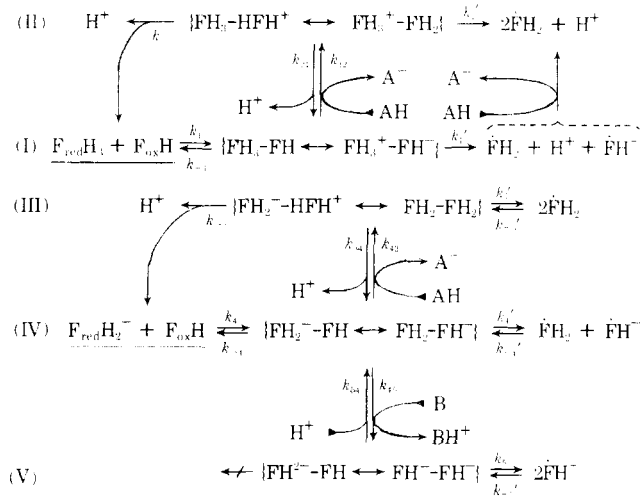
effect from flavohydroquinone. When a strong acid such as perchloric ($pK_{DMF} = 0$) or *p*-toluenesulfonic ($pK_{DMF} = 1.5$) is added to the solution at a concentration comparable to that of the semiquinone at equilibrium, the reaction kinetics are no longer dependent upon the reduction degree and are monophasic and without the appearance of radical anion. Proton donors, including flavohydroquinone and water, have a negative catalytic effect roughly proportional to the difference between their pK_a value and that of the semiquinone ($pK_{DMF} = 12$). For example, 1 *M* benzoic acid ($pK_{DMF} = 12.3$) has practically no effect on the dismutation kinetics.

$$V_i = \{\alpha + (\beta/[F_{red}H_3])\}[F_{red}H_3][F_{ox}H] \qquad (4)$$

Kinetic Evidence for Reaction Intermediates. These results suggest that the effect of neutral flavohydroquinone on the kinetics of the dismutation reaction is an acidic–basic one and that different pathways can be considered for the forward and backward reactions. In other words, intermediates have to be introduced in the reaction. Furthermore, the nature of these intermediates should depend on the initial conditions. Changes in conditions in the course of the reaction can explain the multiphasic character observed in some instances.

The negative catalysis from flavohydroquinone is observed at the early times of the reaction, i.e. without any detectable lag time. The extrapolation of first-order log plots at zero reaction time, to take into account the instrumental dead time, confirms that this effect is completed even before radical formation. Thus, it should be attributed to an intermediary precursor being formed at a much faster rate than the free semiquinone. The concentration of this intermediate is certainly low since the absorbancy of the solution at the extrapolated zero reaction time corresponds simply to the superimposition of that of the fully reduced and of the oxidized starting species.

Scheme I: General Reaction Scheme of Flavine Dismutation.



The effects of acids of different activities suggest that this intermediate possesses at least some radical anion character. The ionic character of the intermediate is further suggested by the lowering effect on the reaction rate of the ionic strength (controlled by the addition of anhydrous tetraethylammonium perchlorate). On the other hand, the parallel initial formation of the neutral and anionic radicals under strictly aprotic conditions suggests that this intermediate is a dimer formed by association of an oxidized and a neutral reduced flavine. The reaction of the weakly acidic flavohydroquinone, $F_{red}H_3$, on this intermediate, that is responsible for the negative catalytic effect, should be distinguished from the secondary neutralization of the radical anion. This neutralization is also due to the presence of the reduced flavine, but this reaction cannot modify the initial rate.

It appears necessary to introduce at this stage of the discussion a reaction scheme corresponding to these observations in order to allow for a quantitative analysis of the kinetics under the various experimental conditions.

Analysis of the Dismutation Kinetics at a Low Degree of Reduction. Under such conditions, the reaction is triphasic (Figure 2). Following the general reaction scheme (Scheme I), the first phase of the reaction should correspond mainly to the forward reaction (I) with parallel formation of neutral and anionic radicals, the initial rate being strictly proportional to the product $[F_{red}H_3]_0[F_{ox}H]_0$. The experimental results obey these conditions. The extrapolation at infinite dilution in flavohydroquinone (Figure 3) defines a specific rate constant for forward reaction I equal to:

$$\frac{k_1 k_1'}{k_{-1} + k_1'} = 560 \pm 40\ M^{-1}\ \text{sec}^{-1} \qquad (5)$$

at 20°C, corresponding to an activation energy of 7.97 kcal/mol and to an Arrhenius factor of $5.3 \times 10^8\ M^{-1}\ \text{sec}^{-1}$. The rapid dissociation of the radical cation $\dot{F}H_3^+$ makes reaction I irreversible.

The second phase of the reaction, which is particularly well defined in the 510–600 nm spectral range (Figure 2),

should reflect the relative importance of the backward reactions involving either a direct dismutation of the neutral semiquinone (reaction III) or a mixed dismutation of the neutral and anionic radicals (reaction IV). The direct dismutation of the radical anion may be neglected under these conditions of low reduction degree and aprotic medium because its relative rate is much slower, as observed under alkaline-buffered conditions. A pseudoequilibrium constant may be defined for the dismutation reaction at the end of the first phase (time (t_1) $\simeq$ 20 msec) (eq 6).

$$K_1 = [\dot{F}H_2]_1^2/[F_{red}H_3]_1[F_{ox}H]_1 \qquad (6)$$

The concentration of radical anion at this reaction time is still comparable to that of the neutral semiquinone, but the latter is more easily measured at 610 nm. K_1 is about 10^{-4}, a value which is comparable to that of the final equilibrium constant K obtained at half-reduction. This result indicates that the mixed dismutation (reaction IV) is slow when compared to the direct dismutation (reaction III), which is mostly responsible for the observation of a second phase during which the radical anion concentration is still increasing, although the neutral radical concentration is already decaying.

The last phase of the reaction, corresponding to a slow and parallel decay of both the neutral and anionic radicals (Figure 2), is due to the changes in the equilibrium conditions associated with the production of anionic flavohydroquinone at the end of the backward reactions III or IV. Then the forward reaction may proceed through reaction IV as well as through reaction I until a final equilibrium depending upon the reduction degree is reached (Figure 3). The effect of the flavohydroquinone concentration appears purely catalytic only for a reduction ratio of 30% or higher, i.e. when its concentration is much larger than that of the semiquinone at equilibrium.

Analysis of the Dismutation Kinetics at a High Degree of Reduction. Under these conditions, the secondary protonation of the radical anion by the neutral flavohydroquinone is fast and only the neutral semiquinone can be observed. The negative catalytic effect from the flavohydroquinone on the dismutation rates can be attributed to an acidic–basic reaction on the intermediate dimers in both the forward (reaction I) and the backward (reaction IV) pathways. The formal analysis of the kinetics of reactions I and IV (see 13–17 in the microfilm issue; see Supplementary Material Available paragraph) allows one to derive an expression for the initial reaction rate as a function of the reciprocal concentration of flavohydroquinone which reflects the relative probabilities of the possible pathways. It appears that the protonation of the primary complex of reaction I is followed rapidly by a heterolytic dissociation ($k_2' \ll k_{-2}$). On the other hand, in the backward reaction (III), the probability for deprotonation of the intermediate complex is much higher than that of a heterolytic dissociation ($k_{34} > 16k_{-3}$). Then the backward reaction can proceed through reaction IV by heterolytic dissociation of the deprotonated intermediate. This dissociation should be slower in the presence of an excess of proton-donating reduced flavine (k_{43}). The overall effect of the reduced flavine is purely catalytic since, at equilibrium, flavohydroquinone is in large excess relative to the radical concentration. Under such conditions, the state of protonation of the radical and the equilibrium of dismutation are no longer dependent on the apparent acidity associated with the flavohydroquinone, $F_{red}H_3$. On the contrary, the dismutation equilibrium should be dependent on the flavohydroquinone concentration at a low degree of reduction (Figure 3) since the apparent acidity under these conditions corresponds to higher pH values. In this pH range, the relative redox potentials for reduction and oxidation of the semiquinone do not follow the same pH dependence (Figure 1).

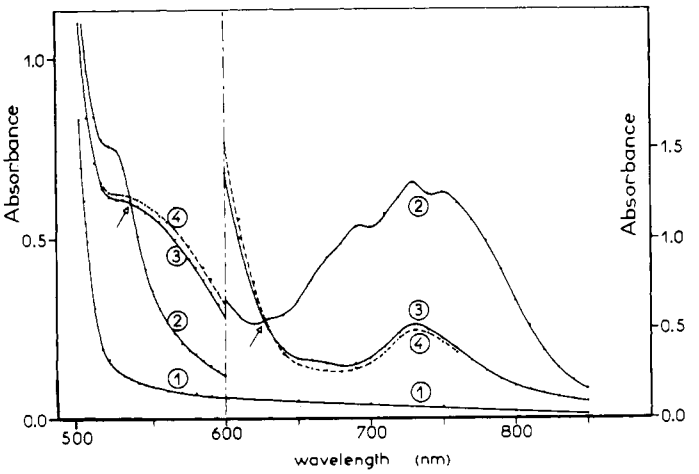


FIGURE 4: Transient absorption spectra for the dismutation reaction of half-reduced Ac_4RF in dimethylformamide buffered at pH 13.4. The various spectra correspond to the initial conditions (1) before mixing, to the time course evolution at (2) 2.5 msec (mixing dead time) and (3) 200 msec, and (4) to the final equilibrium. The spectra exhibit an isosbestic evolution, indicated by arrows, from time 2 to 3. The total flavine concentration was 10^{-3} M for short-wavelength measurements and 2×10^{-3} M in the long-wavelength range. The corresponding half-reaction times for the isosbestic evolution are, respectively, 45 and 35 msec.

Kinetics of Dismutation of Ac_4RF in Alkaline-Buffered Dimethylformamide. The dismutation reaction in dimethylformamide buffered around neutrality ($pH_{DMF} \sim 10$) appears identical with that in unbuffered solvent, but the acidic–basic effects related to the flavohydroquinone disappear. Under acidic conditions, the reaction proceeds very rapidly and cannot be observed kinetically using the stopped-flow technique.

The kinetic behavior under alkaline conditions is characterized by the transitory appearance of a labile intermediate formed during the mixing time of the apparatus and preceding the formation of radical anion (Figure 4). The extrapolation at zero reaction time of the absorbance in the spectral region corresponding to this intermediate indicates its dimeric nature. Indeed, its initial concentration is proportional to the product $[F_{red}H_2^-]_0[F_{ox}H]_0$. Its spectral evolution toward radical anion appears direct and isosbestic and the equilibrium is largely displaced toward radical formation (Figures 4 and 1). The initial rate of radical anion formation increases very rapidly with the pH. However, dimethylformamide is unstable at pH values higher than 13 and a true equilibrium cannot be observed (Figure 1). A slow degradation of the solvent could also explain a slow and non-isosbestic evolution of the solutions following the first phase of decay of the intermediate (Figure 4). The intermediate dimer does not appear during the dismutation reaction in dimethylformamide in the presence of an excess of piperidine ($pK_{DMF} = 10.4$). In other words, the formation of the intermediate dimer is not simply initiated by the deprotonation of the fully reduced flavine.

The kinetics of the dismutation reaction can be analyzed using pathways IV and V in the general scheme since the flavohydroquinone and the semiquinone are in their anionic

Table II: Relative Initial Radical Formation Rates in Direct and Mixed Dismutation Experiments of Various Flavine Derivatives.[a]

	$Ac_4RF_{ox}H$	8α-Morpholino-$Ac_4RF_{ox}H$	iso-$Ac_4RF_{ox}H$	8-Nor-8-chloro-$LF_{ox}H$	3-Benzyl-8-nor-$LF_{ox}H$
$Ac_4RF_{red}H_3$	1.00	50.0	0.84	0.35	3.1
8α-Morpholino-$Ac_4RF_{red}H_3$	1.7	8.2			
iso-$Ac_4RF_{red}H_3$	0.16		<0.02		
8-Nor-8-chloro-$LF_{red}H_3$	0.02[b]			0.14	
3-Benzyl-8-nor-$LF_{red}H_3$	0.08[b]				1.8

[a] The initial radical formation rates were measured by mixing the fully oxidized and reduced flavine derivatives under conditions of low reduction degree, at which the kinetic influence from the acidic–basic catalytic effect of the neutral flavohydroquinones can be neglected. The initial product of the concentrations of the oxidized and reduced starting materials was kept constant. The values of the initial reaction rates are thus directly related to the initial rate constant $k_1k_1'/(k_{-1} + k_1')$ of primary pathway I of the general dismutation scheme (Scheme I). The rate constant of the direct dismutation of unsubstituted Ac_4RF ($560\ M^{-1}\ sec^{-1}$) is taken as unity. [b] Radical formation was preceded by a lag time in these two instances; otherwise it followed immediately the mixing of the reduced and oxidized flavines.

form (100 and 96%, respectively) at pH_{DMF} 13.4. The deprotonation in the forward reaction leading to the radical anion can occur at the intermediate dimer, favoring its homolytic cleavage (reaction V). It can be as well a secondary process after the formation of the neutral radical (reaction IV). The increase with pH of the initial rate of dimer dissociation without an increase of its concentration at the extrapolated zero reaction time favors the first pathway. Furthermore, the relative stability of the dimer ($K_4 \simeq 47\ M^{-1}$) indicates that its pK_a value is much higher than that of the radical, allowing its transient accumulation before loss of a proton. The backward reaction follows the same pathways and is much slower than the dismutation reactions involving neutral radicals. The direct dismutation of the radical anion must involve the protonation of the intermediate dimer of pathway V since the direct formation of a dianionic flavohydroquinone, $F_{red}H^{2-}$, appears to be an energetically unfavorable process considering the two-electron reduction potential of $F_{ox}H$ in aprotic solvent (Sawyer et al., 1971).

Kinetics of Dismutation of Substituted Flavines. The dismutation kinetics of substituted flavines can be carried out with the substituted compounds or in mixed experiments involving two different partners. For example, a substituted reduced flavine can dismutate with unsubstituted oxidized Ac_4RF. As shown later in the discussion, the possibility of specific interactions at the level of the benzenoid ring focused the interest on compounds substituted on this ring, namely 8α-morpholino-Ac_4RF, iso-Ac_4RF (8-nor-6,7-dimethyl-Ac_4RF), 8-nor-8-chlorolumiflavine, and 3-benzyl-8-norlumiflavine. All the experiments were performed in unbuffered dimethylformamide.

The equilibrium of dismutation at 50% reduction for these compounds was found to be identical with that of the unmodified Ac_4RF, with the exception of iso-Ac_4RF which does not exhibit any stable radical formation. The same equilibrium of dismutation was also observed in mixed reactions using Ac_4RF either fully oxidized or fully reduced. In the case of the 8α-morpholino-Ac_4RF, the spectral characteristics of the neutral semiquinone of both flavines allowed one to estimate the proportion of the substituted and unsubstituted radicals at equilibrium. These two radicals were apparently at equal final concentrations.

The equilibrium radical yield following the mixing of reduced (oxidized) Ac_4RF and oxidized (reduced) iso-Ac_4RF was only 50% of that observed in the other experiments. It was not possible to identify the radical in this case, but it may be assumed to be an unsubstituted one. On the other hand, the kinetics of dismutation are different for the symmetrical mixed reactions as observed for the initial formation rate of neutral semiquinone (Table II). The time course of the reaction is also dependent upon the nature of the flavine initially reduced or oxidized. A two-electron transfer between Ac_4RF and iso-Ac_4RF could precede the direct dismutation of Ac_4RF. However, such a transfer requires a proton exchange which should be slow in anhydrous dimethylformamide, while the initial radical formation exhibits no lag time (Table II) and in one case it is nearly as fast as that observed with Ac_4RF alone. The formation of a mixed intermediate dimer, following the general dismutation scheme, could better explain the lack of kinetic symmetry in the two mixed experiments. As a matter of fact, iso-Ac_4RF could more easily acquire the radical anion character in the intermediate complex if it is initially oxidized than it could acquire the neutral radical character originating from the reduced form. This is due to the presence of the 6α-methyl group that certainly hinders the N-5 position to a much larger extent in the planar radical than in the bent flavohydroquinone, as indicated by the lack of stability of the radical.

The dismutation reactions involving 8α-morpholino-Ac_4RF exhibit all the characteristics of that of pure Ac_4RF, e.g. the triphasic time course or the negative catalytic effect of the reduced flavine. However, in the absence of a catalytic effect of the reduced flavine, i.e. at a low degree of reduction, the initial rate of radical formation is increased. This increase (Table II) depends on the nature of the flavine initially reduced and indicates that the substituted flavine is particularly suitable as an electron acceptor from a purely kinetic point of view. The initial radical formation rate does not involve acidic–basic reactions under conditions of anhydrous solvent and low degree of reduction. Thus, the substitution of the 8α-methyl group should accelerate the formation of the intermediate dimer or its homolytic dissociation, especially if the substituted molecule is oxidized.

On the contrary, the 8-nor and the 8-nor-8-chlor reduced lumiflavines do not dismutate with oxidized Ac_4RF since the radical formation is particulary slow and preceded by a lag time of about 15 msec. This lag time corresponds to a two-electron transfer reaction preceding direct dismutation of Ac_4RF. However, these compounds still act as one-electron acceptors in the dismutation reaction either directly or mixed with unsubstituted Ac_4RF as the electron donor, but substitution of a chlorine atom slows the reaction down.

Nature of the Dimeric Intermediate of Dismutation. The existence of intermediate dimers which may be the site of acidic–basic reactions controlling the kinetics of the reaction is firmly established by the kinetic analysis for the dismutation reaction in unbuffered as well as in buffered dimethylformamide. This dimer is even directly observable in

strongly alkaline solutions. Some reactive site of the flavine molecules can be activated under such conditions, resulting in a specific mode of interaction. However, the characteristics of the dismutation equilibrium are qualitatively comparable in the different media.

A dimer of fully reduced and oxidized flavines has been known in aqueous solution for many years (Beinert, 1956; Gibson et al., 1962) and has been sometimes postulated as a possible intermediate in the dismutation reaction (Burn and O'Brien, 1959; Swinehart, 1965; Barman and Tollin, 1972). This dimer, characterized by a broad and structureless absorption band in the near-infrared attributed to charge-transfer interaction, is known as the "flavoquinhydrone" complex and is assumed to be stabilized in a stacked structure mostly by hydrophobic interactions. A similar complex was also postulated as a reaction intermediate in water for oxidation of fully reduced flavine by various quinones (Gibian and Rynd, 1969). It has already been suggested from pulse radiolysis of riboflavine in water (Land and Swallow, 1969) that the quinhydrone dimer could not be formed directly by association of two semiquinone molecules, but only from fully reduced and oxidized flavines. Furthermore, the equilibrium radical yield in aqueous solution is nearly constant at pH 11.4 where all the flavine species are anionic and at pH 5.1 where they are neutral (Ehrenberg et al., 1967). This corresponds to approximately constant relative rates for the forward and the backward reactions. Since the rate constant for the direct dismutation of the radical anion at pH 11.4 is 70% of that for the direct dismutation of the neutral semiquinone at pH 5.1 (Land and Swallow, 1969), the rate constant for the forward reaction cannot differ very much at these two pH values. This could hardly agree with the assumption of the quinhydrone dimer as an intermediate in this reaction since it vanishes at pH values corresponding to ionization of the flavoquinone (Beinert, 1956). In a nonaqueous solvent such as dimethylformamide the quinhydrone dimer may be still present but at an extremely low concentration (Gibson et al., 1962) which does not depend appreciably upon the nature of the flavine. This low concentration could be the limiting factor for the rate of dismutation. The formal analysis of the dismutation kinetics (see supplementary material to this issue) indicates that the formation of the intermediate dimers in dimethylformamide is not diffusion controlled. The rate of the overall reaction is mostly controlled by acidic-basic reactions. These reactions have been shown to occur at the level of the intermediate dimers and to determine the dismutation pathway. The importance of these acidic-basic reactions, which induce an electronic reorganization of the dimers responsible for their homolytic or heterolytic cleavage, is once more against a quinhydrone nature for the intermediates. Furthermore, a stacked structure with π-electron bonding could hardly take into account the large kinetic differences observed for the dismutation reactions of the substituted flavines and for the mixed experiments using the substituted flavines initially either oxidized or reduced.

These experiments suggest rather a more specific interaction which involves well-defined molecular sites. The role of the methyl group in position 8 of the benzenoid ring is already suggested by the experiments using the substituted compounds. It is further supported by the spectral characteristics of the intermediate dimer observed in alkaline-buffered dimethylformamide (Figure 4). The well-defined vibrational structure of the far-red absorption band is in favor of a covalent rather than a charge-transfer interaction. As a matter of fact, the only known flavine species presenting a similar absorption band is the biflavine radical obtained by Hemmerich (Hemmerich et al., 1959; Ehrenberg et al., 1967) under basic conditions and corresponding to the dimerization of two oxidized flavines through the 8α-methyl groups. Both the isolated fully oxidized or reduced Ac_4RF are stable in alkaline-buffered dimethylformamide and the molar absorptivity of the transient dimer ($\epsilon_{730} \simeq 4500\ M^{-1}$) is much lower than that of the biflavine radical. The spectra also differ in their vibrational structure. The correspondence between these dimers is still uncertain because of the different number of electron equivalents involved in their formation. However, considering the lack of reactivity of the pyrimidine moiety of the molecule and the difficulty for a hydrazine-like flavine–flavine interaction at N-5 (Hemmerich et al., 1970) due to sterical hindrance and to the very high pK_a value of the N-5 atom of the reduced flavine, the benzenoid ring remains the best candidate for such a labile but specific interaction.

Conclusion

The dismutation reaction of flavine in an aprotic solvent such as dimethylformamide was slow enough to be investigated kinetically using conventional stopped-flow techniques. The kinetic analysis as well as the spectroscopic observation under alkaline conditions have shown that intermediate dimers are present both in the forward and in the backward reactions, and that the dismutation reaction corresponds to a single electron transfer within the complexes not necessarily accompanied by a proton transfer. However, the dismutation pathway is governed by acidic-basic reactions. The fully reduced flavine itself may play a role in these acidic-basic reactions and negatively catalyzes the reaction. This role is not specifically that of the flavohydroquinone which can be replaced by any mild or strong acid. The rate of the acidic-basic reactions preceding or following the electron-transfer reactions may explain the differences in the overall dismutation rate between water and nonaqueous solvents. The quinhydrone dimer, which is more stable in water, cannot be formed directly from the semiquinone (Land and Swallow, 1969). A reaction scheme which does not involve the quinhydrone complex as an intermediate for dismutation may also account for the kinetic measurements obtained from temperature-jump experiments (Barman and Tollin, 1972). Thus, it appears reasonable to generalize that electron transfer among flavines in a dismutation reaction occurs preferentially through a labile complex involving a σ-type contact rather than π interaction. A specific site in the fully reduced and/or oxidized flavines, probably at the benzenoid ring, could be the preferential, if not the exclusive, site for this interaction.

Most flavoproteins in electron-transferring chains are oligomeric, containing one or more flavines per protomer. Hemmerich et al. (1970) have already suggested that changes in the quaternary structure of these proteins may allow for such a type of flavine–flavine contact more easily than for an interaction involving flavine stacking. As a matter of fact, in the flavoproteins for which the X-ray structure is known, namely the flavodoxins from *Desulfovibrio vulgaris* (Watenpaugh et al., 1972, 1973) and from *Clostridium MP* (Burnett et al., 1974), only the benzenoid end of the flavine ring is apparent at the surface of the protein.

The main role of flavine dismutation in electron-transferring flavoproteins should be to offer an intermediate step between two-electron and one-electron transfer reactions.

The latter involve mostly metal ions, such as in heme, iron-sulfur, or molybdenum containing proteins. Thus, it is necessary to investigate the role of such metal ions in the flavine dismutation reaction before the biochemical implications of these results can be discussed in more detail.

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Supplementary Material Available

The formal analysis of the general reaction scheme of dismutation in unbuffered dimethylformamide, under alkaline conditions, and in the presence of redox-inactive metal ions, will appear following the accompanying paper (part II) in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Business Office, Books and Journals Division, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.50 for photocopy or $2.50 for microfiche, referring to code number BIO-75-4739.

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