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Biphasic Photolysis of Riboflavine with a Low-intensity Light Source

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The photolysis of riboflavine with a low-intensity light source was investigated. Biphasic rate curves were obtained; that is, an induction period was observed before the reaction rate reached its maximum value. The duration of the initial phase depended on the temperature and the concentration of riboflavine. The molecular associations of isoalloxazine nuclei may be responsible for such biphasic photolysis. The addition of xanthine derivatives to an aqueous solution of riboflavine reduced the rate of photolysis. Although the rate was reduced most effectively by theophylline, it appears that the interaction between riboflavine and theophylline differs considerably from that in other systems, *i.e.*, caffeine, theobromine, and hypoxanthine systems.

Keywords——riboflavine; xanthine derivatives; photolysis; spectrophotometric analysis; thermodynamic parameters

The photochemistry of flavins, especially the photolysis of riboflavine, is of continuing interest from various points of view. A number of investigations have demonstrated that the rate of hydrolysis or photolysis of riboflavine can be retarded by the addition of other compounds. For example, the velocity of the base-catalyzed decomposition of riboflavine is decreased by the presence of caffeine.[1] Shin *et al.* found that some phenol derivatives are effective as photochemical stabilizers.[2] They demonstrated that there is a direct correlation between the rate of decomposition and the quenching efficiency of fluorescence, *i.e.*, the rate of decomposition of riboflavine is decreased in the presence of a substance which has a higher quenching efficiency. The formation of molecular complexes between riboflavine and various substances decreases the apparent velocity of the reaction. The stability of riboflavine to light in the presence of additives is pharmaceutically quite important. On the other hand, riboflavine is known to promote the photolysis of other compounds in aqueous solution.[3-6] Riboflavine might be expected to promote the photolysis of a substrate *via* several mechanisms, such as energy transfer, coupled oxidation–reduction and sensitized photooxygenation. Thus, an understanding of the correlation of these properties with the photochemical behavior of riboflavine might provide an insight into some of the energy transfer and storage mechanisms of living organisms, and their biological consequences.

Extensive information on the photolysis of riboflavine has been obtained by using high intensity light sources. Under such conditions, the photodecomposed products of riboflavine complicate the kinetic pattern and the calculation of quantum efficiency. In order to avoid such complications, a microirradiation method was developed by Discher *et al.*[7] They employed a mercury lamp as the light source.

With this point in mind, we have studied the photolysis of riboflavine caused by irradiation with a low-intensity light source. In this study, a tungsten lamp was used as the light source for photolysis. In the present paper, evidence will be presented to indicate that a rapid initial phase occurs in the photolysis of riboflavine. We have also investigated the effect of xanthine derivatives on this reaction.

Experimental

Materials——Riboflavine was a gift from Toa Eiyo Chemical Co., Ltd., and was used without further purification. Caffeine was obtained from Kanto Chemical Co., Inc. Theobromine and theophylline were

from Nakarai Chemicals, Ltd. They were recrystallized from water. Hypoxanthine was obtained from Kojin Co., Ltd. and was used without further purification. All other materials and solvents were of analytical reagent grade. Deionized double-distilled water was used throughout this study.

Methods——Aqueous solutions of riboflavine (5×10^{-5} M) with and without xanthine derivatives were prepared in 0.05 M phosphate buffer (pH=7.0) and shielded from light. The test tubes containing these sample solutions were distributed radially on a disk in a constant temperature bath controlled within ±1°C. The disk was rotated at a constant speed in order to ensure equalized irradiation. The distance from the center of the test tube to the center of the tungsten lamp (100 V, 100 W) was approximately 20 cm. The study was conducted with unfiltered light at an intensity of about 7.2×10^3 erg/s. Pyrex test tubes were used for the irradiation so that ultraviolet irradiation was excluded.

The rate of photolysis was followed spectrophotometrically by measuring the change in absorbance at 445 nm. Most of the rate data were treated by plotting $I_t/I_0\times100$ (percent remaining) *vs.* time, where I_0 and I_t are absorption intensities at 0 and t h of irradiation, respectively.

The absorption and circular dichroism (CD) spectra were taken with a Hitachi 220 spectrophotometer and a Jasco J-400X spectropolarimeter, respectively.

Results

The Photolysis of Riboflavine

The results of kinetic studies on riboflavine at 445 nm fitted a zero-order kinetic pattern rather than a pseudo first-order process (Fig. 1). The major reaction product under our conditions was lumichrome.[8] When riboflavine was decomposed to the extent of more than 35% of the initial concentration, deviation from a linear zero-order kinetic pattern appeared. This is consistent with another study.[2] It should be noted that an induction period or initial rate of photolysis was observed before the reaction rate reached its maximum value. As is clear from Fig. 1, the duration of the initial phase depended on the temperature. It is interest to note that extrapolations of the linear second phases at various temperatures intersected at the same position. The duration was also dependent on the riboflavine concentration, *i.e.*, the higher the concentration of riboflavine, the longer the duration (Fig. 2). Such

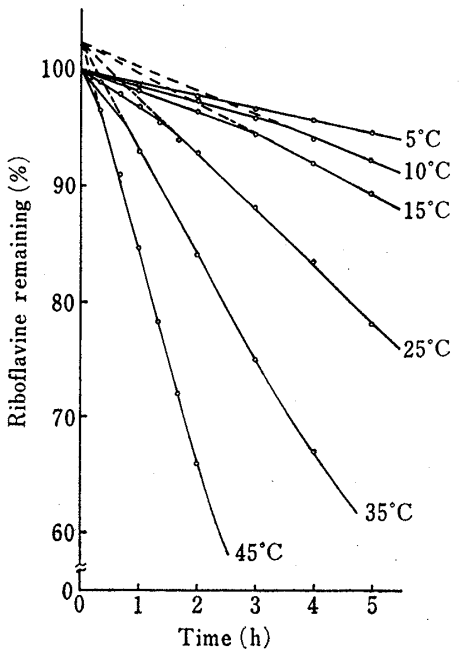

Fig. 1. Temperature Dependence of the Photolysis of Riboflavine in 0.05 M Phosphate Buffer (pH=7.0)

[riboflavine]=5×10^{-5} M.

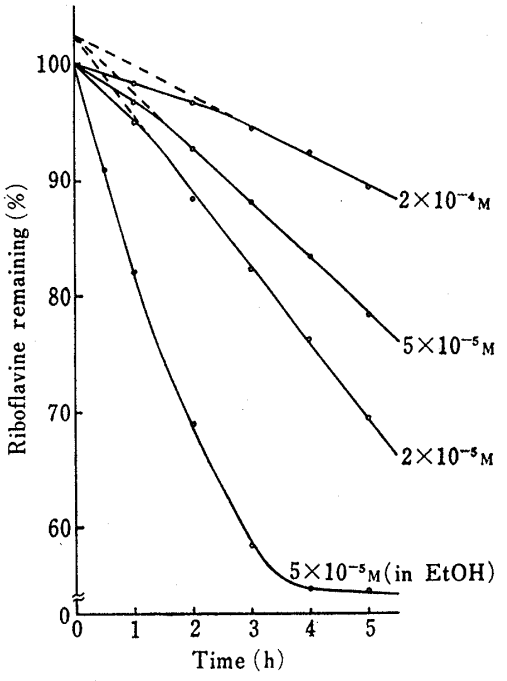

Fig. 2. Concentration Dependence of the Photolysis of Riboflavine in 0.05M Phosphate Buffer (pH=7.0)

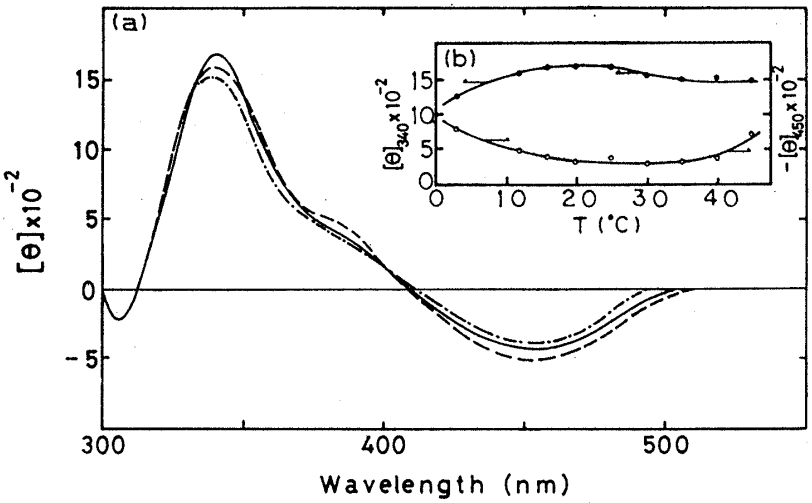

Fig. 3. Temperature Dependence of Circular Dichroism Spectra of Riboflavine in 0.05 M Phosphate Buffer (pH=7.0)

[riboflavine]=5×10^{-5} M; (– – – –): 12°C; (————): 25°C; (– · – · –): 35°C.

biphasic photolysis of riboflavine was not observed in ethanol, even under the same irradiation conditions. In this case, deviation from the linear zero-order relationship occurred when more than 20% of the initial amount of riboflavine was decomposed.

From these results, it appears that the occurrence of the initial phase in the photolysis of riboflavine is related to an association of molecules, namely stacking between isoalloxazine nuclei. The effects of stacking were examined by measuring the CD spectra of riboflavine at various temperatures, and the results are shown in Fig. 3. The CD spectrum of riboflavine is characterized by the negative band at 450 nm, the positive band at 340 nm and a shoulder at about 385 nm. A pseudo-isoelliptic point developed at about 400 nm. This suggests that there is an equilibrium between forms stable at low temperature and at high temperature. The 450 and 340 nm bands undergo biphasic changes with increasing temperature (Fig. 3(b)). Their behaviors, however, are contrasting.

The Effects of Xanthine Derivatives on the Rate of Riboflavine Photolysis

The photolysis of riboflavine in the presence of additives fitted a zero-order kinetic pattern, and the initial rates of photolysis were observed in all systems. Typical results are illustrated in Figs. 4 and 5. As the concentration of a given additive was increased, the rate of photolysis of riboflavine decreased gradually. Complex formation between riboflavine and additives may play an important role in the decrease of the rate of photolysis. The duration of the initial phase depended on the temperature, but seemed not to depend on the concentration of additives. In the riboflavine–caffeine system, extrapolations of the second phase plots at various temperatures intersected at the same position (Fig. 4). In the riboflavine–other additive systems, however, these extrapolations did not intersect at the same positions (Fig. 5).

The apparent amount of riboflavine remaining in the riboflavine–theophylline system at low temperatures showed an unusual increase, as shown in Fig. 6. Figure 7 shows absorption spectra of the theophylline system at various irradiation times. At temperatures higher than about 20°C, only one isosbestic point was observed at about 430 nm. This isosbestic point could not be observed at lower temperature. On the other hand, in the absorption spectra of riboflavine at various irradiation times at 25°C, three isosbestic points were observed at about 401, 390, and 359 nm. The other systems also showed three isosbestic points at around the same positions as in the riboflavine system. In the theophylline system, additional complex formation and/or some other type of photolysis may occur upon irradiation.

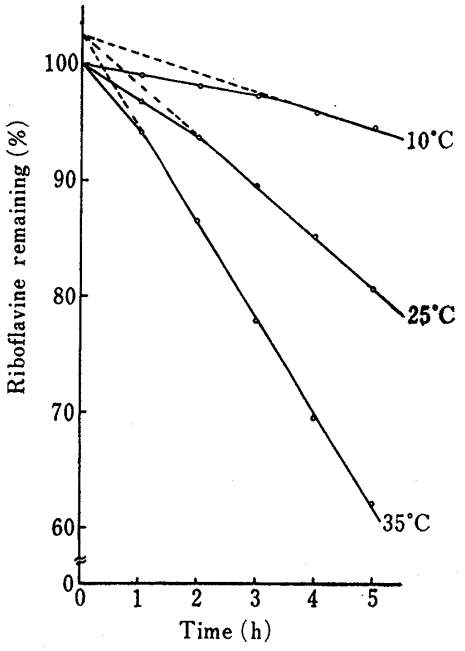

Fig. 4. Temperature Dependence of the Photolysis of Riboflavine in the Presence of Caffeine in 0.05 M Phosphate Buffer (pH=7.0)

[riboflavine]=5×10^{-5} M; [caffeine]=25×10^{-4} M.

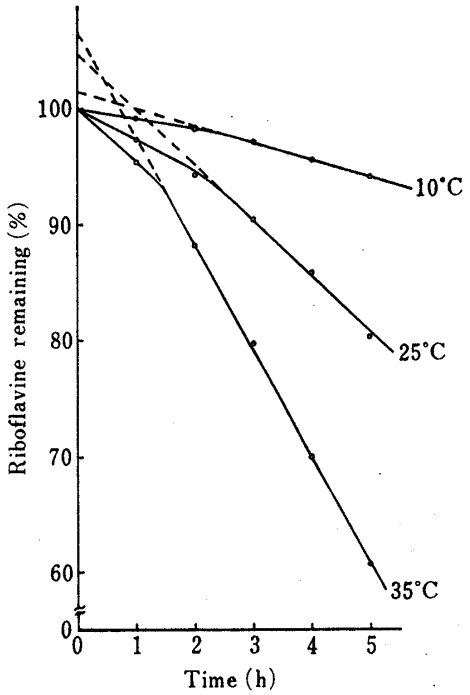

Fig. 5. Temperature Dependence of the Photolysis of Riboflavine in the Presence of Theobromine in 0.05 M Phosphate Buffer (pH=7.0)

[riboflavine]=5×10^{-5} M; [theobromine]=25×10^{-4} M.

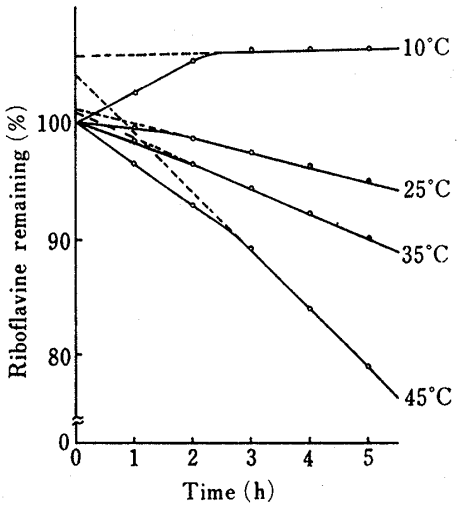

Fig. 6. Temperature Dependence of the Photolysis of Riboflavine in the Presence of Theophylline in 0.05 M Phosphate Buffer (pH=7.0)

[riboflavine]=5×10^{-5} M; [theophylline]=25×10^{-4} M.

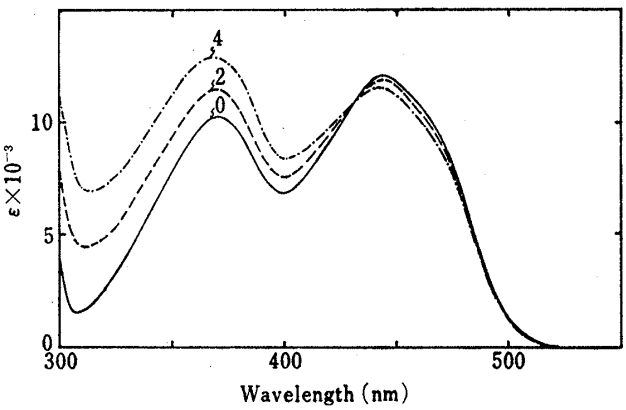

Fig. 7. Effect of Theophylline on the Photolysis of Riboflavine in 0.05M Phosphate Buffer (pH=7.0) at 25°C

Numerical values in the figure are irradiation times (h).
[riboflavine]=5×10^{-5} M; [theophylline]=25×10^{-4} M.

From these results, it seems that ,in the presence of additives, the photolysis of riboflavine in the second phase is also dependent on the stoichiometric concentration of riboflavine in the complexes.

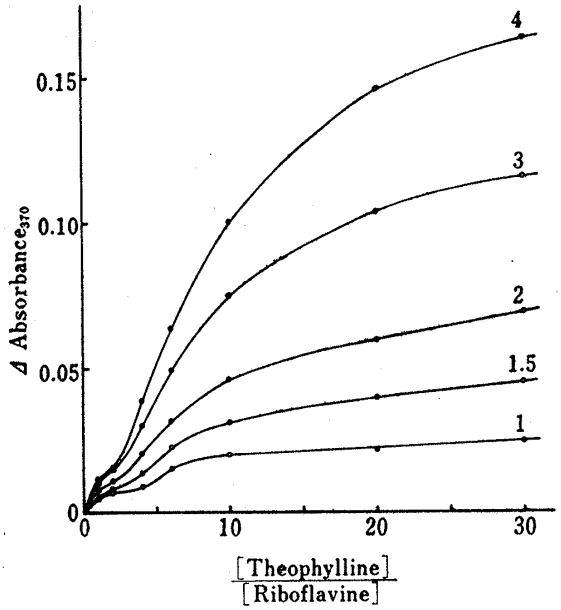

Fig. 8. Mole Ratio Plots for the Riboflavine–Theophylline System at Various Irradiation Times in 0.05 M Phosphate Buffer (pH=7.0) at 25°C

Numerical values in the figure are irradiation times (h).

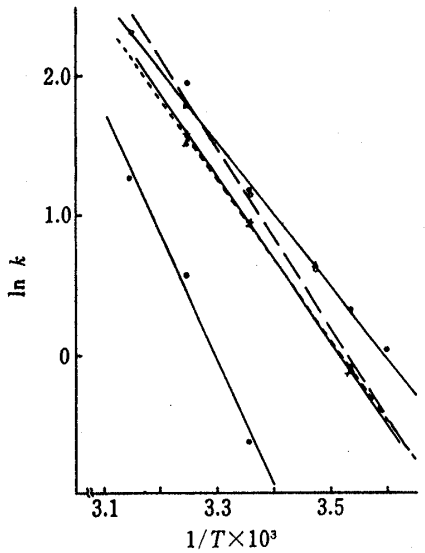

Fig. 9. Arrhenius Plots for the First Phase of Photolysis of Riboflavine in Various Systems in 0.05 M Phosphate Buffer (pH=7.0)

(—○—): riboflavine alone; (—●—): riboflavine–theophylline system; (--△--): riboflavine–theobromine system; (— ● —): riboflavine–caffeine system; (—×—): riboflavine–hypoxanthine system.

Figure 8 shows the results obtained by the mole ratio method applied to the theophylline system at various irradiation times at 25°C. The reactions were monitored in terms of the absorption change at 370 nm, because of its relatively large change (Fig. 7). There were apparently three species of complexes between riboflavine and theophylline in the first phase, *i.e.*, 1:1, 1:4, and 1:7 complexes. At 4 h irradiation, there were two species of complexes, *i.e.*, 1:1 and 1:2 complexes. The 1:4 and 1:7 complexes are presumably transient. Such transient complexes were also observed in the theobromine and hypoxanthine systems. The mole ratio of the complex between riboflavine and theobromine was 1:4 in the first phase, and 1:2 after irradiation for 4 h. In the hypoxanthine system, there were two complexes (1:1 and 1:4) in the first phase, but only one complex (1:1) after irradiation for 4 h. It cannot be ruled out that other additional routes of photolysis may have occurred in these systems. The caffeine system, however, did not show such stoichiometric change. In this system, the 1:1 complex was seen at all irradiation times, though the character of the complex, such as association constant and molar ellipticity, changed.

TABLE I. Thermodynamic Parameters for the First and Second Phases of the Photolysis of Riboflavine in Various Systems

Additive	E (kcal/mol)	ΔS^* (cal/mol·degree)	ΔF^* (kcal/mol)
—	10.3(10.0)	−21.5(−21.5)	16.7(16.4)
Theobromine	11.4(10.8)	−18.3(−19.1)	16.9(16.5)
Hypoxanthine	11.7(11.4)	−17.3(−17.7)	16.9(16.7)
Caffeine	13.3(11.4)	−11.5(−17.3)	16.7(16.6)
Theophylline	17.8(13.5)	−0.2(−12.7)	17.9(17.3)

Numerical values in parentheses are those for the second phase.

Thermodynamic Parameters

From the above results, one can estimate the thermodynamic parameters for these systems. The temperature dependence for the photolysis of riboflavine in the first phase in each system is shown by Arrhenius plots in Fig. 9. The Arrhenius plot was constructed over the temperature range of 25 to 45°C for the theophylline system. Apparent activation energies, E, were graphically estimated. They are summarized in Table I. The values of activation entropy change, ΔS^*, and free energy change, ΔF^*, for each system in the first and second phases are also shown. The activation energies in the presence of additives were increased. These results imply that the rate of photolysis of riboflavine was decreased by the additives. In the second phases, however, the effect of additives on the rate was lowered. These changes may be ascribed to the change of intermolecular association between riboflavine and additives, because the parameters for riboflavine alone remained almost unaltered. These results are consistent with the stoichiometric changes in the riboflavine–additive complexes caused by irradiation.

Discussion

The kinetic behavior in the photolysis of riboflavine by a low-intensity light sourec is not the same as that with a strong light source, but shows an initial phase prior to the faster second phase. Such biphasic photolysis of riboflavine was also reported by Owen and O'Boyle.[8] They observed that the addition of gelatin to an aqueous solution of riboflavine reduced the rate of the aerobic photolysis, and increased the fluorescence quantum yield. Since the kinetic behavior of the photolysis in the present study is very similar to that in their work, we will consider the mechanism of the photolysis by a low-intensity light source on the basis of their discussion. Riboflavine can undergo two types of photoreactions: photobleaching, leading to a degradation of the ribityl side chain, and photoreduction, occurring in the presence of reducing agents such as EDTA. Under our experimental conditions, the major product of the photobleaching reaction was shown to be lumichrome by comparison of its absorption spectrum with that of the authentic product. The aerobic photolysis of riboflavine (Rf) is initiated by singlet oxygen ($^1O_2$). A radiationless transition of the singlet excited state of riboflavine (^{1}Rf) produces a riboflavine triplet (^{3}Rf), which, upon collision with ground state oxygen ($^3O_2$), generates $^1O_2$ *via* energy transfer. Lumichrome can be formed directly from ^{1}Rf by a photoelimination mechanism.[9] Thus, the mechanism of photolysis of riboflavine can be presented simply as follows.[8–11]

$$\mathrm{Rf} + h\nu \longrightarrow {}^1\mathrm{Rf} \quad (1)$$

$$^1\mathrm{Rf} \longrightarrow \text{lumichrome} \quad (2)$$

$$^1\mathrm{Rf} \longrightarrow {}^3\mathrm{Rf} \quad (3)$$

$$^3\mathrm{Rf} + {}^3O_2 \longrightarrow \mathrm{Rf} + {}^1O_2 \quad (4)$$

$$\mathrm{Rf} + {}^1O_2 \longrightarrow \text{photooxidation products} \quad (5)$$

The driving force for reaction (2) is the polarity of the excited singlet state. ^{3}Rf is a long-lived state and a chemically active species. Shin *et al.*[2] found that the quantum efficiency of riboflavine is independent of the intensity and wavelength of light, and of the concentration employed. The number of riboflavine molecules decomposed per unit time is directly proportional to the number of photons absorbed. Although the higher the concentration of riboflavine, the more photons are absorbed and subsequently more riboflavine is decomposed, it was observed in this study that the higher the concentration of riboflavine, the longer the duration of the initial phase and the less the riboflavine decomposition. Under our experimental conditions, although the concentration of each species in the photochemical reactions (1)—(5) should be lower than that with high intensity light sources, the triplet state may make a rela-

tively large contributions to the reactions. It seems unlikely that a decrease in the quantum yield due to products of the reaction causes a break in the rate curve. Therefore, it appears that the effect of molecular associations of isoalloxazine nuclei is much greater than the effect of the species in the photochemical processes. Thus, we consider that the interactions between riboflavine molecules and/or riboflavine and additives occur in the ground states.

Owen and O'Boyle attributed the gradual decrease in the rate on the addition of gelatin to a gradual transition from a reaction in which the singlet state is the important reactive species to one in which the triplet state predominates.[8] The appearence of the initial phase may be due to the fact that the triplet mechanism is catalyzed by lumichrome, which is a product of the singlet reaction. They also proposed that increased rigidity of the solution resulting from the addition of gelatin might explain the increase in fluorescence intensity since it would tend to keep the riboflavine in a planar configuration and so increase the probability of the radiative transition. The above-mentioned explanation for the two consecutive lines in the rate curves is consistent with the results on temperature and solvent effects in the absence and presence of additives. Again, one can say that the molecular associations become more important in photolysis with a low-intensity light source.

In the case of a high-intensity light source, the relative predominance of the triplet mechanism may be lost, even though the photolysis proceeds *via* the photochemical processes of reactions (1)—(5). Thus, the first step may occur so rapidyl that it cannot be observed. Molecular association, *i.e.*, formation of a stacked dimer, should be responsible for the initial phase in the biphasic photolysis. The dimer tends to keep the riboflavine molecule in a planar configuration. In the second phase, the photochemical reactions are the controlling factors. This is also true for the photolysis of riboflavine in organic solvents.

According to the CD spectra, the molecular associations are dependent on the temperature. However, no concentration effects on the temperature difference spectra of flavins were observed in organic solvents.[12] The CD bands of riboflavine are induced by the asymmetric carbons in the ribityl side chain at position 10. A possible explanation for the biphasic and contrary changes of the intensity at 450 and 340 nm bands is the presence of two kinds of stacked dimers. Such temperature dependence of the structural form of the dimer was also pointed out for the dimer of actinomycin D in aqueous solution.[13] The dimer structure of riboflavine is under investigation.

The addition of additives reduces the rate of decomposition of riboflavine by affecting ^{1}Rf and ^{3}Rf. The interaction between riboflavine and caffeine may be explained by a charge-transfer mechanism with riboflavine acting as an acceptor and caffeine as a donor.[14] The charge-transfer complexes should also be formed in the theobromine and hypoxanthine systems. However, the complex formations in these systems are minor because the changes of thermodynamic parameters are small. Although rather large parameter changes were observed in the theophylline system, they cannot be explained only on the basis of a charge-transfer interactive mechanism with riboflavine. The abnormal properties of this system are characterized by extraordinarily large values of ΔS^*, especially in the first phase. The large ΔS^* implies that the increase of degree of freedom in the riboflavine–theophylline system is very large compared with the initial state. The three transient species of riboflavine–theophylline complexes may reflect the stability of riboflavine molecules in this system. In addition, complex formation between theophylline and umichrome may have occurred. Further details of the interaction of riboflavine with theophylline will be reported elsewhere.

In this study, it became clear that in the photolysis of riboflavine by a low-intensity light source the dimer plays an important role. The formation of charge-transfer complex between riboflavine and the xanthine skeleton can stabilize the photochemical reactions. Although theophylline is a most effective photochemical stabilizer, the interaction between riboflavine and theophylline differs greatly from that in other systems. These findings may be significant in relation to the biological activities of riboflavine, and are also of pharmaceutical interest.

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