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Interaction of Flavins and Some Alcohols on the Molecular Level

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Abstract□The effect of some alcohols on the riboflavin derivatives in non-polar solvent was studied by various spectroscopic method in order to support the viewpoint that alcohol may directly interact with the isoalloxazine moiety of FAD, the coenzyme of D-amino-acid oxidase. The most possible association complex between alcohol and riboflavin is the 1:1 complex through the 2-C carbonyl function of the isoalloxazine ring and the hydroxyl proton of alcohol. It is appeared that methanol has a larger association constant than any other alcohols, and the association constant decreases with the carbon number increases and being bulkier in the alkyl group of alcohols.

Keywords□RFTA, RFTB-coenzyme of flavoprotein binding with alcohols-the binding detected by IR, NMR, UV and Fluorescence spectroscopic techniques

The action of flavincontaining D-amino acid oxidase, having a wide substrate specificity, is the mediation of the electron transfer from the substrate to oxygen[1]. Alcohols influence the interaction between substrates and flavincontaining enzymes[2] In fact, ethanol enhances the catalytic activity of D-amino acid oxidase[3~4]. Especially, it is clear that both maximum velocity and the dissociation constant of the enzyme-product complex are increased by alcohols[2,5]. The crystal structure of the oxidized form of flavoenzyme had been studied, and a detailed description of the region around flavin binding site was proposed[6~9]. The spectrophotometric and fluorimetric data on riboflavin in various solvents were also reported[10~13] It has been proved that FAD, coenzyme of D-amino acid oxidase, is surrounded by hydrophobic environment[13~14] Kotaki et al.[15] suggested that FAD partly interact with protein residue(s) or water molecule(s) via hydrogen bonding in aqueous solution, and Ohama et al.[2] proposed that the appearance of vibrational fine structure in the absorption spectra of holoenzyme in the presence of alcohols can be ascribed to the elimination of these hydrogen bonding(s) above mentioned, and these spectral changes may be caused by the intervention of the apoenzyme, not by a direct interaction between alcohol and FAD itself. But, if one might consider that oxidoreduction metabolism of biological system occurs in lipophilic environment rather than in aqueous environment it would be assumed that in nonpolar system the probability of direct interaction between alcohol and isoalloxazine moiety which is the chromophore of coenzyme should not be ignored. So it seemed worthwhile to examine the quantitative

hydrogen-bonded interaction between alcohols and flavins in nonpolar solvent.

EXPERIMENTAL

Materials

Riboflavin-2′, 3′, 4′, 5′-tetraacetate(RFTA) was prepared by the reaction of riboflavin with acetic acid anhydride in pyridine.[16] Riboflavin-2′, 3′, 4′, 5′-tetrabutyrate(RFTB) was obtained from Dae Woong Pharm. Co., Ltd. Its purity was checked by TLC.[17] Alcohols used in the present study were of spectroscopic grade. CCl_4 was treated with methanolic KOH, washed with water, and distilled over P_2O_5. The distillate from the phosphorous pentoxide was tested for water by measuring the infrared spectrum in a quartz cell; a negligible amount of water was found to be present. $CDCl_3$ was purchased from E. Merck, Darmstadt. It was purified by passing through an alumina gel column (5 cm, in length).

Instrumentation

Infrared spectra were measured with a Beckman I.R. 20A infrared spectrophotometer. Fused quartz cell(5mm) and KBr cell(1mm) were used in the 3 μ and the 6 μ region. ^1H nmr spectra were recorded on a Perkin-Elmer R 32 NMR Spectrometer operated at 90 MHz. TMS was used for internal standard. The absorption and difference spectra were measured in a Unicam SP 1750 Ultraviolet Spectrophotometer connected to a Unicam AR Linear Record, using a 10 mm quartz cell. The fluorescence measurement was performed with a Baird-Atomic Fluoricord Spectrofluorimeter equipped with a xenon lamp using 10 mm quartz cell at the excitation wavelength of 474 nm.

RESULTS

Effect of Various Alcohols on the Infrared Spectrum of Riboflavin in Non-polar Solvent

In the spectrum of the 8 mM RFTB solution in CCl_4, a sharp band due to non-bonded 3-N imino stretching vibration is observed at 3380 cm^{-1} (Fig. 1). The spectrum of 8 mM methanol shows narrow band at 3660 cm^{-1}, which is due to non-bonded OH stretching

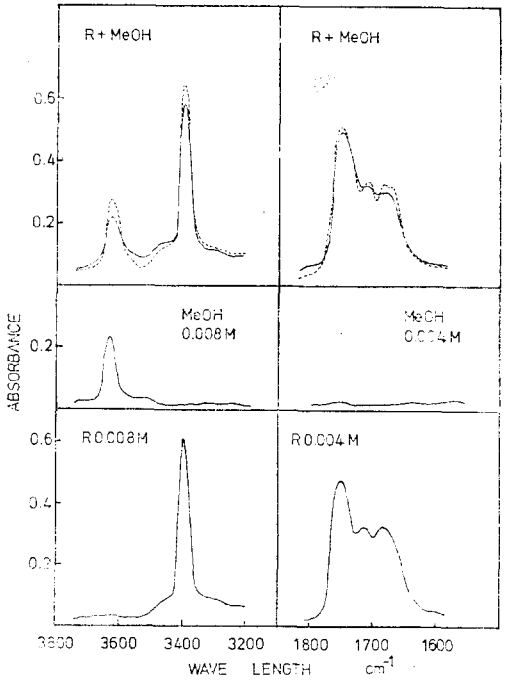


Fig. 1: Infrared spectra of RFTA, methanol and 1:1 mixed solution.
3 μ region: 8 mM in $CDCl_3$, 5 mm quartz cell.
6 μ region: 4 mM in $CDCl_3$, 1 mm KBr cell.
Solid line: observed spectra.
Dotted line: calculated the sum of lower two spectra.

vibration. When both solutions are mixed, the non-bonded bands decrease in intensity. It is considered that these spectral changes are caused mainly by hydrogen bonding. A couple of isosbestic points at 3650, 3600, 3400 cm^{-1} may confirm this point of view.

For the purpose of knowing the possibility of binding at carbonyl groups of riboflavin, the spectra in 6 μ region were studied. The spectrum of 4 mM RFTA in $CDCl_3$ shows a strong band at 1745 cm^{-1} and two medium band at 1715 cm^{-1}, 1695 cm^{-1},. According to Yagi, the 1745 cm^{-1} band is assigned to the carbonyl stretching vibration of the acetyl groups. The 1715 cm^{-1} band and 1695 cm^{-1} band are respectively due to the 4–C carbonyl stretching vibration frequency, and to the 2–C carbonyl stretching vibration coupled with the N–H bending mode as discussed previously[18]. The spectrum of 4 mM methanol shows no band in 6 μ region. When both solutions are mixed, the 1695 cm^{-1} band decreases more conspicuously than 1715 cm^{-1} band remains unchanged. Several isosbestic points are also observed in 6μ region. Similar phenomenon was observed in the case of riboflavin upon addition of ethanol (Fig. 2).

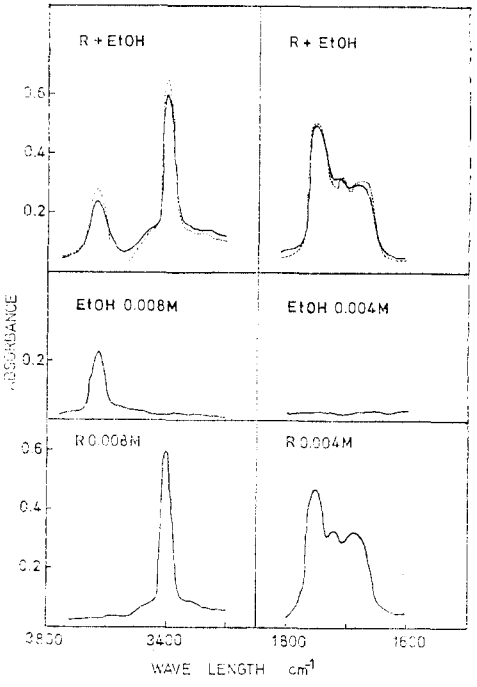


Fig. 2: Infrared spectra of RFTA, ethanol and 1:1 mixed solution.
3 μ region: 8 mM in $CDCl_3$, 5 mm quartz cell.
6 μ region: 4 mM in $CDCl_3$, 1 mm KBr cell.
Solid line: observed spectra
Dotted line: calculated the sum of lower two spectra.

To elucidate the stoichiometry of the interaction, we measured the intensity of the association band at 3450 cm^{-1} in a series of solutions which have varing molar ratios of the two components, while the total concentration remains constant. The intensity of the association band reached maximum at 1:1 molar ratio. Thus 1:1 complexes form in dilute solutions. (Fig. 3).

Effect of Various Alcohols on the NMR Spectrum of Riboflavin in $CDCl_3$

To confirm the formation of hydrogen

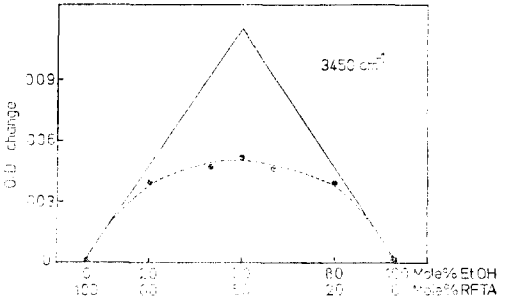


Fig. 3: Change in the optical density of the association bands found at 3450 cm^{-1} as a function of the mole ratio of RFTA and ethanol. The optical density of the pure solution is adjusted to zero.

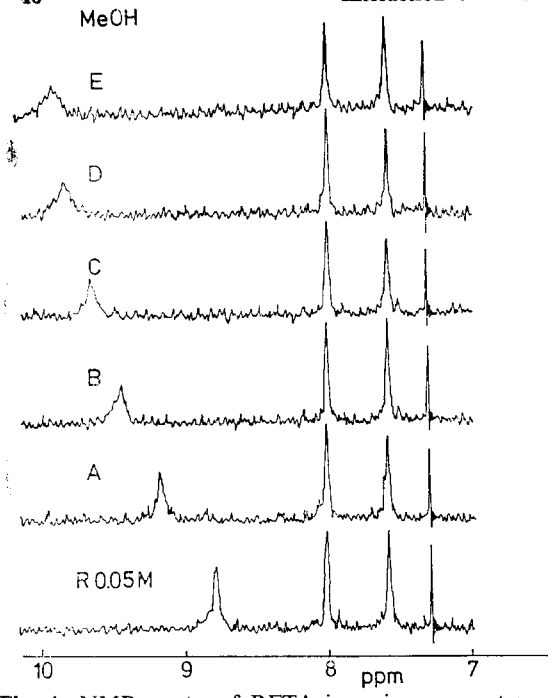


Fig. 4: NMR spectra of RFTA in various concentration of methanol in $CDCl_3$, at 35°C.
RFTA concentration (M); 5×10^{-2}
Methanol concentration (M): 0.223(A), 0.655 0.655(B), 1.077(C), 1.479(D), 1.875(E).

bonds between RFTA and alcohols in $CDCl_3$, the shifts of the imino proton resonance of riboflavin were measured on the addition of alcohols. Then, from the change in the chemical shift of the imino proton, we can evaluate the association constant K of hydrogen bonded complex through 3–N imino group, and the measurement of K values according to the NMR method can be compared with those according to UV method. The resonance signal of the imino proton of RFTA is observed in 8.76 ppm and the NH signal moves downfield as the concentration of methanol increases (Fig.4). The chemical shifts of NH signal are plotted against alcohol concentration at 35°C, keeping the concentration of RFTA constant. (Fig. 5). From these data, the association constants were obtained by using the following folmulas.[19]

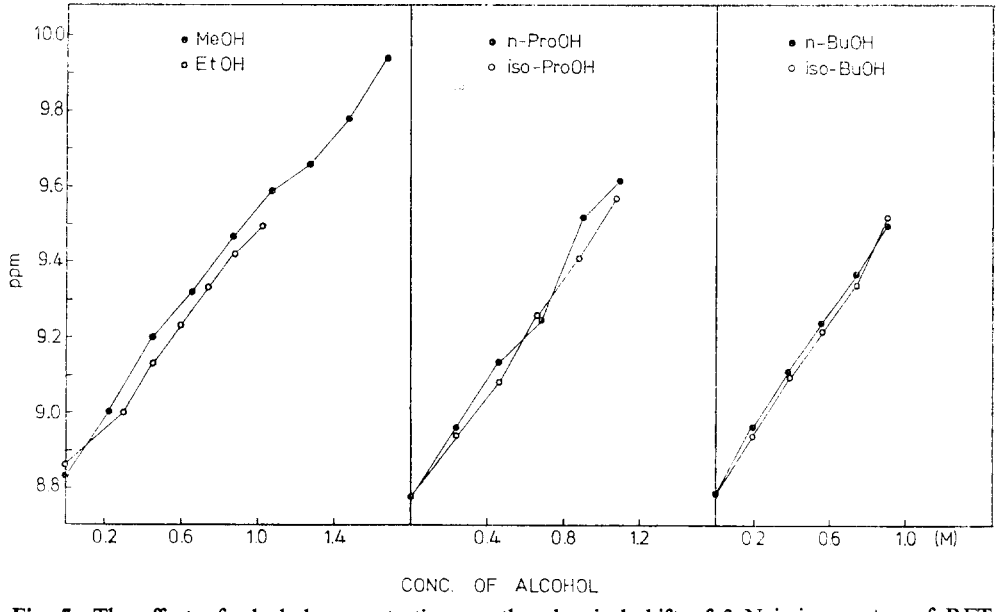


Fig. 5: The effect of alcohol concentration on the chemical shift of 3–N imino proton of RFTA, keeping the concentration of RFTA constant(0.05M).

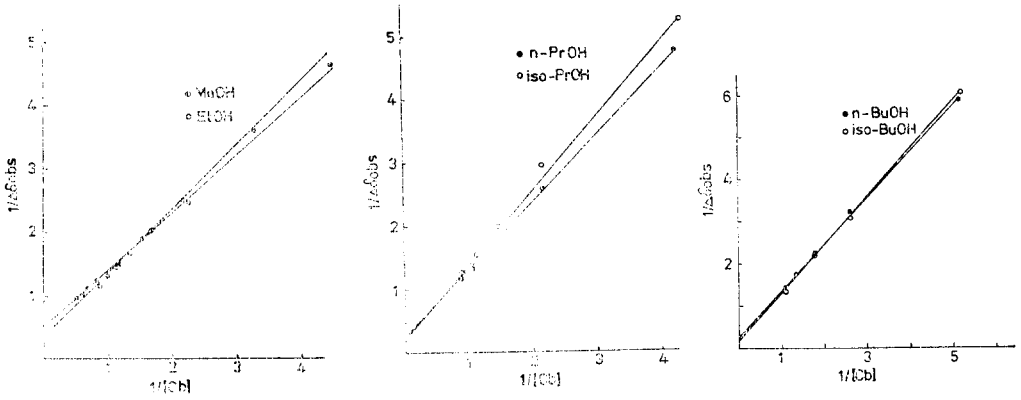


Fig. 6: Relation between $1/\triangle\delta$ obsd and 1/[Cb] values of RFTA–alcohol system in $CDCl_3$, obtained according to Hanna Equation.

Table I: Measured and calculated properties of RFTA and alcohols in $CDCl_3$.

Alcohols	Range of alcohol concentration(M)	Max. obsd ppm	$\delta_c-\delta_a$	K(Association const.) liter of solvent/mole(M^{-1})
MeOH	0.223—1.875	1.15	1.46	0.8718
EtOH	0.304—1.153	0.89	3.02	0.3527
n-Propanol	0.237—1.106	0.87	4.39	0.2108
iso-Propanol	0.233—1.088	0.82	4.89	0.1708
n-BuOH	0.193—0.902	0.71	4.01	0.2278
Iso-BuOH	0.193—0.900	0.75	5.37	0.1696

$$\frac{1}{\triangle\delta\ \text{obsd}}=\frac{1}{Kc(\delta c-\delta a)}\ \frac{1}{Cb}\ \frac{1}{\delta c-\delta a}$$

where $\triangle\delta obsd=\delta obsd-\delta a$, δa is the shift of RFTA imino proton in uncomplexed form, $\delta obsd$ is the observed shift of riboflavin imino proton in complexed media, δc is the shift of riboflavin imino proton in the pure complex, Cb is the concentration of alcohols on some scale to be defined later and Kc is the association constant of the complex. From the above equation, a plot of $1/\triangle\delta obsd$ vs 1/Cb in the case of riboflavin-methanol complex gives a straight line as shown in Fig. 6. For the other complexes, the linear relationship was also obtained. Therefore, K values were evaluated from the slope and the intercept, as summarized in Table I.

Change of UV and Fluorescence Spectra by Hydrogen Bonding

As shown in Figs. 7 and 8, remarkable changes are produced in spectra by adding alcohols to RFTB in non-polar solvent CCl_4. It may be safely assumed that these spectral changes are due solely to the formation of the hydrogen bond between the riboflavin and alcohols.

As indicated in Fig. 7, the shorter wavelength band shifts to the red, while the

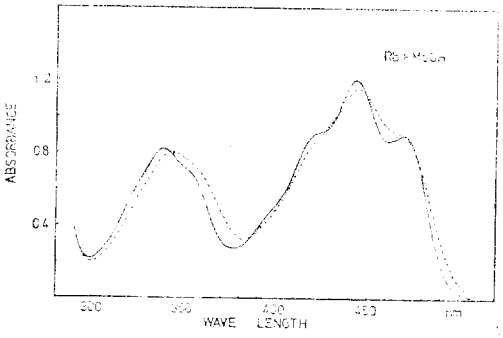


Fig. 7: Effect of methanol on UV absorption spectrum of RFTB in CCl_4. Solution of RFTB, $1x10^{-4}$ M, in CCl_4–methanol solvent, from O to 1% by volume methanol. Solid curve; free molecule, dotted curves: spectra in the presence of methanol in order of increasing concentration of methanol, broken curve: in the presence of 1M methanol.

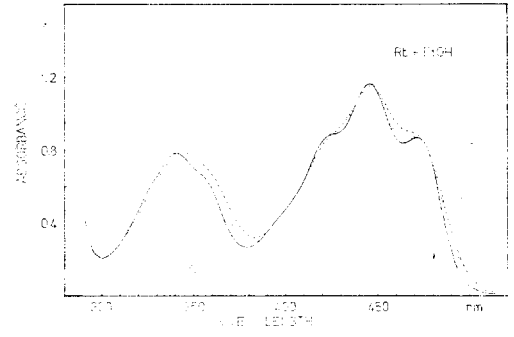


Fig. 8: Effect of ethanol on UV absorption spectrum of RFTB in CCl_4. Solution of RFTB, 1×10^{-4} M, in CCl_4–ethanol solvent, from O to 1% by volume ethanol. Solid curve: free molecule, dotted curves: spectra in the presence of ethanol in order of increasing concentration of ethanol, broken curve: in the presence of 1M ethanol.

longer one does not shift. A vibrational fine structure at 472 nm and a shoulder at 425 nm disappear upon the addition of methanol. Similar phenomena were observed with other alcohols. Riboflavin is fluorescent in non-polar solvents and the decrease in fluorescence intensity is observed when it is hydrogen bonded with alcohols (Fig. 9).

The emission maximum changes, indicating that the complex is emitting. The observed changes of fluorescence intensity may most probably be attributed to the fact that the free and and the hydrogen bonded molecules have different fluorescence quantum efficiency of fluorescence. Determination of the association constant of the complex is possible from spectral data, if a complex shows a significantly different spectrum from those of components. Considering binding of RFTB(R) to alcohol(L), spectral change by complexing is given by;

$$\triangle A=(\varepsilon_{RLn}-n\varepsilon_L-\varepsilon_R)[RLn]l=\triangle\varepsilon_{RLn}[RLn]l \quad (1)$$

where l is the path length of a cell employed, ε's are extinction coefficients of species shown, n is the stoichiometric ratio of the complex. The apparent association constant of R to L is expressed as

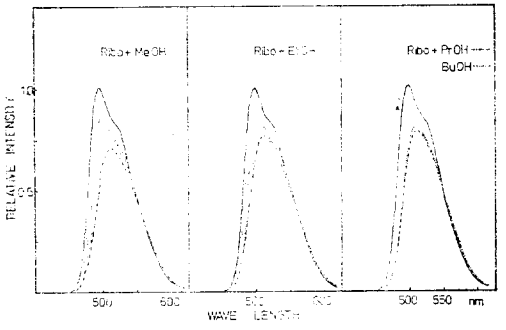


Fig. 9: Effect of alcohol on the fluorescence spectra of RFTB in CCl_4.Solution of RFTB, 2×10^{-5} M, in CCl_4–alcohol solvent, from 0 to 1 M of alcohol. Solid curve: free molecule, dotted curves: spectra in the presence of alcohol in order of increasing concentation of alcohols (top to bottom), lowest curve: in the presence of 1M alcohol.

Table Ⅱ. Measured and calculated properties of RFTB and alcohols in CCl_4 on UV method.

Alcohols	Range of alcohol concentration (M)	K_{RL} (Association const.) (M^{-1})
MeOH	0.05—0.5	5.81
EtOH	0.034—0.343	2.99
n-Propanol	0.027—0.268	2.01
iso-Propanol	0.105—0.264	0.89
n-Butanol	0.066—0.219	0.68
iso-Butanol	0.066—0.218	0.59

$$K_{RLn}=[RLn]/[L]^n[R] \quad (2)$$

Substituting equation 1 into 2 and rearranging the equation, we obtain

$$[R]_0/\triangle A=1/\triangle\varepsilon_{RLn}\cdot l+1/\triangle\varepsilon_{RLn}\cdot l\cdot K_{RL_n}\cdot[L]^n \quad (3)$$

where $[R]_0$ is total riboflavin concentration. The plot of $[R]_0/\triangle A$ against $1/[L]^n$ should yield a straight line for a proper choice of stoichiometric coefficient n, and $\triangle\varepsilon_{RLn}$ and K_{RLn} are calculated from the plots. Such plot is possible by putting [L] into total alcohol concentration $[L]_0$ under the condition $[L]_0 \gg n[R]_0$.

The plots of Eq. (3) are shown in Fig. 8 for n=1 for riboflavig-alcohol systems; other values of n did not yield the expected straight line. These results indicate that riboflavin forms 1:1 molecular complex with alcohol in non-polar solvents. Once the n value is known from the plots as shown in Fig. 10, and K_{RLn} values can be calculated for each experimental point. The $\triangle\varepsilon_{RLn}$ and K_{RLn} values are also calculated from the plots by using Eq.(3). as summarized in Table II.

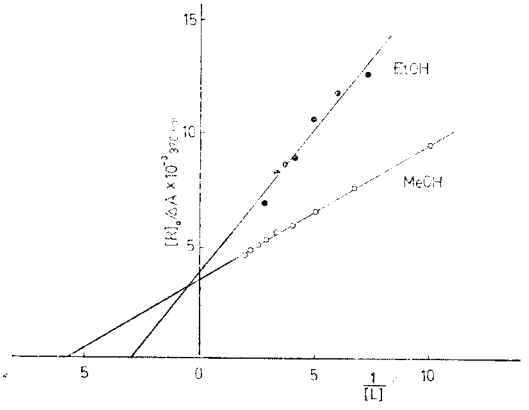


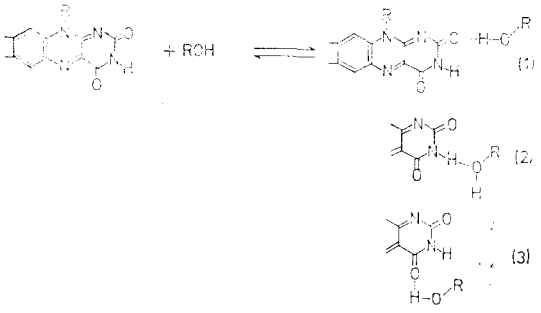
Fig.10: Relation between $[R]_0/\triangle A$ and $1/[L]$ values of RFTB-alcohol system in CCl_4 obtained according to Eq. 3

DISCUSSION

As shown in the 3 μ region of IR spectra, both the hydroxyl proton of alcohol and the imino proton of riboflavin seem to participate in the association. The Association constant calculated from NMR method is much smaller than the one from the UV method, although each method is somewhat different kind, namely, the former is wholely dependent upon the chemical shift change of imino proton while the latter is obtainable from the oscillator strength change of one transition moment of riboflavin. Thus, from not only this point of view but also the geometric aspect of hydrogen bonding, the association through the imino proton of riboflavin might be excluded. In the 6 μ region of infrared spectra, it is easily noticed that only the association through

the 2-C carbonyl group of isoalloxazine is prominent. This is consistent with the fact that in the sequence of the hydrogen bonding on the isoalloxazine ring, the 2-C carbonyl group is preferential to the 4-C carbonyl group, as supposed by Yagi et al.[20] Although the proton affinities of hetero-atoms in the isoalloxazine ring are actually different from one another,[20~22] stoichiometry of the interaction determined by UV method is compatible with the value obtained from infrared method, namely 1:1 complex.

The most probable hydrogen bond is represented by the following equilibrium reaction.



In case 1 and 3, alcohol acts as a proton donor and riboflavin as a proton acceptor, while in case 2, alcohol acts as a proton acceptor and riboflavin as a proton donor. Case 1 has the most possiblity among them.

By comparing the values of the association constants of the complexes (Table 2), it is concluded that the relative stabilities of the complexes decrease with carbon number increase and being bulkier in the alkyl group of alcohol.

The fact that alcohol forms 1:1 association complex with riboflavin in non-polar solvent supports the hypothesis that alcohol could directly interact with riboflavin moiety of FAD, and contributes to the perturbation to the hydrogen FAD, bonding between riboflavin and protein residue(s) of apoenzyme, thus enhance the catalytic activity of certain flavoenzymes.

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