International Journal of Physical Sciences Vol. 2 (3), pp. 068-072, March, 2007
Available online at http://www.academicjournals.org/IJPS
ISSN 1992 - 1950 © 2007 Academic Journals

Full Length Research Paper

Spectrophotometric study of the solution interactions between riboflavin, sodium salicylate and caffeine

Bolotin P. A., Baranovsky S. F., Chernyshev D. N. and Evstigneev M. P.*

Department of Physics, Sevastopol National Technical University, Sevastopol, 99053, Crimea, Ukraine.

Accepted 14 February, 2007

The self-association of Vitamin B_2 (Riboflavin, RBF) and its hetero-association with Sodium Salicylate (NAS) and Caffeine (CAF) have been studied in aqueous solution by UV-Vis spectrophotometry. Using modified Benesi-Hildebrand approach equilibrium hetero-association constants were obtained for RBF-NAS [K = (25 ± 4) M^{-1}] and RBF-CAF [K = (69 ± 4) M^{-1}] systems at T = 298K. Absorption spectra of the vitamin in the mixed solutions with CAF/NAS evidence a bathochromic and hyperchromic shifts which are indicative of the hetero-association process between the dissolved molecules, most likely occurring via vertical stacking of their chromophores. Addition of Urea to the solutions of RBF-NAS and RBF-CAF decreases the stability of the hetero-complexes downto K = (17 ± 3) M^{-1} and K = (51 ± 7)M^{-1}, respectively.

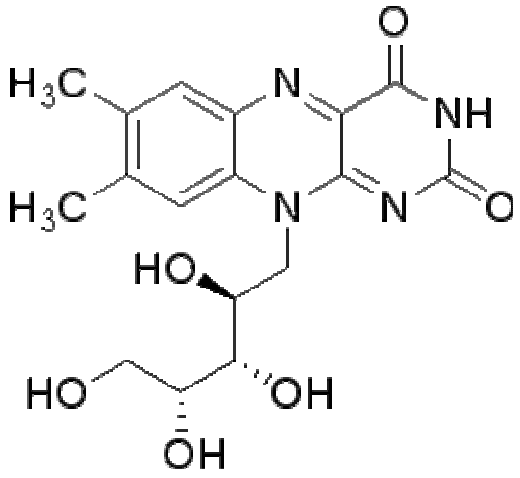
Key words: Spectrophotometry, riboflavin, caffeine, nicotinamide, urea, hetero-association.

INTRODUCTION

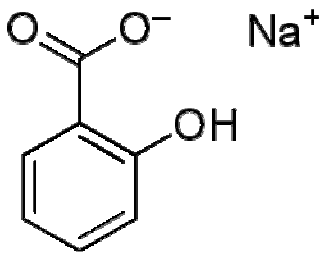
Riboflavin (vitamin B_2, RBF, Figure 1a) belongs to a group of flavoenzymes which catalyze redox reactions and which occur widely in a large number of proteins (Miura, 2001). Although the exact physiological function of this enzyme is not known, many studies have been conducted because of its ability to form complexes with aromatic and heteroaromatic compounds (Miura, 2001; Hermoso et al., 2002; Louie et al., 2002). Thus it has been demonstrated that RBF and its derivatives form stacking-type complexes in aqueous solution with aromatic chemotherapeutic drugs (Munoz et al., 1995; Codoner et al., 1993), antibiotics (Ramu et al., 2000; Evstigneev et al., 2005a; Veselkov et al., 2005), mutagens (Evstigneev et al., 2006a) and other compounds (Datta et al., 2006) containing aromatic planar chromophore, which has led to a proposal that such complexation may be responsible for widely observed alteration both *in vivo* and *in vitro* of biological activity of aromatic drugs administered along with the vitamin. Another view being persued in the literature is that direct aromatic-aromatic interactions with flav in moiety is likely to be the main mechanism for the well-documented solubility enhancement of the vitamin B_2 upon addition of aromatic hydrotropic agents. For example, nicotinamide and Sodium Salicylate (NAS, Figure 1b) increase the solubility of RBF many-fold (Datta et al., 2003; Coffman and Kildsig, 1996); the complexation with RBF of another typical hydrotrope, Caffeine (CAF, Figure 1c), is also thought as a way to improve therapeutic properties of the vitamin (Evstigneev et al., 2005b). Hence, the intermolecular interactions involving RBF and aromatic biologically active drugs are of great importance to understand at molecular level the biological consequences of the Drug-Vitamin co-existence in biological fluid as well as to improve the effectiveness of Vitamin B_2 as a therapeutic agent.

Although the role of Vitamin B_2 interactions with other aromatic compounds has now been recognized, there is practically a lack of quantitative thermodynamical information in literature on that interaction. Apart from common utilization of equilibrium parameters as a way to learn specific features of the association reactions, recently we have demonstrated the use of equilibrium constants to predict behaviour of a more complex multicomponent mixture of aromatic compounds (Evstigneev et al., 2006b; Evstigneev et al., 2006c). Hence the knowledge of equili-

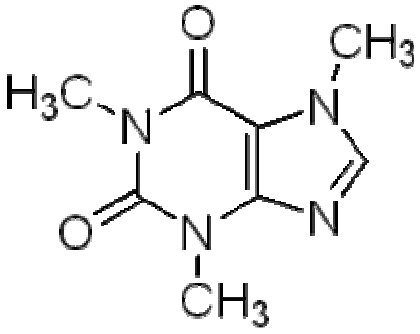
*Corresponding author. E-mail: max_evstigneev@mail.ru Tel: +38(0692)-235251. Fax: +38(0692)-243-590.



a)



b)



c)

Figure 1. Structural formulas of (*a*) riboflavin, (*b*) Sodium Salicylate, (*c*) Caffeine.

brium parameters obtained using similar solution conditions and model approach makes it possible to move towards analysis of more complex systems. In the present work we start series of quantitative studies of binary interactions of Riboflavin with various aromatic drugs exerting biological or biochemical synergism of action when present in solution along with the Vitamin. The equilibrium parameters of RBF interaction with Caffeine and Sodium Salicylate have been obtained and the influence of a third added compound, Urea, on the RBF-CAF and RBF-NAS complexation was qualitatively probed.

MATERIALS AND METHODS

Electronic absorption spectra of the investigated solutions of Riboflavin and the mixed solutions of RBF-NAS, RBF-CAF, RBF-NAS-Urea and RBF-CAF-Urea were measured using SF-46 spectrophotometer in the visible spectrum range (390 ÷ 520 nm).

Riboflavin ("Serva Feinbiochemica GmbH", Heidelberg, Germany), Sodium Salicylate, Caffeine and Urea ("Sigma", USA) (Figure 1) were used without further purification. Initial solutions of the investigated compounds were prepared by addition of the weighted amount of the sample to 0.1M Na-phosphate buffer (pH = 6.86). Due to sensitivity of the samples to day light (blue region of the spectrum) the weighing and sample preparations was accomplished in darkened place. All solutions were made using bidistilled water.

Solutions of RBF for the self-association studies were prepared by sequential dilutions in the range of concentrations $2.5\cdot10^{-3} \div 5\cdot10^{-5}$M. The measurements were made in standard glass cells with an optical path 10.0; 5.01; 2.0082; 1.0077; 0.5062; 0.202; 0.1065; 0.0505 and 0.0207 cm. The temperature in spectrophotometer cell compartment (298K) was stabilized with accuracy ±0.15 ^{0}C.

During the hetero-association studies of RBF-NAS and RBF-CAF the concentration of Riboflavin in the mixed solutions was maintained constant ($C_{RBF} = [B_0] = 5\cdot10^{-5}$M = Const)**.** The range of CAF ($8\cdot10^{-4} \div 6\cdot10^{-1}$ M) and NAS ($2\cdot10^{-4} \div 4\cdot10^{-1}$ M) concentrations were used for RBF titration. The measurements were made in standard glass cells with an optical path 0.0207 and 0.0505 cm.

The studies of the influence of Urea on the formation of hetero-complexes were performed at constant Urea concentration (1M). The measurements were made in quartz cells with an optical path length of 0.998 cm at T=298 ±0.15K.

RESULTS AND DISCUSSION

Self-association of Riboflavin: Dynamic equilibrium in solution was analyzed in terms of the dimer formation:

$$B_1 + B_1 \xleftrightarrow{K_{dB}} B_2\,, \qquad (1)$$

where K_{dB} is equilibrium dimerization constant; B_1 and B_2 are monomers and dimers of RBF.

The overall concentration of the dissolved molecules in solution is given by the mass conservation law:

$$[B_0] = [B_1] + 2[B_2], \qquad (2)$$

where $[B_0]$ is the total concentration; $[B_1]$ is the monomer concentration; $[B_2] = K_{dB}\cdot[B_1]^2$ is the concentration of the dimers.

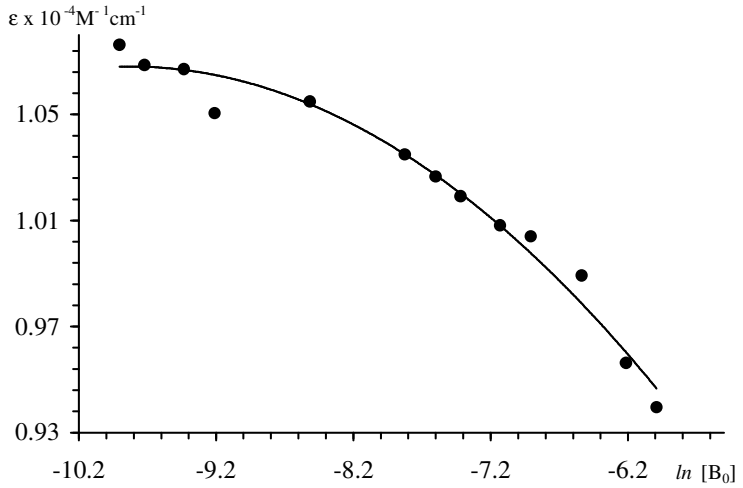


Figure 2. Dependence of molar absorption coefficient of the aqueous solution of Riboflavin (pH = 6.86; T=298K) on logarithm of concentration at λ_{max} = 446 nm

The contribution of the monomers and dimers to the molar absorption coefficient, ε, of the solution is commonly considered to be additive

$$\varepsilon = \varepsilon_m f_m + \varepsilon_d f_d, \quad (3)$$

where ε_m, ε_d are extinction coefficients of RBF monomers and dimers, respectively; $f_m = [B_1]/[B_0]$ is equilibrium mole fraction of the molecules in the monomer form; $f_d = 2K_{dB}[B_1]^2/[B_0]$ is a mole fraction of the molecules within a dimers. Concentration $[B_1]$ can be determined from the solution of the mass conservation law (2). Hence there are three unknown parameters, ε_m, ε_d and K_{dB} in eqn.(3) which can be obtained from numerical analysis of experimental concentration dependence of molar extinction coefficient of RBF (Figure 2). These parameters were calculated as in previous work (Bolotin et al., 2006) by minimization of square deviations of experimental values of molar absorption from the same magnitudes calculated using model (3). The resultant self-association constant of Riboflavin was obtained as K_{dB} = (125 ± 40) M^{-1}, which coincides within the error limits with the same value, K_B = (265 ± 38) M^{-1} when multiplied by a factor of two, obtained from NMR studies of RBF analogue, Flavin-mononucleotide (FMN) in similar solution conditions (Veselkov et al., 2002). It should be noted that in (Veselkov et al., 2002) an indefinite model of self-association was used in analysis, so a direct comparison with a dimer model is only possible if an indefinite self-association constant is scaled down by a factor of two (Davies et al., 1996). Interestingly, the magnitude of the self-association constant of FMN obtained in (Bastian and Sigel, 1997) by NMR and applying similar analytical approach as in (Veselkov et al., 2002) is 10 times lower [K_B = (27 ± 15) M^{-1}]. Though no apparent reason for that can be deduced except a wider concentration range used in (Bastian and Sigel, 1997), the results of the present work along with the work (Veselkov et al., 2002) suggest that the dimerization constant of K_{dB} = 125 M^{-1} is likely to be fair.

Hetero-association of riboflavin with sodium salicylate and caffeine: Electronic absorption spectra of the solution of RBF (C_{RFN} = $[B_0]$ = $5 \cdot 10^{-5}$ M = *const*) underwent changes upon addition of different concentrations of NAS ($2 \cdot 10^{-4} \div 4 \cdot 10^{-1}$M) or CAF ($8 \cdot 10^{-4} \div 6 \cdot 10^{-1}$M), monitored by small bathochomic shift of the absorption maximum of RBF and a decrease in the intensity of the absorption. The latter is commonly treated as a process of intermolecular interaction occuring via vertical stacking of aromatic chromophores of the dissolved molecules (Larsen et al., 1996; Lyles et al., 2001). Along with that the isobestic points were also seen in the absorption spectra which are indicative of the formation of the hetero-complexes RBF-NAS or RBF-CAF.

Analysis of the hetero-association of NAS (CAF) with RBF was accomplished by using Benesi-Hildebrand approach (Benesi and Hildebrand, 1949) under the condition $[A_0] >> [B_0]$, where $[A_0]$ is a total concentration of NAS or CAF. Two-component equilibrium in the mixed solution is given by the reaction scheme (Bolotin et al., 2006):

$$A_1 + B_1 \xleftrightarrow{K} A_1B_1\,,\ A_1 + A_1 \xleftrightarrow{K_{dA}} A_2\,, \quad (4)$$

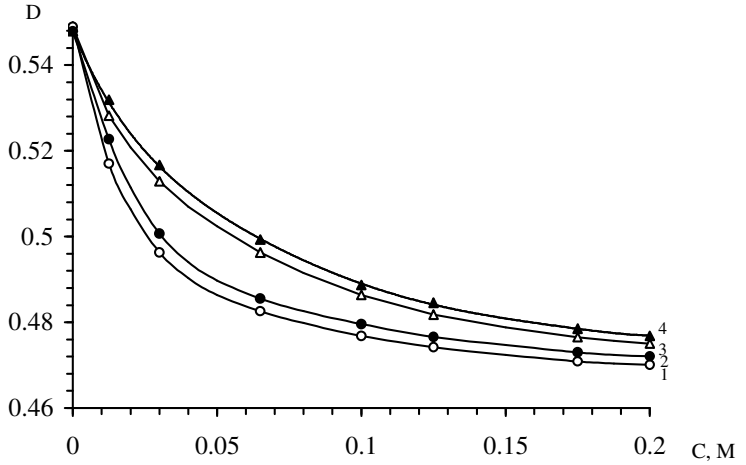


Figure 3. Concentration dependence of the optical density of the mixed solutions (pH = 6.86; T=298K) of: 1) RBF-CAF; 2) RBF-CAF-Urea; 3) RBF-NAS; 4) RBF-NAS-Urea (C_{RBF} = 5·10^{-5} M = const; C_{Urea} = 1.0 M = const) on the wavelength of maximum absorption of RBF λ=446 nm.

where K is the equilibrium constant of NAS (CAF) and RBF hetero-association; K_{dA} *is* the equilibrium dimerization constant of NAS or CAF; A_1 and A_2 are the monomers and dimers of NAS (CAF); A_1B_1 is a hetero-complex. For the scheme (4) the constant K can be written as

$$K = \frac{[A_1B_1]}{[A_1]\cdot([B_0]-[A_1B_1])}, \qquad (5)$$

Where $[A_1B_1]$ is the concentration of the hetero-complex; $[A_1]$ is the monomer concentration of NAS (CAF). The quantity $[A_1B_1]$ can be excluded from (5) by using Beer-Lambert law linking optical density and extinction coefficient

$$D_k = \frac{\varepsilon_k l [B_0] K [A_1]}{K[A_1]+1}, \qquad (6)$$

Where D_k and ε_k are optical density and molar absorption coefficient of Riboflavin molecules within the hetero-complex on the wavelength, which corresponds to the maximum of the absorption of RBF monomers; l is the optical path length in the mixed solution. Evaluation of the optical density quantity for RBF molecules $D = \varepsilon_m \cdot l \cdot [B_1] + \varepsilon_k \cdot l \cdot [A_1B_1]$ with the use of equation.(6), yields a concentration dependence of the experimentally observed absorption:

$$D = D_m - \frac{\Delta\varepsilon l [B_0] K [A_1]}{K[A_1]+1}, \qquad (7)$$

where D_m is the optical density of the solution containing solely RBF molecules; $[B_0]$ = 5·10^{-5} M; $\Delta\varepsilon = \varepsilon_m - \varepsilon_k$; $[A_1] = (-1+\sqrt{1+8K_{dA}[A_0]})/4K_{dA}$ (this equation results from the solution of equation.(2)). Equation.(7) contains three unknown parameters K, K_{dA} and $\Delta\varepsilon$, which can be determined from experimental absorption spectra of RBF on the wavelength of 446 nm (Figure 3).

Equilibrium constants of the formation of RBF-NAS(CAF) hetero-complexes and dimer complexes of NAS (CAF) obtained from modified Benesi-Hildebrand equation (7) were calculated as: K = (25 ± 4)M^{-1}, K_{dA} = (2.7 ± 0.5) M^{-1} for RBF-NAS, and K = (69 ± 4)M^{-1}, K_{dA} = (17 ± 1.5) M^{-1} for RBF-CAF. The self- and hetero-association constants in both systems follow the order $K_{dA} < K < K_{dB}$, which could be expected as three-ring aromatic molecules (Riboflavin in the given case) exhibit typically greater interaction energy than that for two-ring (Caffeine) and one-ring (NAS) molecules (Davies et al., 1996; Ts'o et al., 1963). The intermediate magnitude of the hetero-association constant, K, is also in line with previous investigations (Evstigneev et al., 2005b; Larsen et al., 1996; Davies et al., 2001) and indicates the major role of stacking in the formation of the hetero-complexes.

Influence of urea: Addition of Urea changes the absorption spectra in the mixed solutions of RBF-NAS and RBF-CAF; the resultant hetero-association constants in both systems were calculated using experimental dependences of the optical density on the concentration (Figure 3) in equation.(7) as K = (17 ± 3)M^{-1} and K = (51 ±7)M^{-1}, respectively. It follows that the stability of the

hetero-association complexes is getting lower upon addition of Urea, which is in accord with a qualitative study (Datta et al., 2003). Our view is that a well-known breakdown of water structure upon addition of Urea (Wallqvist et al., 1998) is responsible for the effect observed.

REFERENCES

Bastian M, Sigel H (1997). The self-association of flavin mononucleotide (FMN^{2-}) as determined by ^{1}H NMR shift measurements. Biophys. Chem. 67: 27-34.

Benesi HA, Hildebrand JH (1949). Spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons. J. Am. Chem. Soc. 71: 2703-2707.

Bolotin PA, Baranovsky SF, Evstigneev MP (2006). Spectrophotometric investigation of the hetero-association of Caffeine and thiazine dye in aqueous solution. Spectrochimica Acta. 64A: 693–697.

Codoner A, Medina P, Ortiz C, Jover E (1993). Spectroscopic study of molecular associations between riboflavin and some (dihydro)β-carboline derivatives, Spectrochim. Acta. 49A: 321-327.

Coffman RE, Kildsig DO (1996). Hydrotropic solubilization – mechanistic studies. J. Pharm. Sci. 85: 951-954.

Datta S, Mukhopadhyay C, Bhattacharya S, Kumar BS (2006). Stability and conformation of the complexes of riboflavin with aromatic hydroxy compounds in an aqueous medium. Spectrochim. Acta. 64A: 116-126.

Datta S, Mukhopadhyay C, Kumar BS (2003). Molecular complex formation between riboflavin and salicylate in an aqueous medium. Bull. Chem. Soc. Jpn. 76: 1729-1734.

Davies DB, Djimant LN, Veselkov AN (1996). ^{1}H NMR investigation of self-association of aromatic drug molecules in aqueous solution - Structural and thermodynamical analysis. J. Chem. Soc. Faraday Trans. 92: 383-390.

Davies DB, Veselkov DA, Djimant LN, Veselkov AN (2001). Hetero-association of caffeine and aromatic drugs and their competitive binding with a DNA oligomer. Eur. Biophys. J. 30: 354-366.

Evstigneev MP, Evstigneev VP, Davies DB (2006c). NMR investigation of the effect of caffeine on the hetero-association of an anticancer drug with a vitamin. Chem. Phys. Lett. 432: 248-251.

Evstigneev MP, Evstigneev VP, Hernandez Santiago AA, Davies DB (2006b). Effect of a mixture of caffeine and nicotinamide on the solubility of vitamin (B-2) in aqueous solution. Eur. J. Pharm. Sci. 28: 59-66.

Evstigneev MP, Mukhina YV, Davies DB (2006a). ^{1}H NMR study of the hetero-association of flavin-mononucleotide with mutagenic dyes: ethidium bromide and proflavine. Mol. Phys. 104: 647-654.

Evstigneev MP, Mykhina YV, Davies DB (2005a). Complexation of daunomycin with a DNA oligomer in the presence of an aromatic vitamin (B_2) determined by NMR spectroscopy. Biophys. Chem. 118: 118-127.

Evstigneev MP, Rozvadovskaya AO, Hernandez Santiago AA, Mukhina YV, Veselkov KA, Rogova OV, Davies DB, Veselkov AN (2005b). A ^{1}H NMR study of the association of caffeine with flavin mononucleotide in aqueous solutions. Russ. J. Phys. Chem. 79: 573-578.

Hermoso JA, Mayoral T, Faro M, Gomez-Moreno C, Sanz-Aparicio J, Medina M (2002). Mechanism of coenzyme recognition and binding revealed by crystal structure analysis of ferredoxin-NADP(+) reductase complexed with NADP(+). J. Mol. Biol. 319: 1133-1142.

Larsen RW, Jasuja R, Hetzler R, Muraoka PT, Andrada VG, Jameson DM (1996). Spectroscopic and molecular modelling studies of caffeine complexes with DNA intercalators, Biophys. J. 70: 443-452.

Louie TM, Yang H, Karnchanaphanurach P, Xie XS (2002). FAD is a preferred substrate and an inhibitor of Escherichia coli general NAD(P)H: flavin oxidoreductase. J. Biol. Chem. 277: 39450-39455.

Lyles MB, Cameron IL, Rawls HR (2001). Structural basis for the binding affinity of xanthines with the DNA intercalator acridine orange. J. Med. Chem. 44: 4650-4660.

Miura R (2001). Versatility and specificity in flavoenzymes: control mechanisms of flavin reactivity. Chem. Rec. 1: 183-194.

Munoz MA, Carmona C, Hidalgo J, Guardado P, Balon M (1995). Molecular associations of flavins with betacarbolines and related indoles. Bioorg. Med. Chem. 3: 41-47.

Ramu A, Mehta MM, Liu J, Turyan I, Aleksic A (2000). The riboflavin-mediated photooxidation of doxorubicin. Cancer Chemother. Pharmacol. 46: 449-458.

Ts'o POP, Melvin IS, Olson AC (1963). Interaction and association of bases and nucleosides in aqueous solutions. J. Am. Chem. Soc. 85: 1289-1296.

Veselkov AN, Evstigneev MP, Rozvadovskaya AO, Mukhina YV, Rybakova KA, Davies DB (2005). ^{1}H NMR analysis of the complex formation of aromatic molecules of antibiotic and vitamin in aqueous solution: Heteroassociation of actinomycin D and flavin mononucleotide. Biophysics 50: 20-27.

Veselkov AN, Lantushenko AO, Chubarov AS, Veselkov DA, Davies DB (2002). ^{1}H NMR analysis of the self-association of riboflavin-mononucleotide and its complexation with nicotinamide in an aqueous solution. Russ. J. Phys. Chem. 76: 1350-1356.

Wallqvist A, Covell DG, Thirumalai D (1998). Hydrophobic interactions in aqueous urea solutions with implications for the mechanism of protein denaturation. J. Am. Chem. Soc. 120: 427-428.