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EFFECT OF A MIXTURE OF CAFFEINE AND NICOTINAMIDE ON THE SOLUBILITY OF VITAMIN (B_2) IN AQUEOUS SOLUTION

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ABSTRACT

The effect of Caffeine (CAF) and Nicotinamide (NMD) on the solubility of a Vitamin B_2 derivative (FMN) has been evaluated for mixtures containing either a single hydrotrope (CAF or NMD) or the two hydrotropes simultaneously. A model for analysis of ternary systems, which takes into account all possible complexes between the molecules, has been developed and tested with experimental NMR data on the three-component mixture FMN-CAF-NMD. The results indicate that special attention should be given to the concentration of a hydrotropic agent used to enhance the solubility of a particular drug. A decrease in the efficacy of solubility of the vitamin on addition of large amounts of hydrotropic agent is expected in the two-component systems due to the increased proportion of self-association of the hydrotrope. It is found that a mixture of two hydrotropic agents leads to an increase in the solubility of the vitamin in three-component compared to the two-component system. Rather than using just one hydrotropic agent, it is proposed that a strategy for optimising the solubility of aromatic drugs is to use a mixture of hydrotropic agents.

Key words: Solubility, Caffeine, Nicotinamide, Vitamin B2, Hetero-association

INTRODUCTION

Caffeine (CAF) and Nicotinamide (NMD) are well known as hydrotropic agents and their ability to solubilize a wide variety of therapeutic drugs including Riboflavine [Coffman and Kildsig, 1996a; Rasool et al., 1991; Lim and Go, 2000; Jain et al., 1996] has been demonstrated. A number of mechanisms have been reported to account for the observed solubilizing effect of drugs by CAF and NMD in water, namely, a specific interaction with solvent [Coffman and Kildsig, 1996a], self-association of the hydrotrope [Coffman and Kildsig, 1996b] and complexation between the hydrotrope and drug; the latter mechanism has been the focus of many studies of drug complexation by CAF and NMD using different experimental methods [Rasool et al., 1991; Lim and Go, 2000; Jain et al., 1996; Hussain et al., 1993; Khalil, 1996] and molecular modeling studies [Fawzi et al., 1980; Datta et al., 2003].

Riboflavin (vitamin B_2, RBF) belongs to a group of flavoenzymes which catalyze redox reactions and which occur widely in a large number of proteins. It has been recently shown that a derivative of the vitamin, riboflavin mononucleotide (FMN), preferentially forms stacked complexes in aqueous solution with Caffeine [Evstigneev et al., 2005a], with Nicotinamide [Veselkov et al., 2002] or its close analogue, Salicylic acid [Datta et al., 2003]). In an aqueous environment the aromatic planar structure of RBF and the hydrotrope favour stacking of aromatic chromophores in hetero-complexes, which is considered to be the main factor contributing to the solubilizing effect of these hydrotropes on the vitamin. The degree of aromaticity in the hydrotropic agent is important for its solubilizing power. For example, under the same solution conditions, Caffeine with two aromatic rings affects the solubility of the aromatic antimalarial agent, Halofantrine, to a greater extent than does Nicotinamide with one aromatic ring in the molecule [Lim and Go, 2000]. A similar difference in behaviour between CAF and NMD with respect to solubilising RBF may be expected, because their affinities to the vitamin decrease in the same order, *viz*. $K_{RBF\text{-}CAF}$=160 M^{-1} [Evstigneev et al., 2005a] and $K_{RBF\text{-}NMD}$=65 M^{-1} [Veselkov et al., 2002], T=298K. Hence the operative concentration ranges for CAF and NMD to exert their hydrotropic action differ by an order of magnitude (CAF – tens of mM, NMD – hundreds of mM).

The equilibrium constants for self-association of CAF and NMD differ by at least an order of magnitude (K_{CAF}=11.8 M^{-1} [Davies et al., 2001], K_{NMD}=0.73 M^{-1} [Veselkov et al., 2001a], T=298K). In mixed solutions with RBF the increased aggregation of CAF apparently reduces the total number of binding sites available for RBF compared to NMD, which may 'compensate' the benefit of a greater affinity of CAF to RBF with respect to NMD-RBF in terms of resultant solubility of RBF. Hence, this leads to the question of whether the effectiveness of

the solubilization of RBF by CAF acts over the whole concentration range of the hydrotrope compared to NMD under the same conditions, or whether a decrease in the effect might be predicted because of the increased proportion of self-association of CAF? Recently an improvement of the solubility of various drugs has been reported in multi-component systems including cyclodextrins, which was explained as being due to formation of triple and higher order complexes in solution [Redenti et al., 1999; Ribeiro et al., 2005]. Hence, one may ask whether a *mixture* of CAF and NMD is more effective in solubilizing RBF than a single hydrotropic agent under the same solution conditions and total concentrations?

Quantitation of a three-component mixture of RBF with CAF+NMD requires an analytical methodology which can take into consideration unitary complexes (i.e. the self-association of RBF, CAF, NMD), binary complexes (i.e. the hetero-association RBF-CAF, RBF-NMD, CAF-NMD) and ternary complexes (i.e. the hetero-association RBF-CAF-NMD). To the best of our knowledge the multicomponent systems analyzed so far have used a reduced stoichiometry of the associations (1:1, 1:2, 1:1:1 *etc.*), which is not applicable to the current studies of solubility, because the working concentration range is high enough to anticipate formation of multimers on self- and hetero-association.

In the present work a model for NMR analysis of three-component systems is developed and applied to the experimental and theoretical analysis of the action of a mixture of CAF+NMD on the equilibrium behaviour in aqueous solution of a Riboflavine analogue, Flavine-mononucleotide (FMN). Previously we have reported the complete structural and thermodynamical analysis of the NMR investigation of self-association of all the components (FMN [Veselkov et al., 2002], CAF [Davies et al., 2001], NMD c and two of the three required binary complexations (FMN-CAF [Evstigneev et al., 2005a] and FMN-NMD [Veselkov et al., 2002]) under the same solution conditions. In order to complete the three-component complexation scheme, the hetero-association of CAF-NMD and the three component mixture of FMN-CAF-NMD are investigated.

MATERIALS AND METHODS

Flavin-mononucleotide (FMN, Fig.1a), Caffeine (CAF, Fig.1b) and Nicotinamide (NMD, Fig.1c) from Sigma were used without further purification. The samples were lyophilized from D_2O solutions and re-dissolved in 0.1M phosphate buffer in 99.95% D_2O (pD 7.1) containing 10^{-4} M EDTA.

500 MHz ^1H NMR spectra were recorded on a Bruker DRX spectrometer with the residual water peak saturated during relaxation. Signal assignments of the non-exchangeable

protons of the drugs obtained using both two-dimensional homonuclear TOCSY and ROESY experiments have been reported previously [Veselkov et al., 2001a, 2002, Davies et al., 2001]. Chemical shift measurements of the non-exchangeable protons of the aromatic molecules were made as a function of concentration of the vitamin in FMN-CAF-NMD mixtures and Caffeine in CAF-NMD experiments at *T*=298K. In each mixture the concentration of the component, which possesses the highest self-association constant, was varied whilst maintaining the concentration of other components constant (i.e. CAF in CAF-NMD experiment, Fig.2a; FMN in FMN-CAF-NMD experiment, Fig.3). The thermodynamic parameters of CAF-NMD complexation were determined from the temperature dependences of proton chemical shifts for CAF and NMD measured at constant concentration of drug molecules over the temperature range 273-348K (Fig.2b).

All sets of NMR measurements were made in the fast-exchange condition on the NMR timescale. Chemical shifts were measured relative to TMA (tetramethylammonium bromide) as an internal reference and re-calculated with respect to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate), *i.e.* DSS=TMA+3.178 (ppm). The sample temperature was regulated using the Bruker BVT-3000 unit.

RESULTS AND DISCUSSION

Caffeine-Nicotinamide hetero-association.

In the mixed solution of CAF with NMD, the experimental ^{1}H NMR curves for NMD show an increase in average shielding of NMD protons upon successive additions of Caffeine in solution measured at constant concentration of NMD (Fig.2a). A similar situation has been observed previously for a number of aromatic molecules in hetero-association mixtures with CAF and was explained by the formation of stacked aggregates [Evstigneev et al., 2005a; Davies et al., 2001; Larsen et al., 1996]. Hence the observed shielding in CAF-NMD system most likely results from complexation between NMD and CAF occurring via stacking of their aromatic chromophores.

The dynamic equilibrium in solution containing two types of interacting aromatic molecules *X* (CAF) and *Y* (NMD) may be described using the following scheme of reactions (1) [Davies et al., 2001]:

$$\begin{array}{llll} X_1 + X_i \xleftrightarrow{K_X} X_{i+1} & (a) & Y_1 + Y_j \xleftrightarrow{K_Y} Y_{j+1} & (b) \\ X_i + Y_j \xleftrightarrow{K_h} X_i Y_j & (c) & Y_j X_i + Y_l \xleftrightarrow{K_h} Y_j X_i Y_l & (d) \end{array} \qquad (1)$$

where X_1 and Y_1 correspond to the monomers of CAF and NMD, and X_i, Y_j, Y_l are the aggregates containing i monomers of CAF and j, l monomers of NMD, respectively; K_X, K_Y are equilibrium self-association constants for X and Y and K_h is the hetero-association constant. Analytical expressions for the observed dependence of proton chemical shifts of both X and Y components derived from (1) are given in the form of equation (2) [Davies et al., 2001]

$$\begin{cases} \delta_X = \dfrac{x_1}{x_0}\left[\begin{array}{l} \delta_{mX}\left(2\left(1+K_X x_1\right)-\dfrac{1}{\left(1-K_X x_1\right)^2}\right)+2\delta_{dX}\left(\dfrac{1}{\left(1-K_X x_1\right)^2}-1-K_X x_1\right)+ \\ +\delta_{hX}\dfrac{K_h y_1}{\left(1-K_X x_1\right)^2\left(1-K_Y y_1\right)}\left(1+\dfrac{K_h y_1}{2\left(1-K_Y y_1\right)}\right) \end{array}\right] \\ \delta_Y = \dfrac{y_1}{y_0}\left[\begin{array}{l} \delta_{mY}\left(2\left(1+K_Y y_1\right)-\dfrac{1}{\left(1-K_Y y_1\right)^2}\right)+2\delta_{dY}\left(\dfrac{1}{\left(1-K_Y y_1\right)^2}-1-K_Y y_1\right)+ \\ +\delta_{hY}\dfrac{K_h x_1}{\left(1-K_Y y_1\right)^2\left(1-K_X x_1\right)}\left(1+\dfrac{K_h y_1}{1-K_Y y_1}\right) \end{array}\right] \end{cases} \qquad (2)$$

where x_0, y_0 and x_1, y_1 are the total and the monomer concentrations of CAF and NMD molecules in solution; δ_m, δ_d, δ_h are chemical shifts of X or Y protons in the monomer, dimer and in the hetero-complex, respectively. The values of δ_m, δ_d and the equilibrium self-association constants are known from self-association studies of CAF [Davies et al., 2001] and NMD [Veselkov et al., 2001a]. The monomer concentrations x_1 and y_1 may be derived from the solution of the mass conservation law for scheme (1) [Davies et al., 2001]. It follows that the model (2) is a function of two unknown quantities, δ_h and K_h, which may be determined from the concentration dependences of δ (Fig.2a) using the numerical procedure described previously [Davies et al., 2001].

The thermodynamical parameters, enthalpy ($\Delta H°_h$) and entropy ($\Delta S°_h$), were obtained from the observed temperature dependences of the proton chemical shifts of NMD and CAF (Fig.2b) using van't-Hoff's formalism as described previously [Davies et al., 2001]. The calculated equilibrium, δ_h and K_h, and thermodynamical parameters, $\Delta H°_h$ and $\Delta S°_h$, are summarised in Table 1.

It is seen from Table 1 that the equilibrium hetero-association constant K_h, as well as the enthalpy/entropy values, range between those for self-association of CAF (K_X) and NMD (K_Y), analogous to the results for complexation of other aromatic molecules with Caffeine reported previously [Davies et al., 2001; Larsen et al., 1996], where it was shown that stacking interactions, including both dispersive and hydrophobic interactions, play a major role in stabilization of the sandwich-type hetero-complexes of aromatic molecules in solution. The structure of the CAF-NMD 1:1 hetero-complex was calculated from observed chemical shift changes with concentration using the methodology published previously [Veselkov et al., 2004]

and the results are presented in Fig.4. The structure of the complex clearly demonstrates the co-planarity of the aromatic chromophores of CAF and NMD, which supports the conclusion about stacking as a major stabilizing force in the given system.

Model for the analysis of the triple FMN-CAF-NMD mixture.

There are three types of species present in solution: *X* (*e.g.* CAF), *Y* (*e.g.* NMD) and *Z* (*e.g.* FMN). The interaction between these components may lead to the formation of three hetero-stacks (*X*-*Y*, *X*-*Z*, *Y*-*Z*) and three homo-stacks (*X*-*X*, *Y*-*Y*, *Z*-*Z*) between the molecules in a complex. The number of the hetero-stacks as well as the number of the homo-stacks within an arbitrary selected molecular complex in solution may adopt any possible value ranging from 0 to *N*-1, where *N* is the number of molecules in the complex. It was concluded previously [Veselkov et al., 2001b] that expressions needed to analyse experimental data in two-component system (*X* and *Y*) become highly complicated even if no more than two hetero-stacks are considered. In this case the functional-analytical approach for describing the complex equilibrium for two components in solution was not considered appropriate (reviewed in [Veselkov et al., 2001b]) and a computer-based algorithmical approach was developed. Recently we have reported a Stochastic algorithmical model, which takes into account all possible distributions of homo- and hetero-stacks within a hetero-complex, and have successfully applied it to analyse the NMR data of two-component mixtures of aromatic molecules in solution [Evstigneev et al., 2005b].

The basic idea of the Stochastic algorithm is to represent the hetero-complex, formed from an arbitrary number of *X* and *Y* molecules, as a binary number in which *X* and *Y* correspond to 0 and 1 bits, respectively [Evstigneev et al., 2005b]. Hence, cycling over all *binary* numbers from zero up to the maximum number allowed by the length of a complex means that all statistically possible molecular complexes in solution are generated. It is proposed to extend this algorithm for three-component system; cycling over *ternary* numbers will result in generation of all possible distributions of homo- and hetero-stacks within a hetero-complex formed in a three-component mixture of molecules.

In the present work the computer algorithm developed for binary mixtures [Evstigneev et al., 2005b] has been modified for the cycling over ternary numbers and applied to the analysis of NMR data on the FMN-CAF-NMD mixture (Fig.3). In the analysis of the experimental data on the three-component mixture FMN-CAF-NMD, it is important to test both the modified Stochastic algorithm and the appropriateness of using hetero-association constants derived from previous studies on two-component systems (CAF-NMD, K_{XY}; FMN-CAF, K_{XZ}; FMN-NMD, K_{YZ}; Table 2). Fitting of the experimental data (Fig.3) using the parameters in Table 2 give an average discrepancy of 0.01 ppm per experimental point for each proton of the molecules. The

results confirms the appropriateness of using the Stochastic algorithm to analyse the data for the three-component FMN-CAF-NMD system and so it has become possible to calculate the fraction of each type of complex in the mixture.

The effect of CAF or NMD on the equilibrium distribution of FMN in two-component systems.

In order to compare the hydrotropic action of CAF or NMD on the solubility of FMN in two-component systems, it is necessary to select an appropriate criterion for the efficacy of the solubilization action for both the FMN-CAF and FMN-NMD systems. As reviewed above, the main mechanism of solubilization of FMN in the presence of CAF or NMD is thought to be hetero-association with the hydrotropic agent and so it is logical to consider the concentration of the hetero-complexes as a basic criterion. However, it is desirable that the same criterion should be applied to both two- and three-component mixtures, which invokes the question of what is a hetero-complex in a three-component mixture? In the equilibrium there will be hetero-association complexes of FMN with CAF and with NMD, plus complexes between them or with both of them. Moreover, the fraction of hetero-association complexes in the three-component mixture is determined by at least two independent components, CAF and NMD. It thus possible that some changes in the CAF or NMD equilibrium may lead to a redistribution in the existing hetero-association profile to a much greater extent than the effect on their complexation with FMN, and an erroneous conclusion may be made regarding the solubility of the target molecule. It is proposed that the best candidate for the criterion of solubility in both two- and three-component mixtures is the concentration of the *self-associated* complexes of the vitamin.

One may consider two types of 'self-associated complexes', the concentration of FMN aggregates in the pure form or the concentration of FMN aggregates in the pure form plus those in hetero-complexes. In principal there appears to be little difference between them but, as consideration of the aggregated species of FMN is of primary importance, the total concentration of FMN molecules in self-associated aggregates, complexed or not with CAF/NMD, is used as a basic criterion of the solubilization efficacy of the hydrotropic agents.

Using the basic stochastic model [Evstigneev et al., 2005b] and equilibrium constants in Table 2 , the fraction of the self-associated FMN (F_s) complexes in the two-component mixtures, FMN-CAF and FMN-NMD, have been re-calculated as a function of the hydrotropic agent (CAF or NMD, Fig.5a). It is seen from the Figure that the concentration of associated FMN species at lower concentrations of CAF is smaller in the FMN-CAF mixture than that in the FMN-NMD mixture for the same concentrations of NMD. The result is expected and correlates well with the known ability of CAF to solubilise aromatic drugs better than NMD [Lim and Go, 2000].

However, on increasing the concentration of CAF and NMD past the *ca*. 350mM point (Fig.5a), the F_s curve in the FMN-CAF system goes beyond the F_s curve in the FMN-NMD system, which means that at higher concentrations Nicotinamide *becomes more effective* in solubilising FMN than does Caffeine. This result is important because it leads to the proposition that an increase in concentration of hydrotropic agent may decrease its effectiveness as a solubilising agent. So what is the origin of the effect?

First, one should consider whether the effects observed depend on the criterion used to estimate the hydrotropic action. As discussed above, the criterion for overall 'self-association' may be considered as an alternative to the concentration of the self-association aggregates in the pure state. The latter item may be expressed in a simple form, (3) [Veselkov et al., 2001a]

$$F_s = \frac{z_1}{z_0(1 - K_Z z_1)^2}, \qquad (3)$$

where z_0 and z_1 are the total and the monomer concentrations of FMN molecules in the mixed solutions. Using the basic Stochastic model [Evstigneev et al., 2005b] the calculations for FMN-CAF and FMN-NMD mixtures have been repeated for the quantity F_s expressed in the form of equation (3), and the results gave the same intercept as in Fig.5a, which indicates that the effects under consideration do not depend on the criterion employed.

Second, one should consider whether the observed effect might depend on the model employed, *i.e.* it may be a specific result of the algorithmical approach. So the criterion of hydrotropic action has been calculated using the analytical Generalised model [Veselkov et al., 2001b], which gave the same the intercept of the FMN-CAF and FMN-NMD solubilisation curves as for the Stochastic model (Fig.5a), *i.e*. calculation of the consequences of the hydrotropic effect does not depend on the type of model employed.

It is now possible to give a physical explanation for the relative lowering of the efficacy of the hydrotropic effect for FMN of CAF with respect to NMD in the high concentration region. The relative content of the three main types of complexes affecting the equilibrium in the FMN-CAF and FMN-NMD systems (i.e. monomers, pure self-associates and hetero-complexes with FMN) is shown in Fig.5b. It is seen that the dependence on concentration of the hydrotrope (CAF or NMD) of both the monomers and self-association complexes are similar in the CAF and NMD systems. However, on increasing the concentration of CAF or NMD in the mixture, the fraction of hetero-association complexes in the CAF system decreases but that in the NMD system increases, which explains the effect of a decrease in the efficacy of the hydrotropic action in the FMN-CAF system compared to the FMN-NMD system. The basic reason for the difference in behaviour is given by analysis of the distribution of self-association complexes of CAF and NMD. It can be seen in Fig.5b that in the FMN-CAF system in solution the dominant

species are CAF aggregates (CAFi), whilst in the FMN-NMD system the NMD monomers (NMD1) dominate. It follows that any addition of CAF to the FMN-CAF mixture results in the major redistribution of CAF molecules towards *self-association* of CAF causing the relative lowering of the fraction of hetero-association complexes with FMN and, hence, towards a decrease in the efficacy of solubilisation of FMN by CAF. Conversely, an analogous addition of NMD to the FMN-NMD mixture does not contribute so much to self-association, which results in an increase in the fraction of the hetero-association complexes of NMD with the vitamin. Hence, the difference in hydrotropic behaviour of CAF and NMD is basically determined by the difference in self-association constants of the hydrotropic agents. The self-association constants of CAF and NMD differed by an order of magnitude (Table 2), which explains why in mixtures with FMN in solution, self-association of CAF dominates but not for NMD.

The effect of CAF and NMD on the equilibrium distribution of FMN in three-component systems.

The physical meaning of the cross-over point of the curves in Fig.5a is that at (*ca*. 350mM) CAF and NMD possess equal solubilisation abilities for FMN. In other words, if we take *ca*. 400mM of CAF or 400mM of NMD, theoretically, they would affect the solubility of FMN to similar extent. One can consider what would happen if the quantity 400mM is composed of a *mixture* of CAF and NMD. Evaluation of the ternary mixture using the three-component Stochastic model with incorporation of the condition that CAF+NMD = 400mM, results in a profile (presented in Fig.6) for the fraction (F_S) of the self-associated complexes of FMN. A minimum is clearly seen in the Figure which is lower than both the left boundary (NMD=400mM, CAF=0mM) and the right boundary (CAF=400mM, NMD=0mM) of the graph, which suggests that a mixture of different hydrotropic agents may be more effective in solubilising an aromatic molecule than a single agent at similar total concentrations.

As can be seen in Fig.5b the enhancement of the solubilisation effect in this particular system is too small to be very useful but it shows how the effect depends on the relation between the intrinsic self-association and hetero-association constants in the three-component mixture. In addition the current methodology does not yet take into consideration the possibility of cooperativity in the formation of ternary molecular complexes, recently reported for cyclodextrin-containing systems [Redenti et al., 1999; Ribeiro et al., 2005].

Conclusions.

In the present work the effect of Caffeine and Nicotinamide on the solubility of a Vitamin B_2 derivative (FMN) has been evaluated in mixtures containing either a single hydrotrope (CAF

or NMD) or two hydrotropes simultaneously. A model for quantitative thermodynamic analysis of ternary systems, which takes into consideration all possible complexes between the molecules, has been developed and tested for experimental NMR data on the three-component mixture, FMN-CAF-NMD. The results indicate that special attention should be given to choosing the concentration of a hydrotropic agent used to enhance the solubility of a particular drug, because addition of a hydrotropic agent may cause either a positive or negative effect on the solubility of a drug. Assuming that hetero-association is the dominant mechanism for solubilizing planar aromatic molecules in aqueous solution, it is shown how a redistribution of complexes in the dynamic equilibrium on addition of a hydrotropic agent may explain both the positive and negative effects on the solubility of the molecule. A decrease in the solubilization efficacy on addition of large amounts of hydrotropic agent is expected in two-component systems as a result of increased proportion of self-association of the hydrotrope. On the other hand, it is found that a mixture of two hydrotropic agents in ternary systems may lead to an enhancement of the solubilization effect compared to two-component systems.

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Table 1 Calculated parameters of CAF(*X*)-NMD(*Y*) hetero-association in 0.1M Na-phosphate buffer, pD 7.1, *T*=298K[a]

CAF protons	δ_{hX}, ppm	NMD protons	δ_{hY}, ppm
H8	7.74	H2	8.53
$7CH_3$	3.80	H6	8.46
$3CH_3$	3.31	H4	7.91
$1CH_3$	3.19	H5	7.30
Thermodynamical parameters			
K_h, M^{-1}	$-\Delta G°_h$, kJ/mol	$-\Delta H°_h$, kJ/mol	$-\Delta S°_h$, J/(mol·K)
7.12±0.04	4.02±0.01	18.7±1.9	49±6

[a] The equilibrium parameters of self-association of CAF [Davies et al., 2001] and NMD [Veselkov et al., 2001a] are: CAF: K_X=11.8±0.3 M^{-1}, $\Delta H°_h$=–(21.0±0.4) kJ/mol; $\Delta S°_h$=–(50±1) J/(mol·K); NMD: K_Y=0.73±0.05 M^{-1}, $\Delta H°_h$=–(15.7±2.3) kJ/mol; $\Delta S°_h$=–(55±7) J/(mol·K)

Table 2 Equilibrium self-association and hetero-association constants (M^{-1}) in 0.1M Na-phosphate buffer, pD 7.1, *T*=298K

Constants	CAF (*X*)	NMD (*Y*)	FMN (*Z*)
CAF (*X*)	11.8±0.3[a]	7.12±0.04[b]	160±26[c]
NMD (*Y*)	7.12±0.04[b]	0.73±0.05[d]	65±12[e]
FMN (*Z*)	160±26[c]	65±12[e]	265±38[e]

[a] Ref. [Davies et al., 2001];
[b] This work;
[c] Ref. [Evstigneev et al., 2005a];
[d] Ref. [Veselkov et al., 2001a];
[e] Ref. [Veselkov et al., 2002]

FIGURE LEGENDS

Fig.1 Structural formulas of Flavin-mononucleotide, FMN (*a*), Caffeine, CAF (*b*), Nicotinamide, NMD (*c*)

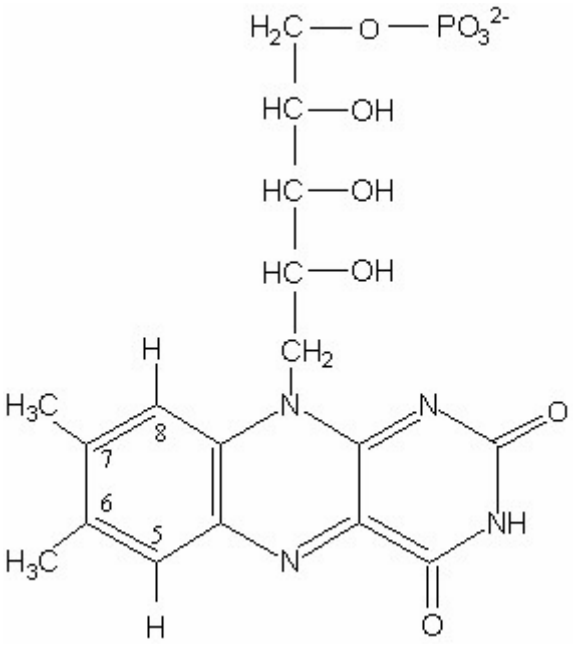
Fig.2 Dependence of proton chemical shifts of NMD (—○—) and CAF (—●—): (*a*) on concentration of CAF (T=298K, C_{NMD}=39.5mM); (*b*) on temperature (C_{NMD}=39.5mM, C_{CAF} = 27.01mM)

Fig.3 Dependence of proton chemical shifts of CAF (—○—), FMN (—●—) and NMD (—■—) on concentration of FMN (T=298K, C_{CAF}=11.65mM, C_{NMD}=41.76mM)

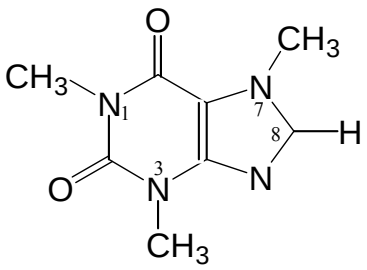
Fig.4 The calculated spatial structure of the 1:1 hetero-association complex of CAF with NMD (side view of the hetero-complex, view looking perpendicular to the planes of the chromophores of aromatic molecules)

Fig.5 Dependence of CAF or NMD in FMN-CAF and FMN-NMD systems on the concentration of: (*a*) self-associated aggregates of FMN; (*b*) different associated complexes in solution. F_S is defined by equation (3)

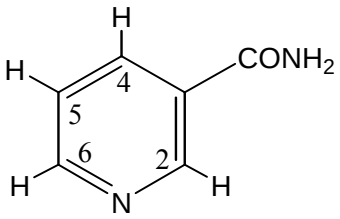
Fig.6 Fraction (F_S) of FMN self-aggregates in FMN-CAF-NMD mixture, depending on the proportion between CAF and NMD, the sum of which equals to 400mM


H2C — O — PO3 2-
HC — OH
HC — OH
HC — OH
CH2
H
H3C
N
N
O
8
7
6
5
NH
H3C
N
H
O

a)

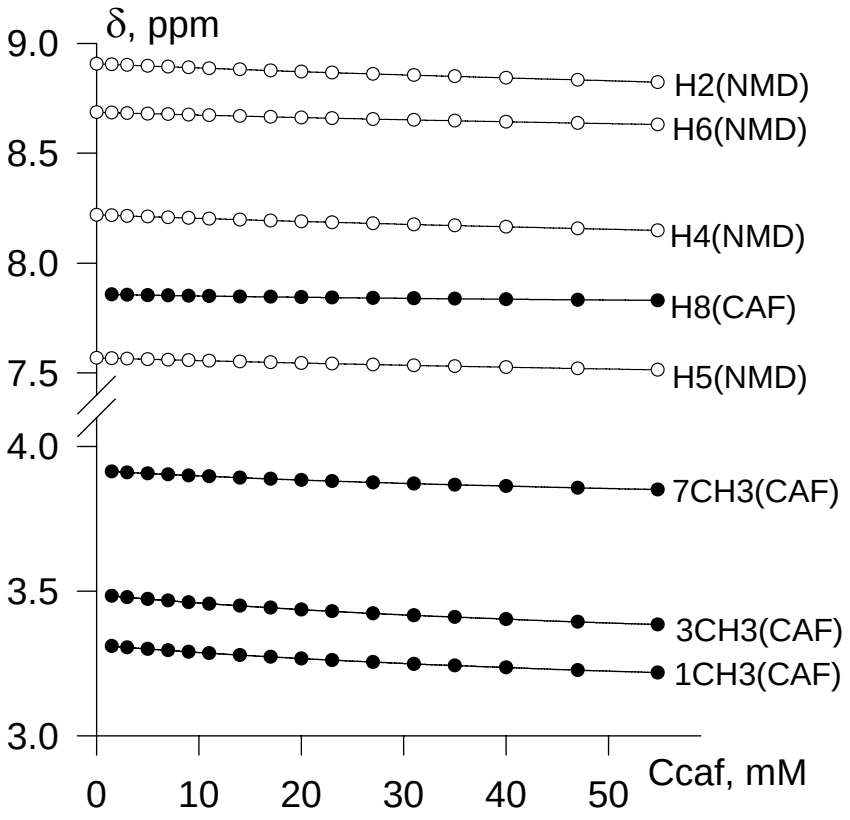

O
CH3
CH3
N1
N
7
8
H
3
O
N
N
CH3

b)

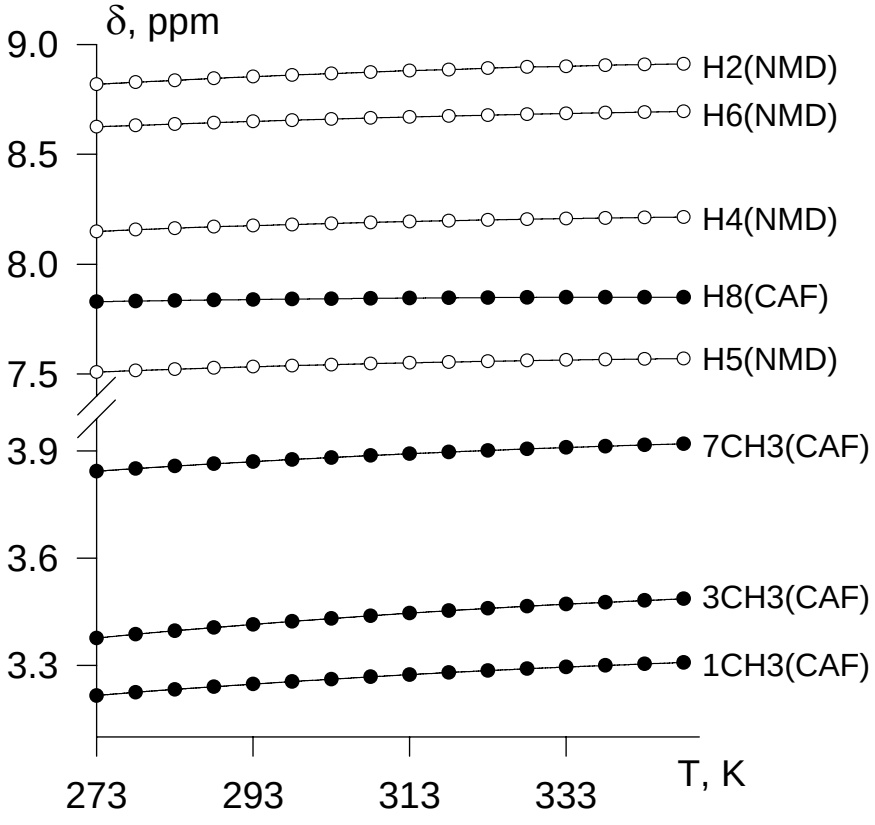

H
CONH2
H
4
5
6
2
H
H
N

c)

Fig.1


δ, ppm
9.0
8.5
8.0
7.5
4.0
3.5
3.0
H2(NMD)
H6(NMD)
H4(NMD)
H8(CAF)
H5(NMD)
7CH3(CAF)
3CH3(CAF)
1CH3(CAF)
0
10
20
30
40
50
Ccaf, mM

a)


δ, ppm
9.0
8.5
8.0
7.5
3.9
3.6
3.3
H2(NMD)
H6(NMD)
H4(NMD)
H8(CAF)
H5(NMD)
7CH3(CAF)
3CH3(CAF)
1CH3(CAF)
273
293
313
333
T, K

b)

Fig.2

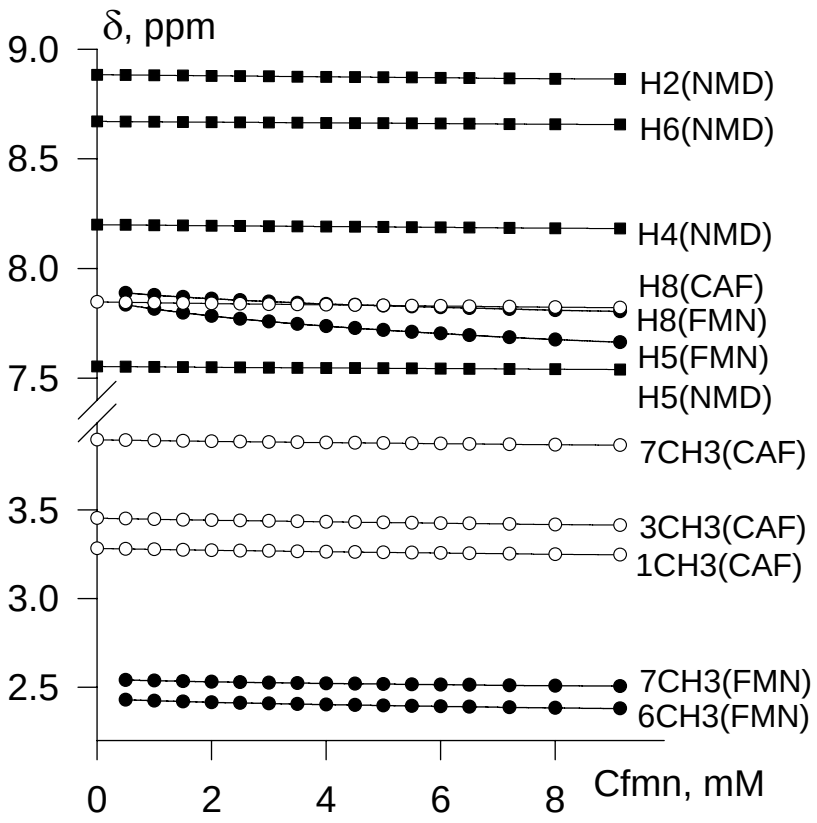

δ, ppm
9.0
8.5
8.0
7.5
3.5
3.0
2.5
H2(NMD)
H6(NMD)
H4(NMD)
H8(CAF)
H8(FMN)
H5(FMN)
H5(NMD)
7CH3(CAF)
3CH3(CAF)
1CH3(CAF)
7CH3(FMN)
6CH3(FMN)
0
2
4
6
8
Cfmn, mM

Fig.3

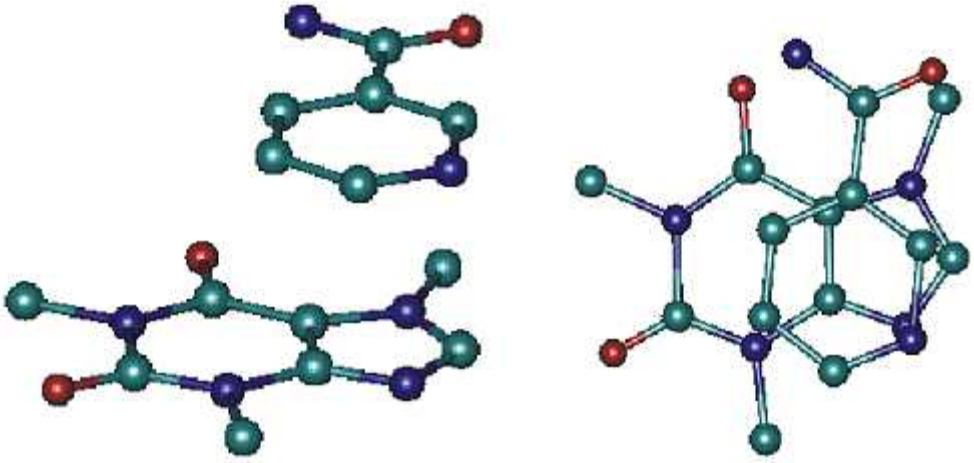
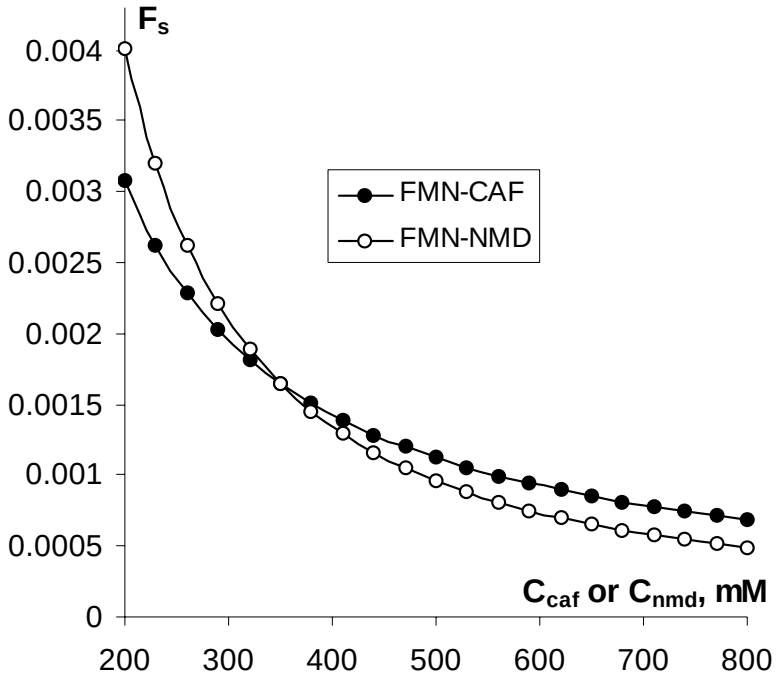
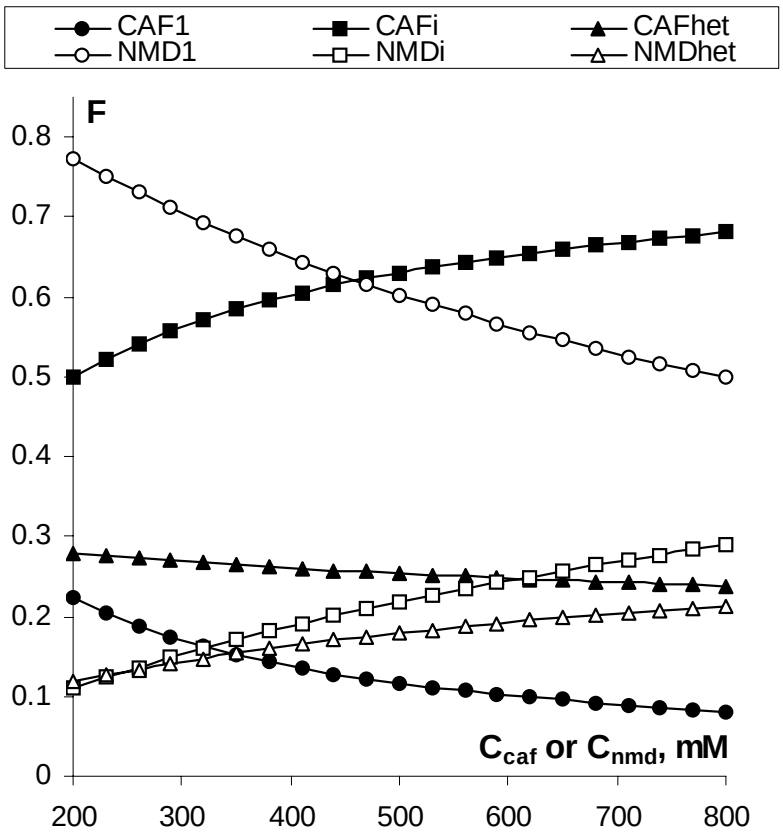


Fig.4


F_s
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
200
300
400
500
600
700
800
FMN-CAF
FMN-NMD
C_caf or C_nmd, mM

a)


CAF1
CAFi
CAFhet
NMD1
NMDi
NMDhet
F
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
200
300
400
500
600
700
800
C_caf or C_nmd, mM

b)

Fig.5

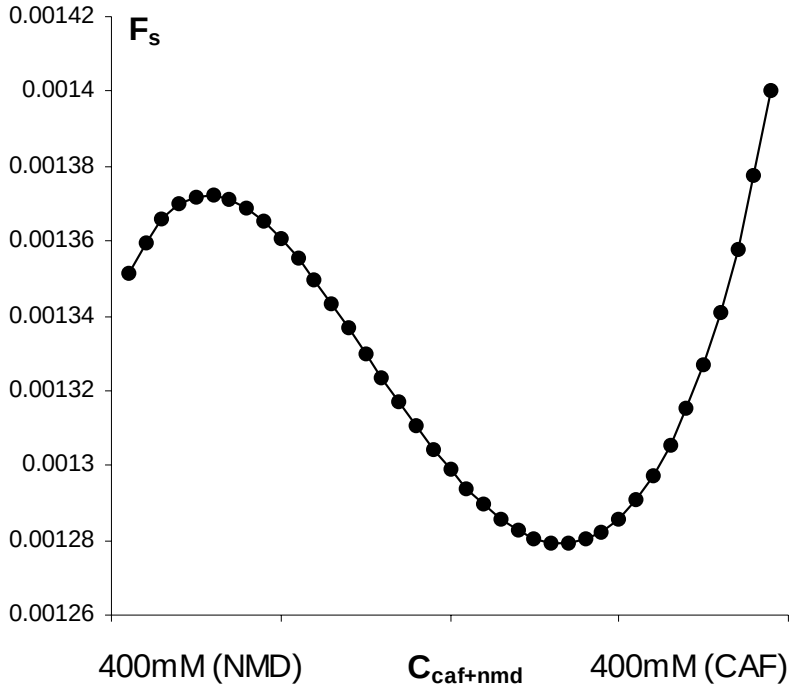

0.00142
0.0014
0.00138
0.00136
0.00134
0.00132
0.0013
0.00128
0.00126
Fs
400mM (NMD)
Ccaf+nmd
400mM (CAF)

Fig.6