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^{1}H NMR DETERMINATION OF THE SELF-ASSOCIATION OF AN ACRIDINE HOMODIMER AND ITS COMPLEXATION WITH ETHIDIUM BROMIDE IN AQUEOUS SOLUTION

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ABSTRACT

^{1}H NMR spectroscopy (500MHz) has been used to investigate the self-association in aqueous buffered solution of a bis-intercalator, Acridine Homodimer (AcrH), and its hetero-association with the aromatic dye, Ethidium Bromide (EB).   The equilibrium constants and thermodynamical parameters (enthalpy and entropy) of self-association have been determined from the observed concentration and temperature dependences of chemical shifts of AcrH protons and the results are consistent with a model consisting of at least four distinct forms of AcrH molecules in solution: unfolded (U), folded (F), a dimer formed from two folded molecules (F_2) and a trimer formed from three folded molecules (F_3). It has also been shown that Ethidium Bromide complexes strongly to the dimer form ($F_{2)}$ of the bis-acridine molecule, AcrH. Comparison of the calculated association parameters of AcrH with the previously studied Ethidium Homodimer (EBH) revealed a correlation between the effectiveness of complexation and the length of chain connecting the chromophores of a bis-intercalator.

Key words: Acridine Homodimer, Ethidium Bromide, self-association, hetero-association, NMR spectroscopy

INTRODUCTION

It has been demonstrated that dimerization of DNA intercalating compounds can lead to molecules able to bind to DNA with a very high affinity constant and be very specific in their base pair recognition [1-7]. Some natural and synthetic bis-intercalators have substantial anti-cancer activity and in certain cases are able to overcome multidrug resistance in cultured cells [6-8]. In addition, they can provide probes for large-amplitude DNA dynamics and may also serve as models for some types of protein-DNA interactions [9].

Over the years a range of bis-intercalators, based on acridine, phenanthridine and anthracycline units connected with linkers of different structure and length, has been synthesized [6,7,10,11] and their complexation with DNA studied, because DNA is considered to be the primary target in their biological action [1-7]. Although a number of models of drug-DNA interaction have been suggested, they are all based on the assumption that a bis-intercalator may exist in an open or unfolded (U) conformation and a closed or folded (F) conformation [3,12]. A few studies on the self-association of acridine and phenanthridine dimeric dyes in solution have provided evidence of the existence of the U- and F-forms in solution [10,11,13,14]. In addition, studies on the interaction of bis-intercalators with mono- and dinucleotides have also been performed in order to gain insight to the specificity of drug-DNA complexation [13,15]. It has been shown that the DNA monomeric units, as well as some other monomeric aromatic ligands, are able to intercalate into the F-form of a bis-intercalator [ref.]. Bearing in mind that monomeric acridines and phenanthridines effectively self-associate in solution via π-π stacking [16], it is likely that some self-association of bis-intercalators can occur in solution.

Early NMR studies on the self-association of Ethidium (EBH) and Acridine (AcrH) Homodimers indicated very little probability of the formation of higher order aggregates than U- and F-forms in solution [11,13]. On the other hand, a spectrophotometric titration of AcrH revealed specific spectral changes attributed by the authors to higher order aggregation involving the complexation of the monomeric forms of AcrH molecules in solution [12,14]. We have recently shown by high field NMR that EBH molecules associate in solution in a complicated fashion resulting in mutually intercalated dimers and trimers formed mainly of the folded form of the molecules [17]. It was also shown that the folded structure of EBH formed strong hetero-association complexes with Propidium Iodide, and appeared to be an effective trapper of the aromatic dye [17]. In the present work we have studied by 500MHz ^{1}H NMR spectroscopic methods the self-association of a Acridine Homodimer and its hetero-association with the phenanthridine dye, Ethidium Bromide. The results obtained in this work, in fact, provide a rationale for analysis of the dynamic equilibrium of structurally-related aromatic bis-intercalators

in solution linked with a spermine-type linker, which also enables to suppose the same mode of self- and hetero-association for another bis-intercalating compound, Acridine Homodimer.

EXPERIMENTAL

Acridine Homodimer ("Molecular Probes") and Ethidium Bromide ("Sigma") were lyophilized from D_2O and re-dissolved in 0.1M solution of HEPES buffer in 99.95% D_2O, pD 7.5, containing 10^{-4} M EDTA. The structures of AcrH and EB are shown in Figure1.

500 MHz ^{1}H-NMR spectra were recorded on a Bruker DRX spectrometer. Signal assignments of the non-exchangeable protons of AcrH and EB were obtained using two-dimensional homonuclear TOCSY and ROESY experiments. Chemical shift measurements of the non-exchangeable protons of aromatic molecules were made as a function of concentration at 298 and 308K in the experiments on the self-association of AcrH (Figure 2a) and at 298K for AcrH-EB hetero-association (Figure 3a); measurements as a function of temperature were made at constant concentration of AcrH (Figure 2b) and EB (Figure 3b) in the temperature range 278-348K. Proton chemical shifts were measured relative to an internal reference TMA (tetramethylammonium bromide) and recalculated with respect to DSS (sodium 2.2 dimethyl 2-silapentane-5-sulphonate). All NMR experiments were made in the fast-exchange condition on the NMR time-scale.

RESULTS

Self-association of Acridine Homodimer. Signal assignments of all the non-exchangeable protons of the ^{1}H NMR spectrum of AcrH in aqueous solution were obtained using both two-dimensional homonuclear TOCSY and ROESY experiments and are in good agreement with the results published previously [13]. The concentration and temperature dependence of proton chemical shifts of AcrH presented in Figure 2a and b, respectively, exhibit normal behaviour for the association process, *i.e.* low frequency shift of the concentration curves on increasing the concentration and high frequency shift on increasing the temperature. The behaviour is markedly different from that reported previously for self-association of the bis-intercalator, EBH, in which the experimental concentration and temperature curves for EBH protons were characterized by a highly non-monotonic profile and explained by a competitive contribution of two processes, the formation of dimers (F_2) and trimers (F_3) of EBH molecules in solution [17]. The competitiveness originated from a pronounced shielding of aromatic protons in the F_2 form

and concomitant deshielding in the F_3 form due to the electric charges located on the linker chain of EBH. In contrast, the bis-acridine molecule studied in the present work has a pK_a significantly lower (<7) than its monomeric acridine unit ($\approx$8) [13]. It follows that AcrD is not charged under the conditions adopted in the present experiment (pD 7.5), which excludes the competitive factor of shielding/deshielding and results in the observed monotonic profile of the experimental chemical shift dependences (Figures 2,3).

The scheme of self-association reactions of AcrH in solution [depicted schematically in Figure 4 and with equilibrium constants defined in equation (1)] includes folded (F) and unfolded (U) forms and the formation of dimers (F2) and trimers (F3) of the folded form, analogous to that in previous work on EBH [17]

$$U \xleftrightarrow{K_h} F \ \text{a}), \quad F + F \xleftrightarrow{K_2} F_2 \ \text{b}), \quad F + F_2 \xleftrightarrow{K_3} F_3 \ \text{c}). \tag{1}$$
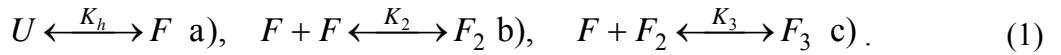
Taking into account the mass conservation law for reactions (1) and the law of mass action, the observed chemical shift (in the fast exchange condition on the NMR time-scale) can be written in the form [17]:

$$h_0 = h_1 + K_h h_1 + 2 K_2 K_h^2 h_1^2 + 3 K_3 K_2 K_h^3 h_1^3$$

$$\delta = \frac{h_1}{h_0} \delta_U + \frac{K_h h_1}{h_0} \delta_F + 2 \frac{K_2 K_h^2 h_1^2}{h_0} \delta_2 + 3 \frac{K_3 K_2 K_h^3 h_1^3}{h_0} \delta_3, \tag{2}$$
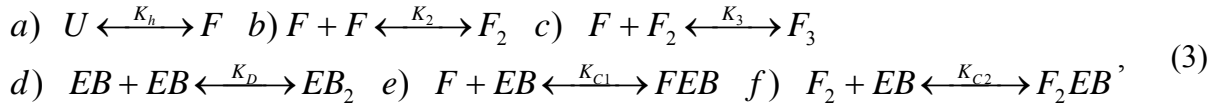
where δ_U, δ_F, δ_2, δ_3 are proton chemical shifts in the U, F, F_2 and F_3 forms of a bis-intercalator, respectively. The proton chemical shifts in the unfolded and folded forms of AcrH, δ_U and δ_F, may be considered approximately equal to each other as in previous work [17], taking into consideration the relatively large spacing between the chromophores in the folded form of AcrH due to a rigidity of the linker which prevents their approaching a distance allowing effective magnetic shielding (0.3-0.4 nm [18]). It follows that the observed concentration dependence of AcrH proton chemical shifts is a function of 6 unknown quantities K_h, δ_F, K_2, δ_2, K_3, δ_3, which can be calculated using the computation procedure described previously [17]. The values of the calculated parameters are presented in Tables 1 and 2.

The thermodynamical parameters, enthalpy (ΔH) and entropy (ΔS), for each of the self-association reactions (1) were determined from the temperature dependencies of the proton chemical shifts of AcrH in solution (Figure 2b) using van't Hoff's formalism [16,17]. The calculated values of enthalpy and entropy of AcrH self-association are summarised in Table 2.

Hetero-association of the Acridine Homodimer with Ethidium Bromide. The concentration and temperature dependence of the proton chemical shifts of AcrH and EB molecules in the mixed solution are shown in Figure 3. As in the case of self-association

experiments (Figure 2), the NMR curves in Figure 3 indicate monotonic behaviour unlike those observed previously for complexation of EBH with Propidium Iodide [17].

A number of models of AcrH-EB hetero-association have been tested in order to give the best description of the experimental data and the minimum discrepancy between calculated and experimental data was obtained using the same scheme of molecular hetero-association [depicted schematically in Figure 5 and with equilibrium constants defined in equation (3)] as in previous work [17];

$$
\begin{aligned}
&a) \quad U \xleftrightarrow{K_h} F \quad b) \, F + F \xleftrightarrow{K_2} F_2 \quad c) \quad F + F_2 \xleftrightarrow{K_3} F_3 \\
&d) \quad EB + EB \xleftrightarrow{K_D} EB_2 \quad e) \quad F + EB \xleftrightarrow{K_{C1}} FEB \quad f) \quad F_2 + EB \xleftrightarrow{K_{C2}} F_2EB
\end{aligned} \tag{3}
$$

where FEB, F_2EB are the most probable 1:1 and 1:2 hetero-complexes between EB and AcrH; K_D, K_{C1}, K_{C2} are equilibrium self-association constant for EB and hetero-association constants for 1:1 and 1:2 complex formation, respectively.

The mass conservation law equations for reactions (3) and the observed proton chemical shift can thus be written in the form [17]:

$$
\begin{cases}
h_0 = h_1 + K_h h_1 + 2K_2 K_h^2 h_1^2 + 3K_3 K_2 K_h^3 h_1^3 + K_h h_1 p_1 K_{C1} + 2K_2 K_h^2 h_1^2 p_1 K_{C2} \\
d_0 = d_1 + 2K_D d_1^2 + K_h h_1 d_1 K_{C1} + K_2 K_h^2 h_1^2 d_1 K_{C2} \\
\delta_h = \delta_U \dfrac{h_1}{h_0} + \delta_F \dfrac{K_h h_1}{h_0} + \delta_2 \dfrac{2K_2 K_h^2 h_1^2}{h_0} + \delta_3 \dfrac{3K_3 K_2 K_h^3 h_1^3}{h_0} + \delta_{C1h} \dfrac{K_h h_1 d_1 K_{C1}}{h_0} + \delta_{C2h} \dfrac{2K_2 K_h^2 h_1^2 d_1 K_{C2}}{h_0} \\
\delta_D = \delta_{mD} \dfrac{d_1}{d_0} + \delta_{dD} \dfrac{2K_D d_1^2}{d_0} + \delta_{C1D} \dfrac{K_h h_1 d_1 K_{C1}}{d_0} + \delta_{C2D} \dfrac{K_2 K_h^2 h_1^2 d_1 K_{C2}}{d_0}
\end{cases} \tag{4}
$$

where d_1 and d_0 are monomeric and total concentrations of EB in solution, respectively; δ_{mD}, δ_{dD} are proton chemical shifts in the monomer and dimer forms of EB; δ_{C1}, δ_{C2} are proton chemical shifts in 1:1 and 1:2 hetero-complexes between the homodimer (h) and ethidium (D) molecules, respectively. The parameters δ_U, δ_F, δ_2, δ_3, K_h, K_2, K_3 and δ_{mD}, δ_{dD}, K_D have been determined from the self-association studies of AcrH (Tables 1,2) and EB [16] under the same experimental conditions. It follows that eqns.(4) contain 4 unknown quantities K_{C1}, K_{C2}, δ_{C1}, δ_{C2}. The computational procedure of determination of these parameters is similar to that used for self-association analysis of AcrH and the calculated parameters of the hetero-association of AcrH and EB are presented in Tables 1,2.

Thermodynamical parameters of the hetero-association reactions (3e)-(3f) were determined from the experimental temperature dependences of the proton chemical shifts of AcrH and EB (Fig.3b), using a similar approach as considered above for the thermodynamical analysis of the self-association of the bis-intercalator.

DISCUSSION

Self-association of Acridine Homodimer.

In the present work on the self-association in aqueous solution of the bis-intercalator AcrH, the same model (1) of the dynamic equilibrium was used as for EBH [17], which included the formation of intercalated dimers and trimers (Figure 4). However the monotonic dependence of the concentration and temperature dependence curves for AcrH (Figure 2) indicates that it may not be necessary to include all the contributing processes (in Figure 4) as used in the study of EBH self-association [17]. A simple dimer model, *i.e.* lacking reaction (1c) in equation (1), has also been tested for AcrH self-association; such calculations resulted in an average induced shielding of AcrH protons in the F_2 form ($\Delta\delta=\delta_F-\delta_2$) equal to $\Delta\delta$ ~2ppm (results are not presented here). If the dimer model is valid, approximately the same shielding would be expected for the aromatic protons of the monointercalator Acridine Orange, which is structurally close to the aromatic moiety of AcrH, when sitting inside an aggregate of molecules. The average induced shielding of Acridine Orange protons is ~1.1ppm [16], which is significantly different from $\Delta\delta$ for AcrH protons derived from the dimer model. On the other hand, the average shielding of AcrH protons in both F_2 or F_3 complexes utilizing the full scheme (1) is no greater than 0.8ppm, which is similar to the shielding parameters of Acridine Orange self-association [16]. It is found that the most appropriate description of the NMR data of AcrH is equilibrium model (1), which includes the formation of dimers and trimers.

It is found that the induced shielding, $\Delta\delta$, in both F_2 and F_3 complexes of AcrH is positive (Table 1) indicating no competition between dimerization and trimerization reactions and resulting in the monotonic dependence of the concentration and temperature curves, in contrast to that found for EBH self-association where the linker chain is charged [17].

Comparison of the thermodynamic parameters of self-association of AcrH and EBH (Table 2) shows that there is a close relation between the energy of aggregation of the molecules in solution and their structures. It should be noted that the linker chain for AcrH is 4 bonds longer than that of EBH, which probably results in a greater spacing between the chromophores in the folded form of AcrH (F in Figure 4a) and a better stacking of the AcrH chromophores in the F_3 complex (Figure 4c) with respect to formation of similar complexes of EBH (the length of EBH linker is critical for stabilization of the trimer with a characteristic inter-chromophore distance of 0.34nm [17]). The stacking of the AcrH chromophores is probably responsible for the much larger equilibrium constant of AcrH trimer formation (K_3) compared to that for EBH resulting from more effective dispersive and hydrophobic interactions in the AcrH trimer

molecules. The high negative values of enthalpy and entropy of reaction (1b) also provides evidence a strengthening of dispersive interactions in the F_2 dimer of AcrH compared to the EBH dimer. It should be emphasised that the enthalpy of self-association of Acridine Orange and Ethidium Bromide, the monomeric analogues of the bis-acridine and bis-ethidium respectively, exhibits the same relation [16].

The calculated magnitudes of the equilibrium self-association constants of Acridine Homodimer (Table 2) were used to calculate the relative content (*f*) of different types of AcrH complexes in solution as a function of concentration of AcrH (Figure 6). Examination of the curves shows that a complicated dynamic equilibrium of AcrH aggregates exists in solution and that it is strongly dependent on the concentration of the bis-intercalator. In particular, at concentrations above 0.6 mM, the total contribution of homodimer aggregates, F_2+F_3, dominates in solution with respect to the monomers, F+U, and the main contribution is given by the trimer form of AcrH aggregates in this range of concentrations (Figure 6).

Hetero-association of Acridine Homodimer with Ethidium Bromide.

In mixed solutions of AcrH and EB it is observed that, on changing the concentration of AcrH, the chemical shifts of EB protons also change (Figure 3a), which is indicative of a complexation process between AcrH and EB molecules in solution. The same pattern has also been observed previously when studying the hetero-association of simple monointercalators [19].

A preliminary search of the model providing the best fit to the experimental data in Figure 3 has resulted in reaction scheme (3), which is the same as that used previously for analysis of the hetero-association of Ethidium Homodimer with Propidium Iodide [17]. However, as a result of a small contribution of the 1:1 hetero-association reaction (3e) to the dynamic equilibrium in solution, the hetero-association constant, K_{C1}, is very small (less than 20 l/mol) and the induced chemical shift, δ_{C1}, seems to be unreliable. Bearing in mind that the K_{C1} value for EBH-PI complexation was estimated as K_{C1}=30 l/mol (*T*=298K [17]), it is possible that the negligible contribution of the reaction (3e) results from the greater length of the linker chain in AcrH, which hinders the approach of the chromophores of the bis-intercalator towards the inserted EB molecule to allow better stacking on formation of the FEB complex (Figure 5a).

Analysis of the complexation parameters (Table 2) reveals that the hetero-association constant K_{C2} is greater than the trimerization constant, K_3. It is likely that a monomeric EB molecule having no linker attached is better accommodated when binding with the F_2 complex when compared with a bulky AcrH molecule forming F_3 complex via reaction (1c). It is interesting to note that the $K_{C2(AcrH-EB)}$ constant is significantly greater than the $K_{C2(EBH-PI)}$ constant (Table 2). As the chain linker of EBH is critical for insertion of two aromatic chromophores into the F-form (Figure 4c) and the chain linker of AcrH linker is longer than that

of EBH, it is possible that its length is sufficient so that the complexation of EB with the dimer of AcrH is facilitated with respect to EBH-PI complexation, thus resulting in more intense dispersive and hydrophobic contact and the greater association constant. An increase in the absolute values of enthalpy and entropy of the hetero-association reaction of AcrH-EB over the same parameters of the formation of F_2 and F_3 complexes of AcrH (see Table 2) supports the assumption of favourable energetics for insertion of EB with the dimer F_2 compared to the binding of the F-molecule to F_2.

The calculated magnitudes of the hetero-association constants were used to calculate the relative content of different types of AcrH associates in the presence of EB (Figure 7). It is easily seen that the equilibrium distribution of the homodimer aggregates is strongly concentration dependent and that, at concentrations of EB greater than about 0.5 mM, the contribution of the F_2EB hetero-complex (~40%) dominates in solution with respect to other associated complexes of the homodimer.

Conclusions.

In the present work it has been shown that a typical bis-intercalator, Acridine Homodimer, exhibits a strong tendency to self-associate in solution, forming an intercalated-like compact complexes of two (F_2) and three (F_3) molecules. It has also been shown that AcrH molecule is an effective trapper of aromatic dye, Ethidium Bromide, which binds with a dimer F_2 of AcrH. A comparative of the calculated association parameters of AcrH with those for Ethidium Homodimer previously studied [17] reveals a correlation between the effectiveness of association reactions and the length of the chain connecting the chromophores of a bis-intercalator. Such models and analyses as in this work should be taken into consideration for investigations of the complexation of bis-intercalating agents with biopolymers and in circumstances when other aromatic species may present in solution.

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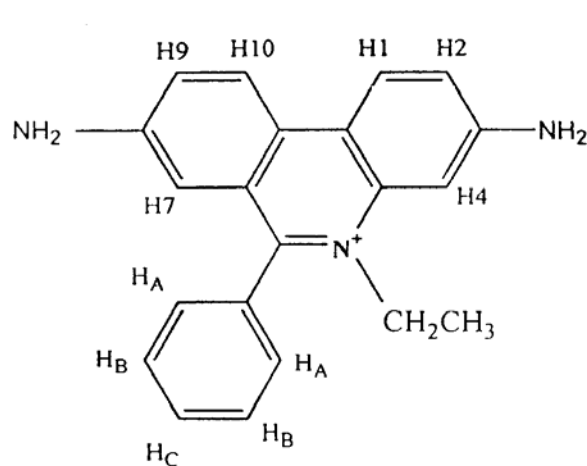Table 1 Induced proton chemical shifts of Acridine Homodimer in different self-association complexes

Protons	H8	H4	H3	H5	H7	H1
T=298K						
$\delta_U \approx \delta_F$	8.04	7.57	7.57	7.49	7.38	7.27
δ_2	7.82	7.31	7.31	7.26	7.25	7.08
δ_3	7.26	6.81	6.81	6.64	6.72	6.45
δ_{C2}	7.77	7.28	7.28	7.18	7.13	6.90
T=308K						
$\delta_U \approx \delta_F$	8.05	7.58	7.57	7.51	7.38	7.28
δ_2	7.76	7.28	7.22	7.28	7.11	7.15
δ_3	7.43	6.91	6.96	6.80	6.82	6.55

Table 2 Comparison of the thermodynamical parameters of self- and hetero-association of Acridine Homodimer (AcrH) and Ethidium Homodimer (EBH) [17] in aqueous solution

| Reaction		K_{298}, l/mol	K_{308}, l/mol	-$\Delta H°$, J/mol	-$\Delta S°$, J/mol·K
$U \leftrightarrow F$	AcrH	42 ± 10	26 ± 6	11000 ± 3000	6 ± 3
	EBH	25 ± 5	—	9000 ± 3000	4 ± 2
$F + F \leftrightarrow F_2$	AcrH	540 ± 130	400 ± 100	32000 ± 5000	50 ± 10
	EBH	730 ± 100	—	23000 ± 3000	22 ± 5
$F + F_2 \leftrightarrow F_3$	AcrH	4200 ± 1000	3200 ± 800	28000 ± 5000	25 ± 5
	EBH	1400 ± 200	—	26000 ± 3000	27 ± 5
$EB + F_2 \leftrightarrow F_2EB$	AcrH	6500 ± 800	—	36000 ± 4000	50 ± 10
$PI + F_2 \leftrightarrow F_2PI$	EBH	160 ± 40	—	17000 ± 3000	15 ± 5
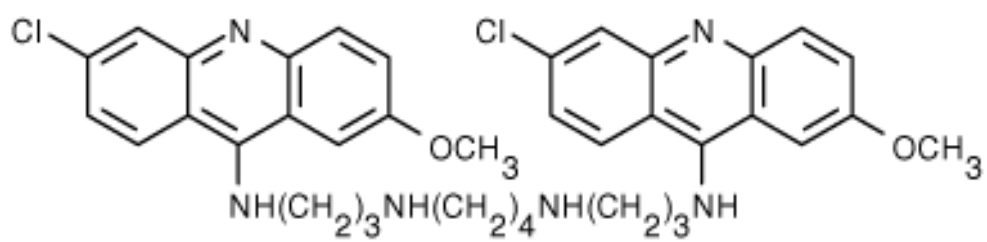
a)

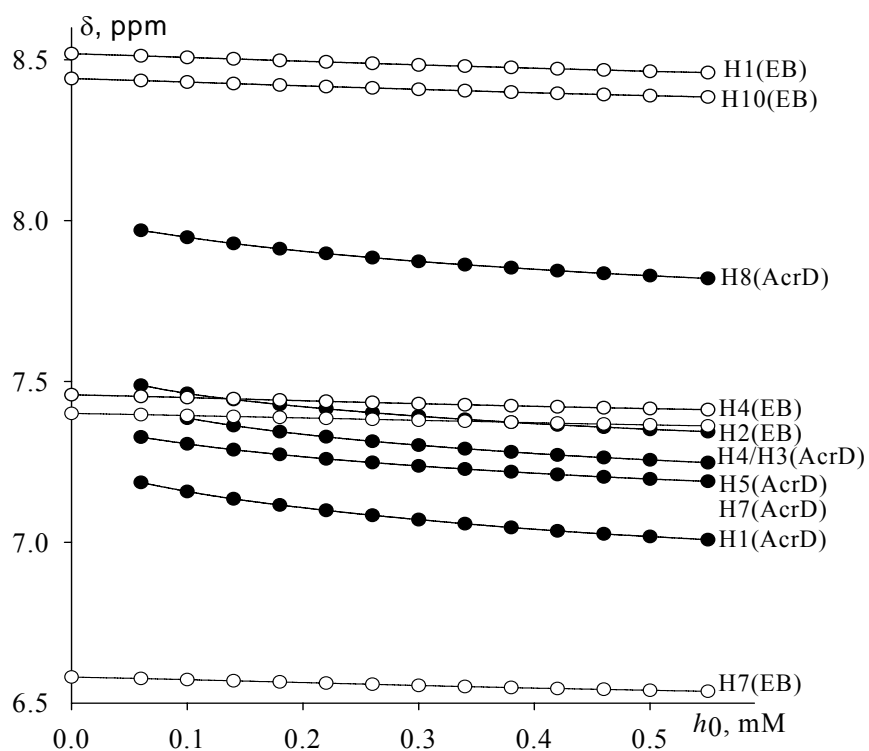

b)
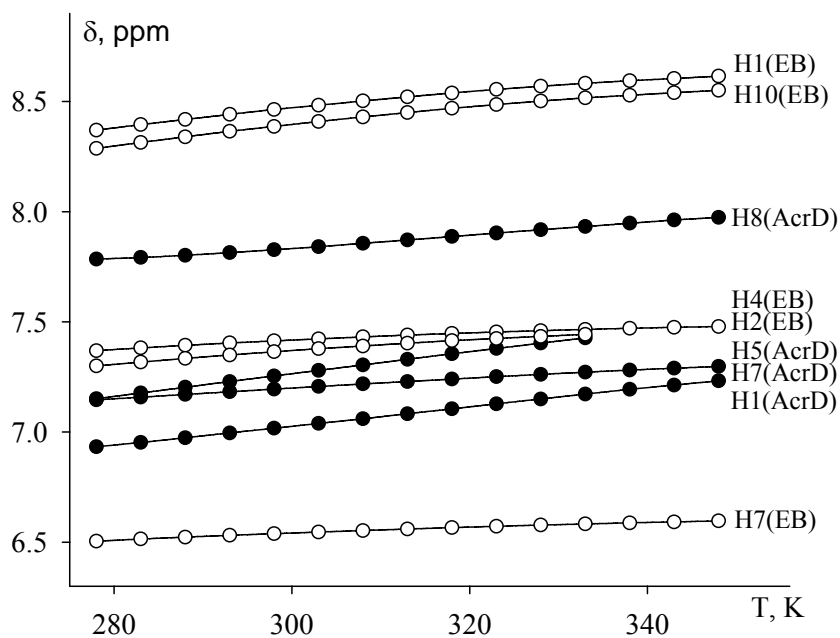
Fig.1 Structure of (*a*) Acridine Homodimer and (*b*) Ethidium Bromide
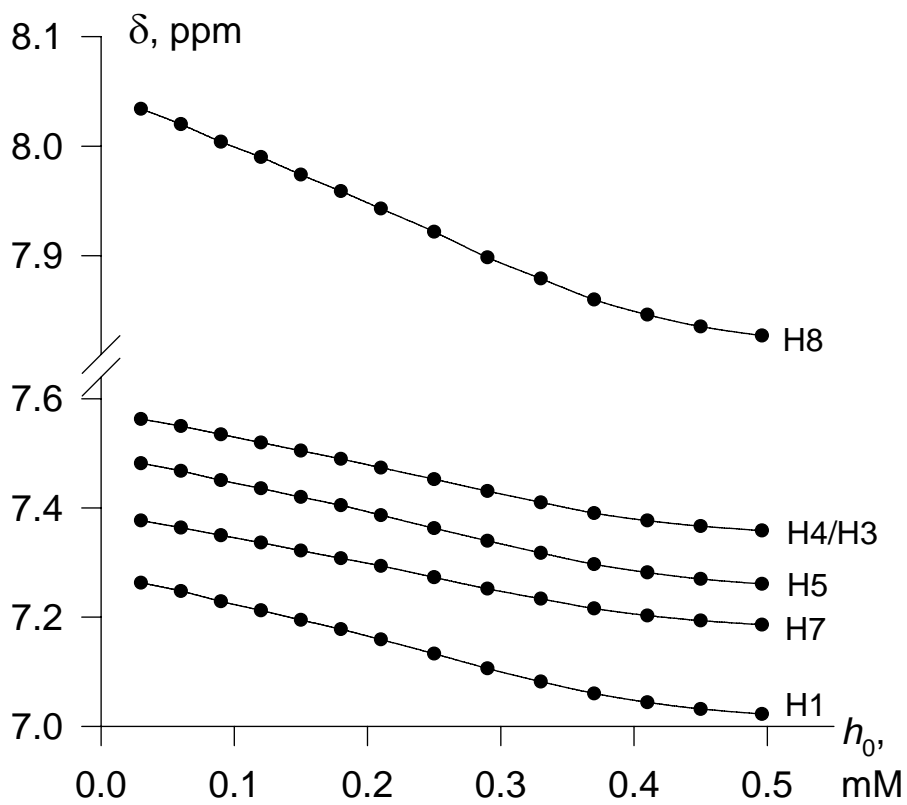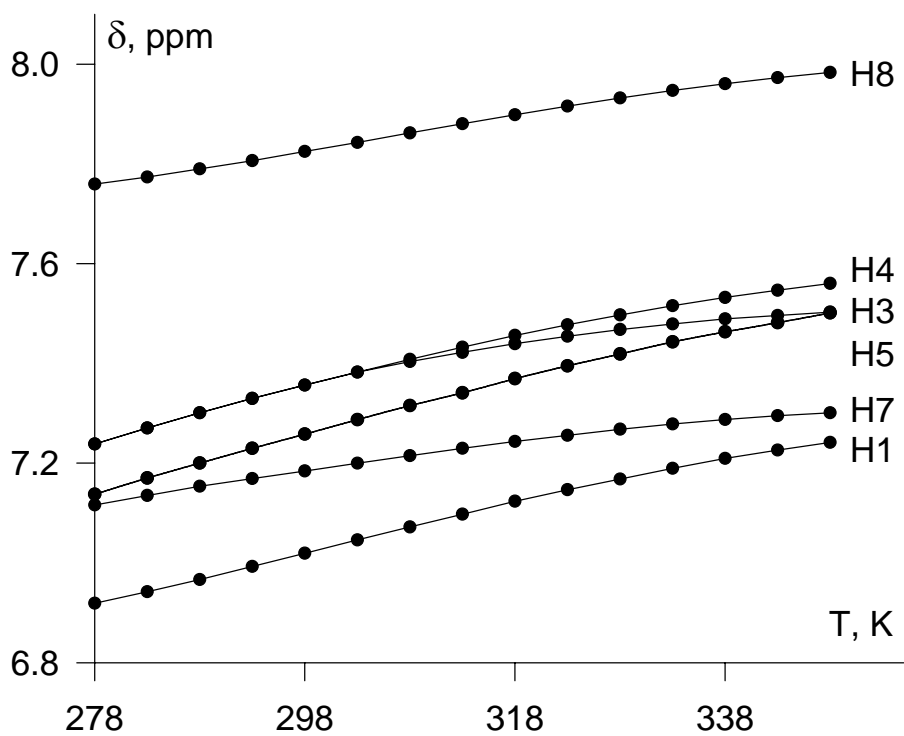
Fig.2 Dependence of proton chemical shifts of AcrH on: (*a*) concentration (*T*=298K); (*b*) temperature (*h₀*=0.496 mM) in aqueous solution, pD 7.5

Fig.3 Dependence of proton chemical shifts of AcrH and EB on: (*a*) concentration of AcrH (*T*=298K, d_0=0.95 mM); (*b*) temperature (h_0=0.55 mM, d_0=0.95 mM) in aqueous solution, pD 7.5
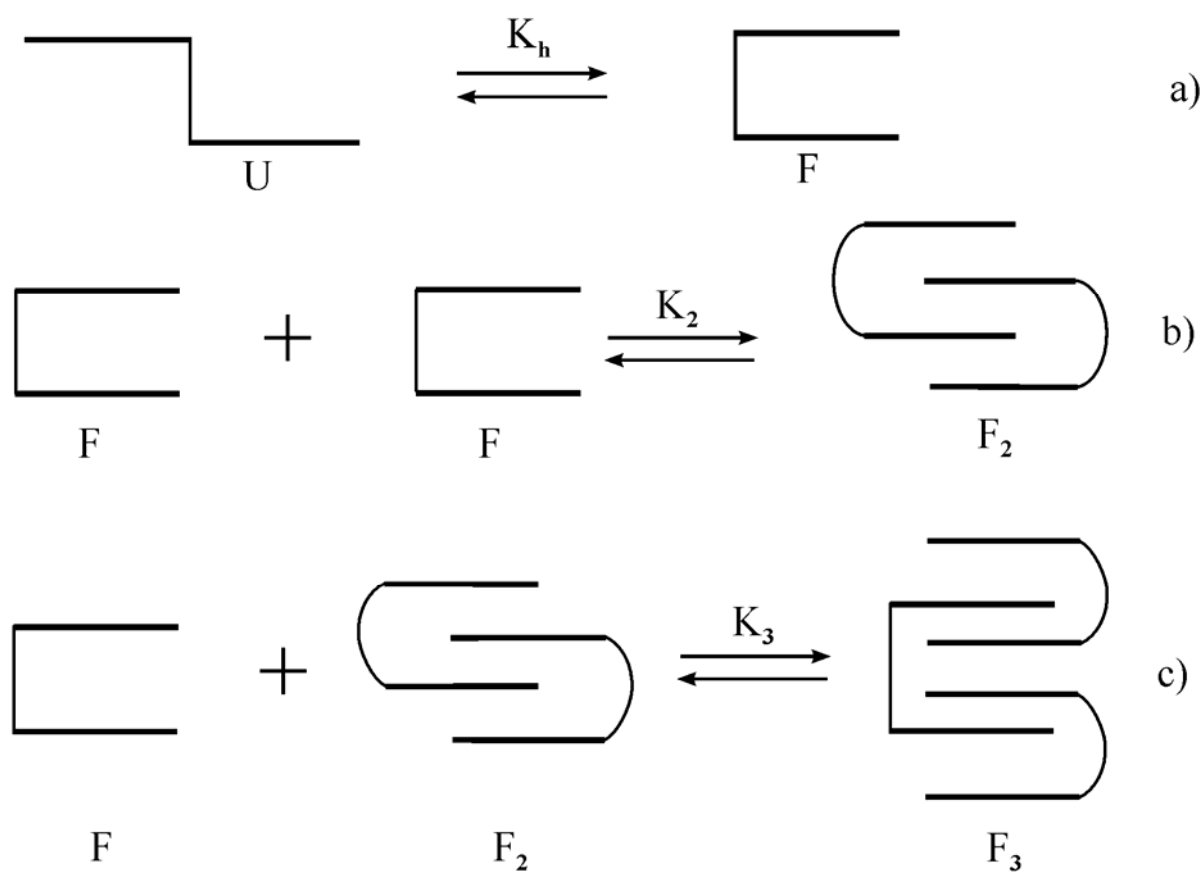
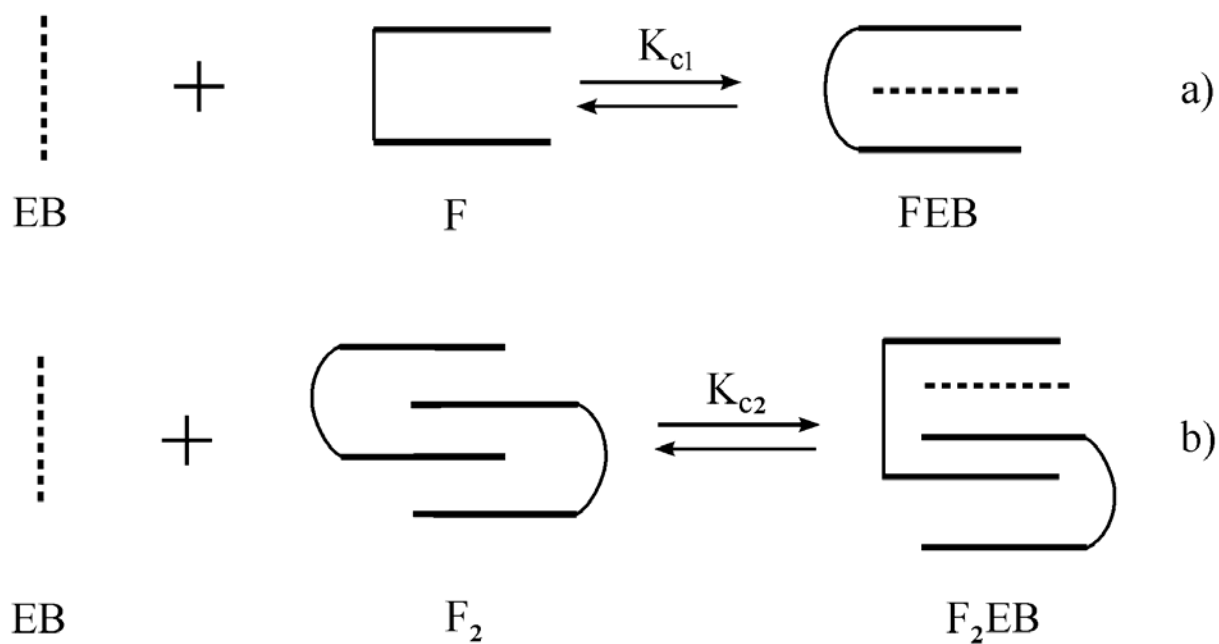Fig.4 Schematic representation of different structures of self-aggregates of AcrH complexes

K_{c1}

K_{c2}

EB

F

FEB

a)

EB

F_2

F_2EB

b)

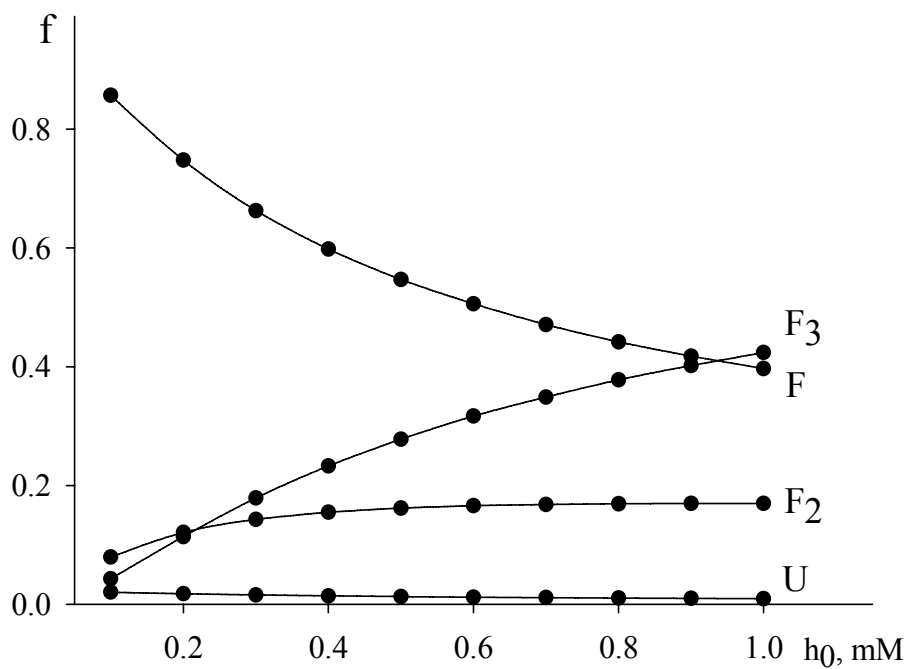Fig.5 Schematic representation of different structures of AcrH-EB hetero-complexes

Fig.6 Relative content (*f*) of different types of AcrH aggregates in solution as a function of AcrH concentration (*h₀*)
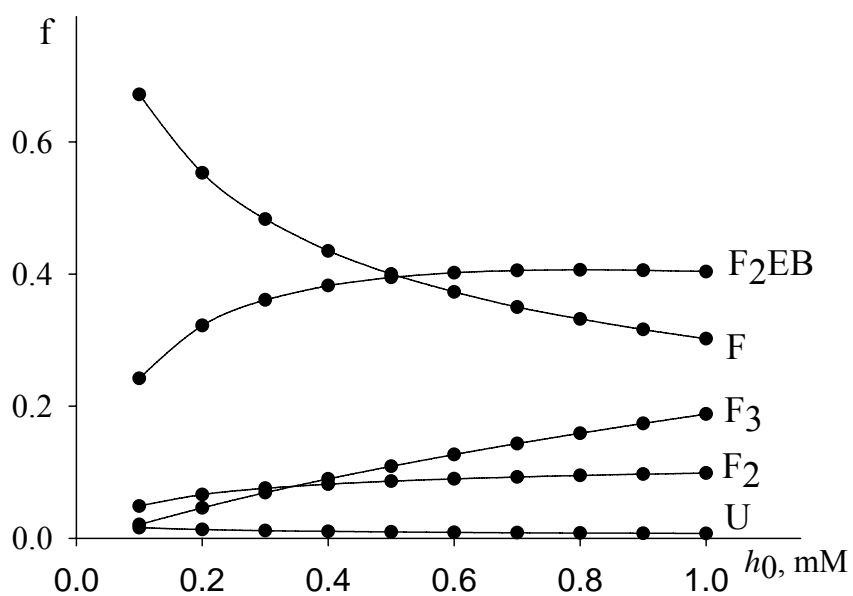

Fig.7 Relative content (*f*) of different types of AcrH aggregates in solution in the presence of EB; the concentration of EB is constant (? mM) and AcrH (*I₀*) is varied.