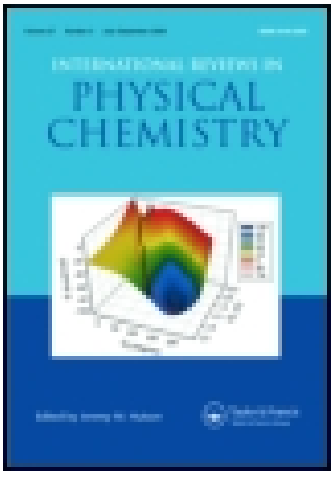

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Hetero-association of aromatic molecules in aqueous solution

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Hetero-association of aromatic molecules in aqueous solution

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Knowledge of the physical chemistry of small molecules complexation (the hetero-association) in aqueous solution is increasingly important in view of the rapidly emerging branch of supramolecular chemistry dealing with the formation of heterogeneous polymeric structures having specific functional roles. In this paper, the 50-year history of scientific studies of hetero-association of heterocyclic aromatic molecules in aqueous solution has been reviewed. Some important correlations of structural and thermodynamic parameters of complexation have been reported based on large data-set of hetero-association parameters accumulated to date. The fundamental problem of 'energetic composition' of π-stacking is extensively discussed. The review has shown that there are some gaps in our understanding of hetero-association, which provides a challenge for further studies in this area.

Keywords: self-association; hetero-association; aromatic molecules; thermodynamics; energetics; structure

Contents

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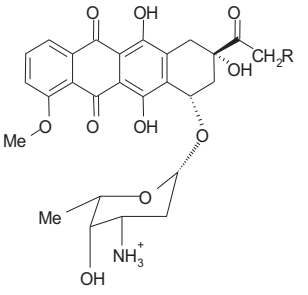
1. Introduction

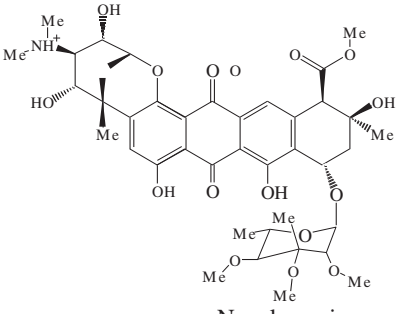
Aromatic compounds constitute an important group of pharmaceutical drugs, extensively utilised in various medicinal applications. Typical examples are the aromatic anthracycline antibiotics (e.g. daunomycin, doxorubicin, nogalamycin, novantrone /mitoxantrone), which are effective against solid tumors and leukemia; quinolone antibiotics (e.g. norfloxacin, ofloxacin), which exert a wide spectrum of antibacterial activity; aromatic vitamins (e.g. riboflavin, nicotinamide), which are used as antioxidants in chemotherapy, and many other aromatic compounds, possessing useful medico-biological properties [1, 2]. Apart from major pharmaceutical applications, aromatic molecules have long been used as model systems to provide insight into the physical interactions, which play an essential role in maintaining the stability of nucleic acids, proteins and many other systems of chemical and biological interest [3–5]. In addition, aromatic–aromatic interactions are currently the focus of great interest due to their role in the formation of supramolecular structures and nano-technological applications [5–7].

Aromatic molecules are represented as rigid planar heterocyclic structures, having an approximately flat chromophore mainly composed of conjugated double bonds (C=C, C=N etc) with delocalised π-electrons (Figure 1). Complexation of such molecules in solution is often considered in terms of a rigid-body interaction with minor contributions from conformational effects such as 'induced fit' interactions well known in host-guest chemistry [8] and polyphenol complexations [9, 10]. It enables the complexation of aromatic molecules discussed in this review to be distinguished from other types of 'structurally flexible' aromatic–aromatic interactions extensively reviewed to date, e.g. [3–5, 8–11]; in addition, this review does not deal with solvent effects on aromatic interactions (e.g. [12, 13]) nor the formation of micellar-type aggregates of aromatic molecules (e.g. [14]).

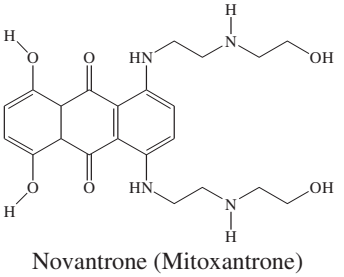
Numerous investigations of the behaviour of aromatic molecules in aqueous solution have shown that for such compounds at pre-micellar concentrations vertical-stacking interactions (also known as face-to-face stacked orientation) are the most important resulting in the formation of 'sandwich'-type aggregates (Figure 2) with more than two monomers in the general case [6, 15–17]. A distinction is usually made



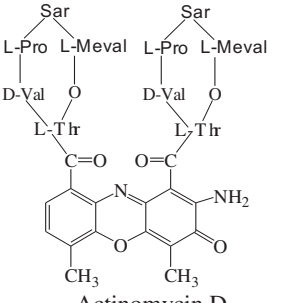
Daunomycin [R=H]
Doxorubicin [R=OH]



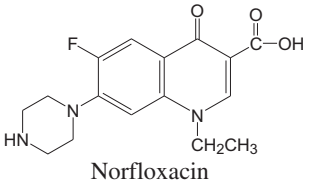
Nogalamycin



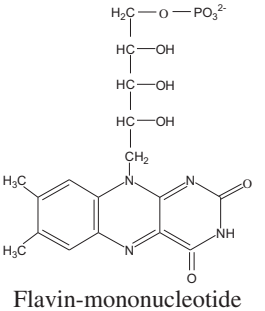
Novantrone (Mitoxantrone)



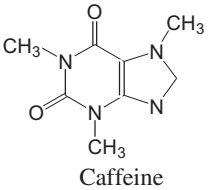
Actinomycin D



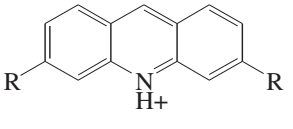
Norfloxacin



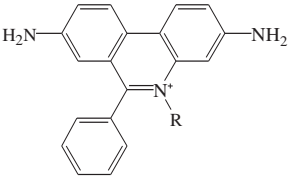
Flavin-mononucleotide



Caffeine



Proflavin [R=NH_2]
Acridine Orange [R=$N(CH_3)_2$]



Ethidium Bromide [R=CH_2CH_3]
Propidium Iodide [R=$(CH_2)_3N^+(CH_2CH_3)_2CH_3$]

Figure 1. Structures of some representative aromatic drug molecules.

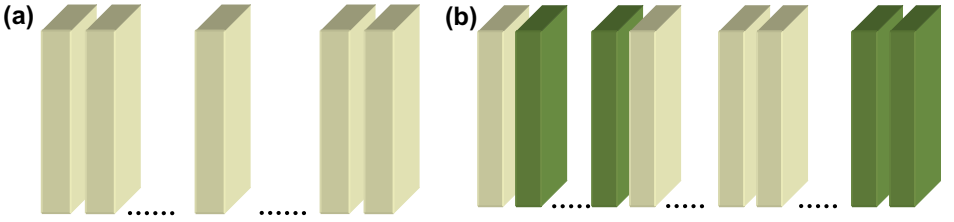


Figure 2. (Colour online) (a) Self-association and (b) hetero-association of planar aromatic molecules.

between self-association (interaction of identical molecules, Figure 2(a)) and hetero-association (interaction of different molecules, Figure 2(b)) [15, 18, 19]. A very specific topical case is the self- and hetero-association of engineered aromatic molecules involving both face-to-face and edge-to-edge interactions [20]. The energies involved in non-covalent interactions are comparable to those of thermal motion; hence, the complexation of aromatic molecules in solution (also known as aggregation, assembly or association) is dynamic in nature, existing in a state of constant rapid formation and disruption of molecular complexes. Although being relatively simple in terms of the physics of interactions, the hetero-association of aromatic molecules provides an excellent template to provide insight into the structure, thermodynamics and energetics of such fundamental and naturally existing molecular constructions as π-stacking.

The self-association of aromatic molecules has been extensively reviewed and various physico-chemical aspects of this process may be considered to be relatively well investigated. The last comprehensive state-of-the-art review on the self-association of aromatic molecules was provided by Martin in 1996 [15], although a few more specific follow-up reviews have appeared since that time [14, 17, 21]. To the best of our knowledge, no review specifically focused on the physico-chemical aspects of the hetero-association of aromatic molecules has been published, except one paper [22] dedicated solely to hetero-association of ionic dyes. Even in the simplest two-component hetero-association system between molecules X and Y, data analysis appears to be complicated because of the experimental observable needs to be taken into consideration and the self-associations of both X and Y and their hetero-association $X+Y$. As a consequence, some aspects of the hetero-association of aromatic molecules, such as the kinetics of aggregation and the influence of metal ions, have not yet been investigated. Charge-transfer interactions and hydrogen bonding between aromatic molecules, commonly investigated in non-aqueous solutions or under specially-arranged conditions, are thoroughly discussed in the literature (e.g. [11, 23–26]) and outside the scope of this review. The review will cover all the currently available analyses of hetero-association in aqueous solutions, starting from probably the first studies dedicated to this subject in the middle of the twentieth century [27–29].

2. Applications of hetero-association

There are at least four mostly well-studied and broadly discussed fields of the applications of hetero-association between aromatic molecules:

(1) *Physical chemistry applications.* The hetero-association of specially selected (synthesised) aromatic molecules has been used as a model system to provide insight into various processes of particular importance in chemistry, such as

arrangement of nucleic acid bases derivatives in stacked complexes [23, 30], binding of drugs to nucleic acids [31] and aromatic residues of proteins [32, 33], protein–DNA and protein–protein interactions [3, 34], exciton and charge-transfer interactions [25, 34, 35], dye chemistry [22], electrostatic complementarity [12, 36], and 'energetic composition' of π-stacking [3, 8, 37].

(2) *Biochemical applications.* The hetero-association in physiological media between aromatic drugs and other aromatic molecules naturally existing in the biosystem or intentionally administered (referred to as the 'guest' molecule) leads to a variety of physico-chemical and biochemical effects such as:
   (a) chemical degradation of the drug due to specific chemical interaction with the guest molecule in the complex. The molecules which exert such effects are sometimes called 'the scavengers' and are extensively reviewed in the biochemical literature [38–40]
   (b) formation of non-covalent hetero-complexes between the drug and the guest molecule leads to diminution of the fraction of active monomeric species of the drug in solution able to bind with bioreceptor and exert a biological effect [39–41]. Such guest molecules, which do not chemically affect the drug, are called 'interceptors' and are currently considered as a means of directed regulation of a biological effect via changing the structure and the concentration of the interceptor [42, 43]. The importance of hetero-association is manifested in a long-established correlation of the hetero-association constant, K_h, with an *in vitro* biological effect for a range of aromatic mutagens [19, 44] – a rare example when a physico-chemical parameter of molecular interaction has a direct link with a biological effect
   (c) complexation between a drug and the guest molecule results in alteration of the bio-kinetics of absorption of the drug in the biosystem and subsequent changes to its medico-biological effect [42, 45, 46]
   (d) the hetero-association of natural aromatic polyphenol molecules with other naturally occurring aromatic substances (such as chlorogenic acid, caffeine and gallic acid) is considered to be a mechanism responsible for or to significantly influence various important biochemical processes, such as co-pigmentation [47, 48] and tea creaming [49]

(5) *Pharmaceutical chemistry application* (*Hydrotropes*). Hetero-association is considered to be the basic mechanism responsible for the widely observed enhancement of the solubility of poorly soluble aromatic drugs in the presence of other aromatic molecules, commonly referred to as hydrotropes [50, 51]

(6) *Medicinal chemistry application.* The effect of hetero-association in certain combinations of aromatic drugs is currently considered to be a potential strategy for the regulation of the medico-biological activity of aromatic drugs in clinical practice, e.g. in reduction of the consequences of overdosing of drugs during chemotherapy [42, 52] or in the production of antimutagenic effects *in vivo* [53–55]. The recent *in vitro* study of cellular effects of a mixture of doxorubicin with fullerene [56], complemented by physico-chemical investigation of their complexation in physiological media [57, 58], has shown that the effect of hetero-association may potentially lie behind new applications of fullerene molecules as elements of combinational chemotherapy of cancer.

A comprehensive review of the well-established fields of the applications of hetero-association summarised above is timely. In addition, there are a growing number of

reports of the successful use of hetero-association of aromatic molecules in the formation of supramolecular structures having specific functional roles (e.g. Refs. [5, 6, 18, 59–61]). It is believed that further understanding of the hetero-association of aromatic molecules will contribute to the future development of the chemistry of functionalised supramolecular structures.

3. Theoretical models of hetero-association

3.1. *General requirements of a model for hetero-association*

The general property of aromatic molecules to form linear aggregates (hydrogen-bonded or π-stacked) in solution allows one to use essentially similar approaches to treat the distribution of aggregates as in supramolecular chemistry. The principal demand of any model of hetero-association is to establish a link between the hetero-association constant, K_h (or enthalpy, ΔH_h, and entropy, ΔS_h) and some experimental observable ξ (e.g. chemical shift in NMR, molecular absorption in spectrophotometry) [18, 61, 62]

$$\xi = \varphi(C^H, \gamma) \tag{1}$$

where C^H denotes the concentrations of possible types of complexes H in solution, and γ is a set of linking parameters, scaling the concentrations C^H to the experimentally detected signals. The concentrations, C^H, can be described analytically by applying the law of mass action, which gives the total concentration of the given type of solute molecule when summed up over all types of complexes in solution. Hence, the C^H values can be found from solution of the system of mass balance equations. Further fitting of the experimental titration curve ξ_{exp} with Equation (1) by adjusting K_h and γ allows their values to be determined when the minimum in discrepancy is reached. Many practical algorithms have been suggested to determine K_h (e.g. [61–64]), and all of them fall within the general scheme outlined above. It follows from Equation (1) that the experimental observable and the mass balance equations are the key components of any model of hetero-association. However, both set of equations depend crucially on the reaction scheme implied by the investigator regarding the processes of complexation in solution and both are commonly linked to the immediate conditions of the experiment. It is this issue that has led to a wide variety of models of molecular hetero-association reported in the physico-chemical literature.

The common approach in chemistry when dealing with non-covalent complexation of two different types of molecules X and Y is, if possible, to reduce the system to simple m:n complexation, where the m and n are finite numbers typically limited to 1 and 2. Such reduced models have been widely used to describe various types of hetero-associations including aromatic molecules, and as they have been thoroughly reviewed (e.g. [15, 18, 61]) they will not be dealt with here. However, the ability of aromatic molecules to bind other molecules on both faces of its chromophore, as well as the use of a wide range of solute concentrations in different experiments, demands that indefinite aggregation of solute molecules needs to be formally accounted for in both the homo- and hetero-association complexes, which, as indicated below, creates the main difficulty in building correct models of hetero-association even in the simplest case of two-component hetero-association. It has been recognised recently that the use of reduced models of self-association (such as the dimer model) of aromatic molecules may not be independent of the solute concentration and, more generally, of the

experimental conditions employed [65]. This should also apply to hetero-association, suggesting that it is mandatory to take into account indefinite aggregation of molecules in both the self- and hetero-association models for aromatic molecules. The main body of current hetero-association studies and those performed during the past decade appears to take indefinite aggregation into account.

3.2. *Analytical approaches to building hetero-association models*

The set of currently developed models of hetero-association can be conditionally divided into three main groups, which differ by the underlying method of mathematical description of the assembly:

- *algebraic approach* in the most complete form is presented in Refs. [64, 66]. The essence of this approach is to write down expressions for the concentrations of sequentially growing complexes using a sequence-generating function method (or any other algebraic method, for instance, combinatorial or probabilistic). The sum of such concentrations in the limit of indefinite association forms a grand partition function, which enables all the necessary properties of molecular distribution in solution to be obtained;
- *matrix approach* in the most complete form is presented in Ref.[67]. The key object is a stochastic matrix raised to the power of the length of the complexes, which is equivalent to enumerating all possible types of molecular complexes in the system, whose contribution to the dynamic equilibrium can be calculated by the methods of matrix algebra. This approach is essentially similar to the general transfer matrix formalism. The algebraic and matrix approaches use analytical evaluations of the main equations of hetero-association model and are commonly referred to as 'analytical' approaches;
- *algorithm approach* is presented in Refs. [62, 68]. All possible molecular complexes are generated by a special computer algorithm, which computes the concentration and contribution to the experimentally observed parameter from every type of complex, thereby ruling out the necessity to deal with analytical expressions.

A general treatment of hetero-association, reducing to all major known partial cases and demonstrating the similarity of the outcome of all these three approaches has been given [67].

3.3. *Evolution of non-cooperative models of hetero-association*

Early models of two-component hetero-association, $X+Y$, used an algebraic approach to derive the main equations and may be referred to as the '1:n' group of models, where the X component does not aggregate but there is no restriction to aggregation of the Y component ($n \in 1\ldots\infty$) (e.g. [27, 32, 69, 70]). Removal of the restriction to aggregation of the X compound was first pioneered by Magar *et al.* [71] and later generalised by Weller *et al*. [72] resulting in the appearance of 'm:n' models. Further generalisation towards N-component hetero-association was first accomplished using either the matrix approach [73] or the algorithmic approach [62], providing a method to study any mixture with any possible types of interacting species. However, in early 2000s [74, 75], it was realised that all the developments cited above, including their partial cases and the matrix approach [73] contain an overestimation of the so-called

'reflected' complexes, i.e. physically identical non-symmetric complexes, resulting from the indefinite summation necessary to obtain the grand partition function in the algebraic approach or from matrix multiplications in the matrix approach. An approximate treatment of the 'reflected' complexes was first accomplished using the algebraic approach [74–76], but it created another difficulty related to the strict treatment of the experimental observable ξ, referred to as the 'edge effects', i.e. the dependence of ξ on the type of neighbouring molecules in the complex. Complete removal of the problem of 'reflected' complexes and 'edge effects' was accomplished with respect to a reaction scheme reduced to no more than two hetero-stacks in all possible hetero-complexes in solution [75, 76]. Removal of all these limitations was made possible by an *N*-component algorithmical model [62, 68, 77] and later by the two-component algebraic hetero-association model [66]. A step forward in expanding the algebraic approach towards *N*-component hetero-association was attempted with respect to mass balance equations, although no link to the experimental observable was provided to account for 'edge effects' [78]. Partial removal of this problem was accomplished [79] resulting in development of the '1:*m*:*n*' hetero-association model specifically adapted to spectrophotometric data. A complete model of *N*-component hetero-association has been developed [67], which accounts for aggregation of all components in solution with formation of all possible types of complexes, which incorporates the matrix and algebraic approaches, which treats strictly the 'reflected' complexes and 'edge' effects, and which reduces to all major known partial cases.

The models reviewed above are all non-cooperative, i.e. the magnitudes of equilibrium self- and hetero-association constants do not depend on the immediate composition of the interacting aggregates. The model of cooperativity in hetero-association is very specific to the system under study. To the best of our knowledge, there are only a few examples in the literature demonstrating the treatment of hetero-association in terms of the empirical cooperativity parameter, introduced with respect to formation of homo- and hetero-aggregates [59, 64, 68, 80].

3.4. *Current state-of-the-art and future perspective in the modelling of hetero-association*

Summarising the information on the development of the hetero-association models given above, it may be concluded that the most strict model of non-cooperative *N*-component hetero-association containing all the necessary equations needed to describe experimental data in solution containing *N* different types of molecules and superior to any other hetero-association model is given in [67]. Reduction of this model to the two-component indefinite '*m*:*n*' hetero-association [66] and some other practically important partial cases, such as '1:*n*' and '1:*m*:*n*' hetero-associations [79] is given in the cited references. A similar strict treatment of hetero-association can also be accomplished within the framework of the *N*-component algorithmical model [62], though it is limited by the fixed maximum length of complexes, which can be handled by the algorithm, and requires more computational time. Based on this summary, it is reasonable to conclude that within the set of common assumptions usually introduced into the reaction scheme (viz. the independence of the equilibrium constant on the length of aggregate and additivity of ξ) and fully covered by the models cited above, it appears that further development of the non-cooperative hetero-association models is not necessary. However, one issue regarding further development of hetero-association models is worth noting.

Table 1. Parameters of hetero-association of aromatic molecules in aqueous solution.

Hetero-system		$\Delta SASA$, 10^{-20} m^2 [237]	ΔA_h, 10^{-20} m^2 [191]	K_h, l mol^{-1}	$-\Delta H_h$, kJ mol^{-1}	$-\Delta S_h$, J (mol K)$^{-1}$	Refs.	Method	Temperature[b]
CAF +	AMD	−379.9	5.91	246 ± 48	27.2 ± 4.3	49 ± 12	[133]	NMR	
	ActIII			297 ± 18	27.0 ± 2.4	43.3 ± 5.0	[143]	NMR	
	AO	−249.4	6.20	264 ± 21	20.4 ± 1.0	22 ± 4	[133]	NMR	
				255.7 ± 4.8			[54]	SP	
				288 ± 37			[53]	SP	
				389 ± 39			[53]	FL	
				258 ± 7			[158]	SP	
				258.4 ± 5.1			[137]	SP	
	DAU	−315.7	5.76	72 ± 4	23.5 ± 1.2	43 ± 4	[133]	NMR	
				149 ± 4			[127]	SP	
	DOX	−317.1	6.81	180 ± 30	22.0 ± 1.0	30 ± 5	[138]	NMR	T = 303 K
				128 ± 10			[42]	SP	
	EB	−258.2	6.44	62 ± 4	22.7 ± 3.0	42 ± 11	[133]	NMR	
				64.6 ± 2.0			[150]	SP	
				60.2 ± 2.3			[161]	SP	
				71 ± 8			[162]	SP	
				84.5 ± 3.5			[137]	FL	
	DAPI			58.2 ± 1.0	46.4 ± 1.7		[150]	SP, MC	
	NOG	−349.9	7.66	180 ± 40	23.0 ± 3.0	33 ± 8	[138]	NMR	T = 303 K
	NOR	−224.9	5.65	30 ± 10	20.3 ± 4.0	40 ± 10	[147]	NMR	
	NOV	−295.4	5.90	256 ± 30	9.3 ± 0.8	−(15.3 ± 4.0)	[145, 146]	NMR	T = 318 K
				356 ± 21	47.3		[42]	SP, MC	
	PF	−219.7	6.77	160 ± 17	24.4 ± 0.5	40 ± 7	[133]	NMR	
				192.4			[160]	SP	
	PI	−304.5	6.57	28 ± 5	21.1 ± 3.6	43 ± 13	[133]	NMR	
				55.2 ± 3.3			[161]	SP	
	MB			200 ± 34	14 ± 10	2.4 ± 0.2	[151, 152]	SP	T = 293 K
	TPT			320 ± 30			[149]	NMR	
					17.6		[148]	MC	
	QM			59 ± 2	0.48	−32.3	[124]	SP, MC	
CAF +	MPTP				0.6 ± 8.4		[126]	MC	T = 303 K
	ICR-170			68.0 ± 2.9			[125]	SP	
	ICR-191			83.0 ± 2.9			[125]	SP	
	Gallic acid			13…33			[170]	NMR	
	Methyl gallate			14…30			[170]	NMR	
	Quercetin			390…775			[170]	NMR	
	Quercetrin			590…820			[170]	NMR	

(*Continued*)

Table 1. (*Continued*).

Hetero-system		$\Delta SASA$, 10^{-20} m^2 [237]	ΔA_h, 10^{-20} m^2 [191]	K_h, l mol^{-1}	$-\Delta H_h$, kJ mol^{-1}	$-\Delta S_h$, J (mol K)$^{-1}$	Refs.	Method	Temperature[b]
	Theaflavin			12…20			[164, 170]	NMR	
	IQ			86.9 ± 2.4	32.4 ± 0.6		[128]	SP	T = 293 K
	IQx			77.0 ± 2.6	18.7 ± 0.2		[128]	SP	T = 293 K
	MeIQx			60.8 ± 1.6	22.1 ± 0.5		[128]	SP	T = 293 K
	7,8-DiMeIQx			95.1 ± 1.5	26.9 ± 0.4		[128]	SP	T = 293 K
	MeIQ			94.4 ± 1.5	29.6 ± 2.3		[128]	SP	T = 293 K
	H_2TTMePP			8390			[157]	FL	
	H_2TMePyP			2900			[157]	FL	
	Cu(II)TTMePP			12060			[157]	FL	
	Cu(II)TMePyP			10610			[157]	FL	
	H_2TCPP			19970			[157]	FL	
	H_2TPPS$_4$			1740			[157]	FL	
	chlorophyll a			11150			[157]	FL	
	Adenine			45.1			[84]	SOL	
THP +	DAU			190 ± 30	24.6 ± 0.4	40 ± 4	[135, 136]	NMR	
				100 ± 5			[127]	SP	
	PF			180 ± 20	34.3 ± 1.0	72 ± 4	[135, 136]	NMR	
	EB			102 ± 6	25.0 ± 2.0	46 ± 6	[135, 136]	NMR	
	NOV			100 ± 10	15.0 ± 1.0	9 ± 3	[135, 136]	NMR	T = 318 K
				223 ± 9			[127]	SP	
	NOR			49 ± 2	21.7 ± 2.0	40 ± 10	[135, 136]	NMR	
	AO			157.0 ± 0.1			[54]	SP	
				300 ± 70			[53]	SP	
				310 ± 50			[53]	FL	
	DOX			365 ± 15			[127]	SP	
	IQ			258.2 ± 7.6	37.4 ± 0.8		[128]	SP	T = 293 K
THP +	IQx			154.7 ± 6.8	15.1 ± 0.1		[128]	SP	T = 293 K
	MeIQx			192.3 ± 12.7	15.7 ± 0.4		[128]	SP	T = 293 K
	7,8-DiMeIQx			108.1 ± 5.1	20.8 ± 0.2		[128]	SP	T = 293 K
	MeIQ			129.1 ± 9.8	34.6 ± 1.0		[128]	SP	T = 293 K
	Adenine			31.1			[84]	SOL	
THP7AA +	DAU			180 ± 30			[134]	NMR	
	PF			250 ± 30			[134]	NMR	
	EB			125 ± 10			[134]	NMR	
THP7PA +	DAU			160 ± 10			[134]	NMR	
	PF			120 ± 15			[134]	NMR	
	EB			120 ± 10			[134]	NMR	
THP7BA+	DAU			150 ± 10			[134]	NMR	
	PF			200 ± 20			[134]	NMR	

	EB			170 ± 20			[134]	NMR	
THB +	DAU			160 ± 40			[134]	NMR	
	PF			250 ± 30			[134]	NMR	
	EB			170 ± 20			[134]	NMR	
	AO			94.5 ± 1.3			[54]	SP	
				141 ± 60			[53]	SP	
				410 ± 110			[53]	FL	
PARA +	DAU			110 ± 20			[134]	NMR	
	PF			155 ± 15			[134]	NMR	
	EB			102 ± 9			[134]	NMR	
	AO			155.3 ± 3.1			[54]	SP	
PTX +	ICR-170			61.2 ± 3.9			[125]	SP	
	ICR-191			87.9 ± 3.3			[125]	SP	
	DAU			173 ± 5			[127]	SP	
	DOX			173 ± 5			[127]	SP	
	NOV			218 ± 7			[127]	SP	
	IQ			84.9 ± 2.1	34.1 ± 1.3		[128]	SP	$T = 293$ K
	IQx			70.9 ± 2.6	20.8 ± 1.0		[128]	SP	$T = 293$ K
	MeIQx			60.4 ± 2.4	21.2 ± 0.7		[128]	SP	$T = 293$K
	7,8-DiMeIQx			96.1 ± 4.0	29.5 ± 1.0		[128]	SP	$T = 293$K
	MeIQ			101.1 ± 1.8	39.8 ± 0.9		[128]	SP	$T = 293$K
FMN +	AMD	−468.0	10.62	890 ± 330	33.5 ± 6.0	49.6 ± 9.9	[186]	NMR	
	CAF	−283.3	6.32	161 ± 26	24.3 ± 2.9	40 ± 16	[153]	NMR	
	THP			180 ± 40	33.5 ± 2.0	70 ± 10	[135]	NMR	
	DAU	−404.7	12.32	453 ± 28	35.8 ± 5.4	69.5 ± 9.1	[184, 187]	NMR	
	EB	−307.5	14.84	640 ± 75	33 ± 2	57 ± 7	[190]	NMR	
	NOG	−468.9	11.24	790 ± 50	30 ± 1	44 ± 4	[19]	NMR	$T = 303$ K
	NOR	−309.2	9.02	180 ± 80	25.5 ± 2.0	42 ± 10	[188]	NMR	
	NOV	−429.8	16.25	33700 ± 100	55 ± 6	87 ± 11	[185]	NMR	$T = 303$ K
	PF	−252.1	13.78	920 ± 80	41 ± 3	80 ± 10	[190]	NMR	
	TPT			1300 ± 200			[189]	NMR	
RBF +	CAF			69 ± 4			[154]	SP	
	NAS			25 ± 4			[154]	SP	
DAU +	AO	−334.6		2470 ± 440	24.5 ± 1.4	17 ± 4	[74]	NMR	$T = 303$ K
	EB	−351.6	11.38	2700 ± 440	42.5 ± 3.3	74 ± 12	[208, 211]	NMR	$T = 303$ K
	EMB			660 ± 100	23.5 ± 2.0	25 ± 5	[209]	NMR	
	EDC			320 ± 65	18.1 ± 3.0	13 ± 4	[209]	NMR	
	NOG	−417.5		2700 ± 1200	33 ± 4	40 ± 8	[19]	NMR	$T = 303$ K
	NOR	−353.8		460 ± 68	28.3 ± 1.9	44 ± 3	[168]	NMR	
	NOV	−406.4	13.39	3333 ± 350	59.5 ± 6.2	95 ± 10	[213]	NMR	$T = 318$ K
	PF	−307.2	12.92	1650 ± 120	34.9 ± 1.1	53 ± 3	[74]	NMR	$T = 303$ K
	PI	−415.6	8.73	560 ± 60	37.0 ± 7.0	70 ± 15	[208, 210]	NMR	$T = 303$K

(*Continued*)

Table 1. (*Continued*).

Hetero-system		$\Delta SASA$, 10^{-20} m^2 [237]	ΔA_h, 10^{-20} m^2 [191]	K_h, l mol^{-1}	$-\Delta H_h$, kJ mol^{-1}	$-\Delta S_h$, J (mol K)$^{-1}$	Refs.	Method	Temperature[b]
	AMD			2750 ± 1100	39 ± 5	58 ± 12	[214, 215]	NMR	
	TPT			2000 ± 340			[189]	NMR	
NMD +	TPT			45 ± 10			[189]	NMR	
	DAU			64 ± 5	26 ± 4	53 ± 14	[197]	NMR	
	NOV			35±5	23 ± 3	44 ± 12	[197]	NMR	T = 312 K
	NOR			23 ± 5	23.5 ± 3.0	53 ± 10	[188]	NMR	
	CAF			7.12 ± 0.04	18.7 ± 1.9	49 ± 6	[225]	NMR	
	FMN			65 ± 12	18.8 ± 4.9	29.8 ± 8.0	[182]	NMR	
drug + drug	NOG + NOR	−378.9		760 ± 280	23.0 ± 2.1	20 ± 3	[168]	NMR	T = 303 K
	NOG + NOV	−363.0		5200 ± 2500	34±4	36 ± 7	[19]	NMR	T = 318 K
	NOG + AMD			3600 ± 600	35 ± 6	47 ± 15	[19]	NMR	T = 303 K
	TPT + PF			3000 ± 300			[189]	NMR	
	TPT + EB			1450 ± 300			[189]	NMR	
drug + drug	EB + PI			123 ± 21	24 ± 4	39 ± 11	[218]	NMR	
	Rutin + rifampicin			1.28×10^4			[220]	Voltammetry	
	PF + NOR			1.41×10^4	38.37	46.56	[207]	FL	
	PF + EB			690 ± 60	24.7 ± 1.5	33.7 ± 4.8	[75, 76]	NMR	
	NOV + AMD			2500 ± 1000	28 ± 7	30 ± 8	[214]	NMR	
AO +	Purine			~2			[54]	SP	
	6-Methylpurine			0			[54]	SP	
	6-Chloropurine			0			[54]	SP	
	1-Methyl-6-aminopurine			~14			[54]	SP	
	3-Methyl-6-aminopurine			44.5 ± 0.2			[54]	SP	
	2-Amino-6-methoxypurine			~2			[54]	SP	
	Guanine monophosphate			0			[54]	SP	
	Xanthine			0			[54]	SP	
	1-Methylxanthine			79.2 ± 2.0			[54]	SP	
	3-Methylxanthine			~10			[54]	SP	
	7-Methylxanthine			0			[54]	SP	
	Aminophylline			596.2 ± 6.5			[54]	SP	
	Uric acid			0			[54]	SP	
	1-Methyl-8-oxyxanthine			0			[54]	SP	
	7-Methyl-8-oxyxanthine			0			[54]	SP	
	1,3-Dimethyl-8-oxyxanthine			38.5 ± 2.2			[54]	SP	
	1,3-Dimethyl-8-chloroxanthine			412.5 ± 11.7			[54]	SP	
	1,3,7,9-Tetramethyl-8-oxyxanthine			551.5 ± 9.0			[54]	SP	
	Adenosine			129 ± 10			[53]	SP	
				138 ± 27			[53]	FL	

CHL +	AO	$(5.6 \pm 0.3) \times 10^5$		[205]	SP	$T = 293$ K
		$(8.4 \pm 2.1) \times 10^5$		[205]	FL	$T = 293$ K
	QM	$(3.1 \pm 0.5) \times 10^5$		[205]	SP	$T = 293$ K
		$(3.3 \pm 0.3) \times 10^5$		[205]	FL	$T = 293$ K
	DOX	$(3.7 \pm 0.2) \times 10^5$		[205]	SP	$T = 293$ K
		$(2.9 \pm 0.3) \times 10^5$		[205]	FL	$T = 293$ K
	Crystal violet	5.6×10^5		[201]	FL	
CHL +	ICR-170	5.3×10^5		[201]	FL	
	ICR-191	$(2.8 \pm 0.5) \times 10^5$		[206]	SP	
		$(3.0 \pm 0.3) \times 10^5$		[206]	FL	
	Rhodamine 6G	3.3×10^5		[201]	FL	
	Quinacrine	6.3×10^4		[201]	FL	
	Trp-P-2	5.9×10^4		[201]	FL	
		$(6.65 \pm 1.39) \times 10^3$		[44]	SP	
	Trp-P-1	5.6×10^4		[201]	FL	
		$(12.74 \pm 1.94) \times 10^3$		[44]	SP	
	Rhodamine B	5.6×10^4		[201]	FL	
	Aflatoxin B1	$(7.0 \pm 2.0) \times 10^5$		[203]	FL	
	Aflatoxin B2	$(5.2 \pm 0.3) \times 10^5$		[45]	FL	
	Dibenzo[*a*,*l*]pyrene	$(5.3 \ldots 10) \times 10^5$		[204]	FL	
	MeIQx	$(10.25 \pm 1.77) \times 10^3$		[44]	SP	
	PhIP	$(8.00 \pm 2.31) \times 10^3$		[44]	SP	
	IQ	$(7.30 \pm 1.69) \times 10^3$		[44]	SP	
	MeIQ	$(5.83 \pm 0.29) \times 10^3$		[44]	SP	
	TriMeIQx	$(5.50 \pm 0.52) \times 10^3$		[44]	SP	
	7,8-DiMeIQx	$(4.25 \pm 1.21) \times 10^3$		[44]	SP	
	4,8-DiMeIQx	$(3.17 \pm 0.85) \times 10^3$		[44]	SP	
	NO_2IQ	<500		[44]	SP	
	Glu-P-2	$(6.63 \pm 3.06) \times 10^3$		[44]	SP	
	Glu-P-1	$(3.07 \pm 1.59) \times 10^3$		[44]	SP	
N-hydroxy-IQ +	Epigallocatechin	1100		[39]	SP	
	Epigallocatechin-3-gallate	1000		[39]	SP	
	Epicatechin-3-gallate	880		[39]	SP	
	Catechin	950		[39]	SP	
	Caffeine	<500		[39]	SP	
Nucleic acid bases	AMP + tyramine	4.73 ± 0.11	18.73 ± 7.30	[97]	MC	
	6-Methylpurine + DMT (N,N-dimethyltryptamine hydrogenic oxalate)	2.5 ± 0.3	22 ± 2	[69]	NMR	
		18 ± 2	20 ± 3	[69]	OSM	
	6-Methylpurine + serotonin	2.7 ± 0.3	22 ± 2	[69]	NMR	
		20 ± 2	21 ± 3	[69]	OSM	
	6-Methylpurine + mescaline	2.6 ± 0.3	21 ± 2	[69]	NMR	
		17 ± 2	16 ± 3	[69]	OSM	

(*Continued*)

Table 1. (*Continued*).

Hetero-system		$\Delta SASA$, 10^{-20} m^2 [237]	ΔA_h, 10^{-20} m^2 [191]	K_h, l mol^{-1}	$-\Delta H_h$, kJ mol^{-1}	$-\Delta S_h$, J (mol K)$^{-1}$	Refs.	Method	Temperature[b]
	AMP + CAF			7.3 ± 1.2			[111]	NMR	T = 306 K
	ATP + CAF			24	16 ± 3	29±10	[115]	NMR	
	ATP + DOX			380	39.7	80	[33]	PRT	T = 310 K
	AMP + DOX			205			[33]	PRT	T = 310 K
	ADP + DOX			320			[33]	PRT	T = 310 K
	NAD + DOX			190			[33]	PRT	T = 310 K
	Adenine + DOX			185			[33]	PRT	T = 310 K
	Cytidine + DOX			35			[33]	PRT	T = 310 K
	Uridine + DOX			25			[33]	PRT	T = 310 K
	Inosine + DOX			100			[33]	PRT	T = 310 K
	Guanosine + DOX			120			[33]	PRT	T = 310 K
	Adenosine + DOX			160			[33]	PRT	T = 310 K
	2′-Deoxyadenosine + DOX			160			[33]	PRT	T = 310 K
	AMD + Deoxyguanosine			2600	43.1 ± 3.3	82.8±10.5	[108]	SP	
	dA + dT			3…6			[87]	OSM	
	dG + dC			4…8			[87]	OSM	
	dA + dC			3…6			[87]	OSM	
	dA + U			3			[87]	OSM	
	dT + dC			0.91			[87]	OSM	
	Pyrimidine + Purine			0.41 ± 0.06			[98]	NMR	T = 308K
	Pyrimidine + 6-methylpurine			0.74 ± 0.05			[98]	NMR	T = 308K
	1,4-Dimethylcytosine + 6,9-dimethyladenine			10	25.9	69	[70]	NMR	T = 307 K
Nucleic acid bases	1,3-Dimethyluracil + 6,9-dimethyladenine			6	18.8	49	[70]	NMR	T = 307 K
	1,3-Dimethyluracil + 1-methyl,4-methylaminopyrazolo-[3,4-d]pyrimidine			3.1	16.7	49	[70]	NMR	T = 307 K
	1,4-Dimethyluracil + 6,9-dimethyladenine			4.3	18	48.1	[70]	NMR	T = 307 K
	1,4-Dimethyluracil + 1-methyl,4-methylaminopyrazolo-[3,4-d]pyrimidine			7.4	20.1	50.2	[70]	NMR	T = 307 K
	2,4-Dimethyluracil + 6,9-dimethyladenine			9.2	25.1	65.7	[70]	NMR	T = 307 K
	2,4-Dimethyluracil + 1-methyl,4-methylaminopyrazolo-[3,4-d]pyrimidine			9	25.1	66.1	[70]	NMR	T = 307 K
	Purine + uridine			3.0 ± 1.3			[99]	OSM	
	CAF + uridinemonophosphate			40.3			[116]	NMR	
	CAF + cytosinemonophosphate			62.8			[116]	NMR	
	CAF + guanosinemonophosphate			96.7			[116]	NMR	
	CAF + AMP			133			[116]	NMR	
	Adenine + Uracil			4.16			[84]	SOL	
	Adenine + 5-fluorouracil			5.44			[84]	SOL	
	Adenine + 5-chlorouracil			6.33			[84]	SOL	

	Adenine + 5-bromouracil	7.04			[84]	SOL	
	Adenine + Uridine	4.03			[84]	SOL	
	Adenine + 5-Iodouridine	9.63			[84]	SOL	
	Adenine + 1,3-dimethyluracil	7.14			[84]	SOL	
	Adenine + Thymine	7			[84]	SOL	
Nucleic acid bases	Adenine + Cytosine	4.89			[84]	SOL	
	Adenine + 8-methoxycaffeine	80.2			[84]	SOL	
	Adenine + Purine	11.3			[84]	SOL	
	Adenine + Adenosine	21			[84]	SOL	
	Adenine + Deoxyguanosine	18.8			[84]	SOL	
	Adenine + Inosine	8.25			[84]	SOL	
	Adenine + Quinoxaline	17.8			[84]	SOL	
	Adenine + Chloroquine	39.2			[84]	SOL	
	Pyrimidine + 5-methylpyrimidine	0.23 ± 0.02			[85]	NMR	
	Pyrimidine + 4,6-dimethylpyrimidine	0.32 ± 0.04			[85]	NMR	
	5-Methylpyrimidine + 4,6-dimethylpyrimidine	0.51 ± 0.04			[85]	NMR	
	Inosine + Cytidine	4.9 ± 2.0			[71]	OSM	$T = 323$ K
	2-Aminopurine + thymidine	5.31	11.5	24.7	[100]	FL	$T = 303$ K

[a]Data have been collected from published material containing quantitative information on hetero-association under comparable solution conditions: pH 6.5…7.5, aqueous solution, medium or high ionic strengths

[b]The temperature equals to $T = 295 \ldots 300$ K, unless specified

The success in building the model of hetero-association using the algebraic or matrix approach is based on the ability to write the mass balance equations and the equation for ξ in a closed analytical form. The overwhelming majority of known applications of hetero-association can be successfully modelled by the analytical approaches (e.g. [32, 42, 61, 64, 67, 73]). Nevertheless, any complication introduced into the reaction scheme may violate analytical derivation of the basic equations, resulting in failure of the analytical model to describe the system. Immediate solution of the problem will always be provided by the algorithmical model, which does not deal with equations at all and is able to treat a reaction scheme of any complexity. We suggest that a similar problem may arise in the future with respect to application of some experimental methods in hetero-association studies, which do not follow the nearest-neighbour principle in writing ξ for each particular hetero-complex, C^{H}, in Equation (1) (such as those measuring diffusion of molecules), thus presumably making great difficulty in obtaining ξ in an analytical form. Another complication might occur if the condition is broken of equal K for self-association and equal K_h for hetero-association and the introduction of the recently developed profile of equilibrium constant [81, 82] and/or the introduction of the cooperativity parameter into the main equations, which is currently considered to be of special importance in supramolecular chemistry [59, 64].

Also, we need to highlight the recent appearance of a novel challenge to further development of the hetero-association models towards description of two- and three-dimensional aggregation of aromatic molecules due to mixed type of interactions involving face-to-face and edge-to-edge bindings [20]. Hence, the story of the development of hetero-association models needs to continue.

4. Structure and thermodynamics of hetero-association

Systematic investigations of hetero-association have been performed for a number of different aromatic systems. A list of hetero-association parameters for these aromatic systems is given in Table 1, providing, where appropriate, information on the equilibrium constant of hetero-association (K_h), associated thermodynamic quantities (ΔH_h and ΔS_h), the change in solvent-accessible surface area ($\Delta SASA$), the change in the area of overlap of the chromophores of interacting molecules in the hetero-complexes (ΔA_h) and the experimental method used.

Investigations of hetero-association have been mainly performed for the following sets of aromatic molecules.

4.1. *Hetero-association of derivatives of nucleic acid bases*

The hetero-association of derivatives of nucleic acid bases historically represents the first example of systematic investigation of the hetero-association phenomenon for aromatic molecules. The aim of these investigations, as in the case of self-association, was to gain deeper understanding of the stacking interactions in stabilising nucleic acids. Quantitative parameters of the hetero-association in solution of various combinations of derivatives of nucleic acid bases have been reported for purine–purine, purine–pyrimidine and pyrimidine–pyrimidine interactions [27, 70, 71, 83–89]. Derivatives of nucleic acid bases tend to form stacking-type complexes in aqueous solutions with minor contribution, if any, from in-plane hydrogen-bonding (base-pairing), as deduced from the monotonic shift of aromatic protons to lower frequency in NMR [70, 83, 85, 86, 88, 89] or the hypochromic effect in UV–vis spectroscopy [90–92] on increasing concentration or decreasing

temperature, and also supported by high-level quantum-chemical computations [93–96]. Lowering the pH of the medium leads to protonation of purine and pyrimidine rings, which is considered to result in weakening of their stacking ability with other molecules [70, 97]. The stacking-type of hetero-association does not disappear even in organic solvents at very low (pH ~1) or very high (pH ~13) pH [89].

In general, the derived magnitudes of the equilibrium hetero-association constants (K_h) have values in between the self-association constants of the interacting compounds [23, 27, 70, 85–87, 98, 99], except for a very few reports [27, 84, 100] which, however, were not linked to the physico-chemical properties of the interacting molecules. It was found that K_h for the interaction of purine derivatives is, on average, higher than that for purine–pyrimidine which, in turn, is much greater than for pyrimidine–pyrimidine interactions [23, 84, 87, 98]. Based on these results, it was concluded that hetero-association of derivatives of nucleic acid bases in water is largely governed by van der Waals interactions between the molecules in hetero-complexes, which are known to correlate with the dimensions of aromatic portion of the interacting molecules [11, 86, 87]. This conclusion is supported by the fact that on average the magnitude of thermodynamical parameters increases with the number of overlapping aromatic rings in the hetero-complex, viz. 2:2 > 2:1 > 1:1 (Figure 3). The magnitude of interaction increases with addition of divalent cations [88] in a qualitatively similar way to that known for self-association [17], although the influence of added metal ions has never been studied systematically with respect to the hetero-association of nucleotides and nucleosides and their derivatives.

The tendency to hetero-association was found to depend strongly on the type of substituents; for example, K_h increases with methylation of purine and pyrimidine bases, which was interpreted in terms of either greater hydrophobic or van der Waals contribution to the thermodynamics of complexation [70, 83–86, 98]. However, methylation also leads to alterations in the structures of 1:1 hetero-complexes due to the steric demands to accommodate the methyl group within the complex (an increase in distance and induction of partial overlap between the chromophores) [85, 101]. As a result of these observations, it was suggested that electrostatics plays an important role in

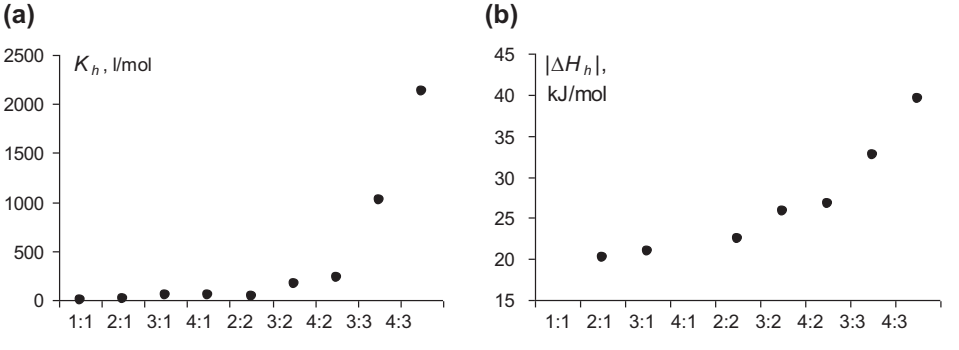


Figure 3. Dependence of mean K_h (a) and ΔH_h (b) on the number of fused aromatic rings *m*:*n* in the structure of $X(m)$ and $Y(n)$ molecules forming hetero-complexes. Mean values were calculated by averaging over data-set collected from Refs. 1:1 [85, 87, 97], 2:1 [70, 84, 87, 97, 99, 100, 188, 225], 3:1 [154, 182, 197], 4:1 [33, 189, 197], 2:2 [69, 84, 135, 147, 166], 3:2 [42, 53, 54, 108, 125, 127, 128, 132–135, 137, 143, 145, 150, 154, 159, 161, 188], 4:2 [33, 42, 127, 134, 135, 138, 149, 168], 3:3 [75, 184–186, 190, 214], 4:3 [74, 189, 210, 211, 213–215] under conditions [pH ~7, $T = 25 \ldots 30$ °C, aqueous solution]. For the source data see also Table 1.

determining the structural features of the hetero-complexes [85, 89] and was later suggested as a general feature of 'electrostatic complementarity' in aromatic stacking [12, 102]. Similarly, 'structural complementarity' was also suggested as being important for the hetero-association of various tautomeric and isomeric forms of nitrogen bases in solution [70]. The same effect as discussed above for the methyl group is also produced by attaching other alkyl groups to the chromophore of the molecule [84, 103], which was interpreted in terms of the assumption that the carbon-bonded alkyl groups enhance the polarisability of the heteroaromatic system and consequently base-stacking through dipole-induced dipole interaction.

Halogen substituents exhibit a marked enhancing effect on hetero-association, as demonstrated for the interaction of 5-halogenated uracils with adenine [84]. The enhancing effect was found to be of the same order as that for increasing the van der Waals radii for the substituents, as well as the increasing polarisability of the molecule, indicating the importance of the contribution of electronic effects and London dispersion forces in stabilising complexes in aqueous media. The addition of a sugar moiety to the structure of pyrimidine or purine commonly reduces the hetero-association constant due to steric hindrance; the existence of a 2′-OH group in the sugar reduces K_h even further [84]. Although stacking interactions in aqueous media are generally considered to be non-specific, the strong dependence of the tendency for hetero-association in a homologous series of compounds on the type of side chains indicates that some structural features may discriminate between the interactions of some pairs of compounds from other pairs [84].

It should be noted that the structures and types of side chains seem to affect the magnitude of K_h to a greater extent than changes in the type or position of heteroatoms in purine or pyrimidine chromophores. Studies on hetero-association involving pyrazole analogues of nucleic acids [70], 2-ring quinoxaline [84], chloroquine [84] and indole [32] derivatives, some aromatic aminoacids [32], and 1-ring sodium benzoate and salicylate [104] reported magnitudes of binding constants similar to or within the same region of discrepancy as for the corresponding purines or pyrimidines.

There are only few reports available in literature on the measurements of enthalpy (ΔH_h) and entropy (ΔS_h) of hetero-association of nucleotides and nucleosides and their derivatives (e.g. [70, 100]), and so general conclusions on the interrelation between these quantities and the physico-chemical properties of the interacting molecules cannot be made. The magnitudes of ΔH_h and ΔS_h fall in the same region of values as previously measured for self-association [23, 27, 100], and are of negative sign, suggesting the importance of van der Waals (of which dispersive forces are considered as the most important) and, possibly, charge-transfer interaction in the thermodynamics of hetero-association. In this respect, it is worth noting that there is no apparent indication of the importance of charge-transfer in hetero-complexes of nucleic acid bases derivatives in neutral aqueous solution, except for a few reports based on analysis of UV–vis spectra (e.g. [101]).

In general, studies of the hetero-association of derivatives of nucleic acid bases do not lead to any new principals about molecular interactions and the results are in line with information deduced from much more extensive and detailed self-association studies of these molecules [17, 23]. The hetero-association of derivatives of nucleic acid bases is mainly a matter of historical interest.

4.2. *Hetero-association of aromatic drugs with derivatives of nucleic acid bases*

The initial aim of investigations into the complexation of derivatives of nucleic acid bases with aromatic drug molecules was to search for a pattern in molecular interactions, in order to utilise this information in studies of aromatic ligand binding with nucleic acids. A more specific goal was linked to the complexation of various drugs to ATP whose intracellular concentrations *in vivo* may reach very high values ~100 mM [105].

The antibiotic actinomycin D [106–109] and the dye ethidium bromide [110] were the first DNA-complexing agents investigated systematically in their binding with nucleosides and nucleotides. It was found that hetero-association of these molecules may be characterised by the stacking type of association, which had led to the suggestion of an intercalation type of binding of these compounds with DNA. Conclusions about the predominant location of the chromophore of the base above the chromophore of the ligand were made from the calculated values of the induced proton and carbon NMR chemical shifts of the bases. In some cases, the approximate orientation of the base with respect to the antibiotic in 1:1 complex was estimated on the basis of comparison between experimental and theoretically calculated proton magnetic shielding [69, 111]. In the majority of cases, stacking between the ligand and mononucleotides obtained in aqueous solution is in line with crystallographic studies [106]. It is important to note that a typical distance, $d = 0.34$ nm, between the chromophores of the drug and mononucleotide in the 1:1 complex comes from X-ray diffraction analysis [112]. Similar results were later obtained for the antibiotics daunomycin [113] and doxorubicin [33], xanthines [111, 114–116], psychotomimetic drugs [69], quinolones [117], polyphenols [118], and other aromatic drugs. It was shown that hetero-association is enhanced by the presence of divalent metal ions due to interaction of the drug with nucleoside phosphates via the ion, as demonstrated for the fluoroquinolone antibiotics [117]. However, the exact mechanism of stabilisation depends on the structure of interacting molecules and does not always require the presence of the phosphate group [119].

The thermodynamical pattern of the binding of drugs with nucleosides and nucleotides appears to be qualitatively the same as previously found for the hetero-association of derivatives of nucleic acid bases, viz. the magnitudes of the hetero-association parameters are intermediate between the corresponding self-association parameters of the interacting molecules and the values of ΔH_h and ΔS_h [33, 111] are both negative. It was realised, however, that the average magnitudes of K_h and ΔH_h are higher in absolute value than those for the hetero-association of the derivatives of nucleic acid bases, which was suggested to be due to a more extended aromatic system of the drug (typically 2–4 aromatic rings in the chromophore) [33, 69]. The key role of hydrophobic and van der Waals interactions in stabilisation of hetero-complexes was postulated [33, 108]. In particular, the importance of hydrophobic interactions was recognised due to increasingly negative entropy of hetero-association in the presence of increasing methanol concentration [108], and the correlation between the K_h and the size of the ring systems of the interacting molecules [69]. However, the electrostatic factor is important in determining the value of K_h [33, 69, 118], unlike the hetero-association of derivatives of nucleic acid bases. A typical example is the incremental enhancement of K_h for binding of the positively charged doxorubicin to the negatively charged adenosine-phosphates in the series AMP < ADP < ATP [33] and the relatively strong binding of the flavylium cation with ATP [118]. The electrostatic factor was also suggested as

important in differentiating the binding of ATP with caffeine, theophylline and theobromine [115], which was correlated by the authors with the π-electron deficiency of the xanthine molecule.

Although characterised relatively well in the past, the use of nucleosides and nucleotides as a model systems to complex with aromatic drug molecules is still extensively used to get insight into various processes of biochemical importance.

4.3. *Hetero-association of aromatic drugs with aromatic interceptor molecules and hydrotropes*

Early investigations of hetero-association mainly provided supporting information to investigations of the complexation of aromatic antibiotics with DNA, and typically did not have their own scientific importance. However, the first reports about a decrease/increase in the toxicity of antitumor aromatic drugs in the presence of caffeine (for reviews see [19, 43]) appeared at the end of the 1970s and early 1980s, which stimulated investigations into the mechanism of the action of caffeine and other methylxanthines. The hetero-association with xanthines (termed the 'interceptor' action) had been first suggested as the principal factor altering the biological activity of the drugs [52, 120, 121] and carcinogens [122, 123], which was supported later on by numerous *in vitro* studies [121, 124–128]. The search for other aromatic interceptor molecules resulted in discovering the interceptor effect of chlorophylline (CHL) [41, 44] and riboflavin [vitamin B2 (RBF) or its analogue, flavin-mononucleotide (FMN)] [129–131]. Subsequent physico-chemical investigations of the hetero-association of aromatic molecules with potential interceptors, already existing or newly synthesised, confirmed that hetero-association is the primary mechanism responsible for the observed biological effects [19, 43].

In addition to the interceptor effect, hetero-association has long been known as the molecular mechanism for enhancing the solubility of various aromatic drugs thereby making hetero-association important in pharmaceutical chemistry. In particular, analysis of quantitative thermodynamic information on hetero-association has established a correlation between K_h (or other binding parameters) and changes in the *in vitro* biological effect for various aromatic mutagens and antibiotics [44, 132], and with solubility enhancement for various aromatic hydrotropes [50, 51].

In summary, the data accumulated on the hetero-association of drugs with interceptor molecules constitute the largest portion of the total published volume of information on hetero-association, which is discussed in more detail.

4.3.1. *Drug-xanthine system*

Hetero-association in Drug-xanthine systems has been extensively studied by NMR, UV–vis, Fluorescence spectroscopies, titration microcalorimetry, partitioning in water–organic mixture, molecular modelling and other techniques for the following aromatic drugs: daunomycin (DAU) [127, 133–136], doxorubicin (DOX) [33, 42, 127, 137–139], actinomycin D (AMD) and its derivatives [133, 140–143], benzene and its derivatives [122, 144], novantrone/mitoxantrone (NOV) [42, 127, 135–137, 145, 146], nogalamycin (NOG) [138], norfloxacin (NOR) [135, 136, 147], camptothecins [139, 148, 149], quinoxaline [84], chloroquine [84], DAPI [150], phenothiazines [121, 151, 152], riboflavin [135, 153–155], porphyrins [156, 157], acridine mutagens [53, 54, 124, 125, 133–136, 158–160], phenanthridine mutagens [133–137, 150, 160–162],

imidazoquinoline-type (IQ) aromatic amines [128], aromatic neurotoxins [126], poorly soluble pharmaceutical drugs [163] and polyphenols [118, 164–166]. It was generally found that the magnitudes of equilibrium hetero-association constants obtained by different methods correlate well with each other and lie in between the self-association constants of the ligand and xanthine [19, 53, 68, 114, 124, 133–135, 138, 158]; a few exceptions (e.g. [166]) were interpreted in terms of specific intermolecular interactions involving water molecules with the 1:1 hetero-complex. A similar situation was also observed for the thermodynamical parameters (enthalpy and entropy) suggesting the absence of any additional stabilisation of the hetero-complexes in solution [68]. The highest magnitudes of K_h were found for the hetero-association of CAF with the antibiotic NOV ($K_h = 324\ M^{-1}$ [145]), TPT ($K_h = 320\ M^{-1}$ [149]), antimalarial agent halofantrine (HAL) ($K_h = 637\ M^{-1}$ [163]) and porphyrins ($K_h > 1000\ M^{-1}$ [156, 157]), and for the mutagen AO with tetramethyluric acid [132] at room temperature and pH ~7. However, the correlation of K_h with the self-association constants of interacting compounds, X and Y (i.e. K_X, K_Y), did not enable the absolute value of K_h to be used in a comparative analysis of various systems by their hetero-association ability. A quantitative measure f_h was developed in Equation (2) to estimate the relative contribution of hetero-association to the dynamic equilibrium in solution, determined as the relation of the equilibrium constants of self-association of the interacting compounds, X and Y (K_X, K_Y), and the constant of their hetero-association (K_h), [68]:

$$f_h = \frac{K_h}{K_X + K_Y + K_h} \tag{2}$$

The highest value of f_h was noted for the CAF-FMN system, which was interpreted as a consequence of additional stabilisation of the 1:1 hetero-complex by intermolecular hydrogen bond, supported by structural modelling [153, 167].

It was concluded that complexation of aromatic ligands with xanthines results from π–π stacking at pH close to neutral, which is based on systematic NMR magnetic shielding of aromatic protons of xanthine (or a drug) on increasing the concentration of the drug (or a xanthine) [115, 122, 133–135, 138, 145, 147, 149, 163], on specific changes in absorption of the drug in the visible range of the spectrum (hypochromic and bathochromic shifts) on increasing the concentration of xanthine [42, 53, 54, 124, 128, 137, 150–152, 157, 159] and on red-shifts in the absorption/emission bands in fluorescence [53, 137, 140, 157]. The overwhelming majority of published NMR work on hetero-association reports that π-stacking affects the value of chemical shift, except in a few very rare examples, e.g. halofantrine-caffeine hetero-association [163], reporting concomitant change in splitting pattern of the signal of the observed nuclei, which may be treated as an exception. Stacking appears to be preserved on lowering the pH, although K_h generally becomes smaller as a consequence of protonation of the interacting molecules [115, 128] (or, much more rarely, becomes higher [163]) or increasing pH [115]. However, at acidic pH the co-existence of stacking and in-plane hydrogen-bonded complexes may be the case, as demonstrated for CAF hetero-association with some polyphenols [118]. The binding of CAF with a range of polyphenolic molecules was generally characterised by stacking-type hetero-association with contributions from intermolecular H-bonding in some cases [164–166]. The majority of published work on hetero-association in drug-xanthine systems in aqueous solution report that the major contribution to their stabilisation is given by hydrophobic and van der Waals dispersive interactions of aromatic chromophores in the π-stacked hetero-complexes. In rare cases

(viz. CAF hetero-association with FMN/RBF [155], actinomycins [141, 142] and porphyrin [157] derivatives) based on the results of molecular modelling, an intermolecular hydrogen bonding between the molecules in 1:1 hetero-complexes was suggested as a source of additional stabilisation. The importance of charge-transfer interactions has also been proposed for drug-xanthine systems [54], although no solid experimental evidence has so far been obtained for such hetero-associations in aqueous solutions. Moreover, measurements of proton spin–spin relaxation times in the hetero-complex of CAF with 6-oxybenzo[*a*]pyrene [122], and the absence of an apparent charge-transfer band in the absorption spectrum [128, 140] suggested that charge-transfer, if any, is not a driving force for the hetero-association reaction. On the other hand, complexation of CAF with aromatic donors in $CDCl_3$ provided evidence that electrostatic interactions and charge transfer may provide the dominant contribution to stability of the complexes [144].

For all the ligands studied, there is general agreement on the most probable spatial structures of the 1:1 hetero-complexes in solution built by molecular modelling methods. The resultant structures are supported, if available, by NMR spectroscopy data, viz. indirect (magnetic shielding, e.g. [133–135, 138, 143, 145, 147, 149, 153, 164]) and direct (in very rare cases intermolecular proton–proton nOes, e.g. [164, 166]) structural information. The chromophores of the drug and xanthine in the majority of cases are oriented in a way to maximise the area of their overlap (i.e. the longitudinal axes of the chromophores of the drug and xanthine molecules tend to be parallel) [42, 124, 125, 133, 135, 137, 139, 159], except for the cases of THP-NOV [135] and, probably, CAF-DOX [139] systems in which the longitudinal axes are nearly orthogonal (the CAF-DOX seems to be the only system for which the early [42, 137] and recent [139] calculations of the structure of 1:1 hetero-complex disagree with respect to the angle of orientation of CAF to the DOX chromophore). The key difficulty in structural modelling of drug-xanthine systems is that the xanthine molecule allows at least two possible orientations differing by 180º in the complex with the drug [133, 137, 139], which sometimes cannot be reliably discriminated by experiment or in structural modelling. In the majority of cases, a typical aromatic stacking distance of 0.33–0.35 nm between the chromophores of the molecules in 1:1 complexes was reported. Due to the large difference in dimensions of xanthine molecules and some drugs (such as the 4-ring DAU, DOX,NOG,TPT molecules), a small deshielding (or nearly zero shielding) of certain peripheral aromatic protons was observed in complexes with CAF and methylxanthines [135, 138], which was interpreted as being due to these protons falling out of the shielding cone of the xanthine chromophore. A similar effect was also observed for the hetero-association of the 2-ring antibiotic NOR with the 4-ring anthracyclines [168]. No apparent evidence was reported for the formation of in-plane complexes due to hydrogen bonding, or simultaneous accommodation of two xanthines on the chromophore of the drug, or other types of arrangements of aromatic chromophores in hetero-complexes (such as perpendicular, suggested to occur in $CDCl_3$ [122]).

It is worth noting that, in the majority of cited papers on the hetero-association of aromatic molecules, aggregations higher than dimer in either 1:1 homo- and/or hetero-complexes are assumed and used in derivation of the model of hetero-association (except for particular cases of very small concentrations of interacting molecules, where the contribution from high-order aggregation may be ignored). The possibility of formation of higher order hetero-complexes, such as 1:2 complexes, for planar aromatic molecules, has long been known (e.g. [169]) and with respect to drug-xanthine systems, indirect evidence has been given from measurements of rotational correlation times by

NMR and comparison of optical absorption and fluorescence spectra of aromatic molecules in DNA intercalated state and in hetero-complexes [122], as well as from recent molecular dynamics simulations in water [155]. Based on fluorimetric titrations, it was also suggested that the aromatic antibiotic AMD can bind into caffeine clusters comprising 8–12 molecules, which may serve as a carrier of the antibiotic to DNA [140]. A qualitatively similar effect was reported [170], where CAF was shown to intercalate into clusters formed by some polyphenolic molecules present in tea, which stimulates tea cream formation.

A correlation between the hetero-association parameters and types of side chains was reported, such that the existence of large side chains in the molecular structures of AMD, AO, NOV results in a pronounced positive hydrophobic contribution to the entropy of hetero-association and subsequent increase in K_h [54, 133, 145]. The most drastic effect on K_h is seen on increasing the number of methyl groups in the structure of xanthines from mono-methylxanthine to di-, tri- and tetra-methylxanthine [54, 115]. Addition of oxygen or chlorine at C8 also increases the binding affinity [54]. A more subtle and not yet completely understood effect on K_h is the variation of the position of methyl groups in the xanthine structure. In mono-methylxanthines the existence of methyl groups at the 1 or 3 position significantly enhances hetero-association, whereas position 7 appears to be unimportant, and in dimethylxanthines [i.e. theophylline (THP), paraxanthine, pentoxifylline (PTX) and theobromine (THB)] variation in the 1,3,7 position of the two methyl groups, as well as addition of a $–(CH_2)_n–COOH$ group as a potential hydrogen bond donor/acceptor, did not result in apparent change in the magnitude of K_h [54, 125, 128, 134].

There is some disagreement in the literature about the influence on K_h of addition of a third methyl group (i.e. CAF) to the structure of dimethylxanthine. Based on spectrophotometric titration of AO, it was found that K_h for CAF is higher than that for dimethylxanthines and explained by a pronounced hydrophobic contribution [54, 132]. (At this point, it is worth noting that the K_h values in AO-THP/THB (e.g. see [53, 132]) and DAU-CAF/THP (e.g. see [127]) systems are not in complete agreement between different authors and different methods, which makes their comparative analysis less informative). A qualitatively similar result was obtained from NMR titration of ATP with CAF, THP and THB [115], but explained by the authors by electrostatic factor (i.e. degree of π-electron deficiency of the xanthine derivatives). Other investigators reported that CAF binding with DAU/DOX/NOV [127] or acridine mutagens [125] may be characterised either by higher or lower K_h as compared with THP (or PTX), although no interpretation was given. And, finally, NMR-based titration of various drugs (viz. EB, PF, DAU [134] and IQ-type amines [128]) by CAF and dimethylxanthines has led to a contrasting conclusion that removing the third methyl group leads to a slight increase in hetero-association ability. The exceptions are (i) the THP-NOV system [127] in which the orthogonal orientation of the chromophores in the 1:1 hetero-complex resulted in minimisation of the overlap area and consequently lower magnitude of K_h as compared with CAF, and (ii) the THP-AO system, featuring lower K_h with respect to CAF-AO due to a decrease in the efficacy of hydrophobic interactions of methyl groups in the 1:1 complex [53, 54, 132]. These results reflect very complex patterns of the interplay between the type of side chains and the magnitude of K_h in hetero-association ability, as previously known from self-association studies of tri- and di-methylxanthines [134] and explained by steric hindrance created by three methyl groups in the structure of CAF.

The importance of steric hindrance in hetero-association is also supported by the facts that the binding of dimethylxanthines by proflavin, which does not contain methyl/ethyl groups, was characterised by the highest magnitude of K_h [134]. A qualitatively similar effect was reported for binding of phenanthridine dyes with CAF [133], viz. an elongation of the alkylamine side chain in EB (transforming EB into PI, see Figure 1) resulted in lower K_h. On the other hand, the predominantly hydrophobic hetero-association of AO with tetramethyluric acid, containing 4 methyl groups, is characterised by relatively high hetero-association constant, $K_h = 552\ M^{-1}$ [132]. Similar predominant effects of hydrophobic interactions were noted when replacing 3,8 imino groups in PF by dimethyl groups in AO (see Figure 1), which gave a marked increase in K_h [133]. An even more complex interplay of the effect of side chains and the structure of chromophore was reported for the case of CAF binding to porphyrins, which additionally involve the coordination of the CAF molecule by zinc in the centre of the porphyrin molecule [156].

These results highlight the key role of 'structural complementarity' in hetero-association and its competition with hydrophobic tendency of stacking in aqueous solution, which is generally in line with what is known to date from hetero-association of the derivatives of nucleic acid bases (see Section 4.1) and, in fact, determine the specificity in structures of 1:1 hetero-complexes. A typical demonstration of the competition of these two factors is the absence of any apparent pattern in the magnitude of K_h for the binding of CAF and THP with AO/DAU/DOX/EB/PF/NOV discussed above, and for the binding of CAF, THP and pentoxifylline to IQ-type amines, which differ in the number of nitrogens in the aromatic chromophore (determining the aromaticity of the molecule and strength of van der Waals interactions) and the number of CH_3 groups (determining the strength of hydrophobic interactions) [128]. On the other hand, the enthalpy of hetero-association, manifesting the contribution of the van der Waals factor, gives the highest values for IQ-type amines with the smallest number of nitrogens in the chromophore, as expected [128].

Thermodynamic parameters (ΔH_h, ΔS_h) have been determined nearly for all the drug-xanthine systems cited above, using the temperature dependence of experimental observable (commonly, chemical shift in NMR or molar absorption in UV-Vis) and van't Hoff analysis. The known calorimetrically determined enthalpy of hetero-association of CAF with TPT [148], DAPI [150], NOV [42], MPTP [126], IQ [128], porphyrin [156] and HAL [163] systems falls within the typical range of van't Hoff derived enthalpies [115]. The magnitudes of ΔH_h and ΔS_h are large negative values, indicating the importance of van der Waals interactions in the net energetics of the hetero-association reactions. The sole exception is the CAF-NOV system, characterised by a small positive ΔS_h, which was explained by a pronounced hydrophobic contribution [145]. The typical range in variation of enthalpy is from −15 to −47 kJ/mol [42, 128], and a clear dependence of ΔH_h on the number of fused rings in chromophore and their aromaticity was reported [128] (see also Figure 3). It should be noted that this correlation is also typical for K_h and was clearly demonstrated for CAF binding with a range of polyphenolic molecules differing in the number of fused rings in the chromophore [170]. Systems falling outside of the typical range of enthalpies are those close to zero value, i.e. $\Delta H_h = -624$ J/mol for CAF-MPTP [126], presumably due to minimal overlap of aromatic moieties in the hetero-complex, and for the CAF-quinacrine mustard system with $\Delta H_h = -486$ J/mol [124], which is difficult to interpret so far.

The range of variation in entropy is approximately −20 to −50 J/mol K and in the majority of cases was related to the presence of hydrophobic groups in the structure of

the drug and xanthine molecules. Interestingly, at neutral pH, no apparent correlation was reported between the existence of charge on the drug molecule with either K_h or ΔH_h, ΔS_h, and the dependence of K_h on counterion concentration seems to be not significant [158, 159], indicating only small contributions from ion-dipole interactions. This is not correct, however, if the electrostatic component is assumed to be dominant as demonstrated for ATP binding with xanthine derivatives at different pH [115]. Moreover, the magnitude of K_h may either increase or decrease on increasing the ionic strength, which is strongly dependent on the electronic properties of the interacting molecules [115, 158]. Unfortunately, data on the influence of di- or multi-valent metal ions on hetero-association are scarce and do not enable any general conclusions to be made. In the study of CAF complexation with a range of polyphenolic molecules [170], it was shown that glucose and calcium have little effect on hetero-association. However, the existence of zinc in the structure of porphyrin molecule, in part, is responsible for the highest known magnitudes of the hetero-association constants ($K_h > 1000\ M^{-1}$ [156, 157]) and relatively high enthalpies ($\Delta H_h = -30 \ldots -40$ kJ/mol [156]) with xanthine derivatives.

4.3.2. *Drug-vitamin system*

There are some early investigations of the hetero-association of the riboflavin vitamins (RBF or FMN) with other aromatic compounds: the complexation of RBF/FMN with a series of betacarbolines, indoles [171–175], simple hydrocarbons [176–180] and some antibiotics [129, 130]. The formation of 1:1 stacked complexes of aromatic molecules was reported, where an increase in stability and lowering of entropy was related to the increase in aromaticity of the drug chromophore in RBF-drug complexes for a series of betacarbolines and some other hydrocarbons [171]. Similar conclusions regarding the type of complexation were also made in studies of the hetero-association of RBF with nicotinamide [181, 182] and with sodium salicylate [154, 183]. Specific changes in spectral parameters (absorption and fluorescence) were interpreted primarily with respect to substituent functions in the aromatic rings. In the majority of cases, early investigations of RBF hetero-association with other aromatic molecules resulted in the view that charge-transfer interactions are likely to be the most important factors stabilising the hetero-complexes.

When antidepressants, which also include a group of betacarbolines (BC), are used in the treatment of depression and Parkinson's disease, some suppression is observed of the enzyme activity of monoaminooxidase (MAO) – a flavoenzyme which catalyses the oxidative deamination of certain important biogenic amines [171]. In order to understand the molecular mechanism of this effect, an investigation was conducted of the interaction of a series of structurally selected BCs and indoles with two typical representatives of the flavin family (FN), riboflavin and flavin-mononucleotide, [171–175]. The formation of 1:1 non-fluorescent molecular complexes was observed and the equilibrium constants and thermodynamical parameters of complexation were calculated. For the series of BC derivatives, it was found that FMN forms more stable hetero-complexes than RBF. The authors tried to correlate the FN-BC complexation with the suppression of MAO, and came to the conclusion that the change in MAO activity was related to the sequence of FN-BC complexation constants that depended on the aromaticity of the BC chromophore.

The most detailed investigations of the hetero-association of RBF/FMN-drug systems were recently carried out by NMR spectroscopy under similar solution conditions (0.1 M ionic strength, pH ~7). The structural and thermodynamic parameters of

hetero-association were determined from analysis of the concentration and temperature dependences of chemical shifts of the non-exchangeable protons of both aromatic compounds in mixed solutions of FMN with the antibiotics, DAU, NOG, AMD, NOV [184–187], NOR [188] and TPT [189], and the mutagens, EB and PF [190]. It was concluded that sandwich-type complexes of aromatic molecules were formed in solution, which were stabilised by stacking-interactions of the chromophores. The FMN-NOV system studied by NMR [185] provided a rare case of the co-existence of homo- and hetero-complexes of the interacting molecules in solution, manifested by a change of a shape of the titration curve compared to the typical concave form into the extremum-containing shape originating from the competition of magnetic shielding in various complexes.

The structures of 1:1 hetero-complexes conform to the general tendency to maximise the overlap area of the chromophores of the interacting molecules. The magnitudes of equilibrium hetero-association constants for FMN interaction with NOG, DAU, NOR and AMD were intermediate in value between the equilibrium constants of the self-association of the individual molecules, whereas the magnitudes of the hetero-association constant for FMN interaction with NOV, EB and PF were greater than the corresponding self-association constants, which indicated that hetero-association of these aromatic compounds was energetically more favourable [185, 190]. Quantitative analysis of the thermodynamic parameters of the interaction of FMN with these molecules, complemented by direct structural information derived from two-dimensional NMR spectra, resulted in the conclusion that intermolecular hydrogen bonds were responsible for the additional stabilisation of the hetero-complexes (Figure 4). These

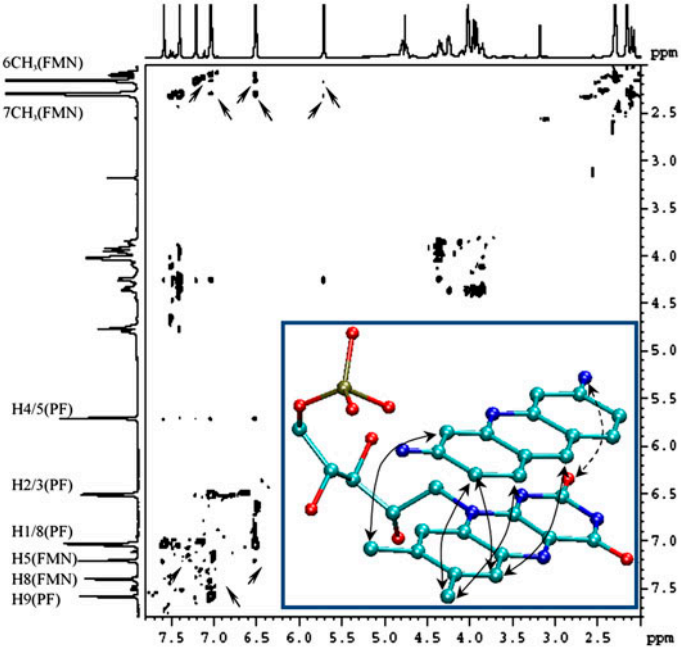


Figure 4. (Colour online) Example of ^{1}H-^{1}H 2D-ROESY NMR spectrum of the FMN-PF mixture in aqueous solution (redrawn from [190]). The inset contains the calculated spatial structure of the 1:1 hetero-complex. Solid lines indicate intermolecular nOe cross-peaks and the dashed line indicates the proposed H-bond.

results were later supported by quantum-chemical calculations [167], statistical analysis of large data-set of hetero-complexes [191] and studies using vibrational spectroscopy [192]. The existence of an intermolecular H-bonding in the RBF-NOR complex was also implied [193] based on high negative values of thermodynamic parameters. Interestingly, the magnitude of the hetero-association constant derived from fluorimetric titration resulted in K_h having magnitudes of the order of $10^5\,M^{-1}$ which is 2–3 orders of magnitude higher than typical values of K_h derived from NMR or UV–vis studies, whereas the enthalpy change appears to be consistent within the three methods. The distance between the chromophores from fluorimetry [193] is also inconsistent with NMR data and molecular modelling [188] and so these results could be treated as an exception, because in more systematic studies of drug-xanthine systems (reviewed above) the quantitative parameters of hetero-association obtained by various methods are in general agreement.

Another type of aromatic vitamin, nicotinamide (NMD) and its derivatives, which possesses effective solubilising properties with respect to aromatic drugs, has been thoroughly investigated [119, 154, 163, 181–183, 189, 194–197]. Using various experimental techniques, it was confirmed that the general mechanism of the solubility enhancement of the drugs in the presence of NMD originates from the formation of 1:1 hetero-complexes. These studies concluded that π-stacking interactions played the predominant role in formation of the complexes, and provided the same qualitative information on the thermodynamics of hetero-association as previously known from interactions of drugs with derivatives of nucleic acids (see Section 4.2). In particular, the hetero-association of NMD with various drugs is always characterised by lower magnitudes of K_h and other thermodynamic parameters compared to RBF/FMN or CAF, which is correlated with a lower solubilising potency of NMD than RBF under similar conditions. The binding of NMD to the antibiotic DOX was significantly enhanced in the presence of Fe(III) ions [119], representing a very rare case where the influence of metal ions on hetero-association was directly investigated.

One specific issue regarding the NMD-drug and FMN-drug hetero-association is worthy of special consideration. It was assumed that two 1-ring nicotinamide molecules could potentially be simultaneously accommodated on one face of the 3- or 4-ring chromophores of some of aromatic drugs (e.g. [163]). Microcalorimetric studies have pointed to the formation of 2:1 NMD-HAL complexes, although no distinction was provided on whether this results from 2:1 or 1:1:1 complexes of two nicotinamides with one halofantrine drug [163]. The NMR investigation of NMD-DAU/NOV hetero-association specifically designed to distinguish between 2:1 and 1:1:1 types of binding indicated that the latter is more probable [197]. The binding of the 3-ring FMN molecule with the 4-ring topotecan drug indicated some preference for 2:1 type of binding, although no firm evidence for this was provided [189]. Hence, the possibility of accommodation of two (or more) aromatic molecules simultaneously on the chromophore of another aromatic molecule is still an open question.

4.3.3. *Drug-chlorophylline system*

Chlorophylline (CHL) is a water-soluble porphyrin which, of all the porphyrins, has been studied most systematically in terms of its hetero-association with other aromatic molecules with the principal goal to get insight into the interceptor properties of porphyrins.

The first detailed investigation of CHL-drug and related systems was performed with respect to various mutagen molecules [39, 44, 198–204], and more recent studies [41, 156, 157, 205, 206] have extended the set of drugs to antitumor antibiotics and some other types of mutagens. It was found that chlorophylls are able to form face-to-face complexes with planar molecules, and the addition of methyl groups above or below the plane of the mutagen molecule interferes sterically with overlapping CHL/mutagen ring systems, thus reflecting the importance of 'structural complementarity' in hetero-association [156]. No clear relationship was observed between K_h and the specific number or positions of methyl groups [44]. The presence of a metal ion in the centre of the CHL chromophore apparently promotes hetero-association, although it is generally believed that the tetra-pyrrole ring of CHL plays a crucial role in determining the strength of hetero-association [41, 44]. The equilibrium hetero-association constant, K_h, in CHL-drug systems was shown to be, on average, two to three orders of magnitude higher than that for drug-xanthine systems [39, 44, 201, 205, 206] and these result have also been supported by calorimetrically measured enthalpies of hetero-association, giving large negative values in the range −30…−40 kJ/mol [156]; this was explained in terms of the more extended aromatic system of the CHL chromophore providing more significant van der Waals and hydrophobic contributions to the net energetic effect of the hetero-association reaction [41, 199, 202, 203]. The order of magnitudes of K_h was found to correlate with the area of aromatic chromophore of the drugs (i.e. with the number of fused aromatic rings, see Figure 3). The general conclusion resulting from these studies and the related functional activity of CHL both *in vivo* and *in vitro* was that CHL must have higher interceptor ability than the xanthine molecules with respect to aromatic drugs [206]. Comparison of the ranges of K_h with the drug-vitamin systems suggests that the interceptor ability of CHL should also be more pronounced when compared to the aromatic vitamins.

4.4. *Hetero-association of aromatic drugs with aromatic mutagens and antibiotics*

The suggested biological role of the hetero-association of aromatic antibiotics and caffeine has stimulated investigations into the interaction of antitumor antibiotics with other types of aromatic compounds, in particular, mutagens and antibiotics. All these molecules typically absorb in the UV and/or visible range of spectrum, which causes a basic difficulty in studying their hetero-association by spectrophotometry. Hence, the hetero-association of these types of molecules has been mainly performed by NMR spectroscopy, except for a few cases when reliable decomposition of UV/Vis was possible (e.g. [207]).

A number of systems have been studied by NMR under similar solution conditions (0.1 M ionic strength, pH ~7.1): the hetero-association of phenanthridine (EB, PI, ethidium mono- and di-azides) [189, 208–211], acridine (AO, PF) [74, 207] and xanthene [212] dyes with the antibiotics DAU, TPT and quinolones; the hetero-association of antitumor antibiotics DAU-NOV [213], DAU-AMD [214, 215], DAU-NOR [168], DAU-NOG [167, 191], NOV-AMD [214], NOV-NOG [167, 191], NOR-NOG [168], NOG-AMD [167, 191], DAU-TPT [189], antimalarial drugs [216] and the hetero-association of mutagens: AO-PF [217], EB-PI [218], PF-EB [75, 76], dyes [219] and some other aromatic drug molecules [13, 220]. These molecules have a planar 3- or 4-ring aromatic chromophore (except the 2-ring NOR molecule) and must potentially be characterised by greater π-stacking interactions than that provided by the derivatives of nucleic acid bases and xanthines. The range of reported magnitudes of K_h, enthalpies and entropies

confirmed this expectation. The general conclusions drawn from analysis of experimental data followed the pattern already described above for the hetero-association of the derivatives of nucleic acid bases and interceptor molecules, i.e. the predominance of π-stacking interactions, intermediate with respect to self-association magnitudes of thermodynamic parameters, large negative values of enthalpy/entropy, systematic magnetic shielding of aromatic protons in the hetero-complex except when *X* and *Y* molecules have significantly different dimensions, e.g. NOR-DAU/NOG [168], resulting in small deshielding of some protons (the effect already discussed in Section 4.3.1). We can, however, distinguish two distinctive features of the drug–drug and drug–mutagen systems.

Unlike the derivatives of nucleic acid bases or porphyrins, which have nearly square-shaped chromophores and so there is some ambiguity in determining the structures of the 1:1 hetero-complexes from NMR proton shielding and/or molecular modelling, the chromophores of aromatic antibiotics and mutagens, listed above, are composed of linear- or angular-fused 3 or 4 aromatic rings (see Figure 1), which results in a more reliable determination of the structure of hetero-complexes from analysis of induced magnetic shielding of aromatic protons of the interacting molecules. The general feature of the structures of these 1:1 hetero-complexes is that the chromophores are always oriented in a way to achieve maximum overlap and no indication of significantly twisted or shifted structures was reported. A tilt and shift of the chromophores was only observed in case of the 1:1 hetero-complexes of antimalarial drugs [216] with relatively weak 2-ring aromatic system and bulky side chains, creating steric hindrance to the formation of a compact π-stacked structure. This fact supports the conclusion that hetero-association is mainly driven by stacking interactions with the major contributions from hydrophobic and van der Waals interactions. In rare cases, the possibility of charge-transfer was inferred from fluorescence spectroscopy [207]. In some cases, the structures of the hetero-complexes appeared to be more compact compared to the homo-aggregates. For example, in the NOG-AMD and NOG-DAU complexes [167, 191], the twist angle between the chromophores is nearly zero, whereas in their homo-aggregates it is 10º or higher. These features most likely reflect the principle of 'structural complementarity' discussed above for the drug-xanthine systems and act as a fine tuning of hetero-association based on stacking as a major driving force. Complementarity in hetero-association has also been manifested by slightly smaller distance between the chromophores in some hetero-complexes (e.g. 0.32 nm in DAU-AMD [214, 215]). But the most general manifestation of complementarity was the fact that nearly all the investigated hetero-complexes had structures with maximum separation of bulky side chains or electric charges on chromophores. In particular, in all combinations of molecules carrying a positive charge (viz. NOG-DAU, AO-PF, EB-PI, PF-EB), the structures of hetero-complexes were always antiparallel. If, however, the interacting molecules are non-rigid or contain bulky side chains comparable in their dimension with the aromatic chromophore, the disruption of the compact π-stacking structure may occur as was clearly shown in dye–dye hetero-complexes [219]. It is difficult to ascertain whether in this context the principle of 'structural complementarity' must be supplemented by 'electrostatic complementarity' recently introduced for aromatic interactions [36], as differentiation of their influence on hetero-association is not possible with the data available. However, there is direct NMR evidence for the formation of two dimer structures of the antibiotic DAU with either parallel (charges on DAU aminosugar are in close proximity to each other) or antiparallel (charges on DAU aminosugar are well separated) orientations of chromophores [221, 222], which suggests that electrostatic factor may not be so significant in aqueous solution.

The second particular property found for some drug–drug and drug–mutagen systems is observation for thermodynamic parameters of hetero-association (either K_h and/or ΔH_h) that are greater than the corresponding self-association parameters. This effect was first reported for the hetero-association of DAU with acridine dyes [74] and then was observed for a range of systems, such as DAU-phenanthridine dyes [208–211], DAU-NOV [213], DAU-AMD [214, 215], FMN-PF/EB [190] and TPT-PF [189]. The effect appeared to be systematic rather than exceptional, as in the case of the hetero-association involving interceptor molecules and derivatives of nucleic acid bases (Sections 4.2 and 4.3). Apart from the increased magnitudes of the thermodynamical parameters, hetero-association of the majority of the above-cited systems was characterised by the existence of intermolecular nOe cross-peaks in 2D NMR spectra, which were not observed in the corresponding homo-aggregates under similar experimental conditions. All these facts, plus structural analysis, lead to the conclusion that these hetero-complexes of drug–drug, drug–mutagen (and mutagen-vitamin discussed in Section 4.3.2) are additionally stabilised by intermolecular H-bonds between NH_2 and C=O groups of the interacting molecules [190, 208–211, 213–215]. Re-investigation of this conclusion compared to an alternative hypothesis of additional stabilisation via charge-transfer interactions has confirmed the hypothesis of H-bonding by statistical analysis of thermodynamical parameters on a large data-set [167, 191], by discovery of the anticooperative character of hetero-association in case of intermolecular H-bonding [80], by sequential withdrawal of H-bond donor groups [209], and by recent experimental studies using vibrational spectroscopy [192]. It was found that the f_h factor (2), $f_h \geq 35\%$ may serve as an indicator of additional stabilisation of hetero-complexes by H-bond and general conditions for the formation of intermolecular H-bond in hetero-complexes of aromatic molecules have been formulated [167].

4.5. *Hetero-association in three-component systems*

Investigations of hetero-association of aromatic molecules so far have dealt with two-component hetero-association, involving complexation of two different species, *X* and *Y*. The question may be asked whether three- or more-component hetero-associations may have some chemical or biochemical significance? Supporting evidence comes from the field of supramolecular chemistry expanding towards the construction of heterogeneous functional supramolecular polymers [64], and studies reporting improvement of the solubility of various drugs in multi-component systems including cyclodextrins and explained as being due to formation of triple and higher order complexes in solution [223, 224]. The following hypothesis was formulated [225]. CAF and NMD molecules are well known as effective hydrotropes increasing the solubility of aromatic drugs via the hetero-association mechanism [163, 194–196]. However, their hydrotropic potency is limited due to their intrinsic ability to self-associate. It was therefore thought that the mixture CAF + NMD may act as a more potent hydrotropic agent than either CAF or NMD alone. NMR investigation of the solubilisation of Vitamin B_2 in a model three-component system FMN-CAF-NMD confirmed this hypothesis [225]. It was suggested that the mechanism of improved solubility was based on optimisation of the interplay between self-association (disfavouring solubilisation) and hetero-association (favouring solubilisation). A qualitatively similar mechanism of the interplay between various types of homo- and hetero-complexes was used to predict the possibility of regulation of biological activity of anthracycline antibiotics in the presence of riboflavin and caffeine [226] and improvement of the solubility of NOV + DAU mixture by the addition

of caffeine [62]. It is concluded that three-component hetero-associations of aromatic drug molecules may find future applications, e.g. in medicinal chemistry.

One issue regarding the three-component systems is worth noting. Although there are few data on multi-component interactions, these studies carried out in aqueous solutions did not report any synergistic enhancement or weakening of the hetero-complexes of the *X*–*Y*–*Z* type. It means that any three-component mixture of aromatic molecules may be described by mass balance equations containing equilibrium constants measured in separate one- (self-association of *X*, *Y* and *Z*) and two-component (the hetero-association of *X*–*Y*, *X*–*Z* and *Y*–*Z*) studies. This statement was verified by theoretical evaluation of the titration curves in three-component mixture by means of the mass balance equations with their further comparison against experiment [62, 79, 225, 226], which led to the important conclusion that the equilibrium self- and hetero-association constants of one- and two-component interactions are transferrable to a multi-component hetero-association system under the same solution conditions. This conclusion, however, must be utilised with caution in cases of additional stabilisation of hetero-complexes by intermolecular H-bonds (see Section 4.4). The formation of H-bonded hetero-complexes of higher dimensions than dimers may be an intrinsically anticooperative process [80], which creates some ambiguity in transferring the equilibrium constants from one-/two-component to multi-component studies.

4.6. *Summary of the structure and thermodynamics of hetero-association*

Although the large data-set on hetero-association reactions in aqueous solutions has been accumulated on very different groups of aromatic molecules, mostly from non-systematic studies, nevertheless some general conclusions can be drawn:

(i) *π-stacking appears to be the major driving force* in the formation of hetero-complexes in aqueous solutions, tending to form compact structures with maximum overlap of aromatic chromophores aligned in parallel or antiparallel. Examples of in-plane hetero-association are known, but should be treated as exceptions. Partial disruption of π-stacking is commonly observed, if the aromatic molecules contain bulky side chains.

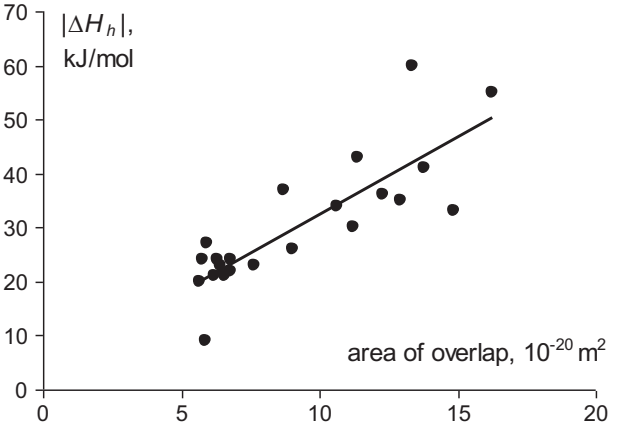


Figure 5. Dependence of enthalpy change on the area of overlap of the chromophores of interacting molecules in hetero-complexes (ΔA_h). Data taken from [191] (see also Table 1).

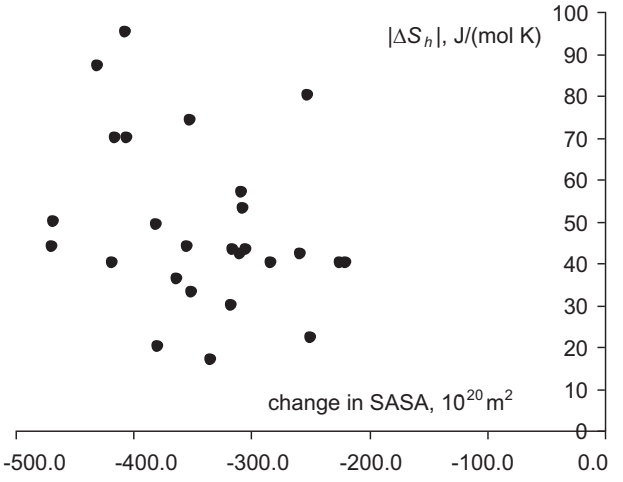


Figure 6. Dependence of entropy change on the change in solvent-accessible surface area (SASA) on hetero-association. Data taken from [167, 191] and Refs. therein (see also Table 1).

(ii) *The principal contributors to π-stacking* in hetero-complexes appear to be van der Waals and hydrophobic interactions, both of which are relatively unspecific for aromatic molecules. It was found that the area of overlap of aromatic chromophores in 1:1 complexes (ΔA_h) is a major determinant of the enthalpy of hetero-association [191]. This property is manifested by a correlation of ΔH_h with ΔA_h in Figure 5, whereas the hydrophobic factor influences the entropy of hetero-association via change in solvent-accessible surface area (SASA). Interestingly, the change in entropy on hetero-association, ΔS_h, does not correlate with the change in SASA (Figure 6), indicating the paucity of our current understanding of the origin of ΔS_h. As a consequence, the overall stability of hetero-complexes is statistically correlated with van der Waals interactions via the area of overlap. The most direct manifestation of the above-mentioned correlations is the increase of K_h on mutual increase of the m and n numbers (reflecting the number of fused aromatic rings in the structure of the interacting molecules) in the m:n hetero-complexes (Figure 3), and increase of K_h on addition of hydrophobic groups.

(iii) *Fine tuning of the structure of hetero-complexes* is determined by two factors, viz. 'structural' and 'electrostatic' complementarity of the interacting molecules within the complex, which cannot be separated based on results of existing investigations. In fact, the structures and stabilities of the hetero-complexes of aromatic molecules in each particular case are determined by an interplay of these complementarities and the tendency to maximise the area of overlap of chromophores in 1:1 hetero-complexes.

(iv) *No specific synergistic enhancement of hetero-association* beyond the dimer stage has been reported, which results in the conclusion that the equilibrium self- and hetero-association constants of one- and two-component interactions are transferrable to a multi-component hetero-association system under the same solution conditions.

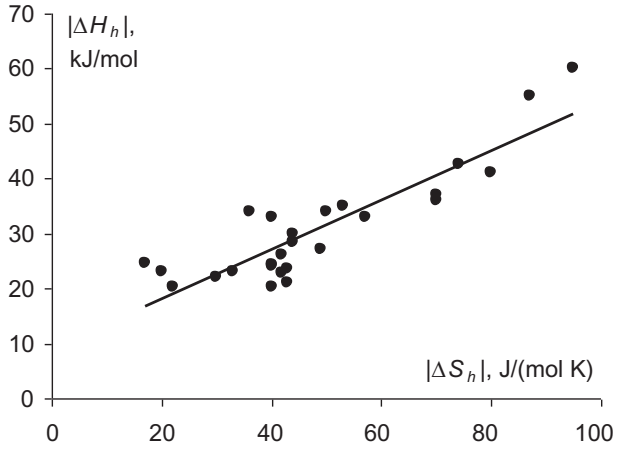


Figure 7. Enthalpy–entropy compensation in hetero-association. Data taken from [167, 191] and Refs. therein (see also Table 1).

(v) An interesting feature in the hetero-association of some systems is *the additional stabilisation of hetero-complexes* by intermolecular H-bonds which intuitively may be considered as sterically less probable in π-stacked structures. Nevertheless, the formation of H-bonds not disrupting stacking has widely been reported. In these cases, it is questionable whether the equilibrium self- and hetero-association constants of one- and two-component interactions are transferrable to the multi-component hetero-association system under the same solution conditions.

(vi) From the investigations reviewed, it is concluded that *the contributions of electrostatic and charge-transfer* factors appear to be relatively small in the thermodynamics of hetero-association in aqueous solutions. This issue is quite contentious, because papers continue to appear reporting on the charge-transfer effect (so far based on indirect evidence); this issue is probably the greatest fundamental problem of hetero-association of aromatic molecules which needs to be resolved.

(vii) As seen from Figure 7, the hetero-association of aromatic molecules follows *the enthalpy–entropy compensation*, well known to date for non-covalent interactions in aqueous solutions, including the self-association of various aromatic molecules (e.g. see [21]).

5. Energetics of hetero-association

So far large efforts have been made with the aim of understanding the major factors governing the π-stacking of aromatic molecules, resulting in recognition of the general importance of hydrophobic, electrostatic and van der Waals contributions (e.g. for review see [8, 11, 24, 34, 36, 37, 227]). The problem of 'energetic composition' of π-stacking is, however, currently far from solution, which is, in part, due to the fundamental ambiguity of thermodynamic analysis (to be discussed below), making a great challenge for further studies. The hetero-association of aromatic molecules provides an excellent model for understanding the nature of π-stacking.

5.1. *A fundamental problem in the thermodynamic analysis*

The wealth of information on the thermodynamics of hetero-complexes summarised above leads to some understanding of the specific factors which stabilise or destabilise the complexes. The experimental data for such analyses are the thermodynamic quantities ΔG_h (or K_h), ΔH_h, ΔS_h supplemented by molecular modelling. There is, however, at least one fundamental problem in using these data in thermodynamic analysis, viz. the physical forces making up the total energies of non-covalent intermolecular interaction are themselves interconnected by the enthalpy–entropy compensation phenomenon [8, 228] (see also Figure 7). Hence, any assumptions about the interrelation of known differences between the systems investigated (e.g. structural) and the net thermodynamical parameters may not be valid. In general, for systems that differ in structural properties and/or electric charge, it is likely that analysis of the experimentally measured total Gibbs free energies, enthalpies and entropies, may not be meaningful. A common manifestation of this problem with respect to stacking of aromatic molecules in aqueous solutions in classical thermodynamical analysis is (i) the inability to separate the contribution from van der Waals and hydrophobic forces, (ii) the lack of clarity of the role of hydration and (iii) contradictory views on the role of electrostatic interactions for aromatic–aromatic complexes [229, 230]. The fundamental reason is that it is not possible to measure independently the contribution of a specific energy term to the total stacking energies, and the questions 'What forces stabilise/destabilise the stacking of aromatic molecules in solution and what are their relative importance?' cannot be addressed unambiguously from analysis of ΔG_h, ΔH_h, ΔS_h. The problems of analysis of thermodynamic parameters can be partly overcome, if the energy term can be deconvoluted, i.e. parsing the experimentally measured values of ΔG_h, ΔH_h, ΔS_h in terms of specific contributions from particular physical factors [8, 231–233]. Then further analysis of the calculated terms would enable the questions formulated above to be addressed, in order to fully understand the 'energetics of hetero-association'.

5.2. *Analysis of energetics at the level of Gibbs free energy change*

There have been a few reports on the analysis of π-stacking energetics for the group of aromatic molecules falling within the scope of the current review (e.g. [230, 234–236]) with the most general contribution given in work specifically dealing with the hetero-association of aromatic molecules [237].The results, which cover more than 40 self- and hetero-association systems in aqueous-salt solution, are briefly summarised.

All the known principal contributors to ΔG_h may be summarised in the following expression:

$$\Delta G_{total} = \Delta G_{vdW} + \Delta G_{el} + \Delta G_{hyd} + \Delta G_{hb} + \Delta G_{entr}, \quad (3)$$

where the subscript indices of ΔG designate the contribution to the energy in (3) from van der Waals (vdW), electrostatic (el), hydrophobic forces (hyd), hydrogen bonds (hb) and specific factors predominantly of an entropic nature (entr), respectively. The entropic factors include the energy equivalent of the loss of translational and rotational degrees of freedom, as well as formation of new vibrational modes in hetero-complexes [238, 239].

Of the five net contributions to the free energy in Equation (3), it was found that the hydrophobic contribution dominates for all systems studied ($\Delta G_{hyd} = -40 \ldots -120$ kJ/mol) and is energetically favourable. The net contributions from 'vdW' and 'el' factors are

comparable to each other and are relatively small (<25 kJ/mol in absolute value). The main destabilisation comes from the net effect of hydrogen bonding ΔG_{hb} dominated by the loss of hydrogen bonds to water, and the loss of degrees of freedom, ΔG_{entr}. The latter term is dominated by unfavourable contributions from the loss of translational and rotational degrees of freedom, whereas the vibrational contribution is favourable.

The relative unimportance of the 'vdW' and 'hyd' terms is a surprise but not meaningful because it is a consequence of consideration of the net contribution of these interactions in Equation (3), composed of the sum of interactions with solvent and intermolecular solute–solute interactions. Further deconvolution of the 'vdW' and 'el' terms into solute–solute and solute–solvent interactions makes it possible to carry out a full analysis of the Gibbs free energies. As examples, the results of such analyses for four selected molecular systems, including self-association, are given below:

- DAU-DAU [236, 237]: the main stabilisation comes from the 'vdW' solute–solute interaction and, to a lesser extent, from hydrophobic interactions and electrostatic interaction with solvent. 'vdW' interaction with solvent and electrostatic solute–solute interaction are unfavourable
- PI-PI [236, 237]: the main stabilisation comes from electrostatic interaction with solvent and, to a lesser extent, about equally from solute–solute 'vdW' and hydrophobic interactions. 'vdW' interaction with solvent and the electrostatic solute–solute interaction are unfavourable.
- CAF-EB [237]: the main stabilisation comes equally from 'vdW' solute–solute interaction and hydrophobic interactions. The 'vdW' interaction with solvent is unfavourable. There is no contribution to the stability of the complex from all types of electrostatic interactions.
- FMN-EB [237]: The main stabilisation comes from the solute–solute 'vdW' interaction and, to a lesser extent, about equally from hydrophobic interactions and electrostatic solute–solute interaction. The 'vdW' and electrostatic interactions with solvent are unfavourable.

The following general conclusions on the energetics of hetero-association may be summarised:

(i) the order of stabilising and destabilising factors depends on the molecular system studied, and may have a 'vdW', electrostatic or hydrophobic nature. The 'el' factor depends strongly on the charges of the interacting molecules and starts to dominate only if they carry double charge; in all other cases 'vdW' and 'hyd' are the principal contributors to aromatic stacking, in full agreement with the review of thermodynamic parameters given in Section 4. The 'entr' factor depends relatively weakly on the type of hetero-system studied, and may be considered as a constant value, to a first approximation.

(ii) the relatively small value of the total Gibbs free energy of hetero-association ($|\Delta G_h|$<30 kJ/mol), as well as the net ΔG_{vdW} and ΔG_{el} terms, is the result of summation of components with large magnitudes but of opposite sign. The complexation of aromatic molecules is governed by the effect of compensation of energy contributions at the levels of physical forces (Equation (3)) and solute–solute/solute–solvent interactions. In part, this provides an explanation of why the 'el' factor is relatively unimportant in the thermodynamics

of hetero-association, viz. the solute–solute and solute–solvent energies nearly compensate one another.

The review of the literature suggests that the energy profile of aromatic stacking is similar to what is currently known from analysis of the energetics of aromatic ligands binding with nucleic acids, also governed by stacking interactions (for reviews see [189, 240–244]).

5.3. *Analysis of energetics at the level of enthalpy and entropy change*

A consequence of analysis at the level of ΔG_h suggests that deconvolution of energetics at the level of ΔH_h and ΔS_h should also be undertaken. However, examples of such analyses for aromatic molecules are scarce in the literature, which creates a challenge for future studies. Nevertheless, some important correlations are known between specific physical factors and the enthalpy/entropy of hetero-association.

General thermodynamics suggests that the 'vdW' and 'el' factors contribute predominantly to ΔH_h, whereas ΔS_h is mainly composed of the 'hyd' and 'entr' factors. As reported above (see Sections 4 and 5.2), for singly charged molecules in aqueous solution the 'el' and 'entr' factors are of lesser importance than 'vdW' and 'hyd'. Indeed, the correlation of ΔS_h with the presence of hydrophobic groups, as well as the correlation of ΔH_h with the area of overlap of aromatic chromophores (responsible, at least, for the solute–solute 'vdW' interactions), has been noted for various hetero-combinations. The importance of the area of overlap for the magnitude of ΔH_h was confirmed for the large data-set of hetero-associations of aromatic molecules (see Figure 5) and, more generally, is a manifestation of the well-known dependence of the stability of π-stacking on area size [11], whereas the 'hyd' factor (and, consequently, ΔS_h) has long been known to correlate with changes in solvent-accessible surface area [245, 246]. However, detailed analysis of energies at the levels of ΔH_h and ΔS_h is required in order to fully link theoretical implications and experiment.

6. 'White spots' of hetero-association

Information on structure, thermodynamics and energetics does provide some general understanding of hetero-association for the set of aromatic molecules and experimental conditions outlined in this review. There remain some gaps in our understanding (white spots) in the physico-chemical literature, which offer challenges for further experimental investigations of hetero-association:

- *kinetics of hetero-association*. Quite a few papers deal with kinetics of hetero-association (e.g. [100, 202]) or with the influence of hetero-association on photo-transformation of the main aromatic drug in the presence of another aromatic compound (predominantly grouped around the photosensitising properties of particular compounds of medical interest, such as Riboflavin and a few other drugs (e.g. [131, 247–249])). These results, however, give little insight into the kinetics of hetero-association;
- *the influence of metal ions* on the stability of hetero-complexes may be based on the intuitive assumption that metal ions enhance the stability of hetero-complexes by analogy with self-association [17], though information on hetero-complexes is generally unknown and needs to be investigated;

- *influence of buffer and other solution parameters.* It is generally believed that the type of buffer in aqueous solution does not significantly affect the structural and thermodynamical parameters of self-association of aromatic molecules, which is based on several direct or indirect investigations (e.g. [221] and Refs. therein). No such studies, however, have so far been performed with respect to the hetero-association. A similar situation is the case with respect to the influence of pH and ionic strength on the hetero-association propensity. As discussed above, few studies on this matter are available in literature (e.g. [159, 220]), which precludes provision of a general view;
- *decomposition of* ΔH_h *and* ΔS_h into the energy contributions from particular physical factors. Solution of this task will enable the role of various physical factors in determining the stability of hetero-complexes to be fully understood.

The list of 'white spots' given above is not complete but will be expanded in the course of further developments in this area.

7. Summary

Although studies of the hetero-association of aromatic molecules have been carried out over the last 50-year period, the subject as a separate physico-chemical process has never been systematically reviewed heretofore. The summary in the present review indicates that there are some important correlations of the ability of aromatic drugs to form hetero-association aggregates in solution, such as the hetero-association nature of hydrotropy and the hetero-association mechanism of biological synergism in combinations of aromatic drugs. Knowledge of the physical chemistry of hetero-association is increasingly important in view of the rapidly emerging branch of supramolecular chemistry dealing with the formation of heterogeneous polymeric structures having specific functional roles. The review has shown that there are some gaps in our understanding of hetero-association, which provides a challenge for further studies in this area.

Abbreviations

AMD	actinomycin D
ActIII	actinocyl-bis(3-dimethylaminopropylamine)
AMP (ADP, ATP)	adenosine mono- (di-, tri-) phosphate
AO	acridine orange
BC	betacarbolines
CAF	caffeine
CHL	chlorophylline
DAPI	4′,6-diamidino-2-phenyl-indole
DAU	daunomycin
DiMeIQx	2-amino-trimethylimidazo[4,5-*f*] quinoxaline)
DOX	doxorubicin
EB	ethidium bromide
EMB (EDC)	ethidium mono- (di-) azide
FL	fluorescence
FMN	flavin mononucleotide
FN	flavin family
Glu-P-1	2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole

Glu-P-2	2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole
H_2TTMePP	5,10,15,20-tetrakis[4-(trimethylammonio)-phenyl]porphine
H_2TMePyP	5,10,15,20-tetrakis(1-methyl-4-pyridyl)porphine
H_2TCPP	4,4′,4″,4‴-(21H,23H-porphine-5,10,15,20-tetrayl)tetrakis-(benzoic acid)
H_2TPPS$_4$	5,10,15,20-tetrakis(4-sulfonatophenyl)-porphine
HAL	halofantrine
ICR-170	1,3,7-propanediamine-N-(2-chloroethyl)-N-(6-chloro-2-methoxy-9-acridinyl)-N-ethyl
ICR-191	2-methoxy-6-chloro-9-(3-(2-chloroethyl)aminopropylamino) acridine
IQ	imidazoquinoline-type aromatic amines
IQx	2-amino-3-methylimidazo[4,5-*f*]quinoxaline
MAO	monoaminooxidase
MB	methylene blue
MC	microcalorimetry
MeIQ	2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAD	nicotinamide adenine dinucleotide
NAS	sodium salicylate
NMD	nicotinamide
NMR	nuclear magnetic resonance
NO_2IQ	2-nitro-3-methylimidazol[4,5-*f*]quinoline
NOG	nogalamycin
NOR	norfloxacin
NOV	novantrone (or mitoxantrone)
OSM	osmometry
PF	proflavin
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
PI	propidium iodide
PRT	partition studies in water–octanol mixture
PTX	pentoxifylline
QM	quinacrine mustard
RBF	riboflavin
SASA	solvent-accessible surface area
THB	theobromine
THP	theophylline
THP7AA (PA, BA)	theophylline-7-acetic acid (propionic acid, buturic acid)
SOL	solubility studies
SP	absorption spectrophotometry
TPT	topotecan
TriMeIQx	2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline
Trp-P-1	3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]-indole
Trp-P-2	3-amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole

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