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ON THE INTERACTION OF CAFFEINE WITH NUCLEIC ACIDS. III. ^{1}H NMR STUDIES OF CAFFEINE–5'-ADENOSINE MONOPHOSPHATE AND CAFFEINE-POLY(RIBOADENYLATE) INTERACTIONS

H. FRITZSCHE, I. PETRI, H. SCHÜTZ, K. WELLER, P. SEDMERA * and H. LANG
Academy of Sciences of GDR, Central Institute of Microbiology and Experimental Therapy, DDR-69 Jena, G.D.R.

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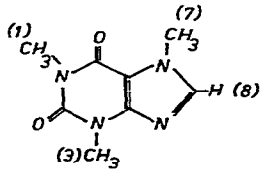
1) The self-association of both caffeine (Cf) and 5'-adenosine monophosphate (AMP) in aqueous solution has been re-investigated by ^{1}H NMR. The self-association process is characterized by an isodesmic model. The apparent self-association constants of the vertical stacking process are $K_{Cf} = (10.6 \pm 1.0)\ M^{-1}$ and $K_{AMP} = (1.67 \pm 0.17)\ M^{-1}$. The arrangement of the monomeric units in the stacked aggregates is discussed in terms of isoshielding curves theoretically calculated by Giessner-Prettre and Pullman. Models are proposed which are consistent with these and further previous NMR data.

2) The interaction of Cf and AMP has been studied by ^{1}H NMR. The apparent association constant of the complex Cf-AMP is $K_{C\text{-}A} = (7.3 \pm 1.2)\ M^{-1}$. Two models of the mutual arrangement of AMP and Cf in the complex are proposed on the basis of the calculated isoshielding curves considering both ring current and local atomic diamagnetic anisotropy effects.

3) The interaction of Cf and poly(riboadenylate), $(rA)_n$, is indicated by a downfield shift of the H-8 line but an upfield shift of the H-2 line in the ^{1}H NMR spectra of $(rA)_n$. The concentration dependence of the ^{1}H NMR shifts of both Cf and $(rA)_n$ can be explained by the existence of two binding mechanisms. We suggest (i) partial insertion of Cf between adjacent base residues of ordered single-stranded regions of $(rA)_n$ and (ii) outside binding of Cf in form of monomeric Cf as well as of self-associated aggregates. The complex geometry of insertion proposed on the basis of the calculated isoshielding curves is characterized by a stronger overlapping of the Cf ring and the H-2 proton of $(rA)_n$ as compared to the H-8 proton.

1. Introduction

Caffeine (Cf, I) is a well-known co-mutagenic agent and a repair inhibitor in bacterial systems [1]. In the presence of Cf the repair system of UV-irradiated bacteria is inhibited and thus mutagenic events can be induced.



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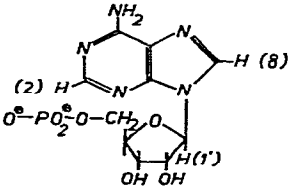

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* Institute of Microbiology Prague of the Czechoslovakian Academy of Sciences.

The previous hypothesis of an enzymatic inactivation by Cf [2,3] has been refuted by Witte and Böhme [4] who have demonstrated the DNA-Cf interaction as the basis of the repair inhibition. Therefore our interest has been focused on the Cf-nucleic acid interaction. Previous results [5–7] have given some insight in the binding process. In this study the NMR is chosen as a method to give detailed information on mobility and mutual arrangement of Cf and nucleic acids including some model systems. Our ^{1}H NMR results of the interaction of Cf with nucleotides and polynucleo-

List of abbreviations:
DNA: sodium salt of deoxyribonucleic acid; Cf: caffeine, AMP: 5'-adenosine monophosphate; dAMP: 5'-deoxyadenosine monophosphate; GMP: 5'-guanosine monophosphate; $(rA)_n$: potassium salt of poly(riboadenylate); EDTA: ethylenediamine tetraacetic acid; UV: ultraviolet; NMR: nuclear magnetic resonance.

tides, respectively, are interpreted in terms of both quantitative and qualitative aspects of the interaction.

The aim of this paper and the succeeding paper in this series [8] is to find out the structure and stability of the complex formed between Cf and nucleic acids. Until now we have little knowledge concerning the detailed structure of the related complexes on the nucleotide and polynucleotide level, respectively. The nucleoside-Cf association has been investigated previously [9].

The knowledge of the self-association of the investigated compounds is a prerequisite to evaluate the interaction between Cf and nucleotides (polynucleotides). Thus we have studied by NMR the self-association of Cf and the nucleotide 5'-adenosine monophosphate (AMP, II), respectively.

2. Experimental and theoretical part

2.1. Materials

Caffeine (pure, VEB Arzneimittelwerk Dresden, GDR), 5'-adenosine monophosphate, 5'-guanosine monophosphate, 5'-deoxyadenosine monophosphate (all in their disodium salts) and poly(riboadenylate) sodium salt (all from Reanal, Hungary) have been used without further purification. The solvent ^2H_2O (Isocommerz, GDR) has an isotopic purity of 99.6% ^2H.

2.2. Methods

The ^2H_2O solutions have been prepared without any buffer or salt except in the caffeine–5'-deoxyadenosine monophosphate experiment in which cacodylate buffer p^{2}H 6.3 was used. 10^{-4} M EDTA was added to remove divalent metal ions. The ^{1}H NMR spectra were recorded by a 100 MHz spectrometer KRH 100 R (constructed by "Zentrum für Wissenschaftlichen Gerätebau der Akademie der Wissenschaften der DDR", Berlin, GDR) working in the continuous-wave mode. The temperature in the probe was $(33 \pm 1^\circ)$. In several spectra the signal-to-noise ratio was enhanced by a time-average computer Spectrostore SPT-1 (ZWG der Akademie der Wissenschaften der DDR, Berlin, GDR). The 5'-deoxyadenosine monophosphate–Cf spectra were run on a JEOL FX-60 MHz instrument working in the Fourier-transform mode. Some spectra were recorded earlier by a Varian HA-100 and a Tesla BS-487 A (80 MHz) spectrometer both working in the continuous-wave mode.

Similarly to recent findings of other authors [10,11] we observed a small shift of the common reference compound sodium 3-(trimethylsilyl)-[$^2H_4$] propionate (TSP) in aqueous solution which is dependent on nucleotide concentration. Therefore, a small amount (0.003 M) of *tert*-butanol was added to the solution as standard. A constant increment of $\delta = 1.266$ ppm was added to these figures thus enabling a comparison to the common chemical shift scale.

The assignment of the caffeine ^{1}H NMR lines follows the studies of Hanna and Sandoval [12]. The assignment of the N-7 methyl protons was confirmed by their splitting due to a long-range coupling of $^4J = (0.65 \pm 0.05)$ Hz between the N-7 methyl and H-8 protons (cf. succeeding part in this series).

2.3. Models

All the ^{1}H NMR shifts of AMP, Cf and the AMP-Cf mixture, respectively, were each collected to a matrix. In the paper of Weller et al. [13] the applicability of the matrix rank analysis to NMR spectra is outlined. The rank of the spectra of AMP self-association as well as of Cf self-association was found to be 2. In the case of mixed association of AMP and Cf, the matrix of the AMP protons exclusively as well as of the Cf protons exclusively shows the rank of 3. The rank of the full matrix of all proton shifts is 5. We made the two assumptions: (i) the interaction shifts in the aggregates are limited to the influence of the nearest neighbours; (ii) the influences of both neighbours are independent and additive.

Thus we can distinguish between the following spectroscopic components: (i) chemical shifts of free AMP molecules in solution; (ii) chemical shifts of free Cf molecules in solution; (iii) interaction shifts of AMP protons caused by one neighbouring AMP molecule in the aggregate; (iv) the analogous interaction shift of Cf protons; (v) interaction shifts of AMP protons caused by a neighbouring Cf and of Cf protons caused by a neighbouring AMP assigned to a AMP-Cf or a Cf-AMP neighbourhood, respectively.

The assumptions concerning the association models are analogous to those of the self-association, i.e. the

interaction free energy of an associate is the sum of free energies of nearest neighbour interactions. The number of monomer units in the associate or aggregate is unlimited. This isodesmic model [14] is used to describe the self-association of both AMP and Cf characterized by the association constants K_{AMP} and K_{Cf}, respectively. The validity of this model has been tested by Solie and Schellman [15] for the interaction of nucleotides in aqueous solution. The model for mixed association is a generalized isodesmic model. The association is unlimited, all types of mixed associates are allowed. The statistical weight of an aggregate with n_A molecules AMP, n_C molecules Cf, n_{AA} neighbourhoods AMP-AMP, n_{CC} neighbourhoods Cf-Cf, n_{AC} neighbourhoods of AMP-Cf, and n_{CA} neighbourhoods Cf-AMP is given by

$$c_A^{n_A} \cdot c_C^{n_C} \cdot K_{AMP}^{n_{AA}} \cdot K_{Cf}^{n_{CC}} \cdot K_{C-A}^{(n_{CA}+n_{AC})} ,$$

c denotes concentration, K_{C-A} is the formation constant for mixed association. Details of the model are described in the paper of Weller et al. [13]. The difference between concentrations and activities is neglected in our calculations.

Admittedly, the differences between activities and concentrations are not zero, but it should be kept in mind that aggregation models are phenomenological to a high extent. In our opinion they allow appropriate extrapolation to the final levels of the curves and an estimation of the interaction shifts. One should particularly exercise caution in comparing K values when covering a broad concentration range using the same measurement technique, and, also, when results from altogether different methods of analysis are compared even in the same concentration range.

2.4. Calculations

A general curve fitting program ALAU (Schütz, unpublished) of the Gauss–Newton type is used combined with a subroutine MST specific for measurements by spectroscopic titrations as described by Schütz [16]. The model specific part of the program for parameter estimation by a least square fit is formed by MST and the models described above. The common procedure consists of selecting protons which determine the system unambiguously by their chemical shifts. In the first step the concentration dependence of the chemical shifts of the selected protons is fitted with respect to the chemical shifts of those protons in the free molecule, their association (interaction) shifts, and the parameters of the model of the system behaviour.

3. Results

3.1. Self-association of caffeine and 5'-adenosine monophosphates

In spite of numerous studies of the self-association both of Cf [17,18] and AMP [18–24] we have reinvestigated the ^{1}H NMR chemical shifts as a function of concentration. It is well known that the self-association of nucleic acid bases in aqueous solution consists in vertical stacking [18,19]. The data are fitted to an isodesmic model in agreement with recent studies of AMP by ^{2}H NMR [24], of N_1-oxide of AMP [25] and ATP by ^{1}H NMR [10,11], and to the results of kinetic studies [26]. Table 1 shows the self-association constants K_{Cf} and K_{AMP}, respectively, and the extrapolated chemical shifts δ of the free and associated species as well as the interaction shifts $\Delta\delta$ (shift per association step), respectively.

Though the NMR data are not sufficient to assign unambiguous models of the stacking arrangements in the associated aggregates, we have ascertained the arrangements which are compatible to the isoshielding curves calculated by Giessner-Prettre and Pullman [27–29]. The isoshielding curves of Cf [30] taking into consideration only the ring current effect have been corrected to the effect of local atomic diamagnetic anisotropies in analogy to the corrections of the four nucleic acid bases [29]. The isoshielding curves of adenosine regarding both ring current and local anisotropy effects [29] are used for AMP as well as for poly (riboadenylate). It should be emphasized that the following assumptions are implicit in the shift calculations: (i) the complex structure is a static one; (ii) the molecules are parallel with interplanar distances of 0.34 nm; (iii) the main contributions to the chemical shifts are the ring current and the local magnetic anisotropy.

Table 1
Extrapolated ^1H NMR shifts δ and self-association constants K_{Cf} of caffeine (Cf) and K_{AMP} of 5'-adenosine monophosphate (AMP), respectively, in neutral aqueous (^2H_2O) solution ($T = 33°C$) under the assumption of an isodesmic association process. The chemical shifts are calibrated in respect to the internal reference *tert*-butanol (δ = 1.266 ppm). "Associated" denotes the shifts of a molecule included by at least two molecules in the stacked associate. Error in the interaction shifts: 5%.

Caffeine (0.01–0.08 M)	Chemical shifts δ, ppm				K_{Cf}, M^{-1}
	H8	H7	H3	H1	
free	7.914	3.983	3.565	3.385	
associated	7.832	3.744	3.170	3.022	10.6
shift per association (interaction shift)	−0.041	−0.119	−0.198	−0.181	± 1.0
5'-adenosine monophosphate (0.01–0.30 M)	Chemical shifts δ, ppm				K_{AMP}, M^{-1}
	H8	H2	H–1'a	H–1'b	
free	8.642	8.296	6.192	6.133	
associated	8.235	7.348	5.894	5.855	1.67
shift per association step (interaction shift)	−0.204	−0.474	−0.149	−0.139	± 0.17

3.2. Mixed association of caffeine and nucleotides (deoxynucleotides)

The chemical shifts of AMP and 5'-deoxyadenosine monophosphate (dAMP) are influenced by addition of Cf and, vice versa, the chemical shifts of Cf are shifted by both nucleotides (fig. 1). Similarly, addition of 0.1 M 5'-guanosine monophosphate (GMP) to a 0.07 M Cf solution induces shifts of the NMR lines of the H8, H7, H3 and H1 protons of Cf by 0.054, 0.035, 0.025 and 0.013 ppm, respectively. These shifts are throughout smaller than those induced by addition of 0.1 M AMP to the same Cf solution which results in upfield shifts of 0.075, 0.044, 0.041 and 0.023 ppm, respectively. On the contrary, Cf induces no shifts in the NMR spectra of either 5'-cytidine monophosphate (CMP) or 5'-uridine monophosphate (UMP). The shifts in the ^1H NMR spectra suggest a decrease of the interaction of Cf and the nucleotides in the order AMP ≈ dAMP > GMP > CMP, UMP. The preference of adenine as the caffeine binding site in nucleic acids has been confirmed by Ts'o et al. [31] in their NMR study of Cf interaction to a self-complementary oligonucleotide AAGCUU.

The self-association of the purine nucleotides in aqueous solution by stacking interaction [19] competes with the formation of mixed Cf-nucleotides complexes. The formation constant K_{C-A} of the complex AMP-Cf has been calculated on the basis of an indefinite stacking model described above (cf. section 2). Complications arose due to small fluctuations of the NMR reference shifts in different sets of measurements Mixed association was measured by two titrations with a fixed concentration of AMP and Cf, respectively. The chemical shifts of the free forms of AMP as well as of Cf (table 1) were correlated from these titrations. This procedure enables the determination of K_{C-A} and the association shifts (interaction shifts) of the AMP protons caused by a neighbouring Cf and vice versa (table 2).

3.3. Interaction of caffeine and poly(riboadenylate)

The interaction studies between Cf and polynucleotides were restricted to poly (riboadenylate), $(rA)_n$, regarding the preferred interaction AMP-Cf on the nucleotide level.

The ^1H NMR chemical shifts of both $(rA)_n$ and Cf are more or less influenced by their interaction in aqueous solution (fig. 2). In comparison to the AMP

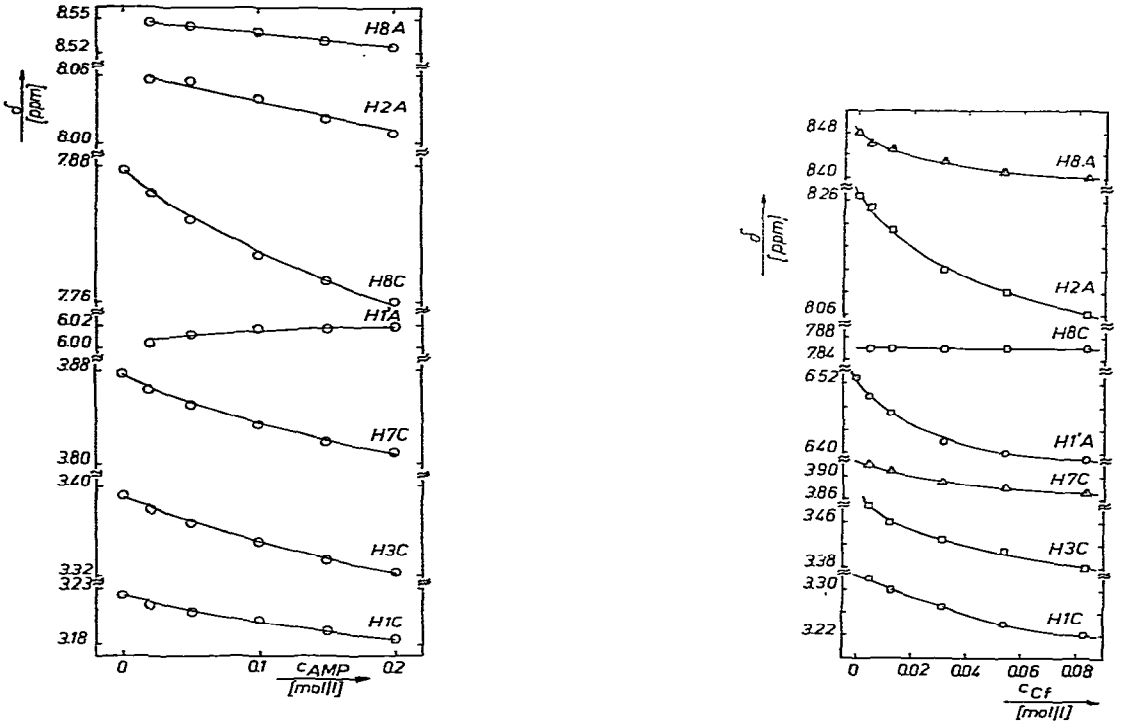

Fig. 1. Chemical shifts δ in ppm of the protons of caffeine–nucleotide mixtures. Solvent ^2H_2O, 33°C, p^{2}H8. (a) Mixtures of 5'-adenosine monophosphate and caffeine (0.13 M) versus concentration of nucleotide. Circles: experimental values; drawn lines: calculated values by the parameters of tables 1 and 2. (b) Mixtures of 5'-deoxyadenosine monophosphate (0.01 M) and caffeine versus concentration of caffeine.

Table 2
Extrapolated ^{1}H NMR shifts δ and formation constant K_{C-A} of the caffeine–5'-adenosine monophosphate complex in neutral aqueous (^2H_2O) solution (T = 33°C) under the assumption of an indefinite isodesmic stacking process. The chemical shifts are calibrated in respect to the internal reference *tert*-butanol (δ = 1.266 ppm). "Complex" denotes the shifts of a molecule included by two molecules of the other complex partner. Error in the interaction shifts: ≤ 10%.

Caffeine 0.13 M AMP 0–0.2 M	Chemical shifts δ, ppm							K_{C-A}, M^{-1}
	AMP			Cf				
	H8	H2	H1	H8	H7	H3	H1	
free	8.642	8.296	6.163	7.914	3.983	3.565	3.385	
complex	8.356	7.602	5.695	7.549	3.661	3.158	3.071	7.25
shift per association step (interaction shift)	−0.143	−0.347	−0.234	−0.183	−0.161	−0.204	−0.157	± 1.19

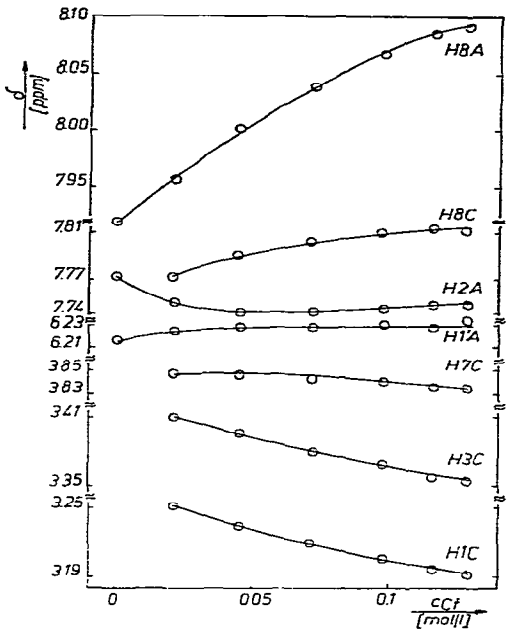

Fig. 2. Chemical shifts δ in ppm of the protons of caffeine–poly(riboadenylate) mixtures. Solvent ^2H_2O, 33°C, p^{2}H 8.5. Circles: experimental values; drawn lines: calculated values on the basis of the parameters $K_1 = 10^{-1}$, $K_2 = 25.3\ M^{-1}$, $\tau = 0.53$ (cf. table 3). As already mentioned (cf. sect. 3), the least square fits on the basis of the other parameter sets of table 3 are nearly identical to the drawn lines presented in this figure.

chemical shifts in the AMP-Cf complex, the strikingly different behaviour of the H-8 line of $(rA)_n$ is evident. In contrast to the upfield shift of AMP (fig. 1) the H-8 of $(rA)_n$ shifts downfield with increasing Cf concentration. Furthermore the concentration dependence of the chemical shifts of the H-2 line of $(rA)_n$ and of some other lines suggests the superposition of two or more binding mechanisms. We suggest two binding mechanisms for the Cf–$(rA)_n$ interaction: (i) Insertion of Cf molecules between two adjacent bases of the polyribonucleotide. This non-cooperative mechanism is described by a binding constant K_1. (ii) Outside-binding of Cf aggregates including monomers. The first attachment of a Cf aggregate to $(rA)_n$ is described by the binding constant K_2, further attachments of the same aggregate by $K_2\tau$. The number of Cf molecules per polynucleotide base is denoted by n. The treatment of this model is given by Weller et al. [13] according to the method of sequence generating functions of Lifson [32].

In the system Cf–$(rA)_n$ all attempts of re-scaling were unsuccessful due to the mentioned fluctuations between different NMR measurement sets. Neither a free-of-model analysis described independently by Halfman and Nishida [33] and Reinert [34,35] nor an exploration of the binding behaviour based upon the most probable model described above yield consistent results.

On account of sterical restriction of the Cf insertion between bases of $(rA)_n$ the value of the ratio $(K_{C-A})^2/K_{AMP}$ valid for nonrestricted insertion of a Cf in an AMP dimer is the upper limit of K_1. Therefore we estimated K_2, τ, and all interaction shifts corresponding to both binding mechanisms by a least square fit assuming a number of $n = 2$ outside-bound Cf per adenine residue of $(rA)_n$ at fixed values of K_1 ($K_1 = 10$, 20, 30 and 60 M^{-1}; table 3). The least square criterium is without any significant dependence on the selected K_1 values.

4. Discussion

4.1. Self-association of 5'-adenosine monophosphate

The self-association of AMP has been studied extensively both by osmometric [36] and NMR measurements [20–24, 36, 37]. Our data support an association to aggregates higher than dimers assuming an isodesmic association process [14, 26]. The association constant $K_{AMP} = 1.67\ M^{-1}$ (table 1) describing the association of AMP to indefinite aggregates is in good agreement with the data of Imoto [22] (dimerization constant of 0.77 M^{-1}; assuming dimerization only, we got a corresponding constant of 0.84 M^{-1}). We are in apparent fair agreement with Egan [24], who observed the $^2H_8$ spin lattice relaxation time T_1 of 8-deuterioadenosine 5'-monophosphate and got a dimerization constant of 2.1 M^{-1}. It should be noted that although Egan got indications to aggregates higher than dimers, he fitted the T_1 values by the assumption of dimerization only. To get an equilibrium constant on the basis of his measurements comparable with our K_{AMP} one has to do the following:

i) Beside T_1^m, T_1^d (for the monomer and the dimer

Table 3
Calculated shifts and binding parameters of the Cf–$(rA)_n$ interaction assuming the two binding processes of (i) insertion and (ii) outside binding. $\Delta\delta_{ins}$ are the shifts of the protons induced by insertion of one Cf between two adjacent adenines of $(rA)_n$. In an A proton chemical shift, insertion of Cf introduces a contribution which is the twofold of $\Delta\delta_{ins}$ because each inserted Cf is neighboured by two adenine residues. $\Delta\delta_{o.b.}$ are the shifts of the protons induced by an attachment of Cf aggregates bound outside under the assumption of $n = 2$ Cf per adenine residue. The calculations are carried out using reasonable values of 10, 20, 30 and 60 M^{-1} of the binding constant K_1 of the insertion process.

K_1, M^{-1}	$\Delta\delta_{ins}$, ppm				$\Delta\delta_{o.b.}$, ppm			
	10	20	30	60	10	20	30	60
H8A	−0.05	−0.05	−0.05	−0.07	0.25	0.29	0.31	0.37
H2A	−0.22	−0.13	−0.11	−0.08	−0.02	−0.01	0	0.01
H1'A	0.06	0.04	0.03	0.02	0.02	0.02	0.01	0.01
H8C	−0.44	−0.23	−0.16	−0.06	−0.19	−0.22	−0.24	−0.29
H7C	−0.60	−0.31	−0.21	−0.08	−0.09	−0.13	−0.16	−0.24
H3C	−0.79	−0.40	−0.27	−0.09	−0.04	−0.10	−0.15	−0.27
H1C	−0.66	−0.32	−0.21	−0.06	−0.03	−0.09	−0.13	−0.24
K_2, M^{-1}					25.3	31.7	38.0	59.1
τ					0.53	0.37	0.30	0.18

form, respectively) one has to introduce one (or more) T_1^a for higher aggregate form(s);

ii) T_1^o (observed) must be formulated as a function of T_1^m, T_1^d, T_1^a depending on the equilibrium concentrations of the monomer, the dimer, and the aggregate form(s) according to the isodesmic model;

Somewhat regrettably curves like those published by Egan (or measured by us) allow the determination of three parameters only.

iii) One has to find out one (or more) relation(s) between the T_1^j ($j \in$ (m, d, a)) to reduce the number of the independent measurement parameters T_1^j to two.

At this stage some arbitrariness is inevitable, because any quantitative relationship between the equilibrium parameters $K_{isodesmic}$ and K_{dimer} depends strongly on the relations chosen between the measurement parameters. Our assumption that the interaction shift of the inner members of an aggregate is the sum of the interaction shift caused by the left hand neighbour plus the interaction shift caused by the right hand neighbour has some justification. Only for this type of assumption does the relation $K_{dimer} = \frac{1}{2} K_{isodesmic}$ [43] hold.

If (i)–(iii) is carried out, the model is ready for use. $K_{isodesmic}$ can be estimated on the basis of Egan's measurements. Without doing (i)–(iii) we can only say that his value is still in the right order of magnitude.

Furthermore the isodesmic association constants of

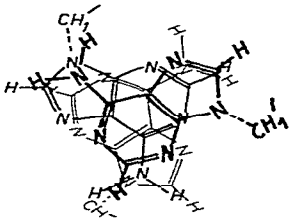

Fig. 3. Model illustrating the proposed arrangement of 5'-adenosine monophosphate molecules in the stacked self-associated species. Upper, middle and lower nucleotides are drawn with heavy, medium and light lines, respectively.

indefinite linear self-association of both adenosine 5'-triphosphate ($K = 0.9$ M^{-1} and 1.3 M^{-1}, respectively) [10,11] and of N_1-oxide 5'-AMP ($K = 2.48$ M^{-1}) [25] are very similar to our result of AMP.

Our proposed model of the stacking geometry of AMP aggregates in aqueous solution (fig. 3) is based on the calculated isoshielding curves [27–29] and on the H8–H2 distance of 0.36 nm evaluated by Imoto [22] using the so-called Desert NMR method [38]. We find the best fit of experimental and calculated shielding data assuming a head-to-tail arrangement of the bases with *trans* position of the ribose moieties and a winding angle of approximately 45°. This model is in contradiction to the head-to-head arrangements

of Schweizer et al. [36] and Evans and Sarma [20] but in agreement with the findings of Guéron et al. [21] and Zens et al. [23]. The essential features of the proposed model should be valid in spite of small deviations in the figures of both the isoshielding curves and the H8–H2 distance between interacting nucleotides. Very recently Poltev and Shulga [39] calculated the most favoured positions of stacked adenines. Their type I is in satisfactory agreement with our model. The winding angle of approximately 50° is very near to our figure (45°) but the translational figures of the mutual arrangement differ slightly. It should be emphasized, however, that their calculations have been done neglecting the influence of ribose and phosphate moieties as well as the influence of water.

4.2. Self-association of caffeine

Thakkar et al. [17] calculated the dimerization constant of Cf in aqueous solution from the ^{1}H NMR data. We, on the contrary, fitted the data assuming the formation of indefinite linear aggregates with equal energy and entropy per step (isodesmic model) in agreement with the results of other methods [40].

Gill et al. [41] as well as Schimmack et al. [42] interpreted the self-association of Cf in aqueous solution by an isodesmic model of indefinite stacking interaction. They report association constants of K_{Cf} = 15 M^{-1} (25°C) [41] and K_{Cf} = 9 M^{-1} (25°C) [42], respectively, which are in good agreement with our result (K_{Cf} = 10 6 M^{-1}, 33°C; table 1). The dimerization constant of Thakkar et al. [17] (8.6 M^{-1}) is likewise near these values for the relation $K_{dimer} = \frac{1}{2} K_{isodesmic}$ [43].

The self-association of Cf is characterized by a very weak association shift of the H8 protons as compared to the shifts of the methyl protons of N7 (table 1). Therefore all models with a strict head-to-head arrangement like the model of Thakkar et al. [17], as well as with a strict head-to-tail arrangement, conflict with the data. The data can be fitted satisfactorily assuming an approximately orthogonal arrangement (fig. 4). Our proposed model is ambiguous as far as the rotation of one of the Cf by 180° in respect to the ring centre results in an arrangement which also fits the data. Nevertheless the model proposed by Thakkar et al. [17] results in a strict disagreement between measured NMR shifts and calculated isoshielding curves of Cf [30].

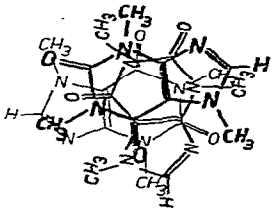

Fig. 4. Model illustrating the arrangement of caffeine molecules in the stacked self-associated species. Upper, middle and lower caffeine are drawn with heavy, medium and light lines, respectively.

4.3. Association of caffeine and 5'-adenosine monophosphate

The association constant K_{C-A} = 7.3 M^{-1} of the Cf-AMP mixed association (table 2) is in the same range as the self-association constants of both AMP and Cf discussed above, but appreciably lower than those found for adenine-caffeine (45.1 M^{-1}) and adenosine-caffeine (39.4 M^{-1}) reported by Nakano and Igarashi [9]‡.

‡ Nakano and Igarashi [9] measured the enhancement of the solubility of adenine and adenosine as a function of the concentration of added caffeine. They estimated their K values by assuming an soluble 1:1 complex. In AMP, however, the charged polar phosphate group is present, and it has a large influence on solubility as well as on aggregation behaviour. No systematic comparison between their K values and our K_{C-A} is possible.

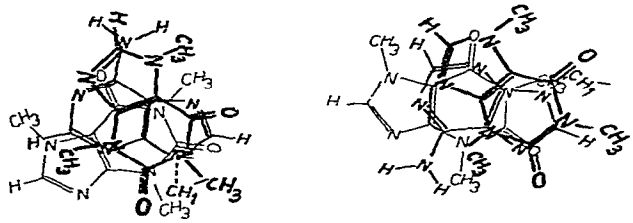

Fig. 5. Models illustrating the proposed arrangement of the mixed stacking complex between caffeine and 5'-adenosine monophosphate. Upper caffeine, middle nucleotide and lower caffeine are drawn with heavy, middle and light lines, respectively. Right: model (a), N9 of both caffeine and nucleotide are on the same side of the stack. Left: model (b), N9 of caffeine and nucleotide are opposite to each other in the stack.

The model of the mutual arrangement of Cf and AMP in the mixed associates (fig. 5) is characterized by the head-to-tail arrangement of the purine rings. The experimental data are compatible with the calculated shifts in two arrangements differing in their relative arrangement in respect to the N9 position: in the model (a) are the related N9 of Cf and AMP on alternating positions but in model (b) on the same side of the stack. A discrimination should be possible by introduction of a paramagnetic label (Mn^{2+}) into the AMP. However, the results of Evans and Sarma [20] demonstrate the ambiguity of the interpretation of this type of experiments.

4.4. Interaction of caffeine and poly(riboadenylate)

Poly(riboadenylate) $(rA)_n$ forms in neutral aqueous solution single-stranded regions by stacking of adjacent adenine residues [44–46]. The shifts of the ^1H NMR lines as a function of the Cf concentration (fig. 2) are interpreted by the existence of two mechanisms of the binding of Cf to $(rA)_n$: insertion of Cf between adjacent adenines and outside binding of Cf aggregates.

Common to both mechanisms is the tendency to destack the ordered regions of $(rA)_n$ to facilitate the binding of Cf to the polynucleotide. Obviously the outside binding of dyes to nucleic acids is different from the outside binding suggested for the Cf–$(rA)_n$ complex. In contrast to the former, the UV spectra do not indicate any interaction between polyphosphate and Cf (G. Löber, unpublished results).

The insertion process is characterized by a stronger influence of the inserted Cf to the H2 protons of $(rA)_n$ compared to the H8. The insertion process involves an increase of the distance of two adjacent adenines. The increasing distance is accompanied by destacking of the adenines as indicated by downfield shifts of the proton NMR lines. The vertical interaction between the inserted Cf and the adjacent adenines is indicated by upfield shifts of the proton NMR lines of both Cf and adenines involved in the interaction. Whether the resulting shift of a proton is upfield or downfield depends on the mutual arrangement of Cf and adenine residues.

We interpret the second binding process as an outside binding of Cf aggregates including monomers. Obviously this horizontal interaction is favoured by destacking of adjacent adenine residues, too, monitored by the monotonous downfield shift of the H8 line of adenine.

As mentioned above (cf. section 3) the two competing binding processes cannot be separated unambiguously. Therefore we calculated the binding parameters of both processes under the assumption of a number of $n = 2$ outside-bound Cf per adenine residue of $(rA)_n$ and a set of reasonable values of K_1, the binding constant of the insertion mechanism. The upper limit of K_1 is given by $(K_{C-A})^2/K_{AMP}$ which results in a value of 31 M^{-1} (table 1 and 2). A rough estimation inspecting the concentration dependence of the H2 line of $(rA)_n$ (fig. 2) results in a somewhat higher value of circa 60 M^{-1}. However, the most reasonable value may be expected to be in the region of $K_1 = 20$ M^{-1}.

The calculated shifts (table 3) of both binding processes under the assumption of $K_1 = 10$, 20, 30 and 60 M^{-1} allow some speculations about the arrangement of Cf in the two types of binding. The insertion of Cf can be described by a model fitting the interaction shifts $\Delta\delta_{ins}$ corresponding to $K_1 = 10$ M^{-1} in respect to the calculated isoshielding curves (fig. 6). In this model we assume that the increase of the distance between adjacent adenines is not accompanied by a change of their mutual arrangement which is described by Saenger et al. [47] on the basis of X-ray studies. As demonstrated in table 4, the $\Delta\delta$ values of the proposed insertion model (fig. 6) and of the model calculation agree very well for $K_1 = 10$ M^{-1}. The deviation between the two sets of $\Delta\delta$ values does not

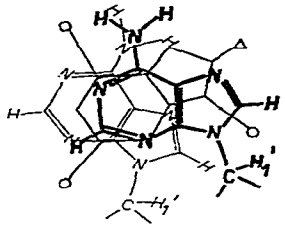

Fig. 6. Model illustrating the proposed arrangement of caffeine insertion between adjacent bases of poly(riboadenylate). Upper and lower base are drawn with solid and broken line, respectively. The inserted caffeine is sketched by the position of its methyl protons (circles) and H8 proton (triangle). It is assumed that the increase of the distance of the adjacent bases which insert caffeine is not accompanied by any significant change of their mutual arrangement in respect to a view along the screw axis [47].

Table 4
Insertion shifts $\Delta\delta_{ins}$ of the model of Cf insertion between bases of stacked regions of $(rA)_n$. The $\Delta\delta_{ins}$ values of the structure model (fig. 6) are estimated on the basis of the calculated isoshielding curves of adenine [29] and Cf [30], respectively. In comparison with these data, the calculated $\Delta\delta_{ins}$ values are included in the table; they are calculated on the basis of the binding model described above (cf. section 2.3) with a presumed value of $K_1 = 10\ M^{-1}$ (cf. table 3).

^1H NMR line	$\Delta\delta_{ins}$, ppm	
	structure model	calc. ($K_1 = 10\ M^{-1}$)
H8A	−0.10	−0.05
H2A	−0.20	−0.22
H1'A	circ. 0	+0.06
H8C	−0.40	−0.44
H7C	−0.65	−0.60
H3C	−0.75	−0.79
H1C	−0.65	−0.66

exceed ± 0.05 ppm which is within the precision of the isoshielding fit procedure.

Outside-bound Cf influences the H2 of adenine, counterbalancing the downfield shift induced by the increase of the distance between adjacent adenines. The result is a $\Delta\delta_{o.b.}$ of nearly zero. On the other hand the H8 of adenine is far from the outside-stacked Cf aggregates. The $\Delta\delta_{o.b.}$ of H8 (adenine) indicate the destacking effect. The Cf proton NMR lines are shifted upfield by the outside binding which may indicate a favourable position in respect to magnetic shielding by ring current or local magnetic anisotropies compared to the association shifts of Cf in its self-associated aggregate (table 1). The favoured position of H8 in respect to H3 and H1 is the most striking result. Obviously the five-membered imidazole cycle of Cf is more engaged in the outside binding to $(rA)_n$ than the six-membered pyrimidine cycle.

5. Conclusions

1) The interaction of caffeine and the four nucleotides decreases in the order AMP, GMP, CMP, UMP, as evidenced by ^1H NMR.

2) The self-association of both caffeine and 5'-adenosine monophosphate has been reinvestigated. The ^1H NMR shifts have been fitted under the assumption of an indefinite isodesmic association process resulting in association constants of $K_{Cf} = (10.6 \pm 1.0)\ M^{-1}$ and $K_{AMP} = (1.67 \pm 0.17)\ M^{-1}$. The geometry of the stacked associates has been obtained by fitting the extrapolated NMR shifts to the magnetic isoshielding curves calculated by Giessner-Prettre and Pullman.

3) The formation constant of the complex Cf-AMP results to $K_{C-A} = (7.3 \pm 1.2)\ M^{-1}$. In the complex the purine cycles are stacked in a head-to-tail arrangement.

4) The interaction of Cf and poly(riboadenylate) is characterized by two binding processes: the insertion of Cf between adjacent adenines and the outside binding of Cf aggregates including monomers. Assuming a value of $K_1 = 10\ M^{-1}$ of the insertion process a model has been proposed fitting the calculated insertion shifts to the isoshielding curves mentioned above.

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