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^{13}C NMR Investigations on the Stacking of 5′-AMP with Tryptamine

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Complex formation between 5′-adenosine monophosphate (5′-AMP) and tryptamine was investigated by measuring ^{13}C chemical shifts and spin-lattice relaxation times. The chemical shift changes observed were attributed to ring current effects originating in the stacking of the two respective aromatic moieties and to puckering changes of the AMP ribose. Differences in the magnitude of the shifts of the aromatic carbons were related to the geometry of the complex and compared with those found for AMP selfassociation. Upon complex formation the relaxation times of especially the tryptamine indole carbons were greatly reduced, this was explained by an increase in the particle size. Small changes found for the AMP carbons in solutions without tryptamine result from AMP selfassociation. Deviations from isotropic motion observed for the non-aromatic moieties are discussed.

Introduction

The planar association, known as stacking, is a well established phenomenon that occurs, in aqueous solutions, between polar aromatic ring systems found in nucleobases, aromatic amino acid side chains and several drugs and dyes It contributes significantly to the stability of biopolymer secondary structure and to biochemical recognition processes. Despite the large amount of work reported in this field, the question has still not been satisfactorily answered regarding the elementary physical forces that contribute mostly and specifically to this interaction. (For review see Refs 1 and 2.)

The aromatic amino acid tryptophan and related indole derivatives have been shown to interact rather strongly with purine nucleotides [3–5]. From a series of investigations, using ^{1}H NMR data in conjunction with considerations of data taken from the literature, it was concluded that mutual polarization (dipole-induced dipole forces) contrifutes significantly to stacking specificity [2, 6–8].

The ^{13}C NMR investigations of the present work were performed in order to clarify whether ^{13}C chemical shift and relaxation time measurements can give specific information concerning complex formation of the stacking type. This is especially interesting, because the complexity of the ^{1}H spectra often allowed only certain of the proton resonances to be used to monitor these interactions; in addition the purine moiety of the common nucleotides possesses only one (guanine) or two (adenine) protons. ^{13}C NMR studies should provide a more powerful tool as all the carbons in the molecule can be observed without interferences from overlapping resonances and purines possess 5 such carbons. Thus ^{13}C NMR investigations should be especially suitable to supply information on geometrical features of those complexes, where significant chemical shift changes due to ring current effects are to be expected upon complex formation.

The quantitative features, *i.e.*, the affinity of complex formation between AMP and tryptamine, have been extensively investigated by ^{1}H NMR spectroscopy in previous work [4, 22].

Experimental

Natural-abundance ^{13}C NMR spectra were obtained on a Varian XL-100-12 spectrometer operating in the Fourier transform mode at 25.16 MHz with proton decoupling. The instrument was locked to the deuterium resonance of the solvent and was controlled with a Varian 620-L computer equipped with a moving head disc together with complementary software.

Tryptamine HCl was purchased from Merck AG and 5′-AMP from Boehringer Mannheim GmbH. D_2O solutions of the respective components or mixtures were adjusted to pD 8.0 and twice lyophilized. Solutions for the T_1 experiments had nitrogen gas bubbled through them to remove paramagnetic oxygen. ^{13}C chemical shift measurements were carried out at 7 °C on samples containing dioxane (0.25 M) and were referred to external TMS. The shifts are accurate to better than ± 0.05 ppm. T_1 measurements were performed at 36 ± 1 °C

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by the use of the modified inversion recovery technique[9, 10] for carbons with short T_1 values (<3 sec), while longer relaxation times were measured by the progressive saturation technique[10]. These experiments and data reduction were performed with Varian software (994120-B). T_1 values of less than 12 sec are accurate to better than ±10%. For the longer T_1 values (in tryptamine solutions B and E) the accuracy is limited to ±20%. This arises from the necessity to use dilute solutions and hence accuracy is limited by the signal to noise ratio and instrumental stability; typical runs for these longer T_1 determinations took 72 h or longer.

Results and Discussion

Chemical shift assignments

The assignments of the resonances of 5′-AMP follow those of Dorman and Roberts[11] as modified by Mantsch and Smith[12] and by Birdsall and Feeney[13]. The ^{13}C spectrum of tryptamine has not been analysed previously. Off-resonance proton decoupled spectra allowed unambiguous assignment of quaternary, tertiary and secondary carbon resonances. Comparison of the chemical shifts with those in 3-methyl indole[14] allowed the assignment of resonances of C(3), C(7), C(8) and C(9). All of the proton-bearing aromatic carbons, apart from C(2), appeared as characteristic doubled doublets in a proton-coupled spectrum from coupling with the directly bonded proton and with a proton 3-bonds removed ($^3J_{CH} \gg {}^2J_{CH}$, $^4J_{CH}$ for aromatic systems)[15]. The resonance of C(2) could thus be assigned as it appeared as a broadened doublet arising from couplings with the directly bonded proton and small unresolved couplings with H(3′). The remaining aromatic resonances of C(4), C(5) and C(6) were assigned by comparison with 3-methyl indole[14] and from selective ^{1}H decoupling experiments (L. Ernst, private communication). The two secondary carbon resonances, C(3′) and C(3″), were assigned from chemical shift considerations[15].

In all the fully relaxed spectra, taken in connection with the T_1 measurements, the resonances of both C(9) and C(2) appeared slightly lower in intensity than those of the other quaternary and proton-bearing carbons, respectively. This gives additional evidence for their assignments and must arise from quadrupolar broadening by the adjacent ^{14}N nucleus. This nucleus does not contribute significantly, however, to the relaxation mechanism of either of these carbons as the areas of all the carbons were equal, within experimental error, in the fully relaxed spectra.

Concentration-dependent chemical shifts of 5′-AMP

A great deal of evidence exists for the concentration-dependent intermolecular association of nucleotides in aqueous solution from ^{1}H NMR chemical shift measurements and various other methods (for review see Ref. 1). Concentration-dependent ^{13}C chemical shift measurements have been reported for 5′-AMP and 5′-dGMP[16–18]. The concentration-dependent ^{13}C shift changes found for 5′-AMP in the present work, illustrated in Fig. 1 (left), are similar to those reported previously by Smith *et al.*[16] at 37 °C. A lower temperature (7°) was used in the present case as complexation is favoured by decreasing the temperature[4]. The chemical shifts have been extrapolated to zero concentration, in order to obtain the shifts of the free uncomplexed 5′-AMP molecule (δ_O). Likewise the shift values were extrapolated to infinite concentration (1/concentration), to obtain the carbon shifts of the pure selfassociated nucleotide (δ_S). These values are listed in Table I, which further indicates the chemical shift changes ($\Delta\delta_{OS} = \delta_O - \delta_S$) generated by the selfassociation of 5′-AMP. These values are of limited accuracy, as the extrapolations were performed over a limited concentration range, but they offer a qualitative impression of what the dif-

Table I. ^{13}C chemical shift changes of 5′-AMP carbons due to selfassociation and complex formation with tryptamine. Units ppm referred to TMS. The chemical shifts were extrapolated to zero AMP concentration (δ_O), to infinite AMP concentration (δ_S) and to infinite tryptamine concentration (δ_C). For full explanation see text. $\Delta\delta_{OS} = \delta_O - \delta_S$ (selfassociation) and $\Delta\delta_{OC} = \delta_O - \delta_C$ (complex formation with tryptamine).

	δ_O	δ_S	δ_C	$\Delta\delta_{OS}$	$\Delta\delta_{OC}$
C(2)	153.75	152.50	152.30	1.25	1.45
C(4)	149.90	148.30	148.20	1.60	1.70
C(5)	119.45	118.10	118.95	1.35	0.50
C(6)	156.35	154.90	155.50	1.45	0.85
C(8)	141.30	140.45	139.80	0.85	1.50
C(1′)	87.90	88.45	88.40	−0.55	−0.50
C(2′)	75.65	76.00	75.35	−0.35	0.30
C(3′)	71.80	71.30	70.55	0.50	1.25
C(4′)	85.90	85.20	84.30	0.70	1.60
C(5′)	64.50	64.50	64.05	0.0	0.45

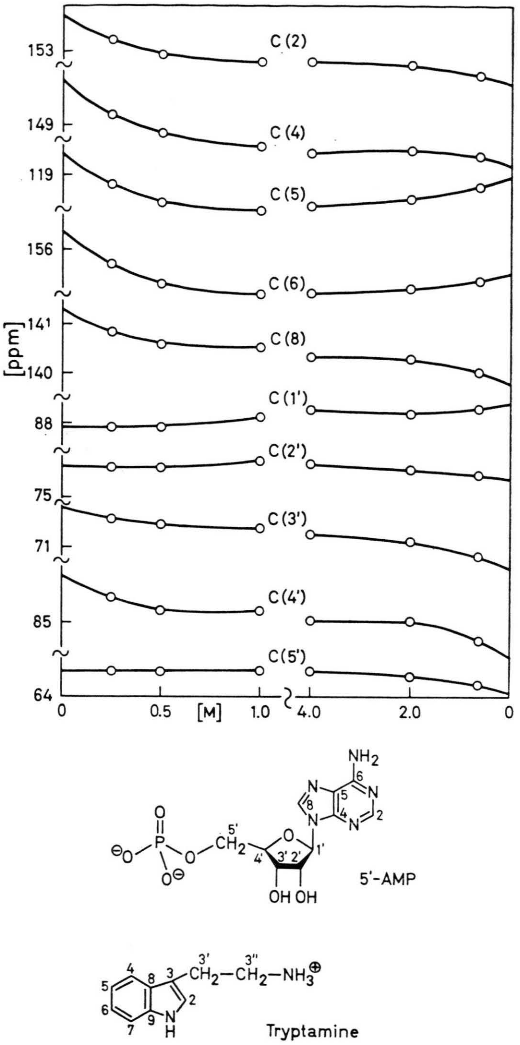

Fig. 1. ^{13}C chemical shift changes of 5′-AMP carbons originating from selfassociation (left) and complex formation with tryptamine (right). The different chemical shifts (in ppm referred to TMS) were plotted *versus* AMP concentration (left) and *versus* the AMP divided by the tryptamine concentration (right). The composition of the three solutions is indicated in Table II.

ferent carbons experience when free 5′-AMP molecules form nucleotide-nucleotide complexes.

The largest upfield shifts upon selfassociation are observed for the base carbons. It was suggested that this was due to base stacking [16], which is the driving force of selfassociation. This is consistent with a ring current effect similar to that extensively discussed for the proton shift changes. It was argued that non-specific solvent effects are not responsible for the observed shifts as the ribose carbons are less affected than the base carbons. This is confirmed in the present study by the use of an internal standard, dioxane, referenced to an external capillary of TMS: there is no shift, within the experimental error, of the carbon of the internal reference. The carbon furthest away from the interacting base moieties C(5′) is affected the least; however, carbons C(1′) and C(2′) of the ribose are shifted downfield with selfassociation, whereas C(4′) experiences a rather strong upfield shift. It is difficult to attribute these changes to solely ring current effects associated with the geometry of the selfassociated complex. More likely, changes in the ribose puckering due to oligomer formation, as observed from ^{1}H coupling constants [19, 4], give rise to the anomalous behaviour of the ribose carbons. Furthermore, regional changes in the solvent sheath and intermolecular electronic effects not specifically arising from ring current effects have to be considered, too.

Chemical shifts changes due to AMP-tryptamine complex formation

In our previous ^{1}H study [4] we showed that selfassociation of tryptamine is small compared to that of 5′-AMP and that the apparent association constant for selfassociation of 5′-AMP is smaller than that for complex formation with tryptamine. The ^{13}C shifts of AMP carbons for different solutions of 5′-AMP and tryptamine are plotted in Fig. 1 (right). The plotting was done to extrapolate to infinite tryptamine concentration (AMP/tryptamine equals zero); the extrapolated values, which represent the chemical shifts of AMP carbons totally complexed with tryptamine (δ_C), are listed in Table I together with the shift changes ($\Delta\delta_{OC} = \delta_O - \delta_C$) generated by this complex formation. Also in this case the constancy of the dioxane resonance again implies that the shifts observed arise from changes in the molecular associations in solution rather than from non-specific solvent effects.

The $\Delta\delta_{OS}$ and $\Delta\delta_{OC}$ values of Table I allow a comparison of the chemical shift changes generated either with AMP selfassociation or complex formation with tryptamine. The magnitude of the shift range is similar for both processes, although for the base carbons differences exist for C(5), C(6) and C(8) (*cf.* also Fig. 1). Assuming the shift changes of the adenine carbons originate mainly from ring current effects one could deduce that unlike AMP selfassociation the center of the adenine-indole interaction lies over carbons C(2), C(4) and C(8) of the adenine system in the proximity of the ribose ring. This is consistent with ^{1}H studies[4] where large upfield shifts for the ribose protons H(2′), H(3′), H(4′) (in decreasing strength) were observed upon association with tryptamine but not with AMP selfassociation.

The chemical shift changes experienced by the ribose carbons extend from medium downfield C(1′) to strong upfield C(4′) shifts. ^{1}H coupling constants revealed[4] that in AMP the ribose moiety in the complex with tryptamine assumes a 3′-endo puckering, whereas in the free state a 2′-ende conformation prevails (selfassociation only partially shifts the puckering equilibrium to 3′-endo). Hence the behavior of the ribose carbons may be explained by two contributions: large shift changes due to the puckering change (downfield for C(1′) and upfield for C(4′)) and an overall upfield shift arising from the ring current of the overlapping indole ring.

Table II contains the chemical shifts experienced by the tryptamine carbons upon the complex formation with 5′-AMP. Again the shifts have been extrapolated to infinite nucleotide concentration (tryptamine/AMP equals zero), in order to estimate the shifts in the pure complex. The $\Delta\delta$ values, indicating the shift changes generated by complex formation, have been obtained from the extrapolated shift values of the pure complex and a 0.25 M tryptamine solution. A correction for tryptamine selfassociation has not been made, because of the limited solubility of tryptamine and the fact that selfassociation is less strong than with 5′-AMP[4, 2].

Table II. ^{13}C chemical shift changes of tryptamine carbons upon complex formation with 5′-AMP. Units ppm referred to TMS. δ_T chemical shifts of a 0.25 M tryptamine solution, δ_C chemical shifts obtained by extrapolation to infinite AMP concentration. $\Delta\delta = \delta_T - \delta_C$.

	δ_T	Chemical shifts for tryptamine solutions containing 5′-AMP of			δ_C	$\Delta\delta$
		0.25 M*	0.5 M	1.0 M		
C(2)	125.23	124.64	124.22	124.14	124.00	1.23
C(3)	109.83	109.68	109.53	109.56	109.60	0.23
C(4)	119.22	118.75	118.50	118.38	118.20	1.02
C(5)	120.32	119.97	119.72	119.65	119.55	0.77
C(6)	123.07	122.70	122.45	122.35	122.25	0.82
C(7)	112.96	112.54	112.21	112.06	111.90	1.06
C(8)	127.29	127.00	126.73	126.65	126.55	0.74
C(9)	137.31	136.93	136.66	136.56	136.40	0.91
C(3′)	23.51	23.67	23.67	23.76	23.85	−0.34
C(3″)	40.59	40.49	40.44	40.49	40.50	0.09

* Tryptamine concentration 0.4 M, otherwise 0.25 M.

The largest shifts are oberved for the aromatic carbons, while the aliphatic carbons are shifted only slightly. Taking the different shift values for construction of the geometry of the complex, based mainly upon the ring current effect of the partner adenine, one can conclude the following: the overlapping of adenine occurs mainly with the carbons C(2), C(9), C(7) and C(4, 5, 6). The aliphatic tail, C(3) and partially C(8) lies outside the adenine plane, probably above the ribose and the phosphate. This would also be consistent with the idea that due to electrostatic interaction the positively charged aliphatic moiety of tryptamine is near to the negatively charged phosphate of AMP.

Spin-lattice relaxation times

In order to compliment the above study, carbon T_1 measurements have been determined and these are shown in Table III. The effects of stacking upon

Table III. ^{13}C spin-lattice relaxation times (T_1, in seconds) for 5′-AMP, tryptamine and complexes hereof. The following solutions were investigated: A, 0.39 M 5′-AMP, 4 mM EDTA; B, 0.32 M tryptamine, 4 mM EDTA; C, 0.4 M AMP, 0.4 M tryptamine, 4 mM EDTA; D, 0.4 M AMP, 0.4 M tryptamine, 10 mM EDTA; E, 0.32 M tryptamine, 0.4 M glucose, 4 mM EDTA. All solutions were adjusted to pD 8.0. For details see experimental section.

Carbons	Solutions A	B	C	D	E
5′-AMP					
C(2)	0.19	—	0.15	0.16	—
C(4)	6.40	—	4.75	6.05	—
C(5)	11.69	—	9.04	10.69	—
C(6)	7.69	—	6.22	5.56	—
C(8)	0.19	—	0.12	0.15	—
C(1′)	0.23	—	0.17	0.16	—
C(2′)	0.25	—	0.20	0.20	—
C(3′)	0.24	—	0.18	0.20	—
C(4′)	0.24	—	0.16	0.16	—
C(5′)	0.12	—	0.12	0.15	—
tryptamine					
C(2)	—	2.12	0.35	0.39	1.59
C(3)	—	37.0	6.91	8.40	30.7
C(4)	—	2.21	0.37	0.39	1.69
C(5)	—	1.65	0.36	0.39	1.50
C(6)	—	1.73	0.33	0.36	1.30
C(7)	—	1.75	0.36	0.38	1.53
C(8)	—	45.5	8.65	10.92	49.1
C(9)	—	39.1	7.55	10.35	39.6
C(3′)	—	1.44	0.36	0.38	1.20
C(3″)	—	1.42	0.44	0.45	1.20

carbon T_1's in 5′-AMP has been investigated by Hamill *et al.*[20] and the mechanisms contributing to these processes have been investigated by Norton and Allerhand[21].

The relaxation of proton-bearing carbons in molecules such as 5′-AMP and tryptamine is dominated by the dipole-dipole interaction of the carbon with its attached protons[20, 21]. Assuming isotropic motion of the molecule, then in the case of motional narrowing the relaxation rate is given by

$$1/T_1 = N \hbar^2 \gamma_H{}^2 \gamma_C{}^2 r_{CH}{}^{-6} \tau_c$$

where N is the number of attached protons, γ_H and γ_C are the gyromagnetic ratios of H and C respectively, r is the C – H bond length and τ_c is the rotational correlation time. The quaternary-carbon relaxation processes will also be dominated by the dipolar mechanism. For tryptamine each quaternary carbon has at least one proton two bonds removed and hence dipole-dipole interactions between the carbon and proton will dominate the dipolar mechanism. A comparison of the integrated areas of the quaternary carbons with those of the proton-bearing carbons indicated that carbon-proton interactions dominated the dipolar mechanism and that this mechanism was the major contributing factor to the relaxation of these carbons. For 5′-AMP in D_2O, however, each quaternary carbon has no proton two bonds removed and it has been shown recently[21] that $^{14}N - {}^{13}C$ dipolar relaxation also makes a significant contribution to the relaxation process.

The changes in the carbon T_1's observed in Table III confirm the existence of an AMP-tryptamine complex in solution. It is possible that the drastic reduction in T_1's of tryptamine arise from paramagnetic impurities associated with 5′-AMP. A repetition of experiment C with 2½ times the quantity of EDTA (Expt. D) shows only a small increase in the T_1's and hence paramagnetic impurities can be discounted as a cause for the observed T_1 changes. Increased EDTA concentration could also to some extent decrease the complex concentration, for it was shown that AMP-tryptamine association is decreased by increase in the ionic strength[22]. Lüdemann and Röder[23] pointed out that it may be possible to explain the T_1 changes observed by Hamill *et al.*[20] for dilution behaviour of 5′-AMP as arising from changes in the viscosity of the medium without needing to invoke a change in particle size. Thus in the present case the changes in T_1 of the tryptamine carbons may arise solely from a macroscopic viscosity change associated with the change in solvent on going from D_2O (Expt. B) to one of D_2O + 5′-AMP (Expt. C). That this is not the case is seen in experiment E where the T_1's of tryptamine are only slightly reduced in a solution containing the non-complexing molecule glucose at the same concentration as the 5′-AMP in experiment D. Thus the 4 – 5 fold reduction in the T_1's of tryptamine must arise primarily from complex formation in the solution containing 5′-AMP. As the dipolar relaxation mechanism dominates the relaxation process of all the carbons in tryptamine, then the reduction in T_1 values must arise from an increase in the correlation time of the molecule associated with an increase in particle size.

The behaviour of 5′-AMP in experiments A and C is consistent with the 5′-AMP existing as a self-associated complex in the 0.4 M solution (Expt. A). The changes in T_1's of 5′-AMP are small compared to those observed for the similar situation with tryptamine. On going from experiment A to C one molecule of 5′-AMP in the selfassociated complex is exchanged for a tryptamine molecule of similar size, thus the size of the aggregate is not significantly changed, hence the correlation times are similar and the T_1 values are little affected. The only other explanation for the maintenance in the 5′-AMP T_1 values would be for a decrease in the viscosity of the solution to occur on going from experiment A to C which is not likely. This conclusion is at variance with the conclusions of Lüdemann and Röder[23] concerning the selfassociation of 5′-AMP at these concentrations. Recently a reinvestigation of the selfassociation of 5′-AMP by the use of ^{2}H NMR by Egan[24] has confirmed the existence of selfassociated complexes and has pointed to apparent reasons for the inconsistency with the previous results.

Thus it can be seen that the most sensitive probe for this type of complex formation is not the observation of the nucleotide but the observation of the partner that shows little selfassociation in free solution[4].

In both the single component systems studied here isotropic motion is not strictly adhered to. Thus for the 5′-AMP the $N T_1$ values of the ribose carbons (0.23 – 0.24 s) are slightly longer than those of the proton-bearing purine base carbons (0.19 s). This has been attributed to pseudorotation in the ribofuranose ring and/or torsional motions about the glycosyl bond caused by base-stacking[20]. For tryptamine the NT_1 values of the proton-bearing ring carbons ($1.65-2.21$ s) are shorter than those of the aliphatic side chain ($2.84-2.88$ s). This arises from the ease with which rotation can occur in this side-chain relative to the bulk of the molecule, *i. e.*, segmental motion, and it results in an effective decrease in the correlation time for these carbons and hence an increase in T_1.

For the complex the difference in NT_1 values between the proton-bearing ribose and base carbons is not as pronounced indicating that the motional freedom of the ribose has become more restricted. For tryptamine, however, the NT_1 values of the side chain and ring carbons show a more pronounced difference, that is the percentage change of the aromatic ring carbons are larger than those of the side chain. Complex formation effectively anchors the aromatic ring system but does not significantly affect the segmental motion of the side chain which is in keeping with the chemical shift differences for these carbons where the environment does not significantly change between the free and complexed molecule.

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