Serotonine-Nucleotide Interaction Studied by PMR. Explanation of the Variability in Association Constants Obtained from Chemical Shifts of Different Protons

R. Lawaczeck and K. G. Wagner

Gesellschaft für Molekularbiologische Forschung, Stöckheim/Braunschweig

(Z. Naturforsch. **29 c**, 488—492 [1974]; received May 6, 1974)

Weak Complexes, Association Constants, Proton Chemical Shifts, Nucleotides, Serotonine

A study of the association of serotonine with 5′-AMP by PMR spectroscopy is described. The association constants obtained from the chemical shift of non-equivalent protons show considerable variation outside experimental error, a fact which was reported by several authors. To support the suppositon that small amounts of complexes of different stoichiometry, — which in general can hardly be detected —, are responsible for this phenomenon, computer simulation of various hypothetical systems were investigated. The results clearly demonstrate that the neglect of minor fractions of complexes of different stoichiometries lead to significant variation in the association constants calculated for different protons, provided that these protons experience different magnetic enviroments in the different complexes.

Introduction

PMR-spectroscopy is often applied for the determination of affinity constants of weak complexes. Several investigations have shown that significant variations in the apparent association constants are obtained, when chemical shifts of non-equivalent protons are observed[1–3]. In a study of the interaction of nucleotides with amines related to the aromatic amino acids we also evaluated different association constants for different protons both in linearized computations according to Benesi-Hildebrand or Scatchard and in non-linear direct least square fits of the data[4]. These evaluations were based on a 1:1 stochiometry which was supported by the appropriate experiments[5]. Minor deviations from this stoichiometry, which are difficult to be demonstrated experimentally but cannot be excluded, are supposed to be the reason for the observed variations in the association constants. This was also suggested by Foreman *et al.*[1].

In the present work the association of serotonine (5-hydroxy tryptamine) and 5′-AMP (adenosine-5′-monophosphate) is described, in order to illustrate an example of different affinity constants obtained for different protons. To show that small deviations from the stoichiometry applied could be the origin of deviations in the apparent association constants, computer simulations were performed. Chemical shifts for protons were calculated based on the association of two different molecules which predominantly form a 1:1 complex but include minor contributions from complexes of different stoichometry. If one now evaluates association constants from these calculated shifts but neglects the contribution from the minor complex fractions, values of significant variation are obtained provided that different protons experience different magnetic environments in the different complexes. These computer simulations were based on experimental data and estimated parameters related to the association of serotonine and 5′-AMP; the results, however, are of general validity explaining a phenomenon which is very often observed.

Results and Discussion

Fig. 1 indicates the chemical shift changes (relative to the shift of the uncomplexed component) of three protons of 5′-AMP in the presence of increasing concentrations of serotonine; Fig. 2 shows the same for different protons of serotonine with increasing 5′-AMP concentrations. The curves of Figs 1 and 2 are directly plotted by a non-linear least square fit taking the mass equation for a 1:1 complex ($A + B \rightleftarrows AB$) and the relationship that the observed chemical shift of a given proton is equal to the sum of the products of chemical shift and proton fraction of the respective species (in the limiting case of fast exchange):

$$\delta_{\text{obs}} = \frac{A_0 - AB}{A_0}\,\delta_{\text{f}} + \frac{AB}{A_0}\,\delta_{\text{c}} \qquad (1)$$

Requests for reprints should be sent to Dr. Karl G. Wagner, Gesellschaft für Molekularbiologische Forschung mbH., *D-3301 Stöckheim*/Braunschweig.

Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung") beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

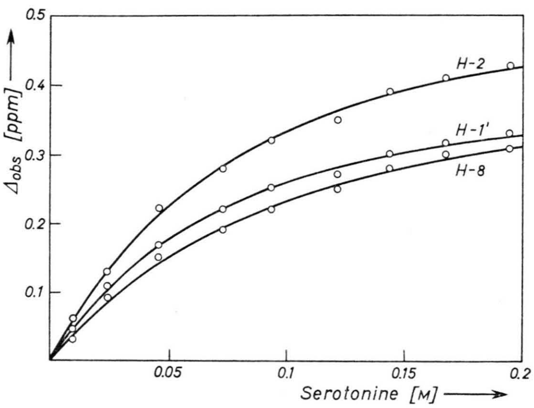

Fig. 1. 5'-AMP-serotonine complex. Differences of the chemical shifts Δ_{obs} plotted *versus* the serotonine concentration. D_2O, pD 7.4 at 15 °C. 5'-AMP protons (5'-AMP 0.037 M).

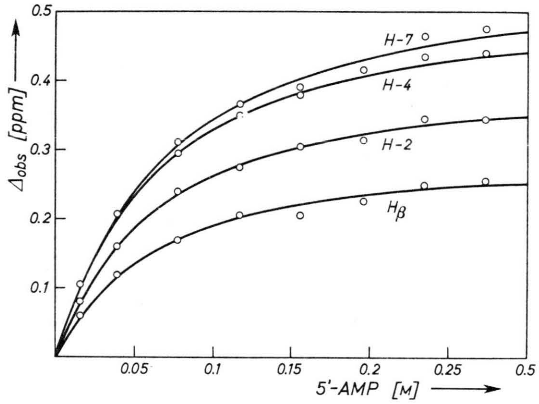

Fig. 2. Serotonine-5'-AMP complex. Differences of the chemical shifts Δ_{obs} plotted *versus* the 5'-AMP concentration. D_2O, pD 7.4, at 15 °C. Serotonine protons (serotonine 0.024 M).

Table I. Apparent association-constants K_{app} [M^{-1}] and extrapolated differences of the chemical shifts Δ_{c} [ppm] for the pure complex 5'-AMP-serotonine. D_2O, pD 7.4, at 15 °C.

	0.037 M 5'-AMP, serotonine concentration varied			
	Curve fitting		Scatchard approximation *	
	K_{app}	Δ_{c}	K_{app}	Δ_{c}
H-8	13.5	0.45	7.8	0.52
H-2	18.8	0.56	10.5	0.64
H-1'	19.8	0.43	11.7	0.47
	0.024 M serotonine, 5'-AMP concentration varied			
H-4	20.9	0.52	14.7	0.55
H-6	17.8	0.45	13.1	0.47
H-7	18.1	0.57	13.0	0.60
H-2	21.6	0.41	16.1	0.43
H_β	22.0	0.29	13.9	0.32

* The relative error of the data from the Scatchard approximation is about 10%.

where δ_{obs}, δ_{f} and δ_{c} are the observed chemical shift, the chemical shift of free and complexed A, respectively; A_0 and AB are the initial concentration of A and that of the complex at equilibrium, respectively. Using chemical shift changes $\Delta_{\text{obs}} = \delta_{\text{obs}} - \delta_{\text{f}}$ and $\Delta_{\text{c}} = \delta_{\text{c}} - \delta_{\text{f}}$, one obtains a simple equation for the complex concentration:

$$AB = \frac{\Delta_{\text{obs}}}{\Delta_{\text{c}}} A_0 . \qquad (2)$$

By non-linear least square curve fitting mentioned above apparent association constants (K_{app}) and upfield chemical shifts (Δ_{c}) of the pure serotonine-5'-AMP complex were determined for each case, and Table I shows the data obtained from the different protons. It is clear that there is a variability in the apparent association constants that is beyond the error of estimation especially for the protons of the nucleotide.

Recently, Lüdemann and von Goldammer[6] reported studies on self-association of nucleosides in water and water-benzene mixtures. These authors normalized the chemical shift changes of the different protons, in that for each proton they divided the observed values by standard chemical shift changes obtained at a reference concentration and temperature. The idea was to eliminate the influence of solvation which they believe to be the reason for the variability in K_{app}. We tested this proposal with some of our data, but without success. The failure is obvious, as always the ratio $\Delta_{\text{obs}}/\Delta_{\text{c}}$ (Eq. (2)) is put into the Scatchard or Benesi-Hildebrand approximation and also into the non-linear direct equation; standard chemical shifts will be attributed as constant factors to both the observed chemical shift changes (Δ_{obs}) and the extrapolated shift of the complex (Δ_{c}) and therefore eliminated.

A more plausible explanation for the origin of the variabality in K_{app} may be found in the appearance of other complexes with stoichiometries different from those applied for the calculations. In order to illustrate this possibility, computer simulations were investigated; the parameters of the present model were chosen as to approximate the association of serotonine and 5'-AMP. The model contains two partners A and B (A representing the nucleotide and B serotonine) which mainly form a 1:1 complex:

$$A + B \rightleftharpoons AB (K_{\text{AB}})$$

but minor contributions from complexes of different stoichiometry may be considered:

$$A + A \rightleftharpoons A\,A \quad (K_{AA})$$
$$A\,B + B \rightleftharpoons A\,B_2 \quad (K_{AB_2})$$

These equilibria were chosen because previous experiments showed that 5′-AMP has a stronger stacking tendency with self-association than serotonine [5] and because there is a possible equalization of the electric charges in the $A\,B_2$ complex (at neutral pH the nucleotide bears two negative charges and serotonine one positive charge).

The initial concentrations of the two partners are defined by the following equations:

$$A_0 = A_f + 2\,A\,A + A\,B + A\,B_2$$
$$B_0 = B_f + A\,B + 2\,A\,B_2 \qquad (3)$$

where A_f and B_f are the concentrations of the free partners, $A\,A$, $A\,B$ and $A\,B_2$ are the concentrations of the respective complexes. Taking a set of appropriate association constants for the three complexes and the stoichiometric and mass equations, the concentrations of the different species of complexes present in the equilibrium can be calculated. This was done for two series of simulated experiments, keeping A_0 constant and varying B_0 and *vice versa*. As a set of association constants values of $K_{AA} = 1$, $K_{AB} = 20$ and $K_{AB_2} = 2.5$ (M^{-1}) were selected, which may well approximate the association of serotonine and 5′-AMP presented above. The computed results are indicated in Figs 3 and 4 illustrating the concentrations of the free components and those of the different complex species as a function of the initial concentrations of B_0 and A_0, respectively. It is obvious that with increasing concentrations of A_0 (Fig. 3) dimerization of A becomes significant, whereas increasing concentrations of B_0 enhance the weight of the complex $A\,B_2$ (Fig. 4).

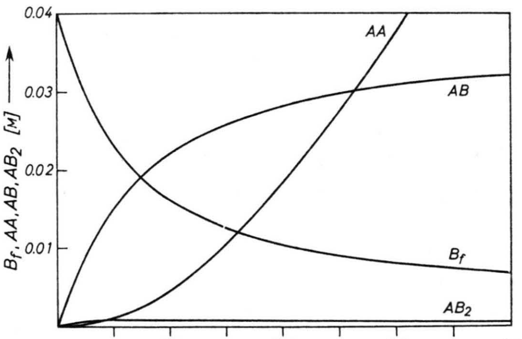

Fig. 3. Concentration of the complexes AA, AB, AB_2, and of B_{free} plotted *versus* the initial concentration A_0. B_0 (0.04 M) is constant. $K_{AA} = 1$, $K_{AB} = 20$, $K_{AB_2} = 2{,}5\ \text{M}^{-1}$.

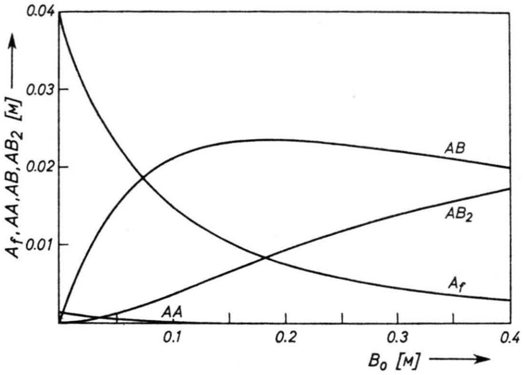

Fig. 4. Concentration of the complexes AA, AB, AB_2, and of A_{free} plotted *versus* the initial concentration B_0. A_0 (0.04 M) is constant. $K_{AA} = 1$, $K_{AB} = 20$, $K_{AB_2} = 2{,}5\ \text{M}^{-1}$.

As the next step sets of appropriate chemical shift differences (Δ_c) for protons in the hypothetical pure complexes were chosen. With each set of parameters (association constants and Δ_c values) hypothetical "observed" chemical shifts can be calculated which are the sum of the products of proton fraction and chemical shift of the respective complex species, *e. g.* for an A proton (fast exchange):

$$\Delta_{\text{obs}} = 2\,\frac{A\,A}{A_0}\,\Delta_{AA} + \frac{A\,B}{A_0}\,\Delta_{AB} + \frac{A\,B_2}{A_0}\,\Delta_{AB_2}\,.$$

These calculated chemical shifts were now used in both the Scatchard plot and the non-linear direct curve fitting procedure to determine apparent association constants (K_{Sc}) and (K_{cf}), respectively, by assuming a pure 1:1 association and neglecting the complexes $A\,A$ and $A\,B_2$. This was done for both cases, illustrated in Figs 3 and 4, keeping A_0 constant and varying B_0 and *vice versa.* A concentration of 0.04 M for the constant partner was chosen and 10 equidistant values in the range of 0.1 to 0.4 M were taken for the variable partner. These values are in the range of concentration used in the experiments described and are also appropriate for satisfying the Scatchard approximation. With these simulations it is possible to look at K_{app} as a function of two sets of parameters:

a. Varying K_i (i = $A\,A$, $A\,B$ and $A\,B_2$) with a fixed set of Δ_i and

b. varying Δ_i with a fixed set of K_i.

Table II contains the data of case a., indicating the computed apparent association constants for the *AB* complex dependent upon different sets of K_i values. It is obvious that the apparent constants obtained by the Scatchard approximation are generally lower than those obtained by non-linear direct curve fitting, presumably because the former method neglects the square term resulting from the mass equation which may especially influence data obtained at low B_0 and A_0 values, respectively. With the set of Δ_i values taken as the basis of the computations of Table II, a comparison of the apparent association constants obtained by varying A_0 with those obtained by varying B_0 shows that generally the former, obtained by varying A_0 (nucleotide) and recording the chemical shift of protons of B_0 (serotonine, tryptamine), are significantly larger than those constants obtained by the reverse procedure. This bias was also found with real measured values in the case of 5′-AMP and serotonine (Table I) and of 5′-AMP and tryptamine [4, 5] and is consistent with the existence of the complex AB_2, which contributes only little in a series of experiments where A_0 is varied but very significantly in those experiments where B_0 is varied (*cf.* Figs 3 and 4).

Table II. Computed results for the *AB* complex. The parameters K_i are varied, the Δ_i are fixed. ($\Delta_{AA} = 0.6$, $\Delta_{AB} = 0.7$, $\Delta_{AB_2} = 0.8$ ppm).

Selected sets of K_i [M^{-1}]			A_0 varied B_0 constant			B_0 varied A_0 constant		
K_{AA}	K_{AB}	K_{AB_2}	K_{Sc}	K_{cf}	Δ_c	K_{Sc}	K_{cf}	Δ_c *
0	20	0	14.5	20.0	0.72	14.5	20.0	0.72
0	20	2.5	16.3	22.6	0.72	11.8	15.9	0.84
1	20	2.5	16.8	23.3	0.68	11.9	16.0	0.84
2	20	2.5	16.5	22.8	0.65	12.0	16.1	0.84
1	20	1	15.7	21.6	0.68	12.6	17.0	0.79
1	20	2.5	16.8	23.3	0.68	11.9	16.0	0.84
1	20	5	18.6	26.0	0.67	12.1	16.6	0.88

* K_{Sc} [M^{-1}] apparent association constant of the catchard approximation. K_{cf} [M^{-1}] apparent association constant of the curve fitting procedure. Δ_c [ppm] differences of the chemical shifts for the pure complex of the scatchard approximation.

Taking a constant set of K_i values and varying the chemical shift changes Δ_i is of much interest because this case simulates – like in real experiments – the situation of different protons in different complexes; the concentrations of which are determined by a set of inherent K_i values. However, different protons in general experience different magnetic environments in the same complex and the same proton also experiences different shielding in different complexes. The data of Table III clearly indicate that the association constants calculated from approximations neglecting minor complex species of stoichiometries different from the major complex greatly depend on the choice of the Δ_i parameters. With the set of complexes as the basis of the present work (AA, AB and AB_2) it is further obvious that the dependence on the Δ_i parameters is much more sensitive in experiments where the proton chemical shifts of A is recorded and B_0 varied than in the reverse experiments. This situation is reflected by the experimental data presented above, apparent association constants related to the protons of 5′-AMP differ more strongly than those related to serotonine protons (Table I). The same was found in the association of 5′-AMP and tryptamine [4, 5]. Table III further shows that the statement discussed above, that due to the contributions of the AB_2 complex apparent association constants obtained by varying A_0 are generally larger than those obtained by varying B_0, holds only if Δ_{AB_2} is larger than Δ_{AB}. This prerequisite may prove correct when A protons are observed and presumably also in experiments where chemical shifts of B protons are recorded.

Table III. Computed results for the *AB* complex. The parameters Δ_i are varied, the K_i are fixed. ($K_{AA} = 1$, $K_{AB} = 20$, $K_{AB_2} = 2.5$ M^{-1}).

Selected sets of Δ_i [ppm]			A_0 varied B_0 constant			B_0 varied A_0 constant		
Δ_{AA}	Δ_{AB}	Δ_{AB_2}	K_{Sc}	K_{cf}	Δ_c	K_{Sc}	K_{cf}	Δ_c
0.1	0.7	0.8				11.5	15.6	0.85
0.3	0.7	0.8				11.7	15.7	0.84
0.6	0.7	0.8				11.9	16.0	0.84
0.6	0.7	0.2	14.5	20.0	0.67			
0.6	0.7	0.4	15.3	21.0	0.67	30.3	49.2	0.58
0.6	0.7	0.6	16.0	22.1	0.67	17.4	24.7	0.7
0.6	0.7	0.8	16.8	23.3	0.68	11.9	16.0	0.84
0.6	0.7	1.0	17.6	24.6	0.68	9.0	11.6	1.0
0.6	0.4	0.8	19.4	27.3	0.39	6.1	7.5	0.76
0.6	0.7	0.8	16.8	23.3	0.68	11.9	16.0	0.84
0.6	1.0	0.8	15.9	21.9	0.96	18.8	27.2	0.97

Fig. 5 illustrates the results of Table III; calculated "observed" chemical shifts related to the same set of K_i values are plotted versus the concentrations of A_0 and B_0, respectively; the Δ_i values of the AB_2 complex are varied simulating different

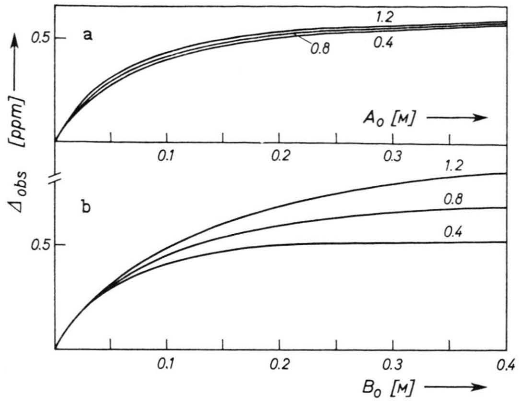

Fig. 5. Differences of the calculated chemical shifts plotted *versus* the initial concentrations A_0 and B_0, respectively. $K_{AA} = 1$, $K_{AB} = 20$, $K_{AB_2} = 2.5\ \text{M}^{-1}$, $\Delta_{AA} = 0.6$, $\Delta_{AB} = 0.7$ ppm being invariable; $\Delta_{AB_2} = 0.4$, 0.8, 1.2 ppm, respectively. a. Proton of B; b. proton of A.

protons. This figure clearly demonstrates that it is mainly the AB_2 complex, bringing its influence to bear in experiments were B_0 is varied, which gives rise to significant changes in the concentration dependence of the "observed" chemical shift. A comparison of the results obtained by simulating calculations (Table III and Fig. 5) and by experiments on the association of 5′-AMP with serotonine (Table I) and tryptamine, respectively, makes clear that the variability in the equilibrium constants is larger with the values of the simulated model than with the real experiments. Smaller values than $2.5\ \text{M}^{-1}$ of K_{AB_2} for the higher complex may be adequate to account for the experimental cases. This illustrates that rather small fractions of complexes with deviating stoichiometries which may hardly be detected can give rise to significant variations in the association constants determined for different protons provided that these protons are differently shielded in the different complexes.

Experimental

5′-AMP was purchased from Boehringer GmbH, Mannheim, and serotonine oxalic acid salt from Sigma Chemical Co. D_2O solutions of these components were adjusted to the desired pD and twice lyophilized. The spectra were recorded on a Varian HA-100 NMR-spectrometer equipped with a variable temperature unit; 0.26 M monodeutero-*tert*-butanol, $(CH_3)_3COD$, purchased from ICN, Isotope & Nuclear Division, was used as internal reference. Computer calculations were performed on a Hewlett Packard Computer (Datendienst Telecomputer, Frankfurt a. M.).

This work has been supported by the Deutsche Forschungsgemeinschaft and by the Bundesministerium für Forschung und Technologie (BCT 16). We are grateful to Dr. V. Wray for reading the manuscript and useful suggestions.

---

[1] M. I. Foreman, R. Foster, and D. R. Twiselton, Chem. Commun. **1969,** 1318.

[2] J.-L. Dimicoli and C. Hélène, Biochimie **53,** 331 [1971].

[3] J. Homer and R. R. Yadava, Tetrahedron **29,** 3853 [1973].

[4] K. G. Wagner and R. Lawaczeck, J. Magn. Res. **8,** 164 [1972].

[5] R. Lawaczeck, Thesis, Technische Universität Braunschweig 1972.

[6] H.-D. Lüdemann and E. v. Goldammer, Z. Naturforsch. **28 c,** 361 [1973].