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Investigation into the Association between Serotonin and Adenosine Triphosphate *in Vitro* by Nuclear Magnetic Resonance and Ultraviolet Spectroscopy

THOMAS NOGRADY,[1] PAVEL D. HRDINA, AND GEORGE M. LING

Department of Chemistry, Loyola College, Montreal 262, Quebec, and Department of Pharmacology, University of Ottawa, Ottawa 2, Ontario, Canada

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SUMMARY

Adenosine triphosphate and serotonin (5-hydroxytryptamine, 5-HT) form micellar complexes in synaptic storage vesicles and blood platelets. The interaction of ATP with 5-HT and the binding forces responsible for micelle formation were investigated by nuclear magnetic resonance and ultraviolet spectroscopy *in vitro*. The principal bond is an ionic interaction between the side chain amino group of 5-HT and the negatively charged phosphate of ATP. This results in the disruption of ATP stacking and overlap of the indole and adenine moiety, forming a contact charge-transfer complex with a low apparent association constant. pH and ionic strength effects on complex formation were also investigated.

INTRODUCTION

Serotonin is known to be an important neurotransmitter stored in synaptic vesicles. Its involvement in depressive states and in the mode of action of tricyclic thymoleptics (antidepressants) has been advocated by several authors (see ref. 1).

5-HT[2] storage organelles can be found in synaptosomes as well as in platelets. Pletscher and co-workers (2) investigated drug-induced changes in 5-HT storage in isolated platelets and platelet storage organelles. They demonstrated (3, 4) by ultracentrifugation studies and ultraviolet spectroscopy that the storage granules contain high molecular weight micelles formed by the association of 5-HT with ATP in a molar ratio of 2–2.5. The size of the micelles is inversely proportional to temperature and is increased by Ca^{++} and Mg^{++} ions. Such micelles form spontaneously in aqueous solutions of 5-HT and ATP and are specific, since much lower apparent micellar weights are seen in mixtures of ATP with tryptamine or histamine. However, Pletscher *et al.* did not examine the 5-HT–nucleotide structure and bonding in these micelles.

The storage mechanism of neurotransmitters in synaptic terminals was investigated recently by Smythies and co-workers (5), who proposed a unified hypothesis for catecholamine and 5-HT storage. On the basis of molecular models, they suggested staircase-like binding of ATP to the glutamate and aspartate residues of a specific helical protein, chromogranin, found in adrenal chromaffin granules. The ATP molecules, held by hydrogen bonding at the N-1

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[1] To whom correspondence should be addressed at Loyola College, Montreal.

[2] The abbreviation used is; 5-HT, serotonin-(5-hydroxytryptamine).

and NH_2-6 sites of adenine, form the rungs of a ladder and can accommodate 4 catecholamine or 2 5-HT molecules by intercalation at each turn of the helix. The side chain amino groups of the neurotransmitter could bind to phosphate or carbonyl groups by electrovalent or hydrogen bonds.

Smythies and Antun (6) also investigated the binding of tryptamine derivatives, including 5-HT, to nucleic acids by fluorescence spectroscopy. No effect of pH or Mg^{++} on complexation was observed; therefore ionic bonding was ruled out and intercalation by hydrophobic binding was suggested. Even though the 5-HT–ATP binding in synaptic vesicles and the tryptamine-DNA binding appeared to be closely related and the molecular features of the interacting species (ATP or DNA with tryptamine derivatives) seemed similar, two different binding mechanisms—ionic bonds and hydrophobic bonds—were proposed. No evidence other than fluorescence quenching, pH and ion effects, and aspects of molecular geometry supports this plausible hypothesis.

Norepineprine-ATP complexes were investigated in detail by Weiner and Jardetzky (7), using nuclear magnetic resonance spectroscopy. By measuring the selective change in relaxation rate of ring and side chain protons of norepinephrine in the presence of ATP (see ref. 8), they demonstrated the existence of a 3:1 norepinephrine-ATP complex at pH 5.6. Ionic bonds between the protonated side chain amino group and the ATP phosphate and an additional hydrogen bond between the side chain hydroxyl and phosphate oxygen stabilize the complex.

Three studies pertaining to nucleotide-neurotransmitter binding were published recently by Muro *et al.* (9) and by Hélène, Dimicoli and Brun (10, 11), using NMR methods. The findings of Hélène *et al.* (11), (published after the completion of our experiments) are in excellent agreement with the results outlined in this paper.

The objectives of the present study were (a) to establish the nature of the 5-HT–ATP interaction *in vitro* to confirm the micelle hypothesis of Pletscher and coworkers, (b) to establish if ionic or hydrophobic bonds are involved, and (c) to determine whether similarity exists between the binding of norepinephrine and 5-HT to ATP. These investigations form a basis for future studies on the influence of thymoleptics and other drugs on intraneuronal 5-HT storage and release.

METHODS

NMR spectra were recorded at ambient temperature in D_2O on a Varian 220-MHz spectrometer (Canadian 220-MHz NMR Center, Sheridan Park, Ontario). Sodium dimethyl silapentanesulfonate was used as internal standard. Serotonin oxalate and ATP $2Na \cdot 4H_2O$ were purchased from Nutritional Biochemicals Corporation.

Since the NMR spectrum of ATP is very sensitive to the presence of metal ions, spectra were determined in unbuffered solutions except for the pH and ionic strength studies, for which 0.1 M Na-phosphate buffer was used. Measured linewidths were corrected for instrumental broadening and are accurate to ± 0.2 Hz.

Ultraviolet spectra were determined in aqueous solutions on a Beckman DK-2A instrument.

RESULTS

The 220-MHz nuclear magnetic resonance spectrum of 5-HT in D_2O (Fig. 1) allows the observation of every proton in the molecule (except OH and NH protons). The side chain methylene group can be considered intermediate between a first-order A_2X_2 and a second-order A_2B_2 spectrum. The side chain proton signal is an intractable multiplet at 60 MHz. When the ATP spectrum was superimposed, no overlap of resonance lines resulted, and most of the chemical shift and linewidth changes could be evaluated in mixtures containing varying ratios of 5-HT oxalate and ATP $2Na \cdot 4H_2O$. The exceptions are the linewidths of H-6 of 5-HT, which is a quartet, and H-2 and H-7 at low 5-HT:ATP ratios, where resonance lines merge, making the measurement of linewidth impossible.

The MNR spectra of various concentrations of ATP at pH 2.9–3.2 show some surprising features (Fig. 2). While the published spectra of ATP (12) show sharp peaks for both adenine protons, with H-8 slightly wider, it is the H-2 proton that be-

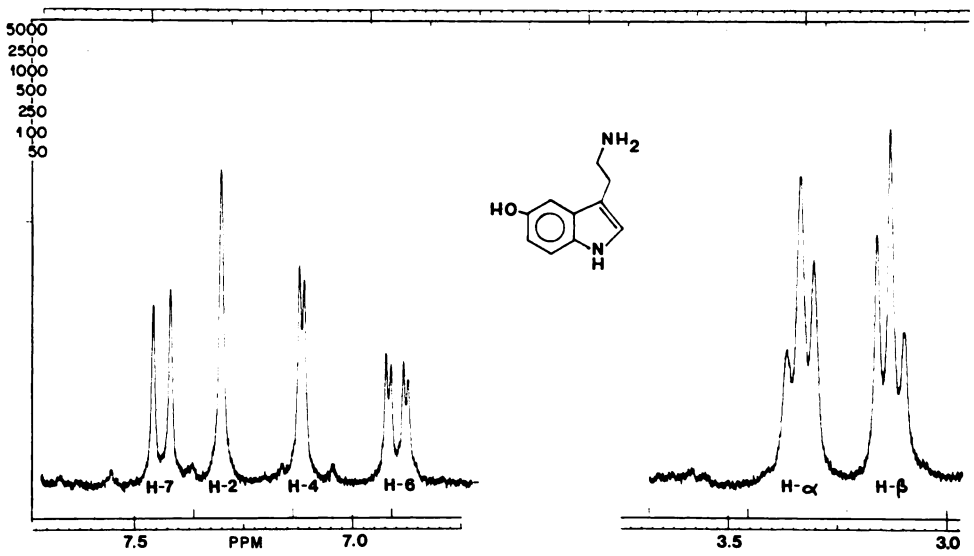

FIG. 1. *NMR spectrum at 220 MHz of 75 mM 5-HT oxalate in D_2O*
For chemical shifts, coupling constants, and linewidths, see Table 1.

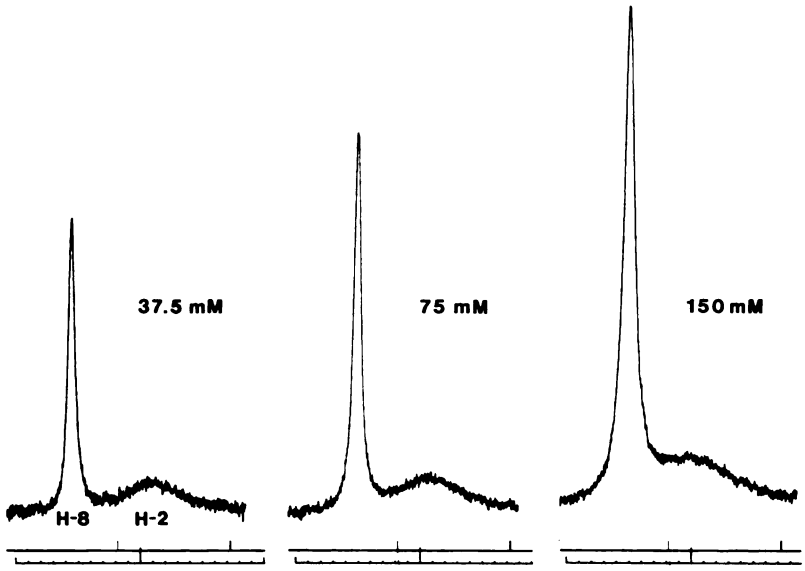

FIG. 2. *NMR spectrum at 220 MHz of adenine protons of ATP $2Na^+$ at different concentrations between pH 2.9 and 3.2 in D_2O at 20°.*

comes extremely broadened in our spectra. All published spectra, however, were recorded at alkaline pH, whereas we used strongly acidic, unbuffered solutions. Upon raising the pH of the solution, the very broad line of the H-2 proton became sharp. The addition of EDTA had no such effect; thus broadening was not due to paramagnetic impurities (see ref. 13).

To evaluate the interaction between 5-HT and ATP, the 220-MHz spectra of mixtures were recorded at 5-HT:ATP molar ratios between 0.5 and 6.0 where 5-HT was held constant (Table 1).

TABLE 1

Chemical shifts and observed spin-spin relaxation rates from 220-MHz spectra of 5-HT, ATP, and their mixtures

Concentration		5-HT: ATP ratio	pH in H_2O	5-HT protons						ATP protons	
5-HT	ATP			2	4	6	7	α	β	2	8
mM											
75			2.80 $\Delta\nu$ (ppm)	7.29	7.11	6.89	7.43	3.33	3.12		
			J (Hz)		2.3	o=8.5	8.5	7.0	7.0		
						m=2.3					
			T_2^{-1}	7.85	6.91		7.85	9.42	6.28		
	150		2.95 $\Delta\nu$ (ppm)							8.46	8.58
			T_2^{-1}							125.6	18.05
	75		3.05 $\Delta\nu$ (ppm)							8.43	8.58
			T_2^{-1}							106.8	11.93
	37.5		3.20 $\Delta\nu$ (ppm)							8.43	8.59
			T_2^{-1}							94.2	11.00
75	150	0.5	2.80 $\Delta\nu$ (ppm)	6.95	6.58	6.43	6.95	3.20	2.86	8.18	8.48
			T_2^{-1}		33.60			39.25	70.96	59.6	14.91
75	75	1.0	2.80 $\Delta\nu$ (ppm)	7.04	6.71	6.55	7.04	3.21	2.91	8.07	8.45
			T_2^{-1}		22.61			31.71	37.99	42.40	15.56
75	37.5	2.0	2.80 $\Delta\nu$ (ppm)	7.12	6.85	6.68	7.19	3.24	2.97	7.99	8.44
			T_2^{-1}	32.97	14.76		26.69	24.80	28.80	32.97	15.56
75	25	3.0	2.80 $\Delta\nu$ (ppm)	7.18	6.93	6.74	7.27	3.26	3.02	7.97	8.45
			T_2^{-1}	18.84	11.62		15.70	18.80	18.50	28.26	11.00
75	12.5	6.0	2.80 $\Delta\nu$ (ppm)	7.24	7.03	6.83	7.36	3.29	3.07	7.95	8.46
			T_2^{-1}	14.13	11.62		14.13	15.10	14.10	20.41	11.00

Chemical shift studies. The aromatic protons of 5-HT and H-2 of ATP show considerable upfield shift in the presence of the other molecules, dependent on their respective molefractions. To interpret our results, we used the Hill equation in the generalized form

$$\log \frac{x}{1-x} = n \log [S] - \log K$$

where x is the variable parameter, S the substrate concentration, n the Hill coefficient (given by the slope), and K a constant. This equation can be recognized as a form of the Michaelis-Menten equation. The Hill coefficient n represents a measure of interaction between ligands, and a simple titration curve gives a line of unit slope (see ref. 14).

When the derivative $\Delta\delta/(1 - \Delta\delta)$ of the chemical shift difference between the free and complexed compounds ($\Delta\delta$) for each proton is plotted against log mole fraction (semilogarithmic Hill-plot), the indole protons display a linear fit to the equation (Fig. 3) while the plot for the side chain protons is curved.[3] A similar representation for the adenine protons is not linear either (Fig. 4), and shows a negligible shift change of the H-8 proton. The ribose protons (not shown in Table 1) display neither a change in chemical shift nor significant broadening.

With the indole protons in Fig. 3 slopes of 2.9, 1.6, 1.25, and 0.80 are found for the H-4, H-7, H-6, and H-2 protons, respectively. It is reasonable to assume that the aromatic protons of 5-HT are more involved in stacking than H-2 or the side chain protons, which deviate from linearity. Also, H-2 of ATP is likely to be more involved than H-8, which yields a slope of zero. This assumption is in

[3] The original Hill plot is a double logarithmic function, and should give sigmoid curves. Our data conform to that shape if plotted accordingly, but we found it more instructive to use the semilogarithmic graphs shown.

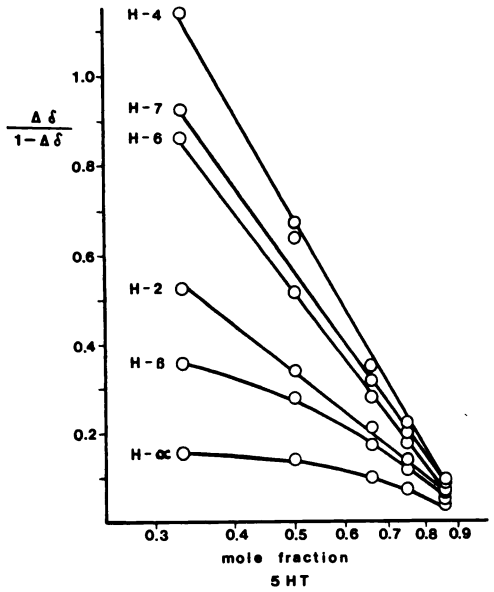

FIG. 3. *Semilogarithmic Hill plot of chemical shift dependence of 5-HT protons at different 5-HT:ATP ratios*

good agreement with the findings of Dimicoli and Hélène (10).

A more quantitative evaluation of these data was attempted by utilizing the Foster-Fyfe modification of the Benesi-Hildebrandt equation for charge-transfer complex association constants (15):

$$\frac{\Delta\delta}{[B]} = -K(\Delta\delta - \Delta\delta_{AB})$$

where $\Delta\delta$ is the chemical shift of the proton in compound A and $\Delta\delta_{AB}$ is the shift difference between the free and complexed forms. If B is present in excess, a plot of $\Delta\delta/B$ against $\Delta\delta$ should be linear with the slope of $-K$. Unfortunately, only the H-2 proton of ATP fulfills these conditions completely. When the data are plotted (Fig. 5), a K value of 1.26 M^{-1} is found, which is somewhat low compared with related data shown by Hélène *et al.* (10, 11). Since ATP was never present in sufficient excess in our complexes, only an approximate value of $K \cong 6.2$ M^{-1} can be obtained for the indole protons. This, however, is a more reasonable value. Different K values for different protons of the same complex would be expected because of the existence of complexes in different molar ratios in the same solution (10).

Proton relaxation rates. Spin-spin relaxation rates, T_2^{-1}, could be estimated relatively easily from linewidths for the aromatic 5-HT protons, with the exception of H-6, which is a quartet. Considerable theoretical and practical difficulties were encountered in determining T_2^{-1} for the two sidechain

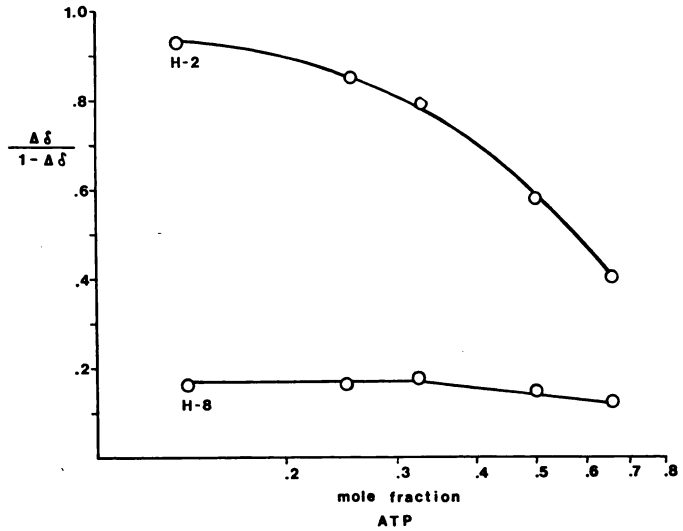

FIG. 4. *Semilogarithmic Hill plot of chemical shift dependence of aromatic ATP protons at different 5-HT:ATP ratios*

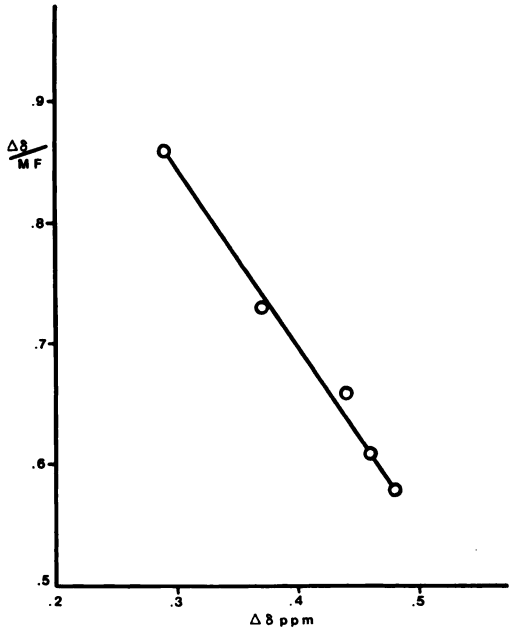

FIG. 5. *Foster-Fyfe plot (11) of ATP H-12 at different 5-HT:ATP ratios*

triplets, which are not first-order even at 220 MHz, but are intermediate between an A_2X_2 and A_2B_2 pattern. The linewidth was determined by computer simulation based on the following assumptions. The coupling constant of the triplets is 7.0 Hz and they are separated by $\Delta\delta$ = 45–75 Hz, which causes the appearance of the two distorted triplets. The expected half-spectrum (16) consists of seven lines. A computer program was devised to simulate the three Lorentzian peaks from the equation

$$y = \frac{a}{(1 + 4/b^2)(x - c)^2}$$

where a is peak height, b is width at half height, c = maximum peak frequency, and x and y are coordinates on the curve. By modifying and interpolating Corio's tables (17), the combined intensities of each peak in the triplet were obtained and used for calculation. It was assumed that J is constant and the linewidth is the same in any one triplet. After correction for baseline drift, the theoretically derived and computer-plotted envelope of the three peaks was compared with the experimental spectra (Fig. 6). The linewidth was obtained by using a b value yielding the least sum of squared deviations. Since the separation of the seven lines composing the triplet is significant, the mean value of this separation was subtracted from the observed linewidth. The T_2^{-1} values shown in Table 1 for H-α and H-β were calculated from these data.

As far as could be observed on the pseudo-first-order side chain spectra, no differential shifts occur, and only line broadening is responsible for the observed effects. The triplets themselves do shift as a result of ring current interactions.

The estimation of the H-2 and H-8 relaxation rates in the ATP spectrum posed another difficulty: because of the experimental conditions, the linewidth of the ATP protons was very broad (Fig. 2) and was not suitable for the determination of linewidths at infinite dilution, necessary for calculating $T_{2(\text{free})}^{-1}$. As can be seen from Table 1 and Fig. 7, the widths of the adenine protons become narrower with increasing ratios of 5-HT. This is obviously due to the disruption of ATP stacks. Therefore the limiting linewidth was calculated by extrapolating the linewidth to zero mole fraction of ATP in the 5-HT–ATP complex. This should theoretically include line broadening due to binding with 5-HT, but is reasonably close to linewidths obtained by extrapolating the aromatic proton linewidths of ATP at pH 7 to infinite dilution (H-2 = 4.8 Hz and H-8 = 2.9 Hz were used).

Linewidths obtained by these calculations were used in the T_2^{-1} values given in Table 1. The relaxation rates of the bound species, however, could not be calculated using the relation

$$T_{2(\text{obs})}^{-1} = AT_{2(\text{bound})}^{-1} + (1 - A)T_{2(\text{free})}^{-1}$$

developed by Jardetzky and Wade-Jardetzky (18), since it is applicable to a fast exchange rate between the free and bound forms, which may not be the case here. Relative relaxation rates, $T_{2(\text{obs})}^{-1}/T_{2(\text{free})}^{-1}$), calculated according to the method of Hammes and Tallman (19), showed a slight preferential immobilization of the side chain protons. However, the present knowledge of 5-HT–ATP interaction does not justify a quantitative treatment, and it was thought best to avoid it.

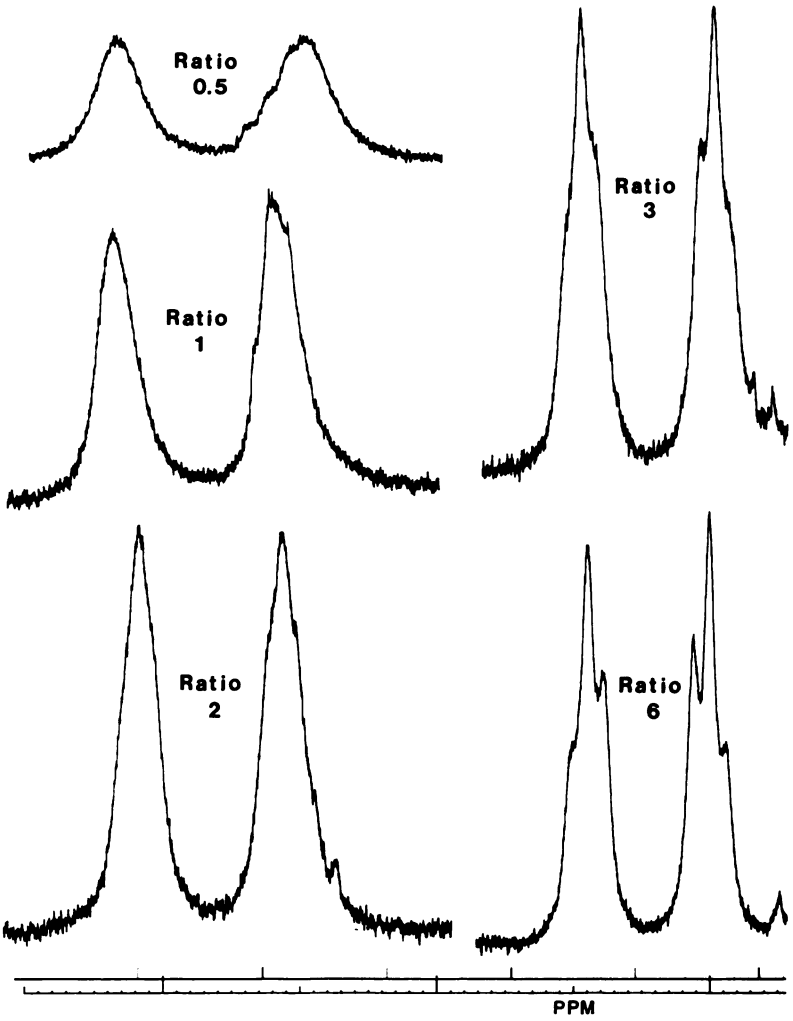

FIG. 6. *5-HT side chain proton resonances at different 5-HT:ATP ratios at 220 MHz*
For 5-HT alone, see Fig. 1. The linewidths were derived by computer simulation (see the text).

Ultraviolet spectroscopy of the 5-HT–ATP complex. Since charge-transfer complex formation between the indole and adenine rings cannot be excluded, we investigated the ultraviolet spectra of 5-HT, ATP, and their mixtures. The absorption spectra of the mixtures show the characteristics of the additive curve only, excluding any true charge-transfer interaction at concentrations of 10^{-4}–10^{-5} M. When the absorbance of the complex at the absorption miximum (265 nm) is plotted as a function of the molar ratio of the components, a simple Job plot (20) is obtained with a weak maximum at a mole ratio of 2.5–3. This seems to indicate that multiple complex species representing different molar ratios are present in solution.

DISCUSSION

The interpretation of the 5-HT–ATP interaction based on our data shows similarities to conclusions reached by Weiner and Jardetzky (7) on norepinephrine-ATP binding and those published by Hélène, Dimicoli, and co-workers (10, 11) on the tryptamine–nucleic acid system.

The 5-HT:ATP ratio in the complexes has

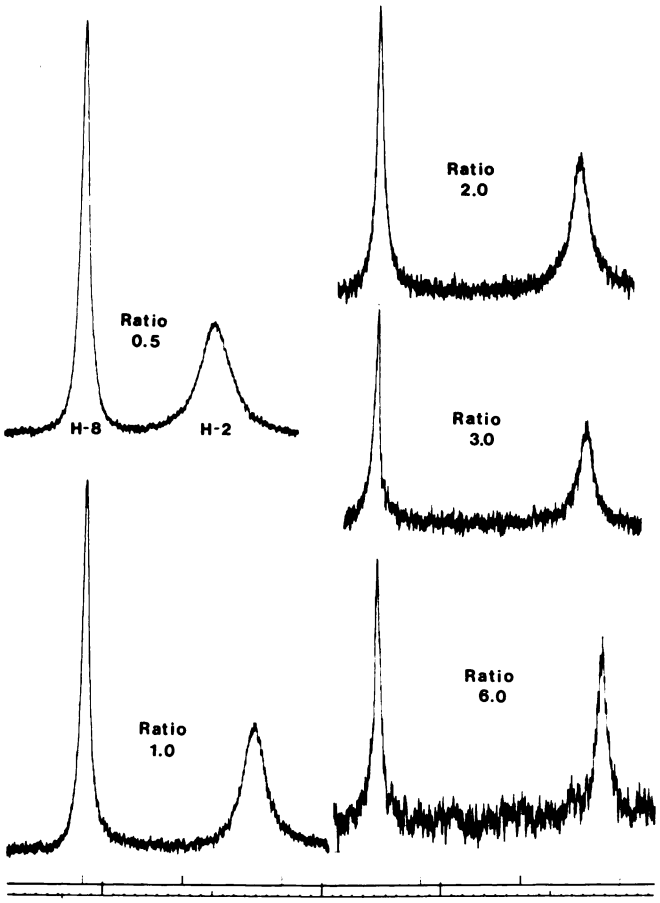

FIG. 7. *ATP adenine proton resonances at different 5-HT:ATP ratios at 220 MHz*

a profound influence on the chemical shifts of the aromatic 5-HT protons and H-2 of ATP but has little effect on the 5-HT side chain protons and none at all on H-8 of ATP. Since all shifts occur upfield, the nature of this interaction can be defined as ring current effects. This must be due to disruption of ATP stacking and the formation of mixed 5-HT–ATP stacks. This concept is illustrated by Fig. 7, which shows narrowing of the H-2 resonance of ATP as the 5-HT concentration increases. The H-8 proton seems to be outside the stacking area and exhibits neither shift changes nor line narrowing.

This line narrowing of the ATP resonances is accompanied by a simultaneous upfield shift, a somewhat puzzling effect. It can be interpreted by assuming that some line broadening of the ATP protons occurs because of 5-HT–ATP binding, but this is far outweighed by the decrease due to dissociation of the ATP-ATP stacks. Similarly, a decrease of ATP self-association does not change the values of the chemical shifts of H-2 and H-8 (as shown in the ATP concentration series in Table 1 and Fig. 2) while the formation of 5-HT–ATP stacks does result in an upfield shift. The over-all result is line narrowing accompanied by upfield shifts.

Since both 5-HT and ATP are good electron donors, the stacks are held together by ring current interaction and not by charge-transfer complex formation; no significant shifts of the ultraviolet absorption maxima were seen. The determination of association constants was attempted from the changes in chemical shifts of the indole

TABLE 2

Effect of NaCl on chemical shifts and linewidths of a 2:1 5-HT–ATP complex

5-HT, 75 mM; ATP, 37.5 mM. Data were obtained at 60 MHz in unbuffered solution at 38°.

NaCl		5-HT protons		
		4	7	$\alpha + \beta$
M				
0	$\Delta\nu$(ppm)	6.85	7.19	3.10
	$\Delta\nu_{1/2}$(Hz)	4.7	8.5	18
1	$\Delta\nu$(ppm)	6.93	7.30	3.16
	$\Delta\nu_{1/2}$(Hz)	4.4	7.4	16
2	$\Delta\nu$(ppm)	7.02	7.34	3.16
	$\Delta\nu_{1/2}$(Hz)	3.8	7.0	16
3	$\Delta\nu$(ppm)	7.04	7.36	3.18
	$\Delta\nu_{1/2}$(Hz)	3.4	6.9	16

protons and ATP H-2, and values of 6.2 and 1.2 M^{-1} were calculated. Hélène *et al.* (11) obtained 6.4 M^{-1} (at pD = 4.9) for the tryptamine-AMP system, a value very close to ours. The lower association constant shown by the ATP proton might be the result of the coexistence of ATP-ATP and 5-HT–ATP stacks. The self-stacking of ATP would change the molar ratio in the 5-HT–ATP complex without becoming noticeable in chemical shift values of ATP, since these are not sensitive to concentration changes (see Table 1). This assumption seems to be supported by the deviation of the ATP protons from linearity in the semilogarithmic Hill plot (Fig. 4), while the aromatic protons of 5-HT give straight lines when plotted this way (Fig. 3).

Spin-spin relaxation rate data indicate a higher degree of immobilization for the 5-HT side chain protons than for the ring protons. Electrostatic interaction between the charged amine group of 5-HT and the phosphate ions of ATP may be responsible for this effect, as already pointed out by Hélène *et al.* (11). This is further supported by the weak interaction of 5-hydroxyindole with ATP, in which no ionic interaction is possible. In the presence of NaCl noticeable downfield shifts and line narrowing can be observed in a 2:1 5-HT–ATP complex (Table 2).

These observations indicate that ionic binding of the charged 5-HT amino group to the ATP phosphates is the primary binding force. This results in the proximity of the indole and adenine rings, forming mixed stacks held together by weak intermolecular forces, as shown by the contact shifts of the aromatic protons but the much weaker shifts of the 5-HT side chain protons, which do not experience ring current effects.

Since several binding phenonema occur simultaneously, it is not unreasonable to assume the existence of multiple complex species as well, in which electrostatic and stacking effects combine to different extents. Since a unique interpretation may not be possible at this time, a quantitative treatment of the data has not been pursued.

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