Interactions of Aromatic Residues of Proteins with Nucleic Acids. I. Proton Magnetic Resonance Studies of the Binding of Tryptophan-Containing Peptides to Poly(adenylic acid) and Deoxyribonucleic Acid†

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ABSTRACT: The binding of tryptamine and oligopeptides containing tryptophan residues to single-stranded poly(adenylic acid) (poly(A)) and double-stranded DNA has been studied by proton magnetic resonance (pmr). Upfield shifts of the indole proton resonances are observed in both cases and ascribed to a stacking of the tryptophan ring with nucleic acid bases. An important broadening of proton resonances is observed in DNA complexes due to a restricted mobility of the bound molecule. Ionic strength and pD effects demonstrate the important contribution of electrostatic interactions in the binding of tryptamine and of the investigated peptides to nucleic acids. Extrapolation of chemical shifts to zero peptide concentration allowed a quantitative analysis of binding isotherms. A comparison of pmr results with results obtained by other techniques indicates the possible involvement of several types of complexes. The poly(A) proton chemical shifts are affected by the binding of tryptamine and tryptophan-containing peptides. At 300°K a downfield shift of H_8 and $H_{1'}$ resonances and an upfield shift of the H_2 resonance of adenine rings are observed except with Lys-Trp-Lys which induces a downfield shift of the H_2, H_8, and $H_{1'}$ resonances. The possible importance of direct interactions between tryptophan residues and bases is discussed with respect to the problem of specific interactions between proteins and nucleic acids.

Many steps of the cellular genetic expression require specific interactions between proteins or enzymes and nucleic acids. This specific interaction may have a structural role (*e.g.*, in ribosomes, viruses, etc.). It may also have a functional role (*e.g.*, repressor–operator, RNA polymerase–promoter, aminoacyl-tRNA synthetases–tRNAs, nucleases–nucleic acids, etc.). The origin of this specificity is still unknown although its importance is widely recognized and documented (Yarus, 1969; Von Hippel and McGhee, 1972). The nucleic acid or the region of it which must be recognized may have a special structure, "complementary" to the structure of the recognition site on the protein molecule. The other source of specificity must rest in specific interactions between amino acid residues of the protein and the bases whose sequence characterizes the recognized region of the nucleic acid. The bases which have to be recognized must not necessarily be in a contiguous sequence. The recognition process might involve different regions of the nucleic acid (and a region may be as short as a single base or base pair).

Several types of direct interactions between amino acids and nucleic acid constituents could be involved in protein–nucleic acid recognition. Electrostatic interactions between basic amino acid side chains and phosphate groups are certainly involved in all protein–nucleic acid complexes. Hydrogen bonds which are essential in maintaining the conformation and the biological properties of DNA might also be involved in amino acid–base recognition. Infrared and proton magnetic resonance (pmr) studies, in our laboratory, have shown that the side chains of tyrosine and carboxylic acids are able to form hydrogen-bonded complexes with nucleic acid bases (Sellini *et al.*, 1973). The peptide linkage as well as ribose and phosphate groups of the nucleic acid might also be involved in hydrogen bond formation (Jain and Sobell, 1972; Cotton *et al.*, 1973).

The role of the aromatic residues of proteins might be of special importance. It is well known that many aromatic molecules, *e.g.*, acridine dyes, are able to stack with purine and pyrimidine bases in single-stranded and double-stranded nucleic acids (Lerman, 1963; Pritchard *et al.*, 1966; Dourlent and Hélène, 1971, and references therein). In several cases, it has been demonstrated that the aromatic molecule intercalates between base pairs and this leads to a change in the DNA structure. If aromatic amino acids were able to intercalate between bases this could, on one hand, participate in the selectivity of the recognition process and, on the other hand, induce conformational changes in the nucleic acid which could be required for further activity of the complexes.

We previously demonstrated that aromatic amino acids and purine or pyrimidine bases form intermolecular complexes in aqueous solutions (Dimicoli and Hélène, 1971, 1973). Luminescence measurements showed that tryptamine, serotonine, and tyramine bind to poly(A) and DNA and that complex formation is accompanied by a quenching of the indole or phenol ring fluorescence (Hélène, 1971a,b; Hélène *et al.*, 1971b,c). Proton magnetic resonance studies indicated a stacking of these aromatic rings with adenine bases of single-stranded poly(adenylic acid) (poly(A)) (Hélène *et al.*, 1971b,c; Razka and Mandel, 1971). Nuclear magnetic resonance (nmr) is a powerful technique to investigate stacking interactions between aromatic molecules because the magnetic anisotropy (ring currents) will induce upfield shifts of the aromatic proton resonances in both molecules. If fast exchange occurs on the nmr time scale between free and bound molecules, only one resonance will be observed for individual protons. The observed frequency will be the weighted average

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of the resonance frequencies in the free and bound states. If a small molecule binds to the polymer, several parameters must be taken into account in analyzing the experimental results: the rotational correlation time of the polymer, the relative mobility of the small molecule (or part of it) with respect to the polymer, the relative concentrations of bound and free molecules, and the exchange rate between the free and the bound state. In most cases, a broadening of the resonance lines of the small molecule will be observed (Jardetzky, 1964a).

We report the results of pmr studies of the binding of oligopeptides containing aromatic residues to single-stranded poly(A) and to double-stranded calf thymus DNA. Lysine residues were introduced in these oligopeptides to increase the electrostatic binding to nucleic acid phosphates. The proton resonances of the oligopeptide as well as those of the polymer when observable were investigated. A preliminary report of these results already appeared (Hélène and Dimicoli, 1972). A pmr study of the binding of several peptides to DNA was also recently published (Gabbay *et al.*, 1972). The present report deals with oligopeptides containing tryptophan residues. The accompanying paper (Dimicoli and Hélène, 1974) describes the binding of phenol derivatives and of oligopeptides containing tyrosine residues to poly(A) and DNA.

Materials and Methods

Poly(A) was purchased from Miles and used as the potassium salt. Calf thymus DNA was sonicated until its sedimentation coefficient $s_{20,w}{}^0$ was equal to 7. Solutions were made in D_2O containing 1 mM NaCl, 1 mM sodium cacodylate, and 0.2 mM EDTA, and the pD was adjusted at the required value by addition of small amounts of concentrated DCl or NaOD (see Results) (pD = pH + 0.4). In some cases citrate ions were added to the solution in order to determine the effect of the solution viscosity on the line width of unbound citrate ions (see Figure 9).

The oligopeptides Trp-Gly, Trp-Lys, and Lys-Trp-Lys were purchased from Schwarz/Mann and used without further purification. Lys-Trp-OMe, Lys-Ala-Lys, and Lys-Ala-Lys-OMe were synthesized in our laboratory by Dr. J. Rossi. Numbering of the residues will always begin with the amino-terminal residue (from left to right).

Pmr spectra were recorded with a Brüker HFX 90-MHz spectrometer equipped with a Fabritek computer. Chemical shifts were measured with respect to an external reference (hexamethyldisiloxane). Corrections for changes in bulk susceptibility with temperature and ionic strength were done by following the resonance of *tert*-butyl alcohol dissolved in the buffer. Concentrations were determined from absorbance measurements.

The binding of a small molecule to a polymer is usually described by a model in which the polymer is assumed to contain n independent and equivalent binding sites. The Scatchard representation is often used

$$r/C_f = K(n - r) \tag{1}$$

where $r = C_b/P_0$, C_b and C_f are the concentrations of bound and free molecules, respectively, P_0 is the total polymer concentration (expressed in moles of phosphate/liter), K is the association constant, and n is the number of binding sites per polymer unit (per phosphate).

When the concentration of bound molecules (C_b) is low as compared to the concentration of available binding sites (nP_0), eq 1 reduces to

$$C_b = \frac{KnP_0C_0}{1 + KnP_0} \tag{2}$$

where $C_0 = C_b + C_f$.

If the binding of the small molecule is followed by pmr spectroscopy and if the exchange rate between free and bound molecules is fast enough on the pmr time scale, only one resonance will be observed for any proton. The chemical shift of proton j (δ_j) will be related to the chemical shifts in the free ($\delta_j{}^f$) and bound ($\delta_j{}^b$) molecules by the relationship

$$\Delta\delta_j = \Delta\delta_j{}^b(C_b/C_0) \tag{3}$$

where $\Delta\delta_j = \delta_j - \delta_j{}^f$ and $\Delta\delta_j{}^b = \delta_j{}^b - \delta_j{}^f$.

If the change in chemical shift $\Delta\delta_j$ is studied as a function of concentration at constant polymer concentration, extrapolation of $\Delta\delta_j$ to zero concentration will lead to a value $\Delta\delta_j{}^*$ such that (using eq 2)

$$\Delta\delta_j{}^* = \Delta\delta_j{}^b\frac{KnP_0}{1 + KnP_0} \tag{4}$$

If $KnP_0 \gg 1$, then eq 4 reduces to

$$\Delta\delta_j{}^* = \Delta\delta_j{}^b \text{ or } \delta_j{}^* = \delta_j{}^b \tag{5}$$

That is, if the binding constant is high enough so that $KnP_0 \gg 1$, the extrapolated chemical shift is equal to the chemical shift of the investigated molecule in the complex.

If several complexes are present simultaneously with association constants K_i and number of binding sites n_i such that $K_in_iP_0 \gg 1$, eq 5 can be written as

$$\Delta\delta_j{}^* = \frac{\sum_i^i n_iK_i\Delta\delta_j{}^{b,i}}{\sum_i n_iK_i} \tag{6}$$

where $\Delta\delta_j{}^{b,i}$ represents the difference in chemical shift of proton j between the free and bound molecule in complex i.

Results and Discussion

Pmr Spectra of the Peptides Investigated. The structural analysis of the Trp-containing peptides investigated here, using fluorescence and pmr techniques, is deferred to another publication. Only the results which are of interest in the binding studies described below will be presented here (see Table I). Several peptides have been studied: Trp-Gly, Trp-Lys, Lys-Trp-OMe, Lys-Trp-Lys, Lys-Ala-Lys, and Lys-Ala-Lys-OMe (see Figure 9 for the pmr spectrum of Trp-Lys and Figures 1 and 2 for the pmr spectrum of Lys-Trp-Lys).

The resonances of $CH_2(\epsilon)$ groups of lysine residues in Trp-containing peptides appear at higher field than in free lysine or in Lys-Ala-Lys at the same pD. The position of the $CH_2(\delta)$ resonance of lysine can be identified by a technique of double irradiation (indor). These resonances also appear at higher field than in free lysine or in Lys-Ala-Lys. It seems thus likely that the upfield shift of the $CH_2(\epsilon)$ and $CH_2(\delta)$ resonances

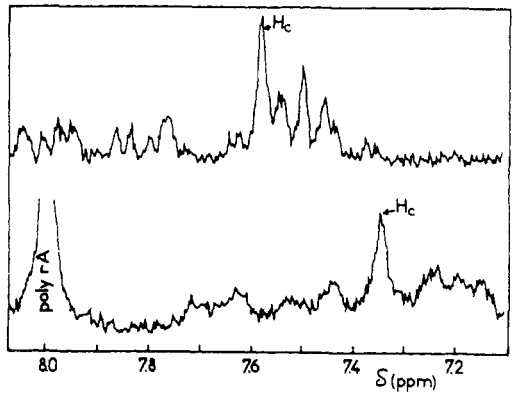
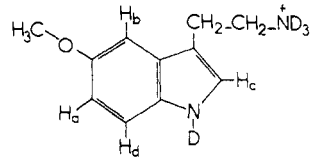


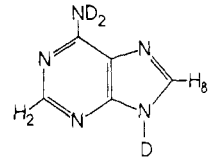
FIGURE 1: Pmr spectra of the aromatic protons of 9×10^{-3} M Lys-Trp-Lys in the absence (upper spectrum) and the presence (lower spectrum) of 2.7×10^{-2} M poly(A) at pD 6.9 and 298°K. (For an analysis of the pmr spectrum of tryptophan see Gerig, 1968.) H_c is the proton linked to C-2 in the five-membered ring of the indole nucleus.

of lysine residues is due to the magnetic anisotropy of neighboring Trp residues.

The pK values of the ND_3^+ terminal groups of peptides (~8.0) are lower than those of lysine ND_3^+ groups. Neutralizing this ND_3^+ terminal group (at pH 9.2) in the peptides containing a Trp residue has an important effect (upfield shift) on the $CH_2(\epsilon)$ resonance of Lys(2) and Lys(3) residues of Trp-Lys and Lys-Trp-Lys, respectively, whereas only a very small effect is observed for lysine or Lys-Ala-Lys. This cannot be due to a direct effect because these groups are separated by several bonds. The observed upfield shifts are very likely due to the magnetic anisotropy of the indole ring. The neutralization of the terminal ND_3^+ group would thus favor a charge–ring interaction involving the lysine ND_3^+ side-chain group.



5-Methoxytryptamine



Adenine

Only the H_c resonance[1] of the indole ring was investigated (coupling of all other protons leads to a complicated spectrum which has been previously analyzed (Gerig, 1968)). This H_c resonance appears at lower field in peptides containing a Trp residue in position 1 (and in Trp-OMe) than in peptides containing a Trp residue in position 2. When the ND_3^+ terminal group is neutralized, the H_c resonance of Trp(1) is shifted to higher fields, indicating an interaction of this ND_3^+ group with the indole ring at pD 6.2. Polarization of the ring π electrons by this positive charge will induce downfield shifts of the ring proton resonances (then, neutralization of this charge would result in an upfield shift).

In Lys-Trp-Lys at pD 6.2, the indole ring probably interacts only weakly with the terminal ND_3^+ group. Neutralization of the latter has only a very small influence on the H_c resonance but introduces an interaction of the ND_3^+ group of Lys(3) with the indole ring as noted above.

The $CH_2(\beta)$ resonance of Trp appears at lower field and the $CH_2(\beta)$–$CH(\alpha)$ coupling in Trp is smaller when this residue is in position 1. This probably also reflects the charge–ring interaction already mentioned.

Binding of Tryptophan-Containing Peptides to Poly(A). PEPTIDE BEHAVIOR. In the presence of single-stranded poly(A) at pD 6.9, the proton resonances of the aromatic ring and of the $CH_2(\beta)$ group of Trp residues are shifted to higher fields (Table II, Figures 1 and 2). These shifts depend on the relative peptide and poly(A) concentrations, on the ionic strength, on the temperature, and on the pD of the solution. Increasing the ionic strength or the temperature leads to a decrease of the upfield shifts (Table III) in agreement with fluorescence studies (Hélène and Dimicoli, 1972) which demonstrated a dissociation of the complexes under these conditions. The concentration and pD dependence will be discussed below. The chemical shifts of other protons of Trp-containing peptides are insensitive to the presence of poly(A) except for the $CH(\alpha)$ resonances of the amino acid in position 1 which undergoes an

TABLE I: Chemical Shifts (ppm) of the Lysine $CH_2(\epsilon)$ and Tryptophan H_c and $CH_2(\beta)$ Protons for Different Molecules.[a]

	$CH_2(\epsilon)$, Lys		H_c, Trp		$CH_2(\epsilon)$, Trp	
	pD 6.2	pD 9.2	pD 6.2	pD 9.2	pD 6.2	pD 9.2
Lysine	3.39	3.37				
Lys-Ala-Lys	3.395	3.37				
Lys-Trp-OMe	3.265		7.61		3.69	
Lys-Trp-Lys (1)	3.28	3.27	7.615	7.61	3.64	3.59
Lys-Trp-Lys (3)	3.31	3.185				
Trp-Lys	3.31	3.175	7.655	7.56	3.75	3.48
Trp-OMe			7.65		3.80	
Trp-Gly			7.645	7.55	3.71	3.445

[a] In D_2O at 300°K measured downfield from external hexamethylsiloxane. The two values reported correspond to pD 6.2 (left) and pD 9.2 (right). Methylated derivatives were not investigated at pD 9.2 because ester hydrolysis takes place.

TABLE II: Change in Chemical Shifts (in Hz) at 90 MHz for Different Protons of the Oligopeptides Lys-Trp-Lys, Trp-Lys, and Trp-Gly (~10^{-2} M) in the Presence of 2.75×10^{-2} M Poly(A).[a]

	H_c, Trp	$CH_2(\beta)$, Trp	$CH(\alpha)$, Residue 1
Lys-Trp-Lys	+22.6	+15.5	−7.4
Trp-Lys	+17.5	+7.4	+1.4
Trp-Gly	+4.7	+3.6	+2.0

[a] In D_2O at pD 6.9; T = 298°K. A plus sign indicates an upfield shift and a minus sign indicates a downfield shift.

[1] H_c refers to the proton linked to C-2 of the five-membered ring of the indole nucleus. Letters were used to label indole protons to avoid confusion with adenine protons labeled with numbers.

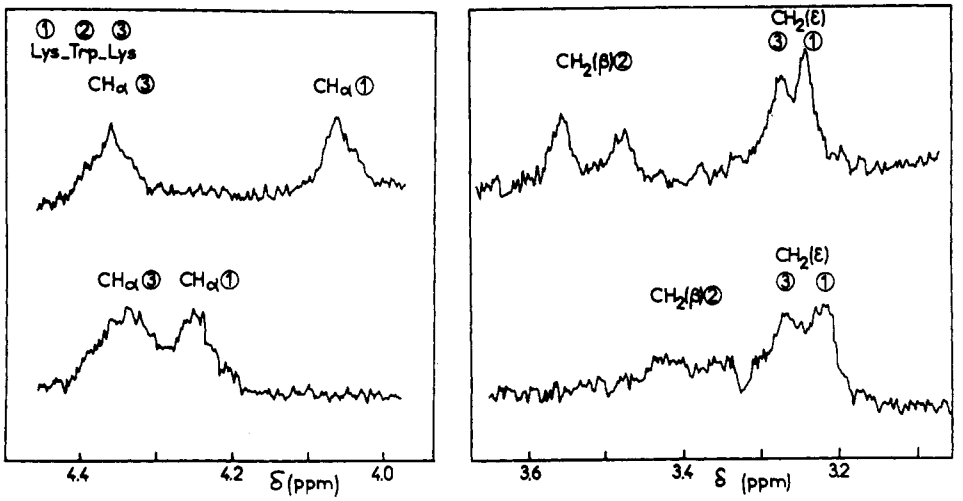


FIGURE 2: Pmr spectra of the CH(α), CH_2(β), and CH_2(ϵ) groups of 9×10^{-3} M Lys-Trp-Lys in the absence (upper spectrum) and the presence (lower spectrum) of 2.7×10^{-2} M poly(A) at pD 6.9 and 298°K.

TABLE III: Ionic Strength and Temperature Dependence of the Upfield Shift ($\Delta\delta$) of the H_ϵ Indole Resonance of the Dipeptide Trp-Lys (9×10^{-3} M) in the Presence of 3.6×10^{-2} M Poly(A).

[NaCl] (M)	$\Delta\delta$ (Hz)[a]	T (°K)	$\Delta\delta$ (Hz)
10^{-3}	22.1	276	28.7
		280	27.8
7×10^{-2}	17.1	286	25.8
		290	24.8
1.4×10^{-1}	11.5	296	23.4
		298	22.1
2.6×10^{-1}	7.5	315	17.4
		335	11.5

[a] At 298°K.

important downfield shift in the presence of Lys-Trp-Lys (see below).

In the case of Lys-Ala-Lys, the resonance of the Ala methyl protons is not shifted in the presence of poly(A). It must be noticed that a general broadening of the resonance lines of the peptides is observed in the presence of poly(A).

Concentration Effects. At constant pD (6.9) and constant temperature (300°K), the upfield shifts of the H_ϵ resonance of the Trp ring which can be conveniently studied depend on the relative concentrations of peptide and poly(A). Precipitation is observed when the ratio of these two concentrations increases beyond about 0.3 indicating a saturation of the polymer (neutralization of the poly(rA) phosphate groups).

At constant poly(A) concentration, the upfield shift of the H_ϵ resonance increases when peptide concentration decreases. Extrapolation to zero concentration leads to the differences in chemical shifts ($\Delta\delta_j$) between free and totally complexed peptide if the association constant is high enough (see Materials and Methods and eq 4–6). These are 0.38 and 0.31 ppm for Lys-Trp-Lys and Trp-Lys, respectively. These values are quite similar to those obtained in the case of tryptamine (0.3 ppm) and of 5-hydroxy- or 5-methoxytryptamine (0.4 ppm). These changes in chemical shifts appear to be much too large to be due to a removal of the peptide positive charges away from the indole ring which could have occurred during peptide binding to poly(A). Such a removal would not be expected to change the chemical shifts of the ring protons by more than 0.1 ppm (see Table I). Interaction of the indole ring with negatively charged phosphate groups would certainly induce upfield shifts of the ring proton resonances but the effect would be expected to be much smaller than 0.3 ppm. Moreover, the results obtained by other physical methods such as fluorescence (Hélène *et al.*, 1971b; Hélène and Dimicoli, 1972) and circular dichroism (Hélène *et al.*, 1973) or by investigating the modifications of the poly(A) pmr spectrum (see below) lead to the conclusion that the upfield shifts of the indole proton resonances are due to a direct interaction of the indole ring with adenine bases whose magnetic anisotropy is large (Giessner-Prettre and Pullman, 1970).

Using the extrapolated values determined above, the concentration of bound peptide can be obtained at any peptide concentration. Then, the results can be plotted according to Scatchard (eq 1). As can be seen in Figure 3, one does not obtain straight lines. However, the first part of the curve can be approximated to a straight line. Association constants fall between 10^3 M^{-1} and 2×10^3 M^{-1}. Thus, the approximation made above ($KnP_0 \gg 1$) in deriving eq 5 and 6 is valid. This means that the extrapolated values reported above correspond to the chemical shifts for the totally complexed peptides.

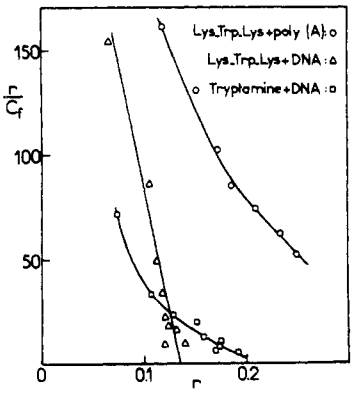


FIGURE 3: Scatchard plot (eq 1) for the binding of tryptamine to DNA and of Lys-Trp-Lys to poly(A) and DNA at pD 6.9 and 298°K. The concentration of bound peptide was determined from the upfield shift of the indole H_ϵ resonance (see text). The concentrations of poly(A) and DNA are constant and equal to 3.9×10^{-2} and 2.7×10^{-2} M, respectively.

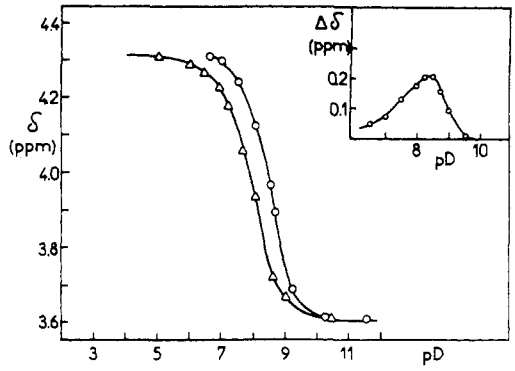


FIGURE 4: pD dependence of the chemical shift of the CH(α) resonance of the ND_3^+-terminal lysine of Lys-Trp-Lys in the absence (Δ) and in the presence (O) of 10^{-1} M poly(A); peptide concentration = 3.4×10^{-2} M; T = 298°K. All chemical shifts are measured with respect to external hexamethyldisiloxane. Inset: change in chemical shift ($\Delta\delta$) between free and complexed peptide *vs.* pD.

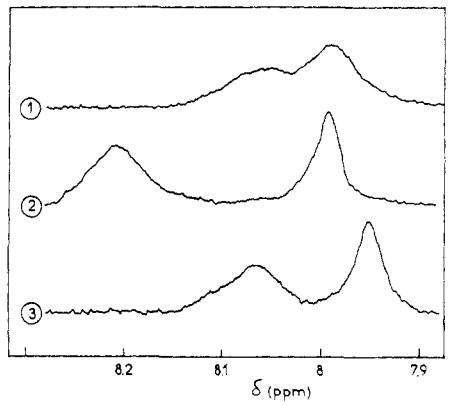


FIGURE 6: Pmr spectra of 3.9×10^{-2} M poly(A) alone (spectrum 1) and in the presence of 1.1×10^{-2} M Lys-Trp-Lys (spectrum 2) and 1.1×10^{-2} M Trp-Lys (spectrum 3) at 295°K and pD 6.9. Low- and high-field resonances correspond to H_8 and H_2, respectively.

pD Dependence of Binding. The changes in chemical shifts of the H_ϵ resonance of Trp residues and of the CH(α) resonance of residue 1 have been followed as a function of the solution pD. The results are summarized in Figures 4 and 5 in the case of Lys-Trp-Lys. As already mentioned, neutralizing the ND_3^+ terminal group of the peptide induces an upfield shift of the CH(α) resonance of the first residue (Figure 4). In the presence of poly(A), the titration curve is shifted to higher pD values and the apparent pK of the terminal ND_3^+ group is increased by about 0.5 unit (from 8.0 to 8.5). This explains why, at constant pD, the CH(α) resonance undergoes a downfield shift when poly(A) is added. This result also demonstrates that the ND_3^+ terminal group is involved in peptide binding to the polymer phosphate groups.

The H_ϵ resonance of Trp in Lys-Trp-Lys is not sensitive to the neutralization of the ND_3^+ terminal group. However, the upfield shift observed in the presence of poly(A) markedly decreases when this group is neutralized (Figure 5). Neutralizing the ϵ-ND_3^+ groups of lysine side chains at higher pD values also leads to a decrease of the upfield shifts of the H_ϵ resonance.

POLY(A) BEHAVIOR. Previous studies have shown that the pmr spectrum of poly(A) in D_2O solutions at pH 7 undergoes a transition between 0 and 30° which can be followed by measuring the total area of the H_8 and H_2 resonance absorption lines (McDonald *et al.*, 1965). This area increases with temperature until it reaches a constant value (which corresponds to the value expected from the polymer concentration) at about 30°. This change in peak area is accompanied by a downfield shift of the resonance lines. This downfield shift is still increasing above 30° (McDonald *et al.*, 1964, 1965; McTague *et al.*, 1964; Jardetzky, 1964b). Its temperature dependence is similar to that observed when following the absorbance or the circular dichroism (Van Holde *et al.*, 1965; Brahms and Van Holde, 1967) and attributed to a decrease in base–base interaction in the polymer upon increasing the temperature. The line width of the proton resonances also decreases when temperature increases. The change in peak area between 30 and 0° may be ascribed to the restricted mobility of the polymer protons leading to severe broadening when dipolar interactions are not averaged to zero. The radius of gyration of single-stranded poly(A) also undergoes a rapid change in this temperature range (Inners and Felsenfeld, 1970). Above 30°, the segments of the polymer and the bases inside each segment have probably acquired a fast enough mobility for the proton resonance lines not to be severely broadened. Also, the polydispersity of the poly(A) sample must be taken into account.

By measuring the area of the H_8–H_2 region of the poly(A) pmr spectrum, a "melting" curve is obtained with a half-transition temperature around 283°K. Addition of Trp-containing peptides to poly(A) at low temperature (277°K) leads to an increase in peak area similar to that observed in the presence of several amino acid derivatives (Razka and Mandel, 1971). This leads to a lowering of the half-transition temperature.

At a higher temperature (300°K), addition of oligopeptides to poly(A) leads to a change in chemical shifts of the polymer proton resonances (Figure 6, Table IV). Tryptophan-containing peptides have been compared to Lys-Ala-Lys-OMe, Lys-OMe, and Leu-OMe (of which the resonance lines of the side-chain protons are not displaced in the presence of poly(A)). Downfield shifts of the polymer proton resonances are observed in the presence of the latter compounds. The effect increases when the number of positive charges increases: Leu-OMe (1+) < Lys-OMe (2+) < Lys-Ala-Lys-OMe (3+). The tripeptide Lys-Ala-Lys which bears three positive charges but also one negative charge behaves like Lys-OMe. Addition of high concentrations (5×10^{-1} M) of Na^+ or ND_4^+ ions also induces downfield shifts of the poly(rA) proton resonances (see Table IV). A complete binding study

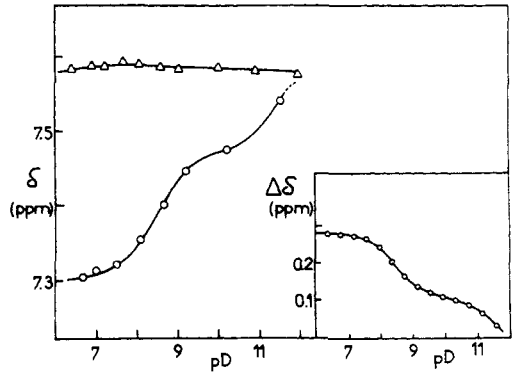


FIGURE 5: Same conditions as in Figure 4. The indole H_ϵ resonance of Lys-Trp-Lys is investigated at different pD values in the absence (Δ) and the presence of poly(A) (O). Inset: change in chemical shift ($\Delta\delta$) between free and complexed peptide *vs.* pD.

TABLE IV: Change in Chemical Shifts of Poly(A) Protons (in Hz) at 90 MHz in the Presence of Different Compounds.[a]

	H_8	H_2	$H_{1'}$
$Na^+(Cl^-)$ (5×10^{-1} M)	−0.6	−1.0	−4.0
$NH_4^+(Cl^-)$ (5×10^{-1} M)	−3.9	−1.6	−2.6
Leu-OMe (10^{-2} M)	−1.0	0	—
Lys-OMe (10^{-2} M)	−7.6	−2.7	−6.1
Trp-Lys (10^{-2} M)	−1.2	+2.3	−1.3
Lys-Ala-Lys-OMe (7.5×10^{-3} M)	−14.2	−3.0	−11.5
Lys-Trp-Lys (7.5×10^{-3} M)	−11.1	−0.4	−7.9
Gly-OMe (5×10^{-2} M)	−2.9	−0.6	—
Ala-OMe (5×10^{-2} M)	−7.0	−2.2	—
Leu-OMe (5×10^{-2} M)	−9.2	−3.3	—
Tryptamine (2×10^{-2} M)	−7.6	+4.8	−4.0
5-Methoxytryptamine (2×10^{-2} M)	−9.1	+5.2	−6.3

[a] In D_2O (pD 6.9) at 298°K. Poly(A) concentration is 3.6×10^{-2} M for the upper part and 2.5×10^{-2} M for the lower part of this table. (Plus and minus signs indicate upfield and downfield shifts, respectively.)

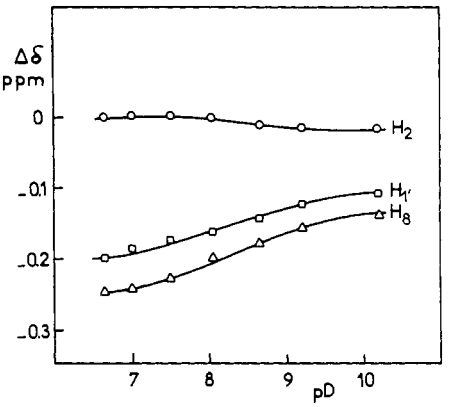


FIGURE 7: pD dependence of the changes in chemical shifts of poly(A) protons in the presence of 3.4×10^{-2} M Lys-Trp-Lys; poly(A) concentration, 10^{-1} M; $T = 298$°K.

TABLE V: Changes in Chemical Shifts ($\Delta\delta$ in Hz) of Poly(A) Proton Resonances per Bound Amine or Peptide at 300°K (See Text).

	H_8	H_2	$H_{1'}$
Tryptamine	−20	+7	−14
5-Hydroxytryptamine	−28	+8	−19
5-Methoxytryptamine	−25	+9	−17
Trp-Lys	−7	+17	−8
Lys-Trp-Lys	−66	−4	−56

should be necessary to determine the effect due to every *bound* molecule. (It is obvious that the binding of Lys-Ala-Lys-OMe with three positive charges is much stronger than that of Leu-OMe with only one positive charge.) The only conclusion that can be reached from measurements at only one concentration is that electrostatic binding of positive charges to poly(A) phosphates affects the stacking interaction between adenine bases.

At still higher temperatures (*e.g.*, 333°K), the binding of indole derivatives induces an upfield shift of all the poly(A) resonance peaks (Hélène *et al.*, 1971a–c).

The effects of Trp-containing peptides on the poly(A) pmr spectrum depend on the solution pD. Whereas the pmr spectrum of poly(A) alone does not depend on the pD in the range 7–10, the peptide-induced shifts decrease when the ND_3^+ terminal charge is neutralized (Figure 7). This demonstrates once more that this charged group is important in peptide binding.

The effect of Trp-containing peptides on the poly(A) spectrum also depends on their relative concentrations. The concentration of bound peptides (C_b) is known from the measured chemical shift $\Delta\delta$ of the H_6 resonance and from the extrapolated values (see preceding section; $C_b = C_0(\Delta\delta/\Delta\delta^*)$. The change in chemical shift ($\Delta\nu_j$) of the poly(A) protons can thus be plotted as a function of $r = C_b/P_0$. As shown in Figure 8, a linear variation is observed. This suggests that the effects of bound peptides on the polymer structure are additive from the point of view of the magnetic environment. It must be noticed that a change in the extrapolated values of the upfield shifts of indole protons ($\Delta\delta^*$) will change the slope of the straight lines but will not affect the linearity of these plots. From the slopes of the straight lines thus obtained (see Figure 8), the values of the changes in chemical shift per bound molecule per phosphate ($\Delta\bar{\nu}_j = \Delta\nu_j/r$) can be calculated for each polymer proton. These values can be thought of as representing the difference in chemical shifts between free poly(A) and a hypothetical poly(A) having one bound molecule per phosphate. These values are given in Table V. Included for comparison are the values obtained with tryptamine and 5-substituted tryptamine derivatives. It can be seen that there exists a marked difference between Lys-Trp-Lys and all other compounds in which the ND_3^+ terminal group is close to the indole ring. This difference is also found in the case of tyrosine-containing peptides (see Dimicoli and Hélène, 1974).

The effect of tryptophan derivatives on the pmr spectrum of poly(A) can be resolved into three contributions: (i) destacking of adenine bases due to insertion of the indole ring between consecutive bases; this will lead to a downfield shift $\Delta\nu_j^P$; (ii) ring current effect of the indole ring on adjacent nucleotides (3′ and 5′ nucleotides); these effects will result in upfield shifts ($\Delta\nu_j^{3'}$ and $\Delta\nu_j^{5'}$); (iii) change in base stacking due to electrostatic interaction of the positive charges of the bound molecule with phosphate groups which will lead to downfield shifts ($\Delta\nu_j^+$) as shown in the case of Lys-OMe or Lys-Ala-Lys-OMe (Table IV).

The net results of these different contributions will depend on the chemical and structural parameters of the Trp derivative investigated as well as on temperature since adenine–adenine interactions (and thus $\Delta\nu_j^P$) decrease when the temperature is raised. In the case of indole amines and of the dipeptide Trp-Lys, it seems likely that only the first two effects have to be taken into account since the presence of one net positive charge has only a very weak effect on the poly(A) pmr spectrum (Table IV).

In contrast the contribution of electrostatic interactions due to lysine residues appears to be important in the effect of Lys-Trp-Lys on the poly(A) pmr spectrum. The main difference between Lys-Trp-Lys and Lys-Ala-Lys effects appears to reside on the H_2 resonance for which an upfield shift contribution due to the ring current effect of Trp is clearly indicated. Quantitative comparison between Lys-Trp-Lys and Lys-Ala-Lys is not possible, however, since poly(A)

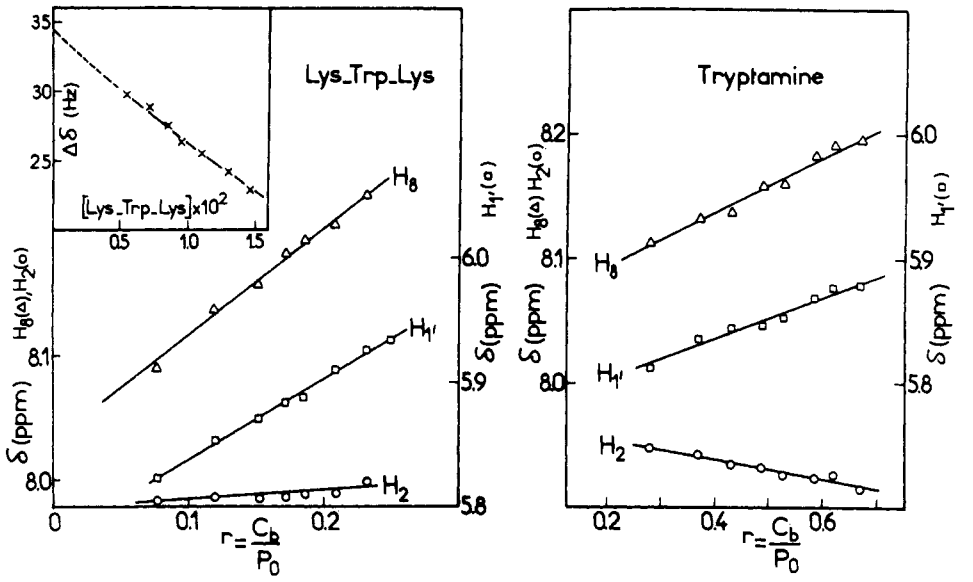


FIGURE 8: Chemical shifts of poly(A) proton resonances as a function of $r = C_b/P_0$ where C_b is the concentration of bound tryptamine (right) or Lys-Trp-Lys (left) molecules and P_0 is the phosphate concentration. C_b was determined, as indicated in the text, from the upfield shift ($\Delta\delta$) of the indole H_ϵ resonance: $C_b = C_0(\Delta\delta/\overline{\Delta\delta^*})$, where C_0 is the total concentration of tryptamine or Lys-Trp-Lys and ($\overline{\Delta\delta^*}$) is the upfield shift extrapolated to zero amine or peptide concentration as shown in the inset in the case of Lys-Trp-Lys.

does not induce any shift in the proton resonances of Lys-Ala-Lys which would have allowed us to determine the concentration of bound peptide for every peptide concentration (the H_δ indole resonance was used in the case of Lys-Trp-Lys). It must be remembered that the values reported in Table V correspond to the changes in chemical shifts per bound peptide molecule and per phosphate. The number of binding sites for Lys-Trp-Lys is estimated to be about 0.35 from precipitation experiments and from Scatchard plots. This means that in order to obtain the difference in chemical shifts between free and saturated poly(A), the values of Table V must be multiplied by 0.35. Different models have been tested (Dimicoli, 1972) but independent determination of the different contributions to the chemical shifts could not be reached. A complete description must await a quantitative analysis of circular dichroism and fluorescence results in order to know the number of different types of complexes formed under similar experimental conditions. The main conclusion that can be reached concerns the important contribution of electrostatic interactions to the change in chemical shifts of poly(A) proton resonances when Lys-Trp-Lys binds to this polymer.

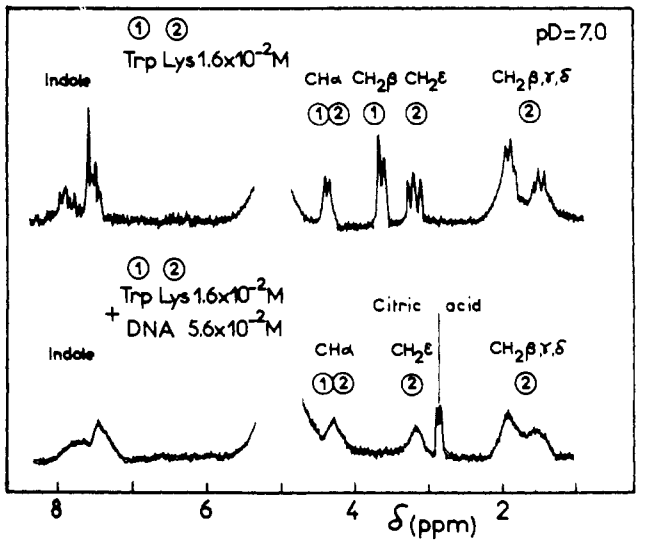


FIGURE 9: Pmr spectra of 1.6×10^{-2} M Trp-Lys in the absence (upper spectrum) and the presence (lower spectrum) of 5.6×10^{-2} M sonicated native calf thymus DNA; $T = 298°K$; pD 7.0. Chemical shifts are measured with respect to an external hexamethyldisiloxane reference. Sodium citrate was added to the DNA solution to demonstrate the absence of line broadening possibly due to the solution viscosity.

Binding of Tryptamine and Tryptophan-Containing Peptides to Double-Stranded DNA. Double-stranded DNA does not give any high-resolution pmr spectrum due to the highly restricted mobility of protons in the native structure. The correlation time for protons in DNA is high and dipolar interactions are not averaged to zero. Only short pieces of a double-stranded helix which have a rotational correlation time short enough exhibit a high resolution pmr spectrum (Cross and Crothers, 1971). At high temperatures, a cooperative transition takes place from a double-stranded to a single-stranded conformation. In the single strands (even for long molecules), the local mobility of bases is high enough to give rise to a pmr spectrum (McDonald *et al.*, 1967). We have followed the pmr spectrum of the peptides in the absence and in the presence of calf thymus sonicated DNA. For the reasons mentioned above, we have not been able to follow the behavior of DNA protons as we had done with poly(A).

In the presence of DNA, the resonances of the Trp ring protons of Trp-Lys and Lys-Trp-Lys are shifted to higher fields and considerably broadened (Figures 9 and 10). The shifts are smaller than those observed with tryptamine. The proton resonance of the Ala residue in Lys-Ala-Lys is slightly broadened but not shifted, indicating complex formation but no stacking interaction with DNA bases.

The $CH_2(\beta)$ resonance of Trp residues in Trp-Lys and Lys-Trp-Lys is markedly broadened and probably shifted to higher fields (although an accurate determination of its field position is prevented by line broadening).

All resonance lines of other aliphatic protons of the oligopeptides are broadened in the presence of DNA. However, it

is difficult to give a measure of this broadening due to the multiplet structure of most lines. Nevertheless, this broadening appears to be smaller than for the Trp ring protons. Broadening is not accompanied by any marked change in chemical shifts.

All changes in chemical shifts of the indole and Trp $CH_2(\beta)$ resonances as well as line broadenings decrease rapidly when the ionic strength is increased. Under these conditions, dissociation of the complexes is observed by fluorescence and circular dichroism measurements (Hélène and Dimicoli, 1972, and unpublished results). This results from a competition of Na^+ ions and lysine residues for the binding to phosphate groups.

When the temperature of the DNA–peptide mixture is raised, several observations can be made: (i) line broadening decreases; (ii) the upfield shift of the H_e resonance decreases in the case of tryptamine and Trp-Lys, whereas it increases slightly in the case of Lys-Trp-Lys. The upfield shifts of the H_e resonance at 300°K are larger if DNA has been previously denatured by heating at 360°K and rapidly cooled to 300°K. The changes in upfield shifts with temperature must arise from several phenomena: the total amount of bound peptides decreases when temperature increases; the structure of the complexes may change with temperature; if several types of complexes are formed, their relative contribution may change with temperature. These different possibilities will be discussed further below in the case of 5-methoxytryptamine binding to DNA.

The chemical shift of the indole H_e proton has been measured at different peptide concentrations while maintaining other parameters constant: DNA concentration (2.5×10^{-2} M), pD (6.0), ionic strength (4 mM), and temperature (315°K). Extrapolation of chemical shifts to zero concentration gives the following changes in chemical shift between the free and bound molecules: 0.62, 0.23, and 0.14 ppm for tryptamine, Lys-Trp-Lys, and Trp-Lys, respectively. It must be noticed that the change in chemical shift for tryptamine is about twice that observed in the presence of poly(A). Only one proton (H_e) of the indole ring is easily identified in DNA complexes. Using 5-methoxytryptamine instead of tryptamine allowed us to investigate different protons of the indole ring due to a simplification of spin–spin couplings. As can be seen in Table VI, the changes in chemical shifts extrapolated to zero concentration are quite different in the complexes of 5-methoxytryptamine with poly(A) and DNA. It can be noticed that the change in chemical shift of the H_e proton in the DNA complex is about twice that observed in the poly(A) complex, a result quite similar to that obtained with tryptamine. Upfield shifts very likely result from the magnetic anisotropy of DNA bases as charge effects would not be expected to induce such large shifts (with the possible exception of Trp-Lys). Among the bases, adenine has the largest magnetic anisotropy (Giessner-Prettre and Pullman, 1970). The difference between poly(A) and DNA very likely reflects a difference in the structure of the complexes, the H_e proton being in a region of higher magnetic anisotropy in the complexes with DNA than in those with poly(A).

TABLE VI: Changes in Chemical Shifts (in Hz) of 5-Methoxytryptamine Protons Extrapolated to Zero Concentration in the Presence of 2.5×10^{-2} M Poly(A) and 2.5×10^{-2} M DNA.[a]

	H_a	H_b	H_c	H_d	CH_3
Poly(A)	70.4	50.4	36.8	70.4	22.4
DNA	56	54	68	56	40

[a] Corresponding values for tryptamine H_e protons are 27 and 56 Hz in poly(A) and DNA complexes, respectively.

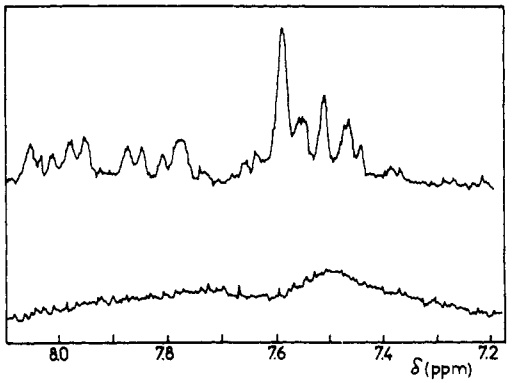


FIGURE 10: Pmr spectrum of the aromatic protons of 1.4×10^{-2} M Lys-Trp-Lys in the absence (upper spectrum) and the presence (lower spectrum) of 6×10^{-2} M native DNA; $T = 298$°K; pD 6.4.

Using the extrapolated values of the chemical shifts of the H_e proton, the concentration of bound peptide can be calculated. Then the Scatchard representation can be used to determine the association constant and the number of binding sites. As can be seen in Figure 3, a straight line is obtained for Lys-Trp-Lys with K and n values of 2×10^3 M^{-1} and 0.14, respectively. For Trp-Lys and tryptamine, Scatchard plots are not straight lines and K and n values could not be determined. Notice that for Lys-Trp-Lys, the value of KnP_0 is about 7. Thus the extrapolated value of the change in chemical shift ($\Delta\delta_j^*$) must be increased by about 10% to obtain the value ($\Delta\delta_j^b$) for the totally bound peptide (see eq 4). An iterative procedure should be used to obtain correct values of $\Delta\delta_j^b$, K, and n, but the precision of concentration and chemical-shift measurements is such that the values reported above can be taken as good approximations.

The marked differences observed between tryptamine, Trp-Lys, and Lys-Trp-Lys probably reflect the presence of different types of complexes at least in the peptide case. Note that the change in chemical shift extrapolated to zero peptide concentration represents an average overall possible types of complexes (eq 6). If in one type of complex the indole ring remains outside the region of high magnetic anisotropy of DNA bases, its chemical shifts will not change (zero contribution). Fluorescence and centrifugation measurements support this idea. The concentration of bound peptides whose fluorescence is quenched is much smaller than the total concentration of bound peptides determined by centrifugation or by competition experiments (results to be published).

In order to get some insight into the mechanism of line broadening of indole derivatives in the presence of DNA, changes in chemical shifts and line widths of 5-methoxytryptamine–DNA mixtures were studied as a function of ionic strength and temperature. This tryptamine derivative was chosen because it gives important upfield shifts and because different protons are easy to investigate and the methyl group resonance is well separated from other resonances. The results are summarized in Tables VII–IX. When the ionic strength increases both upfield shifts and line widths decrease. As shown in Table VII for the methyl group reso-

TABLE VII: Ionic Strength Dependence of the Changes in Chemical Shifts ($\Delta\delta$ in Hz) and in Line Width ($\Delta\delta_{1/2}$ in Hz) for the Methyl Proton Resonance of 1.6×10^{-2} M 5-Methoxytryptamine in the Presence of 6×10^{-2} M DNA at pD 6.5 and $T = 298$°K.

[added NaCl] (M)	$\Delta\delta$ (Hz)	$\Delta\delta_{1/2}$ (Hz)	$\Delta\delta_{1/2}/\Delta\delta$
0	15.4	8.9	0.58
0.02	14.6	8.7	0.59
0.11	11.1	6.4	0.57
0.31	4.6	3.0	0.65

TABLE VIII: Temperature Dependence of the Changes in Chemical Shifts ($\Delta\delta$ in Hz) and in Line Width ($\Delta\delta_{1/2}$ in Hz) for the Methyl Proton Resonance of 2.4×10^{-2} M 5-Methoxytryptamine in the Presence of 5×10^{-2} M DNA at pD 6.5.

T (°K)	$\Delta\delta$ (Hz)	$\Delta\delta_{1/2}$ (Hz)	$\Delta\delta_{1/2}/\Delta\delta$
281	14.2	12.6	0.89
291	12.5	7.2	0.58
297	11.5	5.2	0.45
309	12.0	4.4	0.43
326.5	10.9	1.7	0.15
341	10.0	0.8	0.08
358	8.2	0	0

TABLE IX: Temperature Dependence of the Ratio of Upfield Shifts of 5-Methoxytryptamine Protons (2.4×10^{-2} M) in the Presence of 5×10^{-2} M DNA at pD 6.5.[a]

T (°K)	$\Delta\delta(H_a)/\Delta\delta(H_c)$	$\Delta\delta(H_b)/\Delta\delta(H_c)$	$\Delta\delta(H_d)/\Delta\delta(H_c)$	$\Delta\delta(CH_3)/\Delta\delta(H_c)$
297	1.02	0.91	0.94	0.70
309	1.01	0.93	0.90	0.74
326.5	1.02	1.03	0.90	0.75
341	1.05	1.05	0.96	0.75
358	1.50	1.46	1.45	1.04

[a] The upfield shift of the H_c proton is chosen as a reference as it gives the largest values.

nance, the changes in chemical shift and line width remain proportional to each other when the ionic strength increases (their ratio remains constant). It is known from fluorescence and circular dichroism measurements that increasing the ionic strength leads to a dissociation of 5-methoxytryptamine–DNA complexes (Hélène *et al.*, 1971b, and unpublished results). The above pmr results show that: (i) the line width is directly related to the concentration of bound molecules; (ii) exchange between free and bound molecules does not perturb the line shape; (iii) the relaxation time in the complex does not depend on ionic strength. Only one resonance is observed for each proton and the peak area does not depend on the presence of DNA in spite of the important broadening. Moreover, line widths are smaller than upfield shifts. These results indicate that the exchange between free and bound molecules is fast as compared to the shortest relaxation time in the complex. Thus, line broadening must be due to a restricted mobility of the indole molecule bound to DNA. The observation of sharp lines for citrate protons in the presence of DNA demonstrates that viscosity is not the source of broadening.

When the temperature increases, the proton resonances of 5-methoxytryptamine are less shifted and less broadened in the presence of DNA (Table VIII). But the line width decreases more rapidly than does the upfield shift as shown in Table VIII. This is in contrast to the ionic strength effect (Table VIII). As shown in Table IX, the upfield shifts of the different protons of 5-methoxytryptamine remain proportional to each other when the temperature increases up to the beginning of the DNA melting transition (~345°K). This result strongly suggests that the structure of the 5-methoxytryptamine complex does not change, provided DNA retains its double-stranded conformation. Only the complex concentration decreases. The ratios of the upfield shifts of the different protons change when the double helix begins to melt (see last line of Table IX). As 5-methoxytryptamine binds to single strands (Hélène *et al.*, 1971b), this result shows that the complexes formed with the double helix and the single strands do not have the same structure.

The rapid decrease of the line width of 5-methoxytryptamine protons when temperature increases must therefore be due to a higher mobility of this molecule bound to DNA. This may arise from several sources. The rotational correlation time of the DNA molecule decreases. The local mobility of the indole derivative in its binding site may increase. It is known that DNA structure is changing at premelting temperatures and that the local motion of the bases increases (Studdert *et al.*, 1972; Paleček and Frič, 1972). The relative proportions of different complexes—if they exist—may also change with temperature. This is particularly true for oligopeptides which form different kinds of complexes (see above).

Conclusions

Electrostatic binding of tryptamine and oligopeptides containing lysyl and tryptophyl residues to poly(A) and DNA phosphates makes possible a direct stacking interaction of the indole ring with nucleic acid bases. In the case of poly(A), this interaction leads to a destacking of adenine bases which is due—at least in part—to insertion of the indole ring between neighboring bases. In the case of DNA, it is not possible to follow the behavior of DNA protons (no high-resolution pmr spectrum) as this was done with poly(A). Therefore, information on the type of complexes formed is only fragmentary. The important upfield shifts of the indole ring protons are, however, characteristic of stacking interactions with DNA bases. A similar conclusion was reached by Gabbay *et al.* (1972). The size of the indole ring is similar to that of the purine bases. It seems, therefore, that these stacking interactions might involve only one strand of the DNA double helix as suggested in the case of acridine dyes in the model proposed by Pritchard *et al.* (1966). This should induce a bending of the DNA molecule at the point of intercalation.

Other investigations by physical techniques (fluorescence, circular dichroism, centrifugation, etc.) led to the conclusion that the binding of oligopeptides containing tryptophyl and lysyl residues to poly(A) and DNA involves at least two types of complexes in equilibrium (results to be published). One type of complex involves a stacking of the indole ring with bases and is accompanied by fluorescence quenching and base destacking. In the other type, such a stacking interaction

is not involved and fluorescence polarization measurements indicate a very fast rotational motion of the indole ring in this complex. It is thus possible that fast exchange between these two types of complexes may reduce the line broadening that would be expected for aromatic molecules intercalated between DNA bases. Pmr measurements at different frequencies should help clarify the problem of line broadening in the complexes.

The main force involved in binding interactions is due to electrostatic attraction between positively charged lysine residues and negatively charged phosphate groups. The electrostatic potential of the nucleic acid molecule will decrease when the concentration of bound peptide increases. One must therefore expect a decrease of the binding strength as the binding proceeds. This may explain the curvature observed in Scatchard plots (Figure 3). The presence of different types of complexes (see above) may also contribute to a deviation from the model described by eq 1.

The indole NH group does not form hydrogen bonds with nucleic acid bases in chloroform solutions (Sellini *et al.*, 1973). Thus, it does not seem likely that tryptophyl residues will be involved in hydrogen bonding to bases in nucleic acids.

The fact that the indole ring may interact directly with bases (through stacking interactions) might be of importance in protein–nucleic acid interactions. Insertion of tryptophan residues between bases results in a modification of the nucleic acid structure that might be required for further biological activity of the complexes. We have recently suggested that tryptophan residues of aminoacyl-tRNA synthetases might be involved in the binding of tRNAs by inserting between bases (Hélène *et al.*, 1971a; Hélène, 1971b). It must be noticed that single-stranded and double-stranded regions of nucleic acids will behave differently with respect to the intercalation of the indole ring which is favored in single-stranded structures. This difference might be important when the protein is "choosing" its binding site. Moreover, the stacking of indole derivatives with bases is expected to depend on the interacting base (Dimicoli and Hélène, 1971, 1973). A study of DNAs and polynucleotides of different base composition should allow us to determine whether the selectivity of tryptophan interaction is limited to the structure of the nucleic acid or whether it also involves a base specificity.

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