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PMR STUDIES OF THE SELF-ASSOCIATION OF DNA INTERCALATING ELLIPTICINE DERIVATIVES IN AQUEOUS SOLUTION

A. DELBARRE and B.P. ROQUES
Département de Chimie, Ecole Polytechnique, 17, rue Descartes, 75230 Paris Cedex, France

J.B. LE PECQ
Laboratoire de Pharmacologie Moléculaire, CNRS Villejuif, France

J.Y. LALLEMAND
Ecole Normale Supérieure 75005 Paris, France

and

NGUYEN-DAT-XUONG
Institut des Substances Naturelles, CNRS Gif/Yvette, France

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In aqueous solution DNA intercalating ellipticine derivatives aggregate in *n*-mers. The self-association constants *K* are higher than those of 2-methoxy-6-chloro-9-[3-dimethylaminopropyl-amino]-acridine and ethidium bromide. They are of the same order as that of actinomycin D but inferior to that of acridine orange. The increase of the 9-hydroxy-ellipticine constant by addition of sodium chloride shows the importance of anion participation in the mechanism of stacking in accordance with the high energy of self-association. In the stacked *n*-mers the ellipticine rings are inverted. The geometry shows the importance of the orientation of the quadrupole axis in the intermolecular association of the intercalating drugs.

1. Introduction

Several ellipticine derivatives bind to DNA with a high affinity constant by an intercalating process [1]. Among these drugs, 9-hydroxy-ellipticine (9OHE) shows a high activity on several experimental tumors [1]. Le Pecq et al. [2] have shown that the pharmacological properties of these drugs could be connected to their DNA binding affinity. This affinity depends probably on the orientation of the intercalating molecule relatively to the DNA bases pairs. This problem has been successfully approached by a study of the interaction of the intercalating molecule with mono- and dinucleotides in the solid state by X-ray analysis [3] or in solution by NMR spectroscopy [4,5].

The NMR study of association between ellipticine derivatives and nucleotides first requires the knowledge of the self-association of these drugs. On the other hand, the understanding of the parameters which control the self-association is of interest since some of these parameters also control the affinity for DNA.

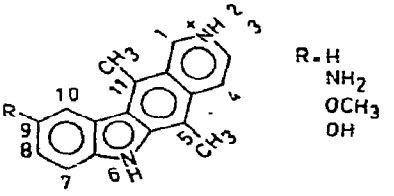

Fig. 1.

In this paper we present informations obtained by PMR spectroscopy on the association of four different

ellipticine derivatives (fig. 1). The geometry of the complexes has been determined. The influence of extrinsic factors such as temperature, pH and ionic strength has been studied.

2. Materials and methods

9-Substituted ellipticines were synthesized by Dr. Nguyen-Dat-Xuong according to procedures which have been published elsewhere [6]. Purified hydrochlorides of ellipticine derivatives were dissolved within deuteroacetate buffer 0.1 M (pD = 4.7 – ionic strength μ = 5.5×10^{-2} M) which is adjusted to the desired pD with deuterated sodium hydroxyde. Since the pK_a values of the studied ellipticines are all superior to 5.5 [2], such a pD value ensures that protonation of all these derivatives is reached. 9OHE was also studied at an upper ionic strength ($\mu = 18 \times 10^{-2}$M) upon addition of sodium chloride. In the case of $9NH_2E$, additional measurements were performed in deuteroacetate solution at pD = 3.2.

pH values were measured with a Tacussel TS 60/N pH meter. pD values were calculated according to the equation pD = pH + 0.4 [7].

PMR spectra were recorded at 90 MHz with a Brucker WH 90 Spectrometer operating on Fourier transform mode and locked on the deuterium (D_2O). Probe temperature was regulated and monitored by observing the splitting in ethylene glycol. Chemical shifts were measured from an external reference (tetramethylsilane in chloroform) and reliable to ±0.01 ppm.

3. Results

3.1. Interpretation of the PMR spectra

The different protons of the spectra of ellipticine derivatives in aqueous solutions are attributed after comparison with respective spectra in deuterated dimethylsulfoxyde [6]. For all compounds, the pyridinic part of the spectrum is always at the first order. The 9-substituted ellipticines shows two coalesced signals corresponding to H_7 and H_8 and an upfield shifted isolated peak for H_{10} (fig. 2). In the case of the ellipticine (R = H) these protons signals have not been attributed. In all cases the shifts of each assigned proton can be followed at any concentration.

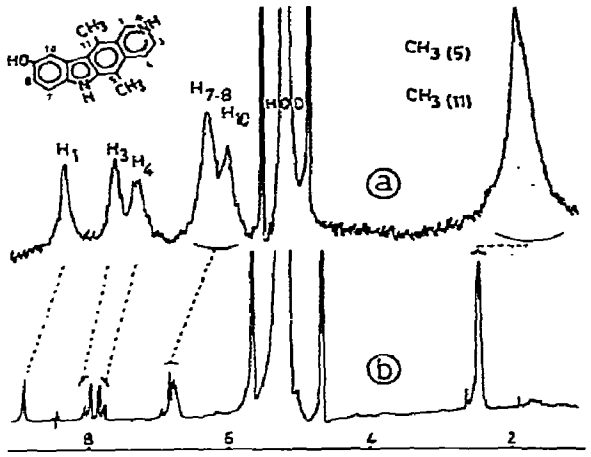

Fig. 2. Chemical shifts (δ) of the 9OHE protons in deuteroacetate buffer at pD = 4.7, $\mu = 5.5 \times 10^{-2}$ M, 27°C. δ in ppm from TMS, external reference (a) $B_0 = 2.9 \times 10^{-2}$ M, (b) $B_0 = 4.5 \times 10^{-4}$ M.

3.2. Dilution shifts of 9-substituted ellipticines

A strong concentration dependence is observed with all the 9-substituted ellipticine protons in aqueous solution. As the concentration of these compounds decreases, the protons progressively shift to low field as shown in fig. 3. All the chemical shifts can be extrapolated at in-

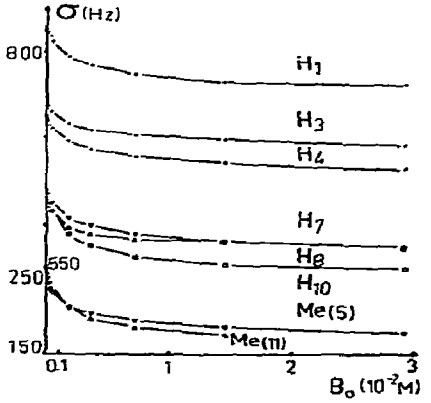

Fig. 3. Concentration dependance of the chemical shifts (σ in Hz from TMS, external reference) of the 9OHE protons at pD = 4,7, $\mu = 5.5 \times 10^{-2}$ M, 27°C. The chemical shifts of the H_7, H_8 and H_{10} were determined from simulated and experimental spectra.

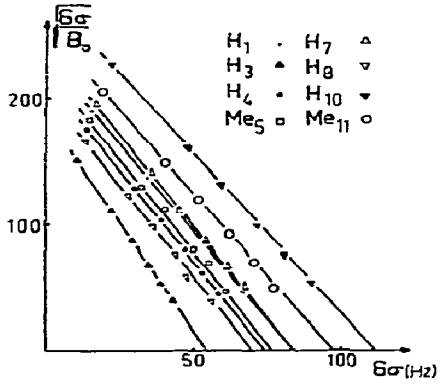

Fig. 4. Self-association of 9OHE in deuteroacetate buffer at 27°C; pD = 4.7. B_0 total concentration of 9OHE. $\delta\sigma$ difference between extrapolated chemical shift at infinite dilution and actual chemical shift at a given concentration. x-axis intercept equals $2\delta\sigma_{B_2}$ and slope $(K/2\delta\sigma_{B_2})^{1/2}$, K being the self association constant and $\delta\sigma_{B_2}$ the difference in chemical shift between the stacked dimer and the monomer.

finite dilution (y intercept σ_0). The hard deshielding of all the protons during the dilution can only be interpreted in terms of formation of stacked n-mers. We calculate $\delta\sigma$ for each concentration and for each proton. $\delta\sigma$ represents the difference between chemical shift σ_0 at infinite dilution and actual chemical shift σ at a given concentration.

3.3. Calculation of the association equilibrium constant K

The equation of Dimicoli and Helene [8] is employed with the following hypotheses: (i) Successive association constants are identical, (ii) effects of magnetic anisotropy are additive, and (iii) only the magnetic anisotropy of the nearest neigbours is taken into account. In a recent work Schimmack et al. have shown that these assumptions are valuable [9].

$$(\delta\sigma/B_0)^{1/2} = (K/2\delta\sigma_{B_2})^{1/2}(2\delta\sigma_{B_2} - \delta\sigma). \qquad (1)$$

B_0 represents the total concentration of 9-substituted ellipticine. A plot of $(\delta\sigma/B_0)^{1/2}$ versus $\delta\sigma$ gives a straight line characterized by a slope (s) and an x-axis intercept (x_0), the values of which being respectively $-(K/2\delta\sigma_{B_2})^{1/2}$ and $2\delta\sigma_{B_2}$. $\delta\sigma_{B_2}$ is the induced shielding of the different protons in the dimer. $2\delta\sigma_{B_2}$ is directly obtained from these plots; the association constant K is calculated ($K = x_0 s^2$) for each proton (fig. 4, table 1). These experiments were performed at different pD values (3.2–4.7) and ionic strengths (5.5×10^{-2} and 18×10^{-2} M).

Table 1
9-R ellipticine self-association constants K

R	K (M^{-1}) (average value)	a: pD = 3.2 b: pD = 4.7 c: μ = 5.5×10^{-2} M d: μ = 18×10^{-2} M
H	732 ± 10	*bc*
NH_2	633 ± 100	*bc*
	76 ± 14	*ac*
OCH_3	697 ± 35	*bc*
OH	657 ± 60	*bc*
	1526 ± 145	*bd*

3.4. Changes in line widths

One point of interest is the somewhat greater line width (16 Hz) obtained for all protons in the case of saturated solutions of hydroxy- and amino-ellipticine (3×10^{-2} M). Such noticeable line broadenings of the ellipticine signals indicate that the process responsible for the shifts changes also affects the relaxation times of the protons, e.g., by slowing the motions of ellipticines as would occur in an aggregate.

3.5. Statistical repartition of molecules within the aggregates

It is of interest to know the number of ellipticine units in n-mers at any concentration. This number n can be determined by use of the following relation. A_n is the molar concentration of ellipticine units stacked in containing each n subunits oligomers.

$$A_n = \frac{n}{K}\left(1 + \frac{1 - (4KB_0 + 1)^{1/2}}{2KB_0}\right)^n. \qquad (2)$$

The curves $A_n = f(n)$ lead to a statistical representation of the state of the n-mers in solution. These curves present a maximum which corresponds to the number n of subunits in the preponderant aggregate in solution

Table 2
Percentage of 9-substituted ellipticine subunits (A_n in the text) in the preponderant aggregate and number n_{max} of subunits in the same aggregate

K (M^{-1})	B_0 (10^{-2} M)	n_{max}	$\%A_n$
1526	3	7	5
	0.24	2	19
	0.02	1	64
657	3	4	8
	0.6	2	19
	0.04	1	68
76	3	2	24
	0.6	1	56
	0.04	1	92

(table 2). The percentage of rings belonging to n-mers is obtained by dividing A_n by B_0.

3.6. Thermodynamical parameters

A temperature variation can affect the ellipticines aggregation in at least two ways: changes in the self-association constant and/or alteration in the geometry of the aggregates. We have studied the upfield shifts of the protons of a solution of 9OH ellipticine (7×10^{-3} M) in a deuterated acetate buffer (pD = 4.7) between 20° and 72°. Successive values of K are obtained with relation (1). A plot of $\ln/K$ versus $1/T$ gave a straight line which permitted to determine the thermodynamical

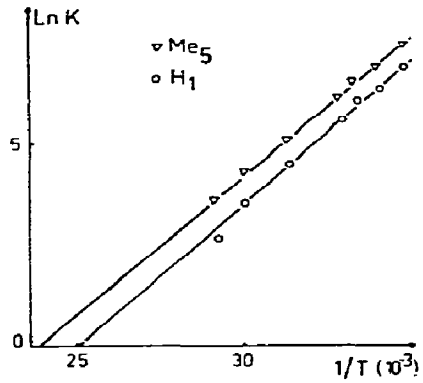

Fig. 5. Plots of ln K versus $1/T$ for Me_5 and H_1 in 9OHE.

parameters $\Delta H° = -15.6$ kcal M^{-1}, $\Delta S° = -39.0$ ue. These datas are valid only if the geometry of the aggregates undergoes little modification during the variation of temperature. A linear relation has been obtained for the protons H_1 and $CH_{3(5)}$ which are far apart. This shows that the above assumption is correct (fig. 5).

4. Discussion

Our results show that following many other intercalating drugs [4,5,10,11,12,14] ellipticines are self-associated in aqueous solution. The large extrapolated shielding values ($2\delta\sigma_{B_2}$) of the different protons within the complexes are an evident proof of the formation of n-mers. The calculated statistical repartition of the complexes is in agreement with this conclusion.

The self-association constants K for the studied ellipticines are higher than those of 2-methoxy-6-chloro-9-[3-dimethylaminopropyl-amino]-acridine (107 M^{-1}) [5] and ethidium bromide (98 M^{-1}) [13]. They are of the same order as that of actinomycin D (700 M^{-1} extrapolated value to 27°) [11] but inferior to that of acridine orange (1×10^4 M^{-1}) [14]. The first constants were determined by a unique method (NMR); therefore they can be compared. The last one was measured spectrophotometrically. Although ellipticine and actinomycin are strongly self-associated, their constants are not the highest. Nevertheless these drugs show the highest affinity constants towards DNA [1,15]. At pD = 3.2 the K value of $9NH_2$ ellipticine dramatically decreases. This probably results from the protonation of the 9-amino group in accordance with the low field shift of the H_8 and H_{10} protons at pD = 3.2. An increase in the salt concentration of a solution of 9OH ellipticine leads to a larger value for the association constant. Such a phenomenon has already been observed for acridine orange [12,13]. In accordance with Robinson et al. [12] we assume this positive ellipticine behaviour is generated by an increase of the anionic surrounding. Negative charges are attracted from the medium and reduce electrostatic repulsions between the rings within the polycationnic aggregates.

The energy of interaction between ellipticines is strikingly high ($\Delta H = -15.6$ kcal M^{-1}) especially since the stacked rings are positively charged. Among possible interpretations of this phenomenon, the salting effect suggests that these charges could be counterbalanced by the proximity of the anions [16].

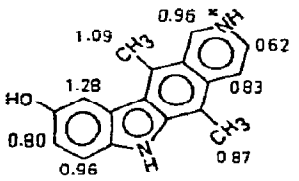

Fig. 6. Different induced shifts $2\delta\sigma_{B_2}$ (ppm) values for the 9OHE protons.

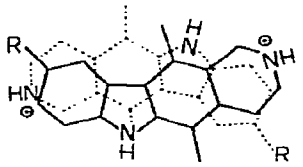

Fig. 7. Proposed inverted geometry for the 9-R ellipticine stacked dimers. R = H, NH_2, OCH_3, OH.

The large shielding differences between the protons of the convex ($CH_{3\ (5)}$) and the concave ($CH_{3\ (11)}$) parts (fig. 6) of the ellipticine rings lead up to propose an inverted geometry for the stacking of the four ellipticines in solution (fig. 7). This stacking geometry is in accordance with the structure determined for the acetate of 9OH ellipticine in the solid state by X-ray analysis [17]. It is of great importance to notice that the inversion of the chromophore is a general phenomenon in the stacking of intercalating drugs [5,10,11]. In the case of the ellipticines the proposed geometry leads to an inverted favourable orientation of the quadrupole axis of these drugs in two parallel planes. The most favourable orientations between the dipole axis of the intercalated drugs and the bases pairs could explain the variation in the unwinding angle of DNA complexed with different ellipticines [2]. Recently similar considerations have been invoked for the observed sequence specificity of new intercalating dyes [18].

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