PROTON MAGNETIC RESONANCE STUDIES OF ETHIDIUM BROMIDE AND ITS SODIUM BOROHYDRIDE REDUCED DERIVATIVE

Gille THOMAS

Service de Physiologie Cellulaire, Institut de Biologie Moléculaire, Faculté des Sciences, 9 Quai Saint-Bernard, Paris 5è, France

and

Bernard ROQUES

Département de Chimie, Ecole Polytechnique, 17 rue Descartes, Paris 5è, France

Received 23 June 1972
Original figures received 24 July 1972

1. Introduction

During the course of investigation on the conformation of intact and modified tRNA molecules [1] we have observed that ethidium bromide (EB) was readily reduced by sodium borohydride, $NaBH_4$. Similar observations have been reported by Wintermeyer and Zachau [2]. Sodium borohydride is a selective reducing agent for some atypical bases of tRNA [3] and has allowed the introduction of an intramolecular fluorescent probe into a number of *E.coli* tRNA [4]. EB is an intercalating dye which has revealed to be of value in the study of nucleic acid conformation (see review in [5]). It interacts with these polymers in two ways: EB intercalates between the bases levels of standard Watson–Crick double helices with high affinity; the EB cation also binds with low affinity to the negatively charged phosphate of single stranded or double-stranded polynucleotides. We have observed that reduced ethidium Et^{red} does not interact with nucleic acids. This reaction seems to be useful in the purification of covalently closed circular DNA.

Based on PMR spectroscopy we have established the structure of the reduced form of EB. This lead us to a reexamination of the PMR datas of Kreishman et al. for EB in aqueous solution [6]. These authors report the formation of an EB complex at high concentration in this medium. We have proposed a model for such stacked EB complex.

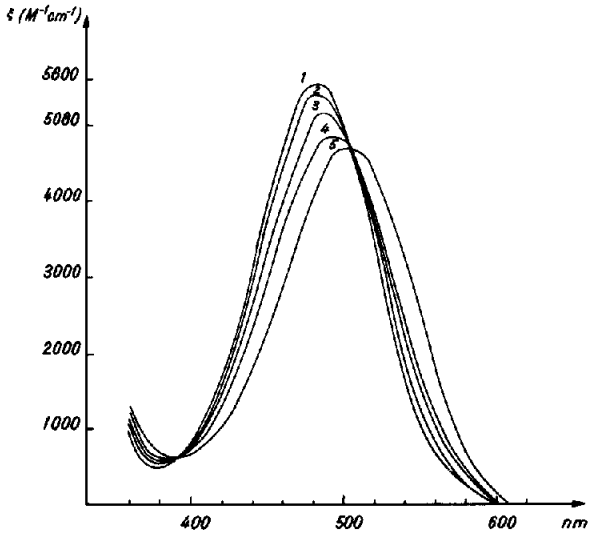

Fig. 1. Absorption spectra of various EB concentrations in aqueous solution 1) 10^{-3} M; 2) 2×10^{-3} M; 3) 5×10^{-3} M; 4) 10^{-2} M; 5) 10^{-1} M. The quartz cuvettes were of 0.5 mm optical length except for 5 where it was of 10 μm.

2. Experimental

2.1.

Ethidium bromide was kindly given to us by Dr. Cobb of the Boots and Pure Drug Co. (Nottingham, England). Sodium borohydride was from Merck Sharp and Dohme.

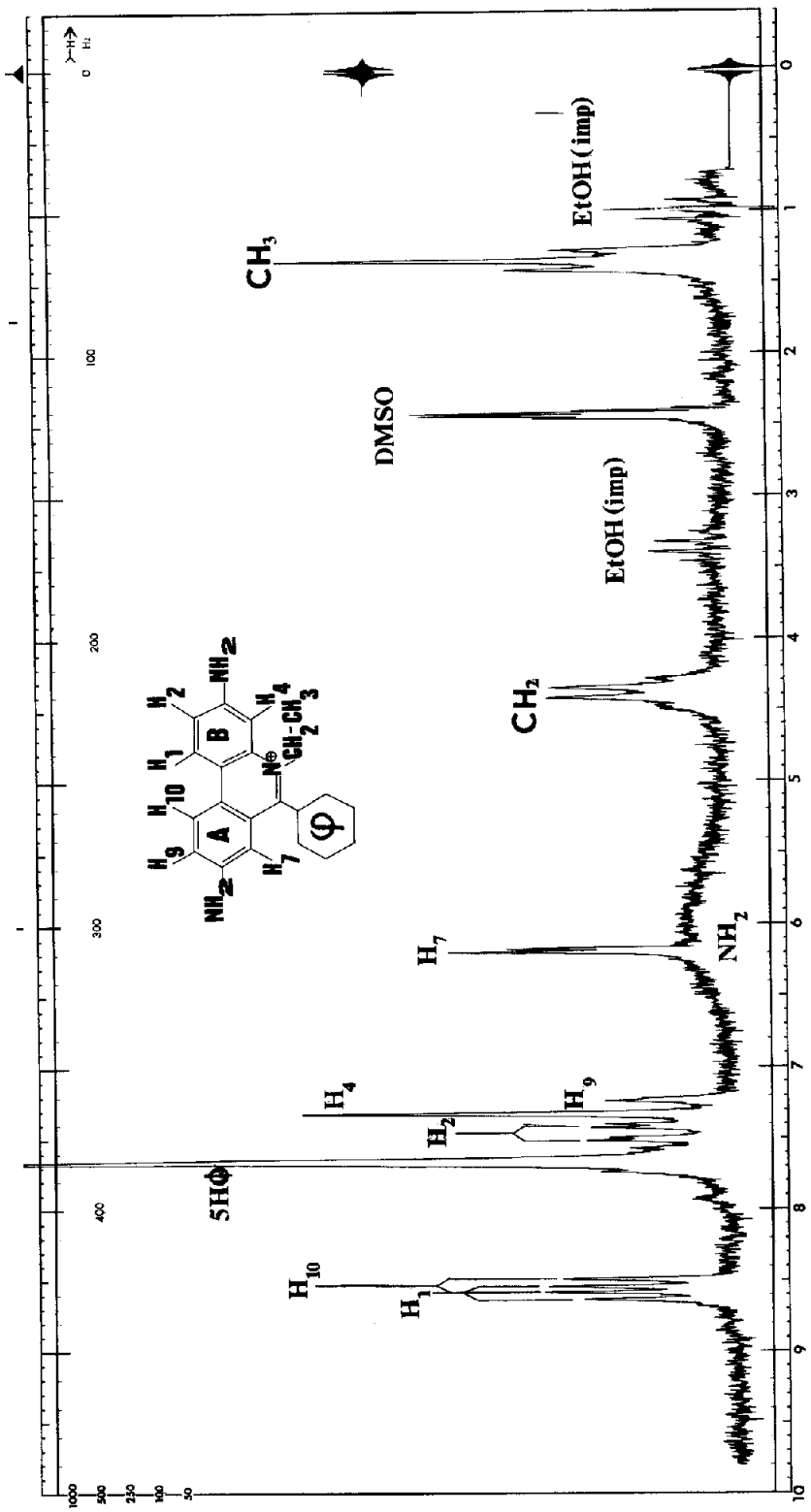

Fig. 2. PMR spectrum of ethidium bromide in DMSO d6 solution. Conditions were as in sect.2.2.

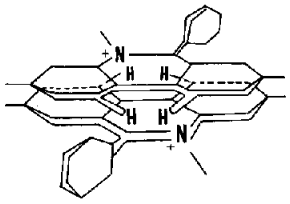

Fig. 3. Proposed structure for the stacked complex between two EB molecules.

2.2.

Absorption spectra were measured with a Cary model 17 spectrophotometer and fluorescence studies were performed with a Jobin Yvon spectrofluorimeter.

The PMR spectra were recorded on a Varian HA 100 spectrometer at a probe temperature of $30 \pm 1°$ with hexamethyldisiloxane (HMDS) as internal lock signal. Concentration of samples were 0.04 M dissolved in DMSO d6. Double resonance and tickling experiments were performed with a Hewlett–Packard 200 AB low frequency generator.

2.3.

Two sets of conditions were used to reduce EB. The products obtained were identical.

2.3.1.

With dilute aqueous EB solution a slight molar excess of a freshly prepared $NaBH_4$ solution (10^{-4} to 10^{-1} M) was added in the dark.

2.3.2.

For PMR studies, large quantities of purified Et^{red} were prepared with a method described for the reduction of quinolinium salts [7]: under nitrogen, in the dark and at 0°, 500 mg of EB in 10 ml absolute methanol were added to 220 mg $NaBH_4$. After 24 hr stirring, methanol was removed by evaporation in vacuum and 15 ml of 3% sodium hydroxide solution were added. After several extractions with ether, the phases were washed with water and dried over anhydrous sodium sulfate. Solvent was evaporated. The precipitate was recrystallized in ether to yield a light brown powder.

3. Results and discussion

3.1. *Ethium bromide stacked complex*

Optical measurements of EB in water confirm the formation of an EB complex at high concentration first observed by Kreishman [6]. Fig.1 shows the absorption spectrum of various EB concentrations in water. There are clearly two isosbestic points (at 392 and 505 nm) indicating the presence of two spectroscopically distinct species.

The absorption changes in this type of interaction are very similar to those observed in the presence of nucleic acids. This allows us by extrapolation to infinite concentration of EB to approximate the equilibrium constant in the formation of EB–EB complexes to about $30 \pm 10\ M^{-1}$ (assuming the complex is dimeric).

Due to the high concentrations used in PMR studies such complex makes spectrum assignment difficult even at 220 MHz. In order to avoid such inconvenience we have studied the PMR spectrum of EB (0.04 M) in DMSO d6 where EB remains as monomer. We find complete assignment of the spectrum (by irradiation of H_7 for the assignment of the signal corresponding to H_2 and H_9 and then by tickling on the four bands situated downfield to assign H_1 and H_{10}) (fig.2). The chemical shifts (δ) and coupling constants are as listed in table 1.

As was reported earlier [6] an important upfield shift of proton H_7 was observed because of its situation in the diamagnetic region of the phenyl. The deshielding of the peri proton H_1 and H_{10} is explained by the paramagnetic effect of the ring current of the phenanthridine. The presence of a charged nitrogen adjacent to cycle B shifts H_2 to lower field compared to H_9.

The considerable downfield shift of proton H_1 and H_{10} in the monomeric form of EB enables us to reinterpret Kreishman data: with increasing EB concentrations, protons H_1 and H_{10} are considerably upshifted although the spectra of other protons were only slightly modified. We therefore propose structure A (fig.3) for the complex between EB molecules.

In this structure hydrogens H_1 and H_{10} are situated over the pyridinic cycle of the phenanthridine and therefore considerably upshifted by its ring current. In structure A both steric hindrance (phenyl) and electrostatic repulsion of the charged nitrogens are minimum, in accordance with crystallographic data [8]. We do not known whether the complex is exclusively in the dimeric form. However structure A can be easily extrapolated to greater aggregates.

Table 1

Chemical shifts of EB and Et^{red} protons (in ppm, internal reference HMDS). For ethidium bromide coupling constants are $J_{1,2}$ = = 9.5 Hz; $J_{2,4}$ = 1.75 Hz; $J_{7,9}$ = 2.5 Hz and $J_{9,10}$ = 9.5 Hz.

	1	2	4	6	7	9	10	5HΦ	NH_2	CH_2	CH_3
EB (δ)	8.58	7.48	7.32		6.18	7.29	8.52	7.66	6.00	4.36	1.32
$Et^{red}(\delta')$	7.22	5.92	5.92	5.24	6.28	6.41	7.28	7.12	4.78	3.16	1.04
$\delta-\delta'$	1.36	1.56	1.40		−0.10	0.88	1.24	0.54	1.22	1.20	0.28

It is known that intercalating dyes tend to form stacked aggregates in aqueous solutions. Proflavine and Acridine Orange complexes are stable enough to explain cooperative binding of these dyes to poly L-glutamic acids [9] and tRNA [10]. In the case of EB the phenyl perpendicular to the phenanthridinium plane imposes an important steric hindrance in the formation of stacked complexes. This might be a reason for its low affinity constant (Le Pecq private communication). However we have found some cooperativity in the electrostatic binding of EB to poly U and tRNA (unpublished results).

3.2. *Reduced ethidium (Et^{red})*

Upon addition of sodium borohydride absorption and fluorescence spectra of EB disappeared and a new spectra appeared (fig.4). The reduced product was stable at basic pH and in the dark. When exposed to light it returned to ethidium. At acidic pH, an irreversible decomposition occurred.

Et^{red} structure is unambiguously characterised by its PMR spectrum (fig.5). Comparing it to EB spectrum we observed the following internal chemical shifts:

1) the CH_2–CH_3 pattern was considerably upfield shifted due to the disappearance of the positive charge on the heterocyclic nitrogen. The methylene protons were magnetically non equivalent and displayed a multiplet because of an asymmetric carbon in the β position [11];

2) the proton H_6 signal was a singlet at 5.24 ppm. The rather high chemical shift of this proton was probably due to the C–N bond and to its position in the paramagnetic region of the phenyl ring;

3) phenyl protons are slightly upshifted as compared with their position in EB;

4) all hydrogens of cycle A and B except H_7 are moved towards high field by 0.9 to 1.6 ppm. This shift cannot be attributed only to the disappearance

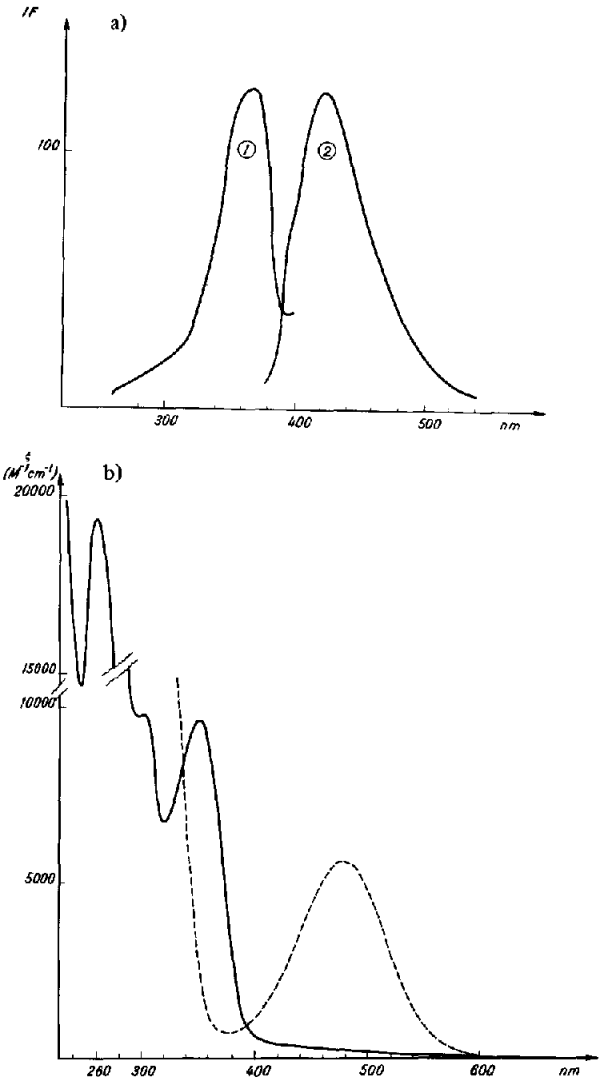

Fig.4. Absorption and fluorescence spectra of Et^{red}: a) non corrected fluorescence spectra (1. excitation λ_{em} = 420 nm; 2. emission λ_{ex} = 365 nm). Arbitrary units. b) absorption spectrum of Et^{red} (——) and EB (- - - - -).

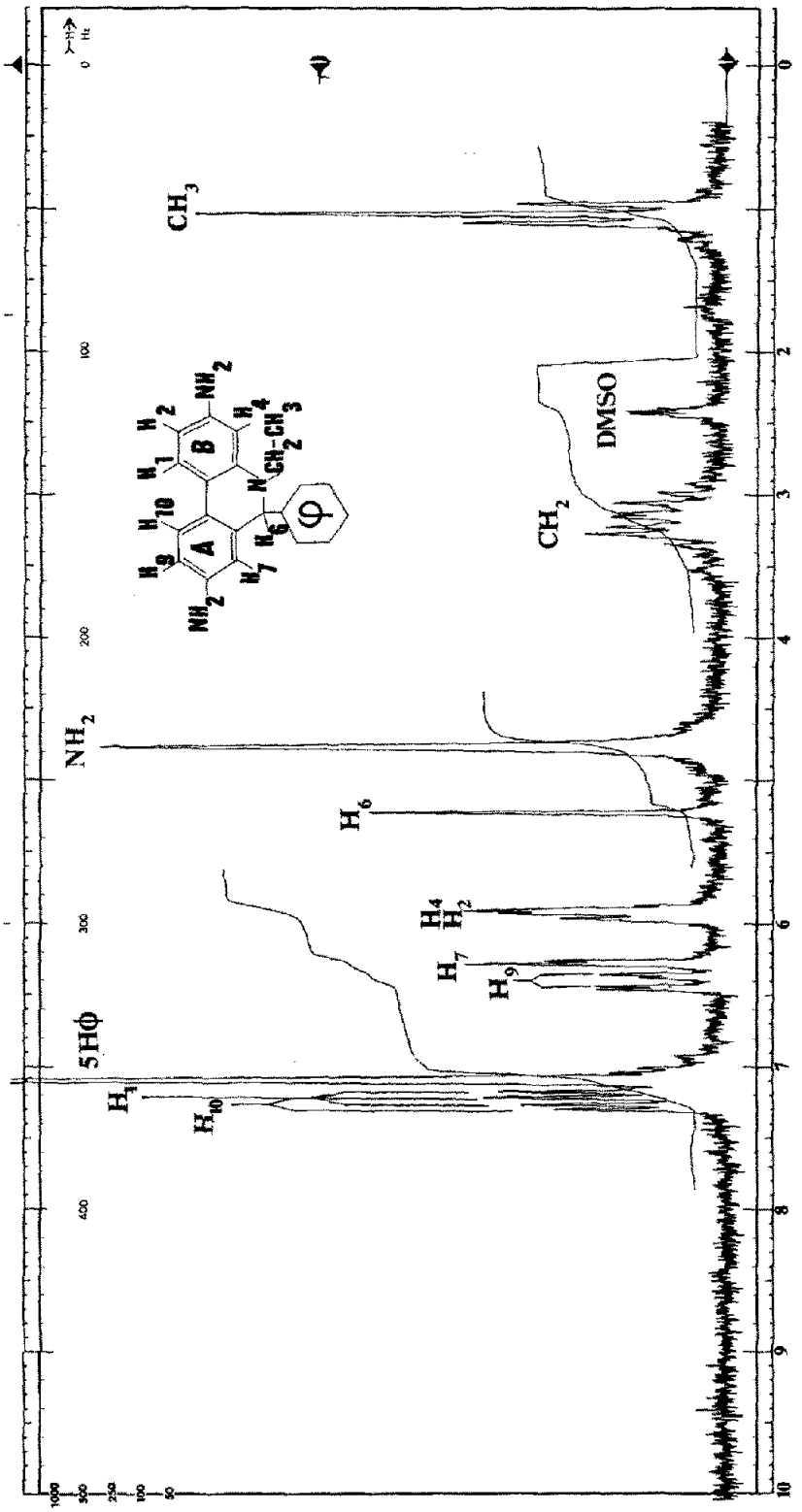

Fig. 5. PMR spectrum of Et^{red} in DMSO d6 solution. Conditions were as in sect.2.2.

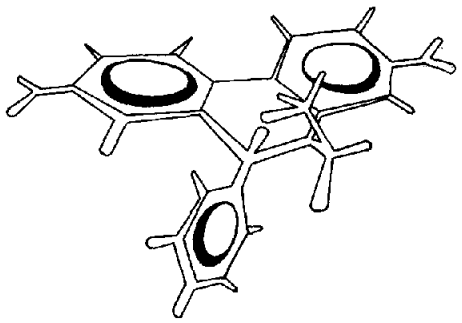

Fig. 6. Proposed conformation for Et^{red}.

of the pyridinic ring current (theoretical shifts computed using Pople approximation [12] yields: 0.75 ppm for H_8 and H_7, 0.28 ppm for H_2 and H_9, 0.65 ppm for H_1 and H_{10}). The very important discrepancy between calculated and experimental values are indicative of a twist between cycle A and B which increases the +M effect of the two NH_2. The upfield shift is maximum for H_2 and H_4 which are influenced both by NH_2 and $N-C_2H_5$ groups;

5) in contrast to all other protons the internal chemical shift of H_7 was negative (0.10 ppm). This deshielding is easily explained by its position out of the diamagnetic region of the substituted phenyl which brings it back to its normal position.

Thus, the H_6 position, the very important shielding of dihydrophenanthridine hydrogens (especially H_1 and H_{10}) with a concomitant deshielding of H_7 brings us to propose conformation B (fig. 6) for reduced ethidium.

In this conformation, cycle A and B are no more coplanar and the substituted phenyl has rotated in a vertical plane over the heterocycle. The intracyclic nitrogen has a strong sp_2 character due to the gain of stability brought by conjugation with cycle B.

No measurable interaction between Et^{red} and nucleic acids have been detected by optical method. Indeed no changes of absorption of fluorescence were observed upon addition of high concentration of nucleic acids. Knowing the structure and conformation of Et^{red}, this can be well understood. First the disappearance of the positive charge on the phenanthridium nitrogen avoids any electrostatic interactions. Second the lack of planearity of the phenanthridine ring and the steric hindrance due to the position of the phenyl residue no longer allow intercalation.

This reaction can be used to remove EB from nucleic acid solution. It is known that, in the dark, common nucleic acid bases are not sensitive to sodium borohydride. Only some atypical bases of tRNA are reactive. Their reduction products have been characterized [3]. In contrast EB reacts with $NaBH_4$ readily and quantitatively; it is therefore possible to reduce selectively EB in solution with nucleic acids. Upon reduction EB no longer interacts with nucleic acids which can then be studied on their own. We have taken advantage of this property in the study of the tertiary structure of tRNA [1].

EB has been widely used to prepare covalently closed circular DNA. However in the final step of the purification, EB extraction by butanol is never complete. We have shown by alkaline band centrifugation and fluorescence that the $NaBH_4$ reduction of EB causes no nicks on circular DNA and allows complete removal of the dye.

Acknowledgements

We are indebted to Dr. A.Favre for stimulating discussions, S.Combrisson for the PMR measurements, J.B.Le Pecq for gift of covalently closed circular PM2 DNA and G.Fonty for help in band centrifugation. We also thank Professor F. Gros for interest and encouragement.

This work was supported by grants from the Centre National de la Recherche Scientifique, the Fond de Developpement de la Recherche Scientifique et Technique, the Commissariat à l'Energie Atomique, the Ligue Nationale Française contre le Cancer and the Fondation pour la Recherche Medical Française.

References

[1] G.Thomas and A.Favre, manuscript in preparation.
[2] W.Wintermeyer and H.G.Zachau, FEBS Letters 18 (1971) 214.
[3] P.Cerutti, J.W.Holt and N.Miller, J.Mol.Biol.34 (1968) 505.
[4] A.Favre and M.Yaniv, FEBS Letters 17 (1971) 236.
[5] J.B.Le Pecq, Methods of Biochemical Analysis, Vol.20 p.41.

[6] G.P.Kreishman and S.I.Chan, J.Mol.Biol.61 (1971) 45.
[7] R.C.Elderfield and B.H.Wark, J.Org.Chem.27 (1962) 543.
[8] M.Hospital and B.Besetta, C.R.Acad.Sci., Paris, série C 268 (1969) 1232.
[9] G.Schwarz, S.Klose and W.Balthasar, European J. Biochem.12 (1970) 454.
[10] M.Dourlent and C.Hélène, European J.Biochem.23 (1971) 86.
[11] M.L.Martin and G.J.Martin, Bull.Soc.Chim.France (1966) p.2117.
[12] J.A.Pople, J.Chem.Phys.24 (1956) 1111.