PMR STUDY OF THE INTERACTION OF ETHAMBUTOL WITH POLYNUCLEOTIDES AND RELATED COMPOUNDS

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1. Introduction

Ethambutol, 2,2'-(ethyleneimino)-di-1-butanol, has been shown to be a considerably more active antituberculous compound than streptomycin against infection of mice with Mycobacterium tuberculosis [1]. The involvement of the nucleic acids in its mode of action has attracted attention, particularly in view of the finding that the total RNA content of ethambutol treated cells is less than in the ethambutol free ones [2]. It therefore became of interest to investigate whether direct interaction of the drug with nucleic acids can take place. The present work is an extension of the electron spin resonance (ESR) study [3]; proton magnetic resonance (PMR) technique is used here to obtain information concerning changes in the magnetic surrounding of protons in complexes of ethambutol with nucleic acids and their components.

2. Material and methods

Ethambutol (Lederle Cyanamid International Corporation) was a gift of Dr. Manuel Rieber. Poly d(A–T), dG:dC, and polynucleotides, were obtained from Miles Laboratories, Inc. and used without further purification. The nucleotides, nucleosides and bases were obtained from Sigma Chemical Company. D_2O (New England Nuclear) was used as solvent in all the experiments. pH was measured on Corning Model 10 pH meter and pD was calculated according to: pD = pH + 0.4.

The proton magnetic resonance spectra were obtained on Varian A-60 and, at 100 MHz, on a Varian HL-100 spectrometer. Unless otherwise indicated the PMR spectra were obtained at 35°. Variation of temperature was accomplished with a Varian Variable Temperature Controller and temperature was monitored with a methanol standard. All mixtures were prepared by pipetting appropriate volumes of stock solutions into Varian PMR tubes. Deuterated TSP (sodium-3-trimethyl-silypropionate-2,2,3,2-d_x $(CH_3)_2SiCD_2CD_2CO_2Na$) was obtained from Merck, Sharp and Dohme of Canada, and was used as internal standard.

3. Results

3.1. *PMR spectra of ethambutol*

Fig. 1 represents the 100 MHz PMR spectrum of 0.1 M solution of ethambutol, pD 5.0. The resonances are identified on the figure. The spectrum is practically pD independent between pD 1 and 6, lines are displaced to higher fields at pD above 6. No line broadening or shifts of proton lines have been observed for ethambutol concentrations between 0.04 M and 0.11 M. At higher concentrations the resonance lines broaden considerably. Such concentrations were however not used in the present experiments.

3.2. *PMR of mixtures of ethambutol with bases and nucleosides*

Equimolar mixtures of ethambutol with the bases or their nucleosides (all at 0.05 M) did not exhibit shifts larger than 0.03 ppm for the resonances of the base and nucleoside protons, nor for the protons of ethambutol. The shifts in all the experiments are rela-

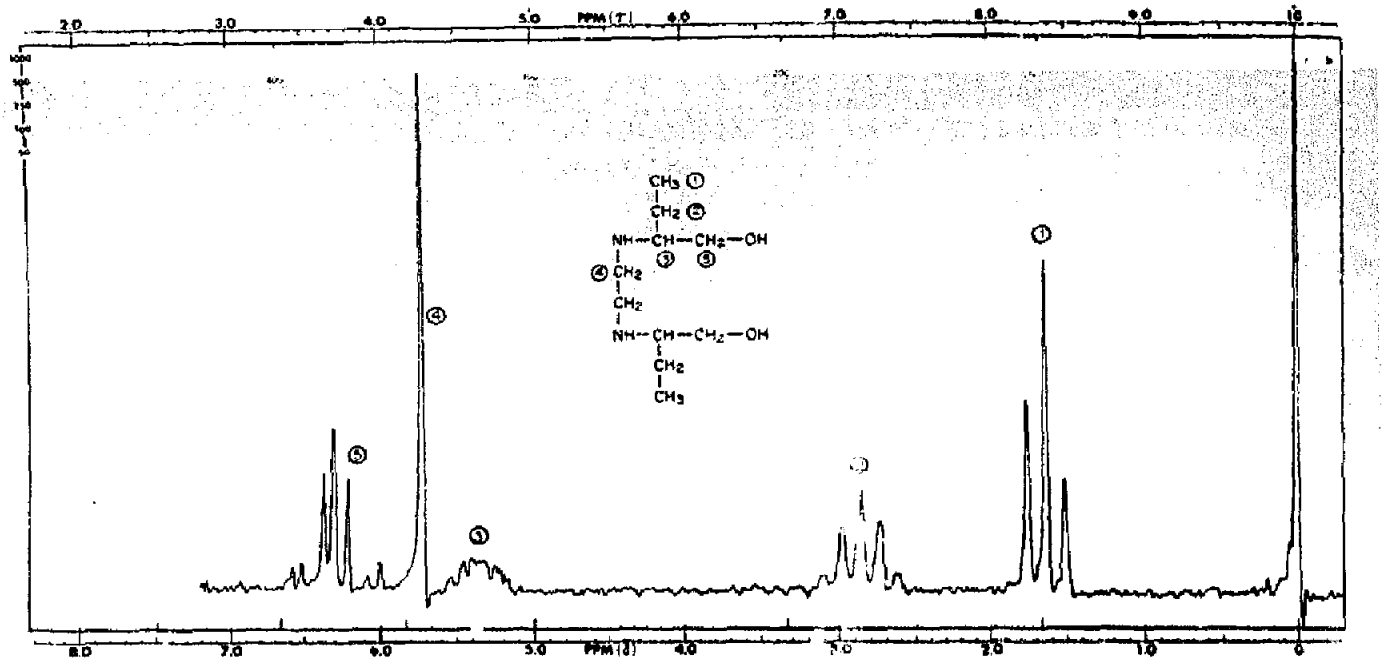


Fig. 1. PMR spectrum of 0.1 M ethambutol in D_2O at 100 MHz, pD 5.0, T = 35°. TSP used as internal standard.

tive chemical shifts of the proton lines compared to the positions of the lines in pure substance of the same molarity and pD. Guanine and guanosine were excluded because of their low solubility.

We have also examined the effect on the PMR of variable relative concentration of cytosine, or cytidine with respect to ethambutol. The concentration of ethambutol was held constant at 0.1 M and the ratio of base (or nucleoside) to drug was varied by changing the concentration of the former. In cytidine maximum shift of 0.04 ppm was observed for CH_2 protons (group 4 on fig. 1) toward higher field. Protons of group 1 and 2 shifted, at 35°, about 0.025 ppm.

Data have also been obtained for equimolar mixture of ethambutol and cytidine at temperatures between 9° and 79°. Fig. 2 is a plot of the difference in chemical shifts of ethambutol protons in the presence and in absence of cytidine. These differences were always positive (toward higher magnetic fields), and were largest between 20° and 35°. At the lowest and highest temperatures the shifts in ethambutol and in the mixture tend to be identical.

3.3. *PMR of mixtures of ethambutol with nucleotides*

Similar experiments performed on mixtures of ethambutol with nucleotides yielded results which are shown in fig. 3. It is apparent that ethambutol protons of group 2, 4 and 5 shift to higher fields in the presence of GMP and CMP, but not in the presence of AMP or TMP. The largest shift is exhibited always by the CH_2 protons of group 4 of the drug, and is

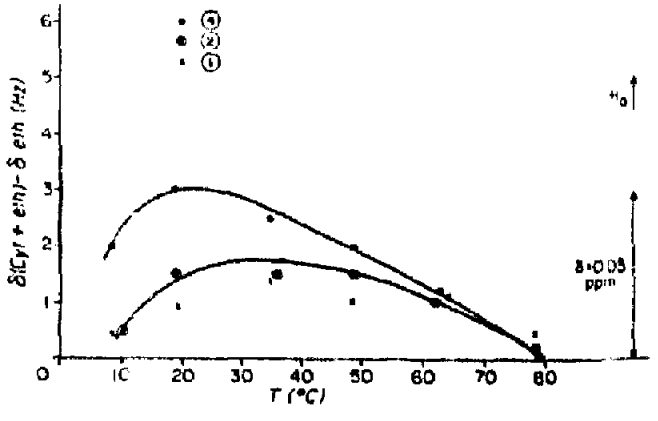
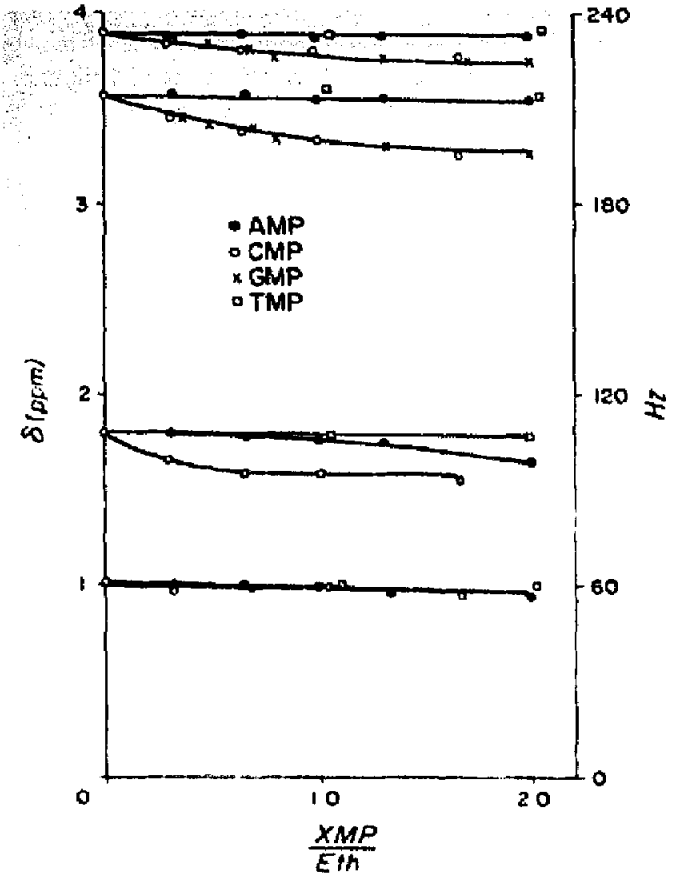


Fig. 2. Temperature dependence of the chemical shifts of ethambutol protons in equimolar mixture with cytidine (0.1 M), pD 5.0. Abscissa indicates the difference of chemical shifts of ethambutol with cytidine and of ethambutol alone.



considerably larger than in the similar series with nucleotides (0.3 ppm *vs* 0.04 ppm). No line broadening, or shifts of the nucleotide proton resonances have been observed (<0.03 ppm).

3.4. *PMR of mixtures of ethambutol with polyadenylic acids*

Fig. 4 shows the observed shift of ethambutol protons of group 4 as a function of the ratio between ethambutol and adenine (or cytosine) bases in poly A and poly C, respectively. The concentration of the latter was 0.1 M in A, or C, pD = 5.5 in D_2O at 35°. It can be observed that the shifts are all toward higher fields, with a pronounced maximum at ethambutol to base ratio of 1:4. The shifts are somewhat larger for poly A than for poly C, and go in the decreasing order for the proton groups: 4 > 5 > 2 > 1.

Fig. 5 represents a plot of the shifts of adenine protons in poly A. The H-2 and H-8 protons of adenine shift toward lower fields while H-1′ protons do not shift on addition of ethambutol. The H-2 and

Fig. 3. Chemical shifts of ethambutol protons as a function of the relative concentration of ethambutol to nucleotides. pD 5.5, ethambutol at 0.05 M, T = 35°, 60 MHz.

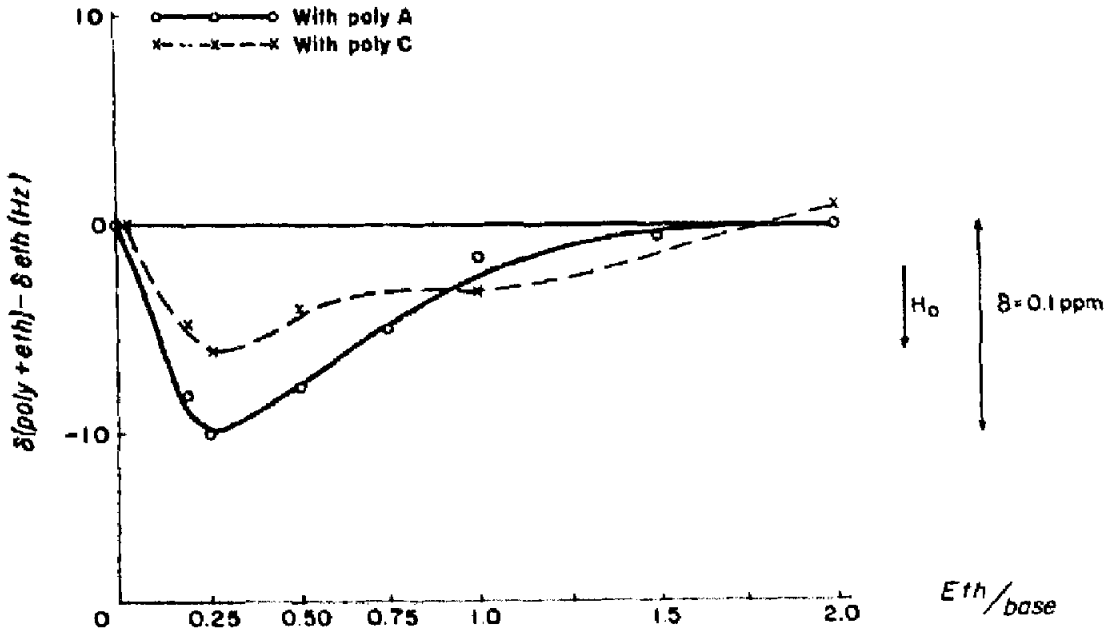


Fig. 4. Chemical shifts of ethambutol CH_2 (group 4) protons as a function of the relative concentration of ethambutol to bases in poly A and poly C. poly A and poly C at 0.1 M in bases, pD 6, T = 35°, 100 MHz.

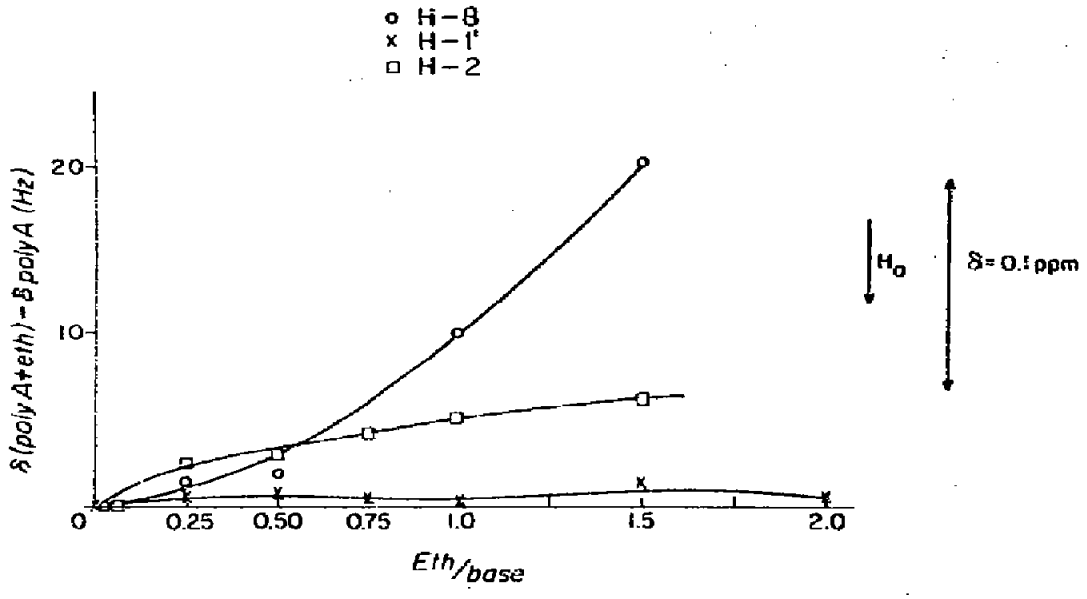


Fig. 5. Chemical shifts of adenine protons in poly A as a function of the relative concentration of ethambutol to adenine in poly A. Poly A 0.1 M, pD 6, T = 35°, 100 MHz.

H-8 resonances broaden progressively with increased concentration of ethambutol and cannot be resolved at the highest concentration.

Cytosine protons in poly C could not be seen in the presence of as little as 0.02 M of ethambutol.

The temperature dependence of the poly A and ethambutol protons showed relative shifts which did not exceed 0.03 ppm at temperatures between 9° and 79°.

4. Discussion

The observed diamagnetic shifts of ethambutol protons are most probably due to the ring currents of the neighboring bases. The smallness of the shifts in mixtures with bases and nucleosides is indicative of poor complex formations in absence of the phosphate group. Among the nucleotides complex formation is evident with CMP and GMP, but not with AMP or TMP. In all cases where PMR shifts are observed, only one set of lines is seen, indicating that the system is of rapid exchange type ($> 10^3$ sec^{-1}). Otherwise two sets of lines, corresponding to free ethambutol and to ethambutol in complex would be observed.

The results obtained with poly A and poly C indicate intercalation of the drug molecule between the bases. Intercalation has been proposed in the cases of several drugs [4–7] where drug–DNA complexes were analyzed. The maximum ratio of the drug molecules participating in the complex in this study is 1 per 4 bases, both in the case of poly A and poly C. The chemical shift toward lower fields of adenine in poly A is consistent with the intercalation. The H-2 and H-8 aromatic protons are normally shifted toward higher fields in the polynucleotides [8, 9] due to the ring currents of the neighbors. Intercalation of the drug molecule between bases will decrease the effect of the neighboring bases, hence will shift the proton resonances toward lower fields.

From the observed shift of ethambutol CH_2 protons (group 4) and the known concentrations of the two species, E (ethambutol) and P (poly A), one can calculate K_p the dissociation constant for the system in rapid equilibrium, as well as the chemical shift of the bound ethambutol, Δ, following Dahlquist and Raftery [10]:

$$E + P \rightleftharpoons EP; \quad K_p = \frac{[E]\,[P]}{[EP]}$$

one obtains $K_p \cong 10^{-2}$ and Δ = 33 Hz (0.33 ppm).

If the ethambutol molecule lines directly between adenine bases one should observe a considerably higher shielding value due to the ring currents in the base.

Since poly A at pD 6 is in random coil configuration [11, 12] the results indicate that the intercalation incomplete in the sense that the drug molecule does not fully penetrate into the space between the bases, the protons of the groups 1, 2, 3 and 5 being at least 6 or 7 Å from the perpendicular to the center of adenine at a distance of 1.7 Å. The group 4 protons would be somewhat closer.

The effect of ring currents in poly C should be considerably less pronounced [8] than in poly A, as observed in the present experiments.

The deshielding effect on H-2 and H-8 protons in poly A are roughly of the right order when compared with [16, 12].

Broadening of adenine and even more so, of cytosine lines in poly A and poly C is indicative of increase of rigidity of the polymer in the presence of ethambutol.

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