An NMR study of the interaction of cytosine arabinoside and lysozyme

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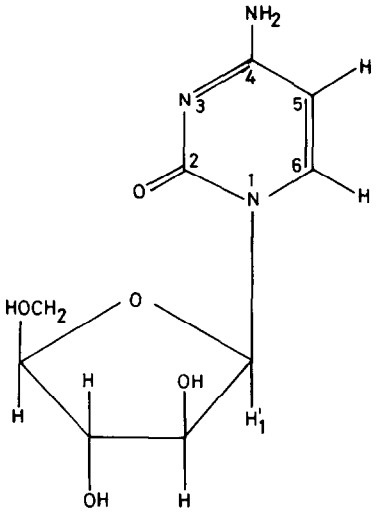
1. INTRODUCTION

Cytosine arabinoside (Ara-C, structure 1) is one of the drugs widely used in the treatment of leukemia. Information regarding its capacity to bind serum and tissue proteins is invaluable in ascertaining bioavailability which is determined by the amount of free drug present in the serum. From fluorescence studies, it is found that cytosine arabinoside associates with both serum albumin and lysozyme with an association constant (K_a) of 2.2×10^4 M^{-1}. In order to characterize the binding site and examine the amino acid residues participating in the binding process, proton magnetic resonance studies were undertaken. These results indicate the involvement of histidine and the cytosine protons of the drug in the association process. The presence of the arabinose moiety also plays an important role in the binding.



STRUCTURE 1.

2. MATERIALS AND METHODS

Hen egg white lysozyme, cytosine arabinoside and cytosine were all purchased from Sigma Chemical Co. (St Louis). 98% Pure heavy water (D_2O) and deuterated hydrochloric acid (DCl) were obtained from the Bhabha Atomic Research Centre (Bombay). The pH of 4.0 was adjusted using DCl and the pH of the heavy water itself was 6.8. All solutions were made in D_2O at the specified pH. The drug concentration was maintained as 3 mM in all solutions and [lysozyme] varied from 3–0.3 mM in the mixtures.

The proton magnetic resonance spectra were recorded on a Bruker WH-270 FT NMR spectrometer operating at 270 MHz for protons at 293 K. The number of free induction decays stored and Fourier transformed with the help of a BNC-12 computer with a core memory of 20 K, in each case is given in the respective figures. The chemical shifts were measured relative to the highest field peak of 3,3-dimethyl-4-silapentane sodium sulphonate (DSS).

3. RESULTS

Studies on the association of Ara-C with lysozyme by fluorescence quenching technique in-

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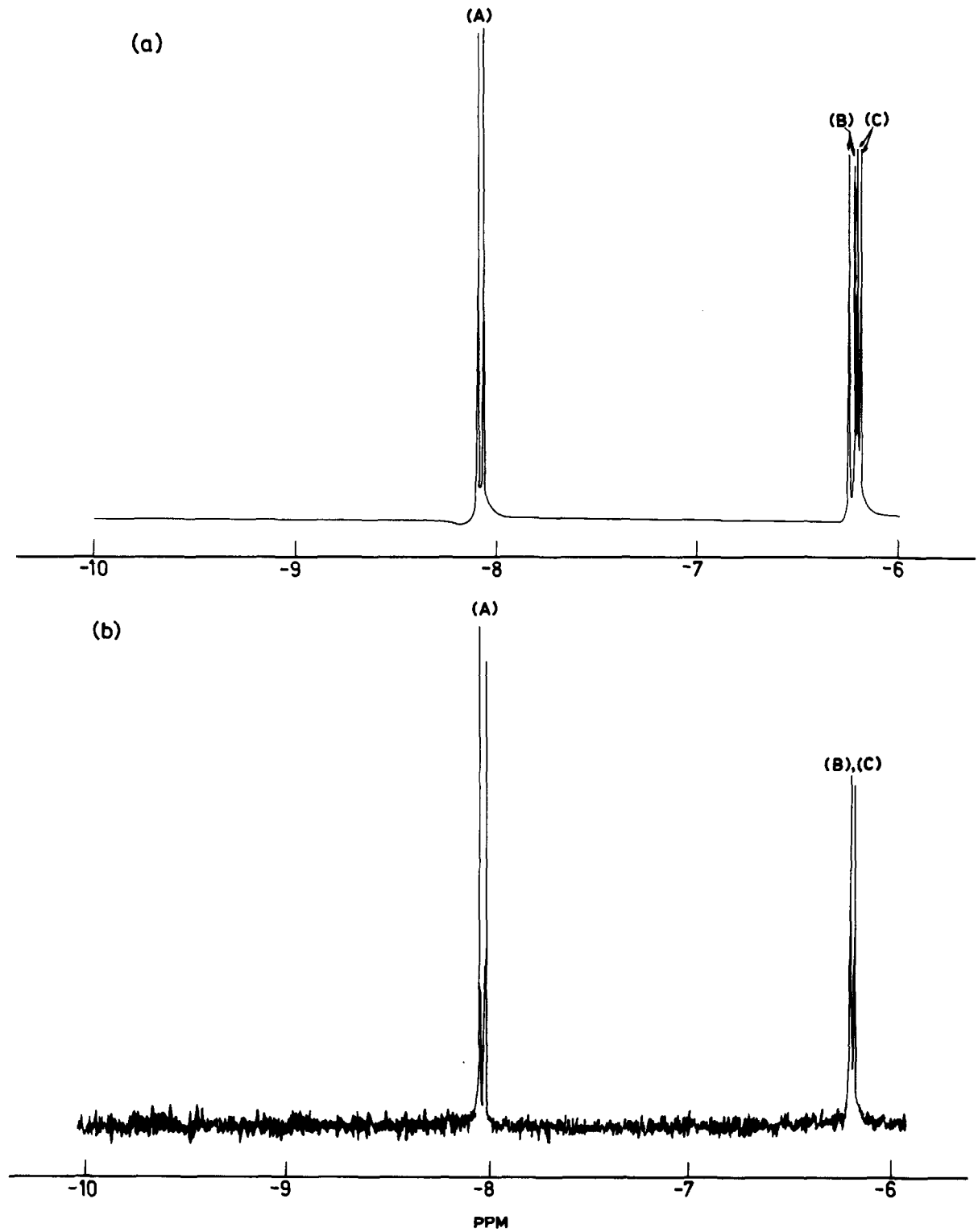

Fig.1. PMR spectra at 270 MHz of 3 mM Ara-C: (A) proton attached to C(6); (B) proton attached to C(5); (C) H_1'; (a) at pH 6.8, 37 scans; (b) at pH 4.0, 234 scans.

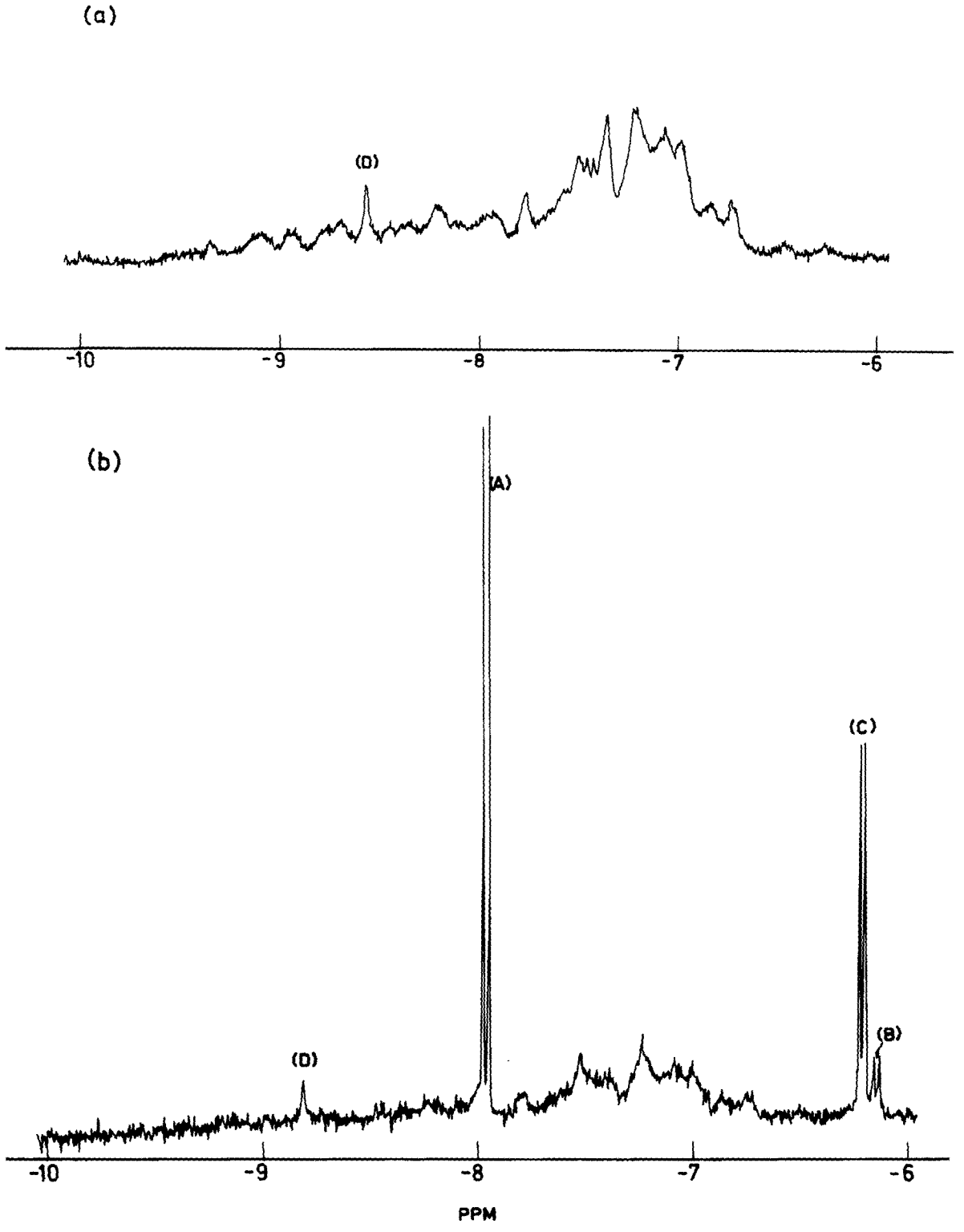

Fig.2 continued

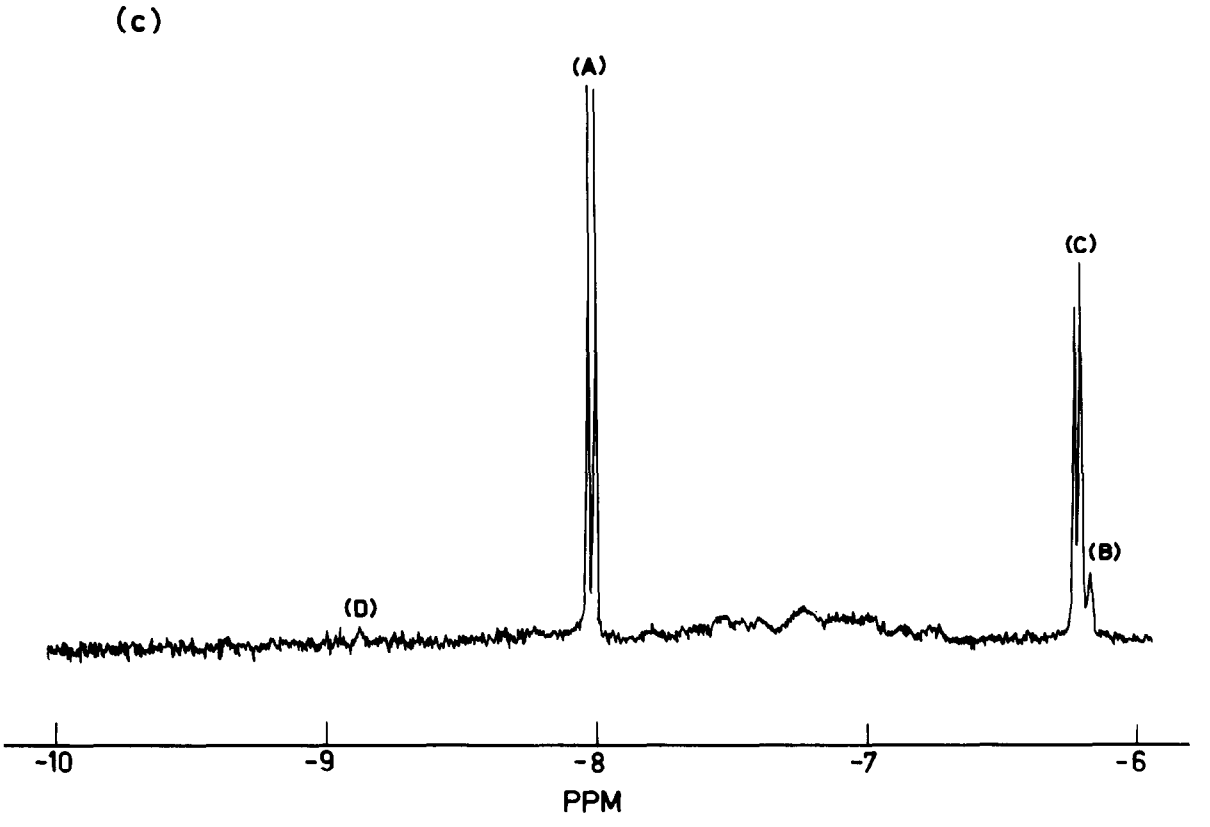

Fig.2. PMR spectra of lysozyme at pH 4.0: (a) 3 mM lysozyme at pH 4.0, 230 scans; (b) 5:1 ratio of Ara-C and lysozyme, 444 scans; (c) 10:1 ratio of Ara-C and lysozyme, 585 scans; (A–C) correspond to those in fig.1; (D) histidine-15 NH.

dicate that the drug–protein binding occurs over pH 3.8–4.0. Therefore, NMR spectra of Ara-C and the various drug–protein mixtures were taken at pH 4.0. The spectra of the drug and the protein were also recorded separately. The portions of the proton spectra between 6–10 ppm downfield from the reference are shown in fig.1 and 2.

The peak assignments shown in fig.1 correspond to those in [1–3]. Each of the cytosine ring protons attached to carbons (5) and (6) and the H_1' proton of the arabinose moiety provides a doublet arising from the indirect spin–spin couplings with the vicinal protons. The resonances due to the proton attached to C(5) and the H_1' overlap (fig.1b). The experiments at pH 6.8 (fig.1a) confirm this point since in this case each of these 2 protons provides separate doublets.

The histidine-15 NH proton is clearly seen in fig.2a [4–6]. The tryptophan NH protons are not detectable probably due to exchange with D_2O. The aromatic region of the spectra appeared as a complex pattern within the region between 6.5–7.5 ppm downfield from the reference (DSS).

An addition of varying [lysozyme] to fixed [drug] (fig.2b) causes a down-field shift of the histidine peak. The shift increases with the increase of drug–protein ratio from −8.556 ppm without the drug to −8.845 ppm when the drug protein ratio is 10:1. The proton attached to C(6) shifts up-field from −8.04 ppm in the drug spectrum without protein to −7.87 ppm in the 1:1 drug–protein mixture. The resonances of the proton attached to C(5) which overlap with those of the H_1' proton in the drug spectrum at pH 4.0 separate out as shown in

fig.2b for the drug–protein ratio of 5:1. With further increase of drug–protein ratio, the resonances due to the proton attached to C(5) slowly move down-field and only one of the lines of the proton attached to C(5) is observed separately from the H_1' proton at a drug–protein ratio of 10:1 (fig.2c). This means that the resonances due to the drug slowly begin to assume the same position as in the spectrum of the drug alone at pH 4.0 due to the increased concentration of the drug.

The aromatic region of the spectrum of lysozyme is also affected though no definite conclusion could be derived from it due to spectral complexity. The high field region between 0 to −5 ppm does not undergo much change and hence it is not shown in fig.2.

At pH 6.8, the shifts in the histidine peak and the resonances of the cytosine and arabinose moieties follow the same trend as at pH 4.0.

Similar studies carried out with cytosine and lysozyme do not show any spectral changes compared with the individual spectra of cytosine and lysozyme.

4. DISCUSSION

The changes seen in the proton magnetic resonance spectra of the drug and the protein as a result of mixing the two indicate that the cytosine protons are sensitive to the environment, especially pH. It is known that the NMR spectra of nucleosides are pH-dependent [7]. The pK_a values of cytosine and cytidine are >4.0 [8] and the cytosine in Ara-C is most likely protonated at pH 4.0. This is shown [7] to cause changes in the π-electron density and the magnetic anisotropy of the lone pair of electrons. Similar changes may be responsible for the differences observed with pH in the Ara-C spectrum (fig.1).

The shift of the cytosine proton resonances of the drug in the presence of the protein at pH 4.0 suggests that the environment around the drug on binding to the protein has changed, i.e.; the pH at the binding site or the microenvironmental pH is different from the bulk pH. This means that the protein is responsible for creating and maintaining a particular pH in its interior.

The observed shifts of the histidine-15 residue and the upfield shifts of both C(6) and C(5) protons indicate that these are involved in the binding process. Some slight changes in the spectra of the aromatic region (such as the broadening and the shifts of the spectral lines in the region between −7.3 to −7.5 ppm) could mean that the aromatic amino acid residues are also participating in the binding. Stacking of nucleotide bases with aromatic amino acids is well-established [9,10] and the upfield shifts of the drug resonance point to stacking interactions in this case also.

Cytosine itself does not seem to interact with the protein whereas Ara-C does cause several changes in the protein spectrum. This suggests that the arabinose moiety could be involved in positioning the cytosine of the drug at the binding centre.

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