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^{13}C-N M R ANALYSIS OF THE EFFECTS OF DISSOCIATION OF THE HYDROXYL GROUPS OF 1-β-D-ARABINOFURANOSYLCYTOSINE AND ITS O′-METHYL DERIVATIVES ON CONFORMATION

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ABSTRACT

The ^{13}C-n m r spectra of 1-β-D-arabinofuranosylcytosine (AraC) and its various *O′*-methyl derivatives in neutral aqueous and strongly alkaline media have been analysed Replacement of a given hydroxyl proton by a methyl group was accompanied by characteristic regularities in changes in the ^{13}C chemical shifts The marked changes in conformation of the sugar ring, in going from neutral to increasingly alkaline medium, previously reported on the basis of ^{1}H-n m r data, have been confirmed The ^{13}C-n m r data allowed the effect of the initial stages of ionisation of HO-5′ on conformation to be studied The data were applied to an analysis of the glycosidic bond torsion-angle which, for 2 ,3′-di-*O*-methylAraC, exhibited a preference for the *anti* form The conformations of the *O′*-methyl groups in this compound were also evaluated and compared with those previously proposed on the basis of the ^{1}H-n m r data

INTRODUCTION

In neutral, aqueous media, the conformations of the pentose moieties of AraC (**1** 1-β-D-arabinofuranosylcytosine) and AraU, as determined by ^{1}H-n m r spectroscopy, correspond to a C-2′*endo* ⇌ C-3′*endo* equilibrium, with only a small preponderance of the *gauche–gauche* rotamer of the exocyclic CH_2OH group If the solutions are basified to pH ~14, where the hydroxyl groups are ionised the conformation of the sugar ring becomes predominantly C-2′*endo*, and that of the exocyclic group predominantly *gauche–gauche*[1,2] This is precisely the conformation observed for the neutral forms of AraC and AraU in the solid state, as determined by X-ray diffraction, with concomitant formation of an intramolecular hydrogen-bond, O-2′-H O-5′-H (**2**), which presumably stabilises such a structure[3–5] By analogy, it was proposed that the C-2′*endo*, *gauche–gauche* conformations of AraC and AraU in alkaline media are stabilised by formation of an intramolecular

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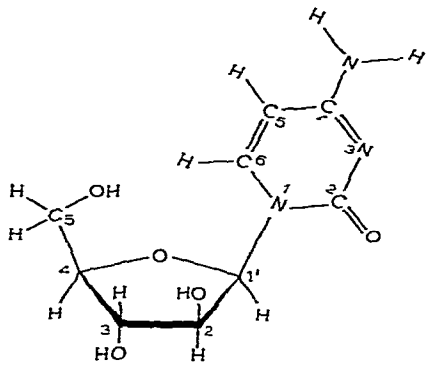

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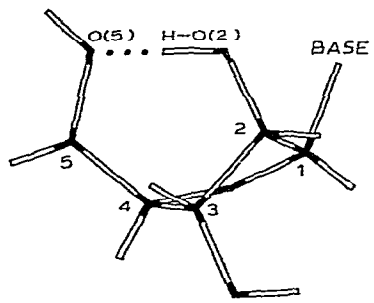

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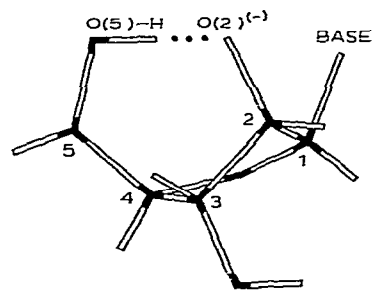

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hydrogen-bond O-5'-H O-2' (3), bearing in mind[6,7] that the pK of HO-5' is well above 14, so that it is available under these conditions to act as a donor in hydrogen bonding The foregoing conclusions were confirmed by experiments in which one or more of the hydroxyl groups were methylated

Stepwise basification of the solutions made it possible to monitor the dissociation of HO-2' and/or HO-3' by ^{1}H-n m r spectroscopy, and this approach should be applicable generally[8]. We now report on the feasibility of employing ^{13}C-n m r. spectroscopy for monitoring the dissociation of the hydroxyl groups of arabinonucleosides, and the accompanying modifications in conformation

RESULTS AND DISCUSSION

Proton-decoupled, ^{13}C-n m r spectra were recorded for all the *O'*-methyl derivatives, both in neutral and strongly alkaline media For selected derivatives, the spectra were recorded without proton decoupling, and a typical spectrum is that for 2',3'-m_2AraC*, shown in Fig 1

*Abbreviations *m*, methyl, AraC, 1-β-D-arabinofuranosylcytosine, 3'-*m*AraU, 3'-*O*-methyl-β-D-arabinosyluracil, 2',5'-m_2AraC, 2',5'-di-*O*-methyl-β-D-arabinosylcytosine, *etc*, 1-*m*C, 1-methylcytosine

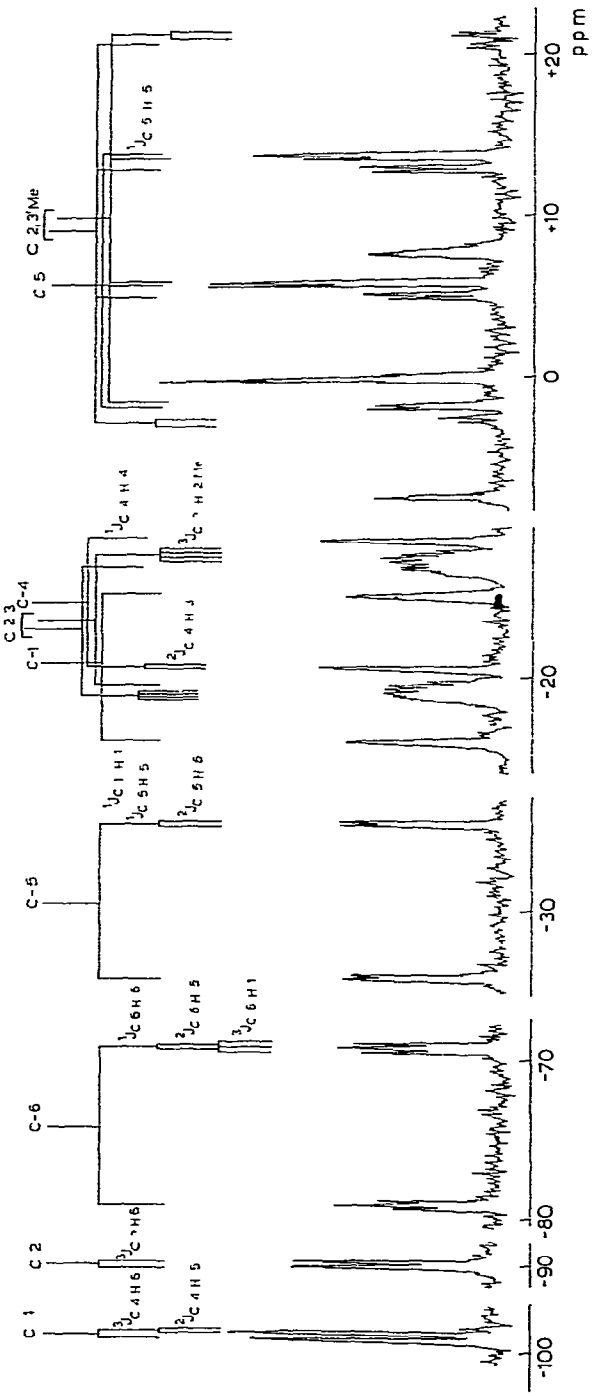

Fig 1 Proton-coupled, ^{13}C-n m r spectrum of 2 ,3 -*m*$_2$AraC in neutral, aqueous medium at 34° Chemical shifts are in p p m relative to internal 1,4-dioxane

TABLE I

CHANGES IN CHEMICAL SHIFTS (IN P P M) OF THE SIGNALS FOR THE CYTOSINE AND PENTOSE CARBONS OF THE NEUTRAL FORM OF AraC, RESULTING FROM METHYLATION OF THE SUGAR HYDROXYLS[a]

Derivative	*Changes in chemical shifts relative to AraC*								
	C-2	*C 4*	*C-5*	*C 6*	*C-1′*	*C-2′*[b]	*C-3′*[b]	*C-4′*	*C-5′*
2′ *m*AraC	−0 06	−0 05	−0 16	+0 03	+1 43	−9 54	+2 07	+0 84	+0 45
3′ *m*AraC[c]	—	—	—	—	−0 7	+2 4	−9 8	+0 9	−0 5
5′-*m*AraC	−0 13	−0 11	−0 04	+0 14	−0 08	+0 18 (−0 38)[d]	−0 26 (+0 30)[d]	+1 80	−10 64
2′,3′-*m*$_2$AraC	+0 15	−0 07	+0 09	−0 10	+0 58	−7 32 (−7 93)[d]	−7 81 (−7 10)[d]	+1 50	−0 17
2′,5′-*m*$_2$AraC	[e]	[e]	−0 13	0 00	+1 22	−9 83	+1 31	+2 42	−10 41
3′,5′-*m*$_2$AraC	+0 11	−0 03	+0 09	+0 01	−0 63	+2 58	−10 31	+2 94	−10 99
2′,3′,5′ *m*$_3$AraC	+0 04	−0 23	−0 10	−0 02	+0 38	−7 02 (−8 26)[d]	−8 14 (−6 90)[d]	+3 29	−10 78

[a]Changes in chemical shifts relative to carbons of AraC, *viz* , C-2, −90 59, C 4, −99 49, C 5, −28 63, C-6, −76 23, C-1′, −19 49, C 2′, −9 09, C-3′, −8 97, C-4′, −16 68, C-5′, +5 64 [b]The data in these columns are based on the identification of C 2′ and C 3′, see footnote (*a*) If these assignments are inverted, the change in C-2′ is increased by 0 12 p p m , and that of C-3′ decreased by 0 12 p p m [c]Values calculated on the assumption of additivity of effects of *O′* methylation (see text) [d]Because of their close proximity, C-2′ and C-3′ could not be identified unambiguously, values in brackets correspond to inverse assignments [e]Signals not recorded

Assignments of the ^{13}C signals for neutral forms The ^{13}C signals for the neutral form of AraC were identified on the basis of the assignments of Jones *et al* [9] and by comparisons with data reported for other arabinonucleosides[10]

Identification of most of the signals of the *O'*-methyl derivatives of AraC were based on the following observations (Table I) Methylation of HO-2′ or HO-3′ caused a downfield shift of the signal for the corresponding (α) ring carbon of −7 to −10 p p m , and an upfield shift of 0 5–3 0 p p m for the signal of the corresponding proximate (β) carbon These effects were additive to within ~0 3 p p m and agree well with the findings[11 12] for methylated methyl arabinofuranosides The closer similarity of our results with those for the methyl α-D-arabinofuranosides may reflect the marked differences in conformation between AraC and its *O'*-methyl derivatives on the one hand, and methyl β-arabinofuranosides on the other AraC and its *O'*-methyl derivatives, exhibit a slight preference for the *S* conformation of the sugar ring[13] In contrast methyl β-D-arabinofuranoside exhibited appreciably higher $^3J_{H\,H}$ coupling constants (relative to those of AraC), pointing to a clear preference for the *N* form

The ^{13}C-chemical shift data for the *O'*-methyl derivatives of the neutral form of AraC are shown schematically in Fig 2 In some instances signal identification

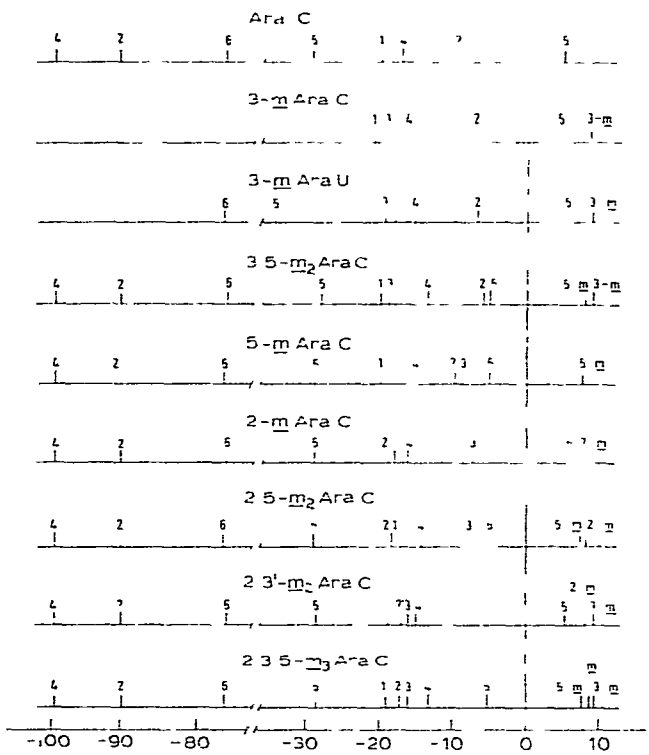

Fig 2 Diagrammatic representation of the chemical shifts (in p p m relative to internal 1 4-dioxane) of the carbon atoms of 3′-*m*AraU, and of AraC and its various *O'*-methyl derivatives

was achieved by spectral measurements under conditions of ^{13}C-[^{1}H] off-resonance spin-decoupling

Sugar-ring carbons bonded to protons which exhibit sufficiently large chemical-shift differences (1 5–4 p p m) in the ^{1}H-n m r spectrum were distinguished by application of a simplified graphical procedure[14], using the relationship

$$J_R/(J_O^2 - J_R^2)^{1/2} = \Delta\nu/\gamma H_2,$$

where J_R is the residual coupling constant, J_O is the coupling constant, $\Delta\nu$ is the frequency difference between the corresponding decoupled proton and the decoupling frequency, and H_2 is the strength of the decoupling field At a constant intensity of decoupling, the magnitude of the residual coupling constant is proportional to $\Delta\nu$

This procedure made it possible to distinguish readily the signal for C-1′ from those of the other sugar carbons, since the chemical shift for the H-1′ signal differed appreciably from those of the other ring protons (see, for example, Fig 3)

However, the signals for C-2′ and C-3′ could not be distinguished when *both* carried OH or OMe, *i e* , for AraC or 2′,3′-m_2AraC

Assignments of the ^{13}C signals for ionic forms at pD ~14 Stepwise basification of AraC solutions by the addition of NaOD was accompanied by changes in chemical shifts of the ^{13}C signals Identification of some of the signals at pD ~14 required the use of the method noted above, particularly for C-2′ and C-3′, the signals of which exhibited differences in chemical shifts of ~3 p p m , as compared to 0 1 p p m at pD 7 This problem was resolved by application of the procedure of Roth *et al* [15] which, in contrast to the graphical method, involves recording a series of off-resonance spectra at a constant frequency-difference $\Delta\nu$, but with a stepwise decrease in decoupling field-strength to zero Linear relationships are obtained in this way for the individual carbons To obviate the need for measurement of the strength of the decoupling field, the values of $J_{R_i}/J_{O_i}^2 - J_{R_i}^2)^{1/2}$ are determined in terms of some function $J_R/J_{O_i}^2 - J_R^2)^{1/2}$ for a selected carbon, in this instance C-6, the signal of which is most readily identifiable in all the spectra This leads to a series of lines with different slopes, even for carbons bonded to protons having closely similar Larmor frequencies

From the relationship

$$\frac{J_{R_i}}{(J_{O_i}^2 - J_{R_i}^2)^{1/2}} = \frac{\Delta\nu_i}{\Delta\nu} \frac{J_R}{(J_O^2 - J_R^2)^{1/2}},$$

it is clear that the slopes of the lines represent the differences between the frequency of the decoupling field (which is kept constant) and the Larmor frequency of the proton bonded to a given carbon (and denoted by i)

The results of such an analysis, which make possible the unequivocal identification of the signals of the individual carbons in AraC in strongly alkaline media, are shown in Fig. 3

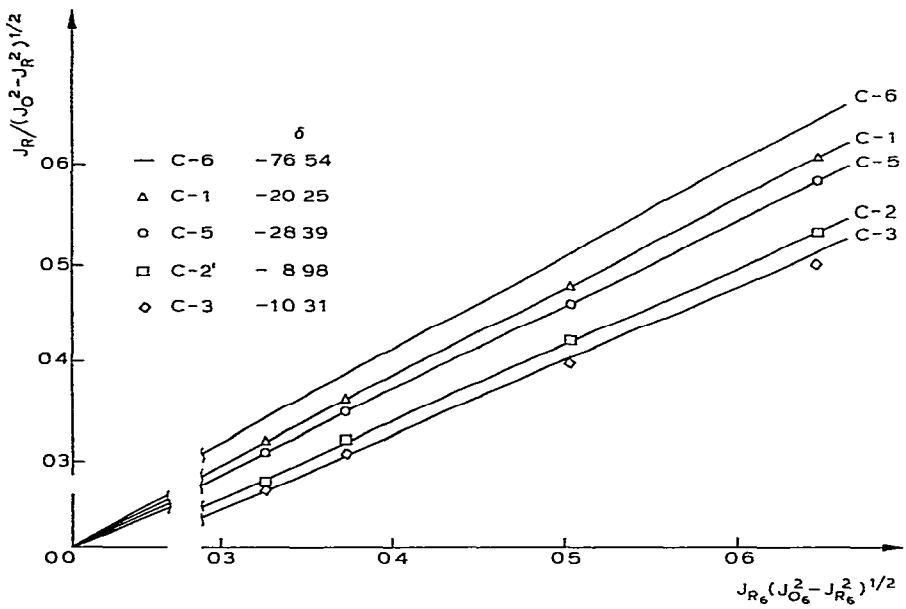

Fig 3 Identification of the individual carbon signals of the sugar ring of AraC in aqueous medium, pD ~ 14, by the procedure of Roth *et al* [15], as described in the text

TABLE II

CHANGES IN CHEMICAL SHIFTS OF THE SIGNALS FOR THE PENTOSE CARBONS OF AraC AND ITS *O*-METHYL DERIVATIVES, AND FOR 3-*m*AraU, RESULTING FROM A CHANGE IN pD FROM 7 TO ~ 14 BY ADDITION OF 10–20 MMOL OF NaOD

Derivative	*Change in chemical shift (p p m)*				
	C-1'	*C-2'*	*C-3'*	*C-4*	*C-5*
AraC	−2 50	−1 78	−4 92	−5 56	−1 97
2'-*m*AraC	−0 03	−2 56	−2 51	−3 56	−1 85
3'-*m*AraC[a]	−2 7	1 1	−3 4	−2 1	−0 8
5'-*m*AraC	−0 88	−3 40	−3 40	−1 91	−1 54
2',5'-*m*$_2$AraC	0 02	−2 49	−2 23	−2 31	−1 42
3',5'-*m*$_2$AraC	−1 24	−3 07	−3 11	0 40	−0 94
2',3'-*m*$_2$AraC	0 02	−0 61	0 03	−1 74	−1 15
2',3',5'-*m*$_3$AraC[b]	0 08	−0 01	0 00	0 04	−0 01
3'-*m*AraU	−3 25	1 09	−3 53	−1 96	−1 20

[a]Based on the use of calculated values for the chemical shifts at neutral pD (see text for details)
[b]Amount of NaOD added was only 3 mmol, so that pD was slightly less than 14, since further addition of NaOD led to precipitation

Changes in chemical shifts accompanying ionisation of sugar hydroxyl(s) The changes in chemical shifts of the signals for the pentose carbons, resulting from an increase in pD from 7 to 14 (where HO-2' and/or HO-3' are partially or totally

ionised), are listed in Table II for AraC and some of its *O'*-methyl derivatives, and for 3'-*m*AraU

For 2'-*m*AraC 2',5'-m_2AraC, and 3',5'-m_2AraC, ionisation of a given hydroxyl was reflected by downfield changes in chemical shifts of the signal for the α-carbon and the two β-carbons The chemical shifts of γ-carbons were virtually unchanged In contrast, the chemical shift of the signal for the exocyclic C-5' was modified by ionisation of HO-2' or HO-3' These changes in chemical shifts, which were all downfield relative to those for the neutral forms, resulted exclusively from the effects of hydroxyl ionisation, since the alkali-induced changes for 2',3',5'-m_3AraC (see Table II) were negligible ($\leqslant$0 1 p p m) Furthermore, from ^{1}H-n m r measurements for these compounds, there are no marked alkali-induced changes in conformation of the pentose rings[2]

The situation is quite different for AraC (Fig 4) 3'-*m*AraC and 3 -*m*AraU, where sugar-hydroxyl dissociation is accompanied by marked changes in conformation of the pentose ring ^{1}H-N m r spectroscopy has shown that the C-2'*endo* $\rightleftharpoons$ C-3'*endo* equilibrium in neutral media undergoes a very pronounced shift to the C-2'*endo* conformation in strongly alkaline media[2]

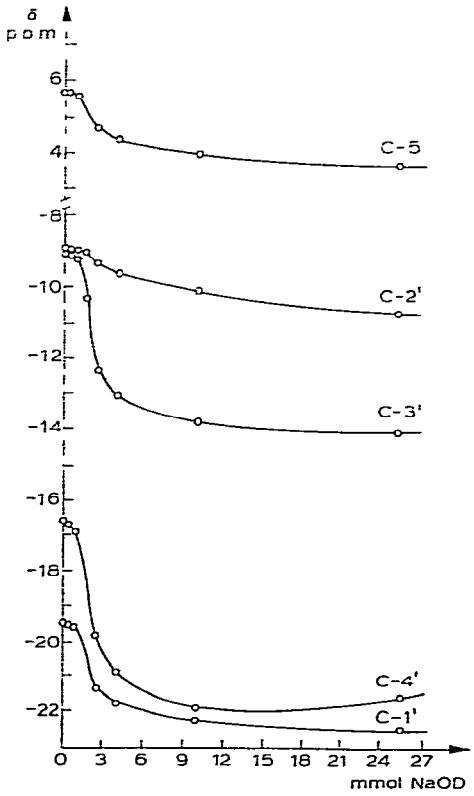

Fig 4 Dependence of ^{13}C-chemical shifts (in p p m relative to internal 1,4-dioxane) on amount of NaOD (in mmol) added to a 2-ml sample of M AraC

The characteristic *upfield* shifts (+1 1 p p m) of the signal for C-2′ in 3′-*m*AraC and 3′-*m*AraU as a result of basification must be due, in part, to a change in conformation of the pentose ring A similar effect was observed for AraC, where the change in chemical shift (—1 78 p p m) for the signal for C-2′ was the result of a change in conformation and ionisation of HO-2′ and HO-3′ The change in chemical shift of the signal for C-2′ due to ionisation alone is ~—3 p p m [*e g* , —3 4 p p m for C-2′ of 5′-*m*AraC (see Table II), the conformation of which is essentially unchanged in going from neutral to alkaline medium], so that the change in conformation from C-2′*endo* ⇌ C-3′*endo* at neutral pH to C-2′*endo* at strongly alkaline pH leads to an upfield shift of the signal for C-2′, thus accounting for the decrease in $\Delta\delta$C-2′ from approximately —3 to —1 78 p p m

The alkali-induced changes in conformation of the pentose rings of AraC, 3′-*m*AraC, and 3′-*m*AraU, previously established from analyses of the ^{1}H-n m r spectra[2], are also reflected by downfield shifts for the signals for C-1′ (see Table II) as compared to the remaining AraC derivatives

A comparison of the changes in chemical shifts accompanying an increase in pD for 2′,3′-m_2AraC and 2′,3′,5′-m_3AraC demonstrates that some ionisation of HO-5′ in the former occurs and maximally affects the chemical shifts of the signals for C-4′ and C-5′ The chemical shift of the signal for C-2′ was also affected, to a smaller, but still significant, extent Hence, for all derivatives with HO-5′ unsubstituted, chemical shifts due to its partial ionisation cannot be ignored In particular for those derivatives that form an intramolecular hydrogen-bond in alkaline media the changes in chemical shifts of the signals for C-4′ and C-5′ result not only from the accompanying change in conformation, but also to a lesser extent from ionisation of HO-5′

Thus, neglecting the smaller effects of partial dissociation of HO-5′, the chemical shifts of the sugar-ring carbons in AraC are sensitive to ionisation of HO-2′ and HO-3′, as well as to the accompanying changes in conformation of the sugar ring A change in conformation of the latter from C-3′*endo* to C-2′*endo* increases the shielding of C-2′ and deshields C-1′

For all the nucleosides studied, dissociation of a proton from a sugar hydroxyl leads to deshielding of the α and β carbons In ^{1}H-n m r spectroscopy[2], hydroxyl dissociation results in shielding of the protons bonded to the corresponding carbons, and this illustrates the opposing changes in chemical shifts of the signals for protons and other nuclei (most frequently ^{13}C and ^{31}P) resulting from ionisation[16,17] For ^{1}H and ^{13}C, this has been attributed to polarisation of the C-H bond[16] An additional factor, which may operate in the immediate vicinity of the dissociating hydroxyl groups, is a change in excitation energy[18]

Conformation of O′-methyl groups The ^3J values for the grouping HCOMe associated with positions 2′, 3′, and 5′ can be used to determine the rotamer population associated with Me-O and C-O bonds To a first approximation, the dependence of $^3J_{COCH}$ on the rotational angle about the C-O bond, as established for ethers[19] and methyl glycosides[20], may be applied to 2′,3′-m_2AraC The value of $^3J_{H,C}$, 5 3 Hz

for MeO-3′, is close to that for dimethyl ether[19], and there is free rotation about the C-3′–O-3′ bond, but the value (3 5 Hz) for MeO-2′ is well below that for free rotation and indicates a preference for the *gauche* (H-2′) conformation

These results contrast with those obtained by ^{1}H-n m r spectroscopy[21] However, conformational assignments based on analyses of proton chemical shifts are less accurate Of particular interest is the difference in conformation between MeO-2′ and MeO-3′, not revealed by ^{1}H-n m r spectroscopy The preference of the MeO-2′ for the *gauche* (H-2′) conformation may be accounted for by steric hindrance to rotation about the C-2′–O-2′ bond, which is readily seen in molecular models Such steric hindrance does not occur with MeO-3′

Conformation about glycosidic bond The relationship established by Lemieux *et al*[22] for the dependence of $^3J_{C\text{-}2\,H\,1}$ on the glycosidic torsion-angle for cyclic uracil nucleosides was subsequently extended by Davies[23] to pyrimidine nucleosides in general, with the *syn* conformation predominating for $^3J_{C\,2\,H\,1} > {}^3J_{C\,6\,H\,1}$ and the *anti* conformation preferred for the inverse relationship

For 2′ 3 -m_2AraC an average value for $^3J_{C\text{-}6,H\text{-}1}$ of ~4 Hz was obtained which is close to the values reported[23] for cytidine (3 3 Hz), uridine (3 7 Hz), and thymidine (3 9 Hz) The value of $^3J_{C\,2\,H\text{-}1}$ could not be measured directly, because of the low spectral resolution, but its upper limit could be established as 2 Hz, as compared to values of 1 8 Hz for cytidine, 2 4 Hz for uridine, and 2 0 Hz for thymidine[22] On this basis, the conformational preference of 2′,3′-m_2AraC about the glycosidic bond is *anti* in accord with results obtained by ^{1}H-n m r spectroscopy[2 21]

An independent evaluation of the conformational preference about the glycosidic bond of pyrimidine arabinosyl nucleosides may be obtained from the chemical shift of the signals for C-6 with increase in pD, as previously reported[2] for the alkali-induced chemical shift of the signal for H-6 For those arabinonucleosides having HO-2′ unsubstituted, ionisation should affect the chemical shift of the signal for C-6 in the *anti* conformation, since these are then closest to each other

The changes in chemical shifts of the carbon-nuclei signals of the aglycon following addition of 4 mmol of NaOD (Table III) may be ascribed to ionisation of the sugar hydroxyls in particular HO-2′ The influence of the ionisation of HO-2′ is especially pronounced on C-6 and, for all derivatives having HO-2′ unsubstituted, there is an appreciable downfield shift (~1 p p m of the signal for C-6) This is accompanied by an upfield shift of ~0 5 p p m of the signal for C-5 For those derivatives having HO-2′ substituted, the changes in chemical shifts of the signal for C-6 are much smaller (Table III) Also, the effect of the dissociation of HO-2′ on C-2 is significantly less than on C-6, consistent with the proposed *anti* conformation about the glycosidic bond In general, the dependence of the chemical shifts of the signals of the carbons of the nitrogenous-base moiety of AraC derivatives on the amount of added NaOD is rather complex Increasing addition of NaOD leads to large changes in chemical shifts of the signals of the base carbons, in a direction opposite to that produced by lower concentrations of NaOD (associated in this case with sugar hydroxyl dissociation), accompanied by appreciably smaller changes

TABLE III

CHANGES IN CHEMICAL SHIFTS OF THE CYTOSINE RING CARBONS IN AraC AND ITS *O'*-METHYL DERIVATIVES RESULTING FROM A CHANGE IN pD FROM 7 TO ~14[a]

Derivative	*Total NaOD added (mmol)*	*Change in chemical shift (p p m)*			
		C-2	*C-4*	*C-5*	*C-6*
AraC	4 0	−0 35	0 01	0 63	−1 00
	18 0	−0 54	[b]	[b]	−0 65
2 -*m*AraC	4 0	−0 16	−0 01	0 01	−0 38
	18 0	−0 22	−0 30	−0 57	−0 11
3'-*m*AraC	2 0	[b]	[b]	0 9[c]	−1 1[c]
5 -*m*AraC	4 0	−0 37	0 15	0 30	−0 82
2 ,3 -m_2AraC	4 0	[b]	[b]	−0 17	0 21
	18 0	[b]	[b]	−1 24	1 13
2 ,5 -m_2AraC	4 0	[b]	[b]	0 08	−0 32
3 ,5 -m_2AraC	4 0	−0 49	0 00	0 63	−1 18
1-*m*C	1 5	[b]	[b]	−0 08	0 00

[a]Addition of 4 mmol of NaOD brings the pD to ~14 With up to 18 mmol of NaOD, the pD is at least 14 and probably somewhat higher [b]Signal not observed in the spectrum [c]Based on the use of calculated values for chemical shifts at pD 7 presented in Table I

in chemical shifts of the sugar-ring carbons It is most likely that this effect is due to the initial stage of dissociation of the cytosine amino group[24] Experiments with AraU demonstrated that changes in chemical shifts of the signals of the carbons of the base occurred only on addition of up to 4 mmol of NaOD The beginning of dissociation of HO-5' at pD ~14 also affects the chemical shifts of the signals of the carbons of the base

The consistency of the data in Tables I and III and the proton chemical-shifts previously reported[1,21] point to the similarity in conformation about the glycosidic bond of the various *O'*-methyl derivatives *O'*-Alkylation only minimally affects the conformation of the sugar rings[21] However, on the basis of chemical-shift data alone, it is difficult to exclude some variation in the populations of the *syn* and *anti* states between individual compounds, of the order of 20%

From the ^{13}C-n m r , proton-coupled spectrum for AraC in strongly alkaline media, despite the observed, appreciable broadening of the signals, the value of $^3J_{C\text{-}6\ H\text{-}1}$ may be estimated as 4 Hz, *i e* , a value similar to that for compounds preferentially in the *anti* form, including 2',3'-m_2AraC It follows that both introduction of an *O'*-methyl substituent, and dissociation of sugar hydroxyls, do not appreciably affect the conformation about the glycosidic bond in *O'*-methyl derivatives of AraC

EXPERIMENTAL

AraC and its *O'*-methyl derivatives were synthesised as described elsewhere[25],

and 3′-*m*AraU was obtained[26] by deamination of 3′-*m*AraC in M acetic acid D_2O (99.75 mole% D) and NaOD (99 mole% D) were used

^{13}C-N m r spectra were obtained at 34° for 0 4–2M solutions with a Varian-CFT-20 spectrometer, using 8K data points with a resolution of 0 37 Hz/point The solvent deuterium resonance was used as a field-frequency lock Solutions of increasing alkalinity were obtained by stepwise addition of measured volumes of 15M NaOD, using calibrated Carlsberg micropipettes DO^- concentrations were determined from the amount of NaOD added

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