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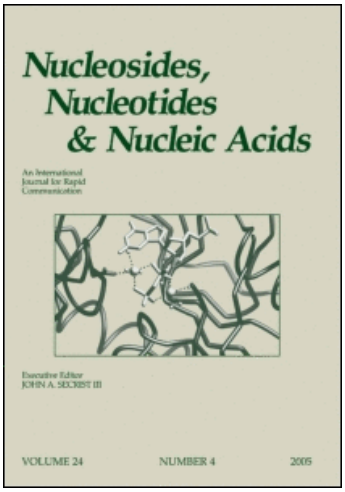

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HALOGENATED NUCLEIC ACIDS: STRUCTURE AND CONFORMATIONAL STUDIES OF 5-FLUOROCYTIDINE BY X-RAY CRYSTALLOGRAPHY AND NMR SPECTROSCOPY

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HALOGENATED NUCLEIC ACIDS: STRUCTURE AND CONFORMATIONAL STUDIES OF 5-FLUOROCYTIDINE BY X-RAY CRYSTALLOGRAPHY AND NMR SPECTROSCOPY

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Abstract

The stereochemistry and the conformational equilibria of 5-fluorocytidine (5FCyd) have been determined by X-ray crystallography and correlated with NMR spectroscopy in the solution state. Crystals of 5FCyd have unit cell dimensions a=9.854(1), b=15.012(2), c=15.290(2)Å, $\alpha=\beta=\gamma=90°$ with two molecules in the asymmetric unit. Both molecules are in the anti-conformation, C3′-endo sugar pucker and a Υ (C4′-C5′) of g^+. The two molecules in the asymmetric unit show slight variation in their bond distances and bond angles but their overall solid state conformation is similar. The NMR results indicate the 5FCyd has an anti-conformation, a mixed sugar pucker of 36% C2′-endo and 64% C3′-endo and an exocyclic furanose conformation (Υ) of 74%(g^+), 19%(t) and 7%(g^-).

Certain purine and pyrimidine analogues readily replace the natural bases in nucleic acid if they are present during replication. Halogenated nucleic acids have been known for the past thirty years when for the first time it was found that 5-iodouracil could be incorporated into the nucleic

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acid of E•coli[1]. This provided the basis for the synthesis of 5-fluoro-nucleic acid bases and their use as a chemotherapeutic agent.

The biological properties of 5-halogenated uracil derivatives have been examined in detail. The incorporation of the halogenated base into mRNA could lead to errors in the reading frame which would alter the phenotype without permanent change in DNA genotype[2-4], also it can substitute for uracil into mRNA and may exert a mutagenic effect[5] or can occasionally lead to the production of altered protein[6-7]. A substitution of uracil by fluorouracil in the mRNA and a preferential pairing of the 5FUra with a guanosine in the tRNA anti-codons can account for the first substitution. This is because 5FU assumes properties similar to cytosine when the base ionizes. This occurs readily for 5-FU (pKa = 7.8) due to the electronegative fluorine atom at C5 instead of hydrogen. 5FCyd shows antifungal activity against Saccharomyces cerevisiae and Candida species. It is the drug of choice for systemic infections. In order to obtain a more basic understanding of the potential of such processes, the present study was undertaken as the first step using solid phase study by X-ray crystallography and its structure in solution by NMR spectroscopy[8].

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Crystallography: 5FCyd was obtained from Hoffman-La Roche Inc., courtesy of Dr. W.E. Scott. Crystals of 5FCyd were obtained from methanol and water. A crystal of dimension of 0.3 x 0.1 x 1.0 mm mounted on a glass fibre was used for data collection.

Crystals are orthorhombic with unit cell constants at (22+3°C) given in Table 1.

Systematic absences in 0k0, k odd; h00, h odd; 00l, l odd established that the space group was $P2_12_12_1$. The observed density of 1.51 $g.cm^{-3}$ indicated that there are two molecules in the asymmetric unit. Accurate unit cell parameters were determined with Enraf-Nonius CAD-4 automatic diffractometer using 25 reflections with $\theta > 51°$. Three dimensional data (to the limit $2\theta = 154$ for CuKα radiation) were collected by the $\omega/2\theta$ scan; scan widths calculated using the expression $(0.5 + 0.15 \tan\theta)$, aperture widths using $(3.0 + 1.2 \tan\theta)$mm. The maximum time spent on any reflection measurement was 100 seconds and the background count time was half the scan time. Faster scans were used for stronger reflections. The intensities of three reflections were monitored after every hour of x-ray exposure and the variation in intensities was less than 3%

Table 1

CRYSTAL DATA

Empirical Formula	$C_9N_3O_5H_{12}F$
F.W.	261.2
Crystal System	Orthorhombic
Space group	$P2_12_12_1$
a	9.854(1)Å
b	15.012(2)
c	15.290(2)
α	90°
β	90°
γ	90°
Volume	2261.7 A^3
Z	8(2 molecules/asymmetric unit)
D_o	1.52 $g.cm^{-3}$(flotation in bromoform/benzene)
D_C	1.534 $g.cm^{-3}$
μ	11.4 cm^{-1}
$\lambda CuK\alpha$	1.5408Å
Total no. of reflections	2728 (2333>3σ)
Final R value	0.040

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during the time of data collection. The orientation matrix was checked every 100 reflections. A total of 2728 unique reflections were measured of which 2333 had intensities greater than 3σ and these were used in structure determination and refinement. The intensity data were corrected for Lorentz-Polarization effects. The intensities of three reflections close to χ at 90° were measured for all values of ϕ from 0° to 360°, and the resultant curve of transmission as a function of ϕ was used to calculate the absorption corrections for all the reflections.

The structure was solved by the multisolution technique with the help of the program MULTAN[9]. The E-map with the best figure of merit gave the positions for all the non-hydrogen atoms of the two bases and a part of sugar moiety. The positions of the remaining atoms were obtained from successive difference electron density maps. At the end of the isotropic refinement, the R factor was 0.051. The locations of all the hydrogen atoms, excepting that of the amino nitrogen of molecule B, were obtained from difference electron density maps. The hydrogen atoms attached to the amino nitrogen of molecule A has higher temperature factors than the remaining hyrogen atoms, indicating that these hydrogen atoms are less accurate than the remaining hydrogen

atoms in the structure. The structure was refined using the full matrix least-squares refinement with individual anisotropic temperature factors for the non-hydrogen atoms and isotopic temperature factors for hydrogen atoms. The final R value was 0.044 for the 2333 observed reflections ($I>3\sigma$). The quantity minimized in full matrix refinement was $w(|F_O| - 1/k |F_C|)$ where $w = 4|F_O|^2/(\sigma(|F_O|^2)^2$, and $\sigma(|F_O|)^2 = [\sigma^2(I) + p^2I^2]^{1/2} / LP$ and p is an "ignorance factor" to reduce the weights of intense reflections ($p = 0.05$), $\sigma(I)$ is the standard deviation in intensity I based on counting statistics and k is the scale factor. The atomic scattering factors are taken from International Tables for X-ray Crystallography[10]. Fourier and torsion angles programs written by Dr. S.T. Rao and ORTEP by Johnson were used (1965)[11].

The final atomic coordinates are given in Table 2.

NMR Spectroscopy

Samples were prepared to a volume of 0.4-0.5 ml. Tertiary butyl alcohol (TBA) was added in trace amount (approximately 0.1% of total volume) as an internal chemical shift reference. Chemical shifts are expressed relative to DDS by adding TBA reference as 1.542 ppm at 30°C. Aqueous solutions contained 0.04 M phosphate buffer in D_2O and 2-4 mM (ethylenedinitrilo) tetracetic acid (EDTA). Proton magnetic resonance spectra were obtained from either a Bruker AM-400 or WP-200 spectrometer (Fig. 1).

RESULTS AND DISCUSSION

(A) Bond lengths and angles: The bond length and angles for 5FCyd are given in Figures 2 and 3 for the two independent molecules A and B respectively. The average standard deviation in a bond length is 0.0015Å and in a bond angle is 0.015°. The bond lengths and angles in the base are very similar to the values found in other nucleosides and nucleotides[12-17]. In 5Fcyd, the nucleoside is in the anti conformation with glycosidic torsion angles of 15 and 20° in molecules A and B respectively.

The torsion angles in the sugar ring are given in Table 3. Following the analysis of Altona and his coworkers[18] the conformation of the sugar ring is C3′ endo (^3E) in both the molecules, with C(3′) deviating by -0.62 and 0.59 Å from the mean plane in molecules A and B respectively, with

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Table 2

TABLE OF FINAL FRACTIONAL POSITIONAL PARAMETERS AND THEIR ESTIMATED STANDARD DEVIATIONS IN PARENTHESES

	X	Y	Z	B(Å²)†
MOLECULE A				
N1	0.4314(3)	0.1108(2)	0.437(2)	2.43(5)
C2	0.3878(4)	0.0235(2)	0.0557(2)	2.82(6)
O2	0.3636(3)	-0.0221(2)	-0.0097(2)	3.43(5)
N3	0.3733(5)	-0.0084(2)	0.1378(2)	4.04(7)
C4	0.4025(5)	0.0425(3)	0.2077(3)	3.81(8)
N4	0.3904(8)	0.0102(3)	0.2876(2)	7.60(1)
C5	0.4479(4)	0.1309(2)	0.1947(2)	2.90(6)
F1	0.4813(3)	0.1791(1)	0.2654(1)	4.18(5)
C6	0.4614(4)	0.1637(2)	0.1134(2)	2.69(6)
C1′	0.4572(4)	0.1383(2)	-0.0482(2)	2.76(6)
C2′	0.3298(4)	0.1655(2)	-0.0966(2)	2.89(6)
O2′	0.3504(5)	0.1395(2)	-0.1856(2)	4.76(7)
C3′	0.3335(3)	0.2666(2)	-0.0859(2)	2.37(5)
O3′	0.2540(3)	0.3107(2)	-0.1498(2)	2.80(4)
C4′	0.4846(4)	0.2859(2)	-0.0932(2)	2.37(6)
C5′	0.5294(5)	0.3741(3)	-0.0552(3)	4.17(8)
O5′	0.4873(4)	0.3866(2)	0.0317(2)	5.58(8)
O4′	0.5461(2)	0.2129(2)	-0.0465(2)	3.23(4)
MOLECULE B				
N1	0.1769(3)	0.6232(2)	-0.0493(2)	2.76(5)
C2	0.2196(4)	0.5350(2)	-0.0577(2)	2.88(6)
O2	0.2244(3)	0.4868(2)	0.0081(2)	3.62(5)
N3	0.2547(4)	0.5042(2)	-0.1387(2)	3.10(5)
C4	0.2622(5)	0.5601(3)	-0.2066(2)	3.42(7)
N4	0.2932(5)	0.5304(3)	-0.2854(2)	4.95(9)
C5	0.2293(6)	0.6514(3)	-0.1946(3)	4.06(8)
F2	0.2419(5)	0.7055(2)	-0.2654(2)	6.87(7)
C6	0.1853(5)	0.6818(2)	-0.1176(2)	3.60(7)
C1′	0.1211(4)	0.6500(2)	0.0372(2)	2.70(6)
C2′	0.2264(4)	0.6743(2)	0.1037(2)	2.81(6)
O2′	0.1686(4)	0.6534(2)	0.1855(2)	3.78(5)
C3′	0.3380(4)	0.7750(2)	0.0925(2)	2.31(5)
O3′	0.2947(3)	0.8163(2)	0.1669(1)	2.89(4)
C4′	0.0901(4)	0.8004(2)	0.0756(2)	2.55(6)
C5′	0.0678(4)	0.8866(2)	0.0276(3)	3.27(7)
O5′	0.1382(3)	0.8904(2)	-0.0530(2)	3.76(5)
O4′	0.0384(3)	0.7268(2)	0.0240(2)	2.89(4)

† Isotropic equivalent thermal parameter defined as: $(4/3)[a^2B(1,1) + b^2B(2,2) + c^2B(3,3) + ab(\cos\gamma)B(1,2) + ac(\cos\beta)B(1,3) + bc(\cos\alpha)B(2,3)]$.

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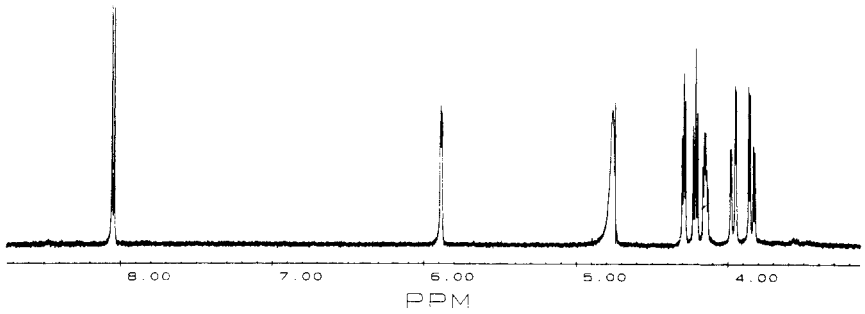

Fig. 1. 400 MHZ spectrum of 5-fluorocytidine in D_2O (10 mM, 30°C). (The absorption at ~ 4.75 ppm is residual un-suppressed HOD).

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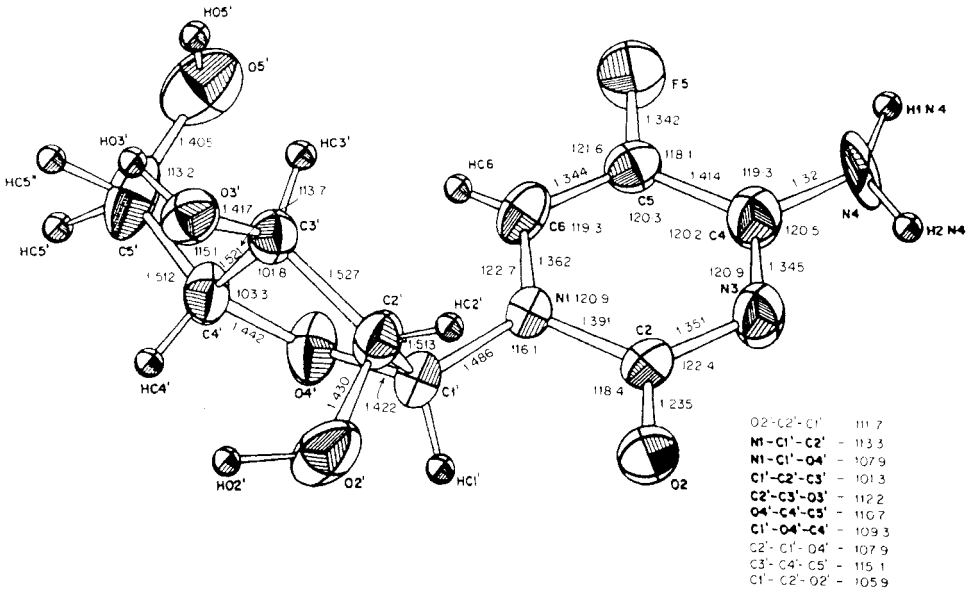

Fig.2. Bond distances (Å) and bond distances (°) in 5-fluorocytidine for Molecule A

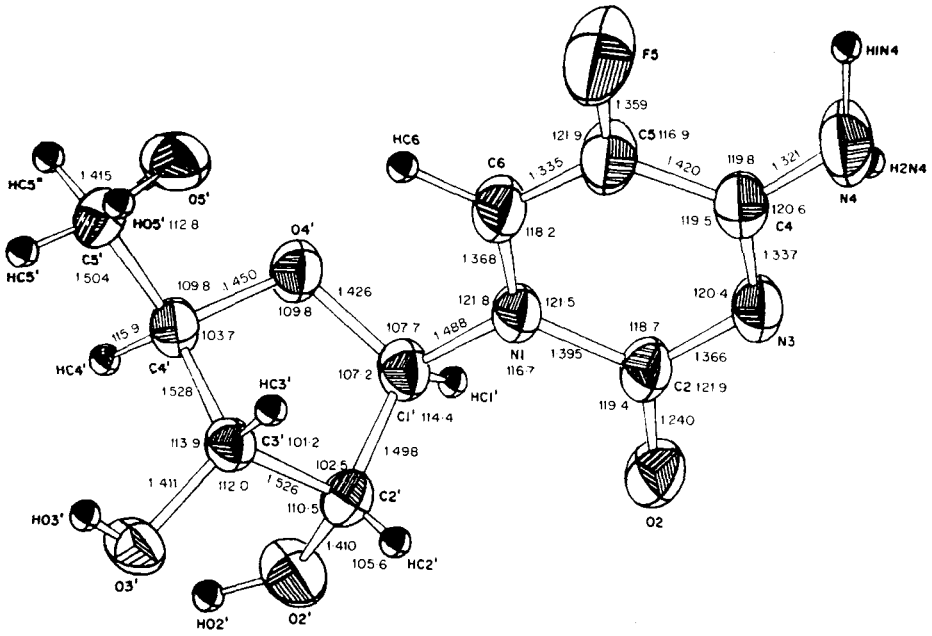

Fig.3. Bond distances (Å) and bond angles (°) in 5-fluorocytidine for Molecule B

reference to C(5'). The conformation across C(4′)-C(5′) is g^+ in both the molecules with values of 51.4 and 54.7 for Φ_{0C} and -64.5 and 62.2 for Φ_{oo} in molecules A and B respectively.

The pseudorotation parameters[19] found in this structure are τ_m = 40.4°(3) and 39.4(3)° and P = 20.5(4) and 14.4(4) respectively. These τ_m value can be compared to the average τ_m value of 39° from other nucleosides and nucleotides[20].

Hydrogen Bonds and molecular Packing :

The intermolecular hydrogen bonding scheme is shown in Fig. 4 which is a projection down the crystallographic a-axis. Solid bonds are used for molecule B in order to distinguish it from molecule A which is represented with open bonds. The fluorine atoms of both molecules A and B are not involved in any hydrogen bonding. The hydrogen bond distances and angles are given in Table 4. The crystal structure is stabilized by a network of O-H...O and N-H...O hydrogen bonds involving both the molecules A and B. N(3) of molecule B is the acceptor of a hydrogen bond from O(3') of molecule A. The N-H...O hydrogen

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TABLE 3

TORSION ANGLES (°) OF 5-FLUOROCYTIDINE

Torsion Angle		Molecule A	Molecule B
C(6)-N(1)-C(1')-O(4')	χ	15.2	20.0
C(2')-C(1')-O(4')-C(4')	ζ_0	-1.5	1.9
O(1')-C(1')-C(2')-C(3')	ζ_1	-23.0	-25.0
C(1')-C(2')-C(3')-C(4')	ζ_2	37.6	36.9
C(2')-C(3')-C(4')-O(4')	ζ_3	-39.4	-36.1
C(3')-C(4')-O(4')-C(1')	ζ_4	25.9	21.8
O(5')-C(5')-C(4')-C(3')	Φ_{OC}	51.4	54.7
O(5')-C(5')-C(4')-O(4')	Φ_{OO}	-64.5	-62.2

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bonds involving the amino groups of the cytosine of both molecules A and B could not be well characterized as the coordinates of the hydrogen atoms of the amino group of molecule A are not accurate and that of molecule B could not be located unambiguously. The furanose ring oxygen atom O(4') of both the molecules A and B is not involved in any hydrogen bonding [11].

The molecular packing and stacking is very much different as compared with other halogenated nucleosides and nucleotides[21]. Minimal base stacking is seen as illustrated in Figure 5. The closest contact between screw related pyrimidine bases are N(3A).....N(3B) = 3.759Å, N(3A).....C(2B) = 3.648A; N(1B).....N(3A) = 3.709Å, the remaining interbase distances are greater than 4.0Å.

B NMR SPECTROSCOPY

The chemical shifts (δ) and coupling constants (J) of 5-fluorocytidine are listed in Tables 5 and 6 respectively. The chemical shifts were assigned by comparison to the published values of cytidine[22] and by computer spectral simulation[25]. The values of the furanose ring are all typical and very similar to cytidine (Table 7). The H6 resonance of FCyd is shifted down field slightly from the

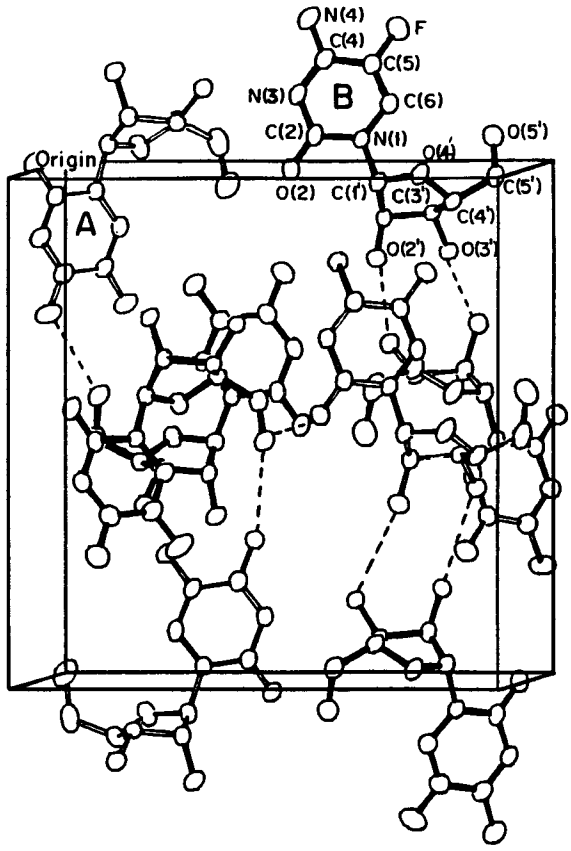

Fig.4. An ORTEP[11] diagram showing the packing of the molecules in the unit cell. Some of the intermolecular hydrogen bonds are shown by dashed lines.

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H6 of Cyd. This is similar to the shift observed for 5-fluorouridine and uridine and is attributed to the electronegativity of the fluorine atom at C5. The J values of FCyd (Table 6) are generally quite similar to those of Cyd, qualitatively indicating a similar conformation.

A more detailed conformational analysis of the NMR coupling constant data was performed to evaluate the furanose and exocyclic conformations. The procedure described by Davies and Danyluk[26] was used to calculate the conformational preferences listed in Table 7. A comparison of γ(C4′-C5′) and C2′-endo/C3′-endo distributions of FCyd and Cyd show little difference between them. This indicates that the presence of the fluorine atom at the

Table 4

GEOMETRY OF THE HYDROGEN BONDS

A	H	B	A.....B	A---H	H...B	A---H...B
O3′A	HO3′A	N3B	2.910[i]	0.82	2.10	170°
O3′B	HO3′B	O2′A	2.759[ii]	0.81	2.26	121°
O2′A	HO2′A	O3′B	2.759[iii]	0.90	2.03	137°
O2′B	HO2′B	O3′A	2.686[iv]	0.76	2.01	149°
N4A**	H1N4	O5′B	2.872[v]	0.76	2.16	156°
N4B**	H1N4	O2′B	2.820[vi]	1.23	2.30	123°
N4B**	--	O2B	3.173	--	--	-
O5′B	HO5′B	O2A	2.664[vii]	0.88	1.81	164°

**The hydrogens of the amino groups cannot be well located from difference maps.

Subscripts denote the following transformation.

I	x	y	z
ii	x	y	z
iii	½ - x	1 - y	-½ + z
iv	½ - x	1 - y	-½ + z
v	½ - x	1 - y	-½ + z
vi	½ - x	1 - y	-½ + z
vii	x	-1 + y	z

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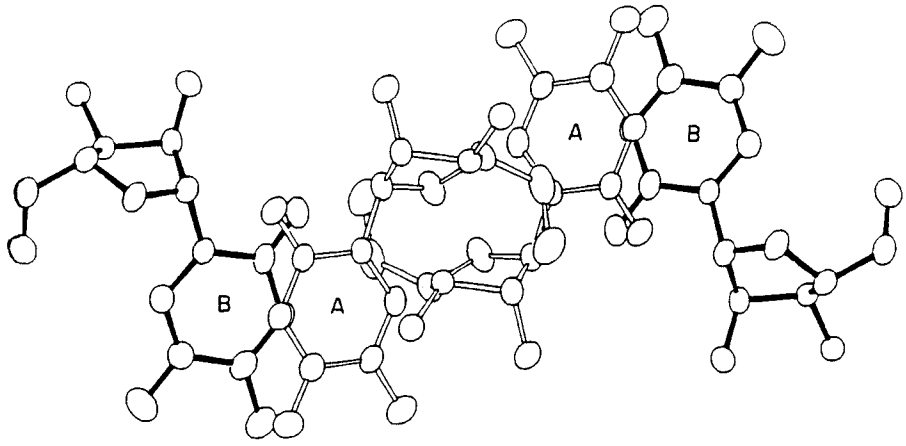

Fig.5. An ORTEP[11] diagram showing the base stacking pattern observed in 5- fluorocytidine.

Table 5

CHEMICAL SHIFTS[A] OF CYTIDINE AND ITS 5-FLUORO-DERIVATIVE

NUCLEIC ACID[B]	pH	H1′	H2′	H3′	H4′	H5′	H5′	H5	H6
CYTIDINE[C]	8.0	5.912	4.315	4.215	4.142	3.942	3.828	6.054	7.857
5-FLUOROCYTIDINE	7.0	5.849	4.258	4.186	4.116	3.939	3.819	---	8.029

[A] In PPM, EXPRESSED RELATIVE TO INTERNAL DDS.
[B] AT 27°C IN D_2O; 0.1 M.
[C] Hruska et al (1975), 23°C

Table 6

COUPLING CONSTANTS[A] OF CYTIDINE AND 5-FLUORO-DERIVATIVE

NUCLEIC ACID[B]	pH	1′,2′	2′,3′	3′,4′	4′,5′	4′,5″	5′,5″	1′,F5	6,F5
CYTIDINE[C]	8.0	4.0	5.2	6.0	2.8	4.3	-12.7	---	---
5-FLUOROCYTIDINE	7.0	3.5	5.0	6.2	2.7	3.9	-12.8	1.6	1.6

[A] In Hertz, 23-27°.
[B] At 27°C in D_2O; 0.1 M.
[C] Hruska et al. (1975), 23°C.

Table 7

CALCULATED CONFORMATIONAL PREFERENCES FOR CYTIDINE AND 5-FLUORO-DERIVATIVE

	γ(C4′C5′)			FURANOSE		
NUCLEIC ACID	pH	Fg^+	Ft	Fg^-	$F_{C2'}$-ENDO	$F_{C3'}$-ENDO	K_{eq}
CYTIDINE	8.0	0.68	0.24	0.08	0.40	0.60	0.67
5-FLUOROCYTIDINE	7.0	0.74	0.19	0.07	0.36	0.64	0.57

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C5 position has essentially no effect on these conformational states. The effect of fluorine substitution on furanose-base conformation has been evaluated[24] from the heteronuclear coupling constants between the H1′ of the furanose and the C2 and C6 of the base. The nearly identical J-values indicate similar anti conformations for both Cyd and FCyd. The similarity of NMR solution conformational results for Cyd and FCyd is analogous to the situation observed for Urd and FUrd.

The conformational results observed for FCyd in the solid and solution states are quite similar. The pseudorotational phase angles for the two FCyd molecules in the asymmetric unit are 14.4 and 20.5°. These values are very near to a pure C3′-endo conformation in which P ≠ 18. The NMR results indicate a C3′-endo / C2′-endo equilibrium with a clear preference for C3′-endo. For the exocyclic bond (C5′-C4′), the solid state torsion angles for the two FCyd molecules is 51.4 and 54.7. A pure g^+ conformation has a value of 60. The NMR results indicate a 74% preference for the g^+ rotamer. The sugar-base torsion angle for the FCyd molecules is 15.2 and 20.0; both are in the low-anti range. The NMR results simply indicate a preference for the anti conformation.

In summary, both the x-ray and NMR studies indicate that 5Fcyd exists in the anti conformation with a C3′ endo sugar pucker and the preferred g^+ across the exocyclic C4′-C5′ bond. It appears that the presence of the fluorine atom at the C5 position of the base, has very little effect on the general conformation of the nucleoside or its base-stacking properties.

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