Fluorinated Nucleic Acid Constituents: A Carbon-13 Nuclear Magnetic Resonance Study of Adenosine, Cytidine, Uridine, and Their Fluorinated Analogues†

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ABSTRACT: Adenosine, cytidine, uridine, and their fluorinated analogues 2-fluoroadenosine, 5-fluorocytidine, and 5-fluorouridine have been analyzed by carbon-13 nuclear magnetic resonance (NMR) spectroscopy. All carbon resonances of the sugar and base moieties are assigned. The carbon–fluorine coupling constants of the base and the carbon–proton coupling constants between carbons of the base and protons of the base and the anomeric proton of the sugar have been assigned. Effects of the fluorine atom on carbon chemical shifts of the nucleoside are expressed as $\Delta\delta_F$ values [$\Delta\delta_F = \delta$(fluorinated nucleoside) − δ(normal nucleoside)]. Theoretical charge density calculations (CNDO/2) of the fluorinated and nonfluorinated base carbons are compared [$\Delta E_T = E$(fluorinated nucleoside) − E(normal nucleoside)]. The $\Delta\delta_F$ and ΔE_T values are shown to correlate very well, except where a nitrogen atom is situated β to the fluorine atom. This apparent deviation is attributed to a lone-pair electron (LPE) effect of the nitrogen. Contributions of the LPE effect appear to vary $^1J_{C,H}$ and $^1J_{C,F}$ values in a predictable way. Long-range (four- and five-bond) carbon–fluorine coupling constants are observed in the base moiety. At these experimental conditions, introduction of the fluorine atom has no measurable conformational effect on the sugar–base torsion angle.

Fluorinated nucleic acid constituents are used extensively in chemotherapy and in vivo as modulators of aberrant cell growth (Heidelberger, 1975). The formation of nucleotide derivatives is usually required for these fluorinated constituents to be metabolically active. In the active form they can function as inhibitors of essential enzymatic processes (Heidelberger, 1973) and become incorporated into the ribonucleic acid (RNA)[1] of the host cell (Heidelberger, 1965; Mandel, 1969). Once incorporated into RNA their detailed mechanism of action is not known, although it is conceivable they could induce conformational changes (Moore & Kaiser, 1977) that may result in mutations (Abdulnur, 1976) or improper maturation of the RNA molecule (Wilkinson & Pitot, 1973).

In order to evaluate the effect of fluorinated derivatives on nucleic acid structure and biochemical function, we have been studying model nucleic acid constituents using nuclear magnetic resonance (NMR)[1] spectroscopy (Alderfer & Hazel, 1978, 1980). This spectroscopic technique is quite useful for elucidating conformational details as well as electronic structural properties of biomolecules. It seems likely that alterations in both nucleic acid conformation and electronic structural perturbations may be important determinants underlying the biochemical and biological properties observed for fluorinated nucleic acid constituents. The present study on normal and fluorinated nucleosides of uracil, cytosine, and adenine (Figure 1) forms the basis for the study of more complicated models in the future. In this study assignments of all base and sugar carbons are made, as well as carbon–fluorine and carbon–proton coupling constants of the base and to H1′ of the furanose. Charge density calculations of the fluorinated and normal bases are compared. The results indicate a good correlation between ^{13}C chemical shifts and charge density changes induced by the fluorine atom. The fluorine atom is shown to produce electronic perturbations throughout the entire base moiety of each nucleoside. The magnitude of the effect of the fluorine atom on the base chemical shift, $^1J_{C,H}$, and $^1J_{C,F}$ values has been correlated with the number of directly bonded nitrogen atoms. At the experimental conditions used in this study, the fluorine atom has no measurable effect on the nucleoside conformation.

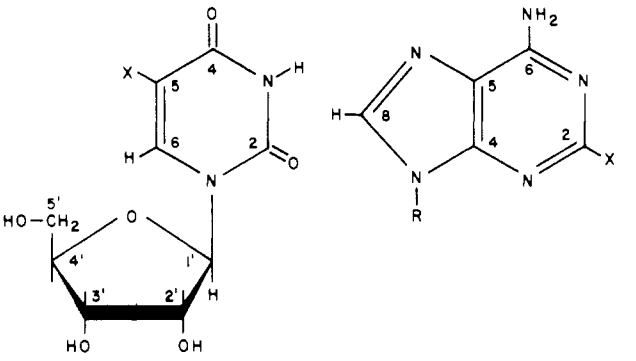

FIGURE 1: Structure of uridine, 5-fluorouridine, adenosine, and 2-fluoroadenosine.

Materials and Methods

Materials. Uridine, cytidine, and adenosine were obtained from Boehringer Mannheim, Inc. 5-Fluorocytidine was chemically synthesized (Robins et al., 1976) or obtained from Hoffman-La Roche Inc., courtesy of Dr. W. E. Scott. 5-

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[1] Abbreviations: RNA, ribonucleic acid; ^{13}C NMR, carbon-13 nuclear magnetic resonance; FUra, 5-fluorouracil; FCyt, 5-fluorocytosine; FAde, 2-fluoroadenine; FUrd, 5-fluorouridine; FCyd, 5-fluorocytidine; FAdo, 2-fluoroadenosine; LPE, lone-pair electron; Me_2SO, dimethyl sulfoxide; NOE, nuclear Overhauser effect; Me_4Si, tetramethylsilane; CW, continuous wave.

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Table I: Carbon-13 Chemical Shifts[a] of Uridine, Cytidine, Adenosine, and Their Fluorinated Analogues

	chemical shifts (ppm, from internal dioxane)									
nucleoside	C1′	C2′	C3′	C4′	C5′	C2	C4	C5	C6	C8
5-fluorouridine[b]	22.75_3	7.25_3	2.72_0	17.75_2	-5.99_5	83.61_5	92.82_7	74.19_7	59.04_8	
uridine	22.84_1	7.09_2	2.85_1	17.63_5	-5.82_0	85.09_1	99.61_2	35.70_9	75.29_4	
$\Delta\delta_F$[c]	-0.08_8	$+0.16_1$	-0.13_1	$+0.11_7$	-0.17_5	-1.47_6	-6.78_5	$+38.48_8$	-16.26_6	
5-fluorocytidine	23.70_4	7.70_6	2.57_4	17.29_9	-5.98_1	89.20_1	91.89_1	71.00_9	59.17_2	
cytidine	23.79_2	7.42_9	2.82_2	17.27_0	-5.70_3	91.05_8	99.80_2	29.74_3	75.16_2	
$\Delta\delta_F$[c]	-0.08_8	$+0.27_7$	-0.24_8	$+0.02_9$	-0.27_8	-1.85_7	-7.91_1	$+41.26_6$	-15.99_2	
2-fluoroadenosine	21.62_5	7.12_8	3.86_3	18.97_5	-5.11_1	92.17_3	83.37_0	50.79_9	90.72_1	74.06_2
adenosine	21.78_8	7.25_3	4.12_4	19.24_4	-4.49_2	85.92_5	81.80_1	52.46_7	89.02_5	73.99_3
$\Delta\delta_F$[c]	-0.16_3	-0.12_5	-0.16_1	-0.26_9	-0.13_9	$+6.24_8$	$+1.56_9$	-1.68_8	$+1.69_6$	$+0.06_9$

[a] Obtained in aqueous solution, at 30 °C and pH 7. [b] At pH 4.4 (see Discussion for explanation). [c] $\Delta\delta_F = \delta$(fluorinated nucleoside) − δ(normal nucleoside).

Fluorouridine was purchased from Calbiochem, Inc., and 2-fluoroadenosine was obtained from Merck Sharp & Dohme, Inc., through the courtesy of Dr. D. Cochran. 5-Deuteriouridine was synthesized as described by Wataya & Hayatsu (1972). NMR solvents were obtained from Bio-Rad (D_2O) and Aldrich (Me_2SO-d_6). The purity of all materials was verified by their carbon-13 NMR spectrum.

NMR Measurements. The ^{13}C NMR data were obtained from a Bruker WP-200 (50.288 MHz) by utilizing the Fourier-transform/quadrature phase detection mode with 10 mm diameter sample tubes. Broad-band proton-decoupled spectra were obtained by collecting 8K data points and 8K zero filling or collecting 16K data points with a sweep width of about 6K Hz, yielding a computer resolution of 0.7 Hz. Proton-coupled ^{13}C spectra were obtained without proton irradiation or by using a gated decoupling routine (proton coupling with NOE enhancement) with a 2-kHz sweep width, yielding a computer resolution of about 0.2 Hz. Sample temperature was controlled with the BVT-2000 temperature controller of the WP-200. The temperature controller was set at 30 °C, unless otherwise indicated.

NMR Samples. Samples were prepared to a volume of 1.1–1.4 mL. Dioxane was added in trace amount (approximately 0.1% volume) as an internal chemical shift reference. Chemical shifts can be expressed to the Me_4Si reference with $\delta(Me_4Si) = \delta$(dioxane) + 67.22 ppm. Aqueous solutions contained 0.04 M sodium phosphate, 2–4 mM (ethylenedinitrilo)tetraacetic acid (EDTA), and usually about 20% D_2O.

Charge Density Calculations. Charge densities are calculated by the CNDO/2 method (Pople & Beveridge, 1970), using the Quantum Chemistry Program Exchange, computer program no. 141 (Dobosh, 1970). This method provides charge distributions and dipole moments for molecules containing the elements hydrogen to chlorine in the closed-shell case. This program is adequate for molecules containing up to 35 atoms or 80 basis functions, whichever is smaller. Each hydrogen atom in a molecule requires one basis function, and each heavier atom up to chlorine requires four basis functions.

Results

Assignment of Resonances. Carbon-13 spectra of uridine, 5-fluorouridine, adenosine, and 2-fluoroadenosine are illustrated in parts A, B, C, and D of Figure 2. The general features of cytidine and 5-fluorocytidine are similar to parts A and B of Figure 2, respectively. The assignments of these resonances (Table I) are accomplished by (1) the proton off-resonance broad-band decoupling technique, (2) evaluation of resonance multiplicity and magnitude of splitting of the proton-decoupled spectra, and (3) evaluation of the proton-coupled spectra.

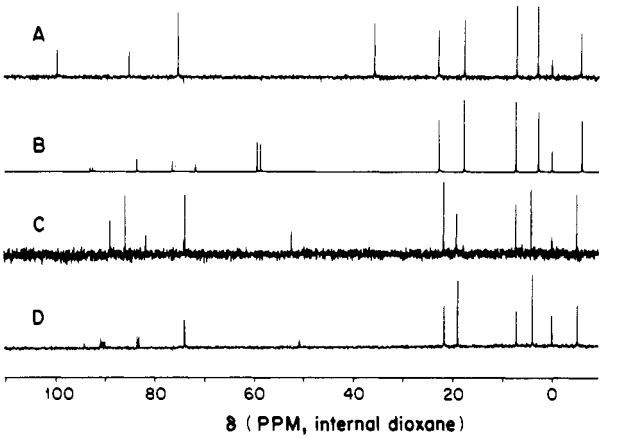


FIGURE 2: Carbon-13 (broad-band proton-decoupled) NMR spectrum of (A) uridine, (B) 5-fluorouridine, (C) adenosine, and (D) 2-fluoroadenosine in D_2O at 30 °C.

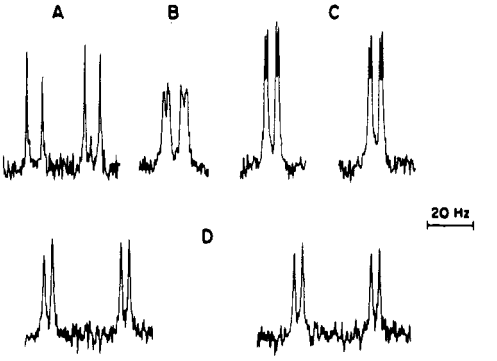


FIGURE 3: Carbon-13 (proton-coupled) NMR subspectrum of (A) C4, (B) C2, (C) C5, and (D) C6 of 5-fluorouridine in D_2O at 30 °C. In (C) and (D), the base line between the multiplets is eliminated to conserve space. The scale bar applies to all multiplets with a continuous base line.

Table II: Carbon–Fluorine Coupling Constants[a] of the Base Moiety of Fluorinated Nucleosides

nucleoside	C2	C4	C5	C6	C8
5-fluoro-cytidine	<0.2 (4)[b]	14.6 (2)	242.1 (1)	32.7 (2)	
5-fluoro-uridine	0.5 (4)	26.1 (2)	232.9 (1)	34.9 (2)	
2-fluoro-adenosine	211.5 (1) [204.2][c]	18.9 (3) [20.2]	4.0 (4) [4.0]	19.8 (3) [21.2]	2.6 (5) [2.8]

[a] Obtained in aqueous solution. [b] Number (n) of bonds between interacting nuclei ($^nJ_{C,F}$). [c] Values in brackets obtained in Me_2SO-d_6 solution.

The carbon of the base moiety that is directly bonded to the fluorine is easily identified by the large $^1J_{C,F}$ value (range of

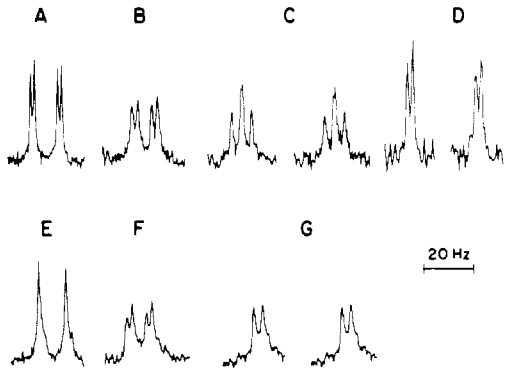


FIGURE 4: Carbon-13 (proton-coupled) NMR subspectrum of (A) C4, (B) C2, (C) C6, and (D) C5 of uridine and (E) C4, (F) C2, and (G) C6 of 5-deuteriouridine in D_2O at 30 °C. In (C), (D), and (G), the base line between the multiplets is eliminated to conserve space. The scale bar applies to all multiplets with a continuous base line.

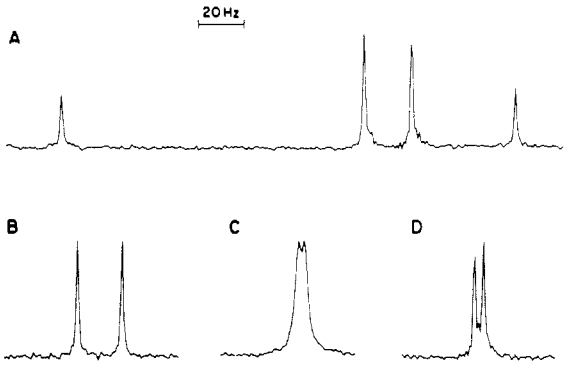


FIGURE 5: Carbon-13 (broad-band proton-decoupled) NMR subspectrum of (A) C2 and C6, (B) C4, (C) C8, and (D) C5 of 2-fluoroadenosine in Me_2SO-d_6 at 30 °C. The scale bar applies to each subspectrum.

211–242 Hz) (Figure 2B,D and Table II). The remaining carbons of the base moiety of 5-fluorouridine are assigned from the proton-coupled spectrum (Figure 3) and from the spectra of uridine and 5-deuteriouridine (Figure 4). The C6 is identified by the large $^1J_{C,H}$ value (184.1 Hz), C4 by the single proton coupling, $^3J_{C4,H6}$, and C2 by two proton couplings, $^3J_{C2,H6}$ and $^3J_{C2,H1'}$. The assignments of the carbons of 5-fluorocytidine are made in an analogous procedure since the carbon–fluorine and carbon–proton splitting patterns of 5-fluorocytidine and 5-fluorouridine are qualitatively identical. The assignments of the resonances of the base moiety of 2-fluoroadenosine are made from the decoupled spectrum (Figure 5) and proton-coupled spectrum (Figure 6). The C2 is assigned by the large carbon–fluorine splitting (Figure 5 and Table II). The C8 is identified by the large $^1J_{C,H}$ value (213.3 Hz), C6 by no proton coupling, C5 by three proton couplings, $^3J_{C5,H8}$, $^3J_{C5,NHa}$, and $^3J_{C5,NHb}$, and C4 by two proton couplings, $^3J_{C4,H8}$ and $^3J_{C4,H1'}$. The assignments of the base carbons are consistent with those of 2-fluoro-6-aminopurine (Thorpe et al., 1974). The assignments of the furanose carbon resonances are accomplished by off-resonance proton decoupling, which is based on complete analysis of the proton spectra of the compounds listed in Table I (Alderfer & Hazel, 1978).

Assignment of Coupling Constants. Assignments of the interacting nuclei are straightforward for most of the observed coupling patterns. For uridine, C6 has a large $^1J_{C6,H6}$ and two smaller values from $^2J_{C6,H5}$ and $^3J_{C6,H1'}$ (Figure 4C). The values of these spin–spin coupling interactions were unambiguously determined by comparison of the spectrum with 5-deuteriouridine where the $^2J_{C6,H5}$ is removed, leaving only the $^3J_{C6,H1'}$ splitting (Figure 4G). There are other long-range

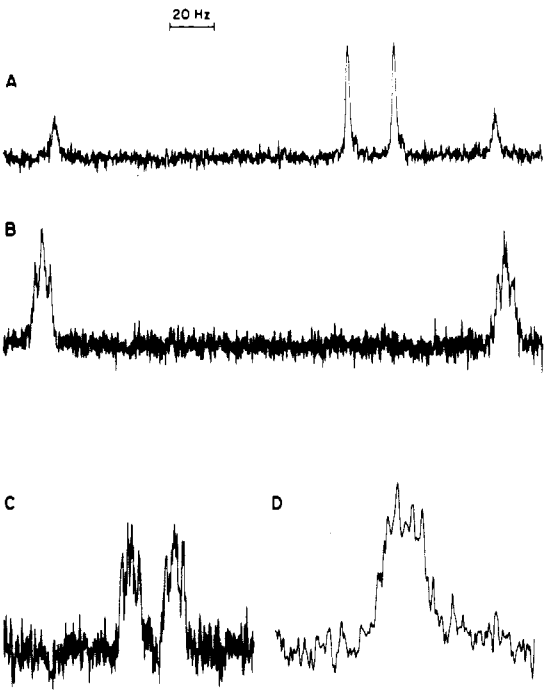


FIGURE 6: Carbon-13 (proton-coupled) NMR subspectrum of (A) C2 and C6, (B) C8, (C) C4, and (D) C5 of 2-fluoroadenosine in Me_2SO-d_6 at 30 °C. The scale bar applies to each subspectrum.

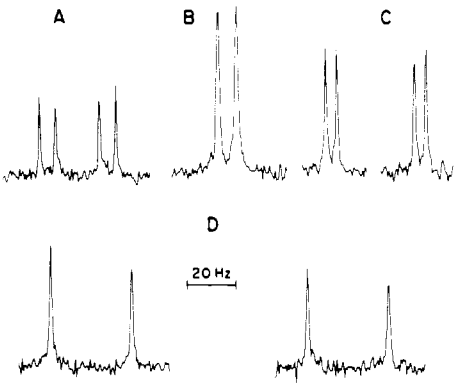


FIGURE 7: Carbon-13 (H1′ proton-decoupled) NMR subspectrum of (A) C4, (B) C2, (C) C5, and (D) C6 of 5-fluorouridine in D_2O at 30 °C. Comments in Figure 3 apply here.

(four-bond) couplings observed for C2 and C5 (FUrd and FCyd). The smallest observed spin–spin splitting of C2 in FUrd is 0.5 Hz (Figure 3B). This is also observed in the proton-decoupled spectrum and is assigned as $^4J_{C2,F5}$. The smallest splitting observed for C5 (1.0 Hz) disappeared with specific CW irradiation of H1′ [as well as the 2.1-Hz splitting of C2 and the 3.6-Hz splitting of C6 (Figure 7)]; this was assigned as $^4J_{C5,H1'}$.

In the proton-decoupled spectrum of FAdo, C5 and C8 both appear as doublets (Figure 5) and are assigned as $^4J_{C5,F2}$ (4.0 Hz) and $^5J_{C8,F2}$ (2.6 Hz), respectively. In the case of the proton-coupled spectrum, FAdo becomes considerably more complex, especially C4 (Figure 6) and C5. The C4 resonance is an apparent eight-line pattern resulting from $^3J_{C4,F2}$ (20.3 Hz), $^3J_{C4,H8}$ (4.8 Hz), and $^3J_{C4,H1'}$ (3.2 Hz). These assignments are made from the specific proton-decoupling experiments illustrated in Figure 8, where the smallest splitting (3.2 Hz) disappeared when H1′ was irradiated and the 4.8-Hz coupling was eliminated when H8 was irradiated. The C5 resonance is consistent with a 12-line pattern—four triplets (a doublet of doublets of triplets). This pattern arises from C5 coupling with H8, H2 or F2, and NHa and NHb (since the purine

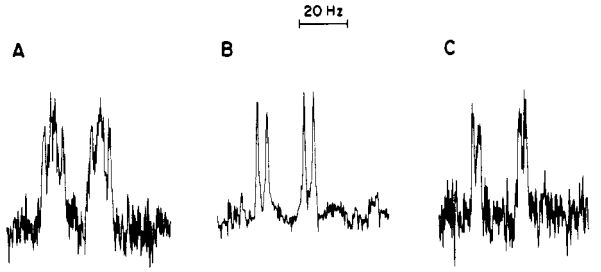


FIGURE 8: Carbon-13 NMR subspectrum of C4 with (A) proton coupling, (B) H1′ proton decoupling, and (C) H8 proton decoupling, of 2-fluoroadenosine in Me_2SO-d_6 at 30 °C. The scale bar applies to each subspectrum.

spectra were obtained in Me_2SO-d_6). Addition of D_2O to the solution reduces the pattern to a doublet of doublets. The amino protons are therefore assigned equal values of 4.3 Hz each. The remaining two coupling constants were assigned by comparing C5 of adenosine and that of 2-fluoroadenosine since they only have $^3J_{C5,H8}$ in common and $^3J_{C5,F2}$ in 2-fluoroadenosine is known from the proton-decoupled spectrum. The $^3J_{C5,H8}$ was also determined by specific proton CW irradiation of H8, collapsing the doublet of doublets to a simple doublet due to $^3J_{C5,H2}$ or $^3J_{C5,F2}$.

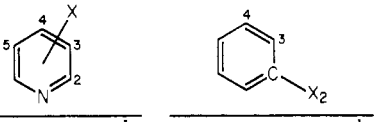
Discussion

The carbon resonance assignments of cytidine, uridine, and adenosine (Table I) are similar to those reported by Jones et al. (1970), except that their C2′ and C3′ assignments should be interchanged. Also, our assignments for 5-fluorouridine are similar to those made by Triplett et al. (1977), except the C2′ and C3′ resonances are interchanged. A critical comparison of the data in Table I with those of Triplett et al. (1977) indicates significant differences ($\Delta\delta$, ppm) in the chemical shifts: +0.10 (C1′), +0.05 (C2′), +0.22 (C3′), −0.19 (C4′), +0.33 (C5′), +1.76 (C2), +4.84 (C4), +3.72 (C5), and −0.93 (C6). These differences are most readily understood by comparing the solution pH. In Table I the 5-fluorouridine data is reported at pH 4.4, while Triplett et al. (1977) reported their data at pH 7. Since the pK_a of N3–H in 5-fluorouridine is about 7.57 (Wempen et al., 1961), the base moiety is partially charged at pH 7, whereas at pH 4.4 (Table I) the base is neutral. Therefore, an effect of fluorine substitution at the C5 position of the uracil base is to lower the pK_a about 1.7 units (Wempen et al., 1961), which in turn has large effects on the chemical shifts of the base carbons.

So that the effects of fluorine substitution on the carbon-13 chemical shifts can be more clearly elucidated, the $\Delta\delta_F$ values [$\Delta\delta_F$ = δ(fluorinated nucleoside) − δ(normal nucleoside)] are determined in Table I. The furanose carbons (C1′–C5′) of both pyrimidine nucleosides show an alteration in the sign of the $\Delta\delta_F$ value as the cyclic carbon chain proceeds from C1′ to C4′ and then to the exocylic C5′. In contrast, all the $\Delta\delta_F$ values of the furanose carbons of the purine nucleoside are negative. On the other hand, the $\Delta\delta_F$ values of all the base carbons, both purine and pyrimidine, indicate a consistent trend where the $\Delta\delta_F$ values alternate in sign as a function of intervening bonds between the fluorine and carbon atoms (Table I). Thus, odd numbers of intervening bonds have a positive $\Delta\delta_F$ value and even numbers are negative.

A more quantitative evaluation of these $\Delta\delta_F$ values can be provided by theoretical calculations of charge densities. Numerous studies have shown correlations between the carbon-13 chemical shifts and calculated charge densities (Jones et al., 1970; Tarpley & Goldstein, 1971; Pugmire et al., 1973). In Table III are listed the calculated (CNDO/2) total residual

Table III: Carbon–Proton and Carbon–Fluorine Coupling Constants in Benzene, Pyridine, and Their Fluorinated Analogues



	X = H[a]	X = F[b]	X = H[c]	X = F[d]
$^1J_{C2,X2}$	+177.6	−236.3	+157.5	−245.3
$^2J_{C3,X2}$	8.5	37.6	1.1	21.0
$^3J_{C4,X2}$	6.3	7.5	7.6	7.7
$^1J_{C3,X3}$	+163.0	−255.1		
$^2J_{C2,X3}$	3.1	22.6		
$^2J_{C4,X3}$	0.7	17.7		
$^3J_{C5,X3}$	6.6	3.7		
$^1J_{C4,X4}$	+162.4	−261.8		
$^2J_{C3,X4}$	0.8	16.1		
$^3J_{C2,X4}$	6.8	6.4		

[a] Hansen & Jakobsen (1973). [b] Lichter & Wasylishen (1975). [c] Tarpley & Goldstein (1972). [d] Weigart & Roberts (1971).

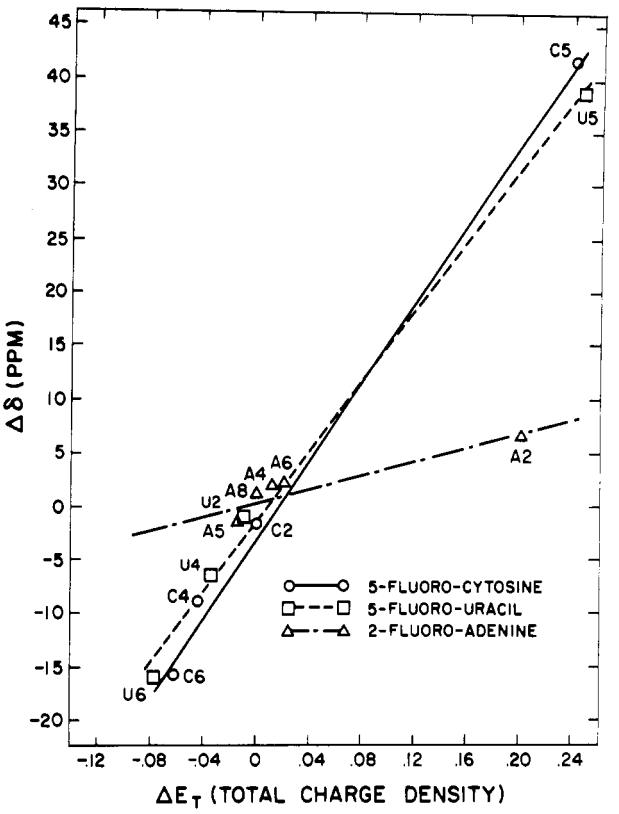


FIGURE 9: Plot of fluorine-induced carbon-13 chemical shift change ($\Delta\delta_F$) vs. CNDO/2 calculated total residual charge density change (ΔE_T) of 5-fluorocytosine (○), 5-fluorouracil (□), and 2-fluoroadenine (△).

charge densities for the carbon atoms of uracil, cytosine, adenine, and their fluorinated analogues. So that the effect of fluorine substitution on charge density can be emphasized, the ΔE_T values [ΔE_T = E(fluorinated base − E(normal base)] are calculated. A comparison of the signs of these ΔE_T values with those of the $\Delta\delta_F$ values in Table I reveals a similar alternation in sign with the number of intervening covalent bonds. These $\Delta\delta_F$ and ΔE_T data are plotted in Figure 9, and the lines drawn have correlation coefficients of 0.999, 0.997, and 0.936 and slopes of 164, 190, and 31 for fluorouracil, fluorocytosine, and fluoroadenine, respectively. The apparent correlation of fluoroadenine is not as good as that of fluorouracil and fluorocytosine. This could be a result of differential base–base stacking interactions in aqueous solution, which are more likely

Table IV: Calculated[a] Total Residual Charge Densities for Base Carbons of Uracil, Cytosine, Adenine, and Their Fluorinated Analogues

	net charge (E)				
base	C2	C4	C5	C6	C8
5-fluorouracil	+0.439	+0.335	+0.092	+0.098	
uracil	+0.449	+0.368	−0.157	+0.173	
ΔE_T[b]	−0.010	−0.033	+0.249	−0.075	
5-fluorocytosine	+0.419	+0.291	+0.057	+0.130	
cytosine	+0.423	+0.323	−0.174	+0.194	
ΔE_T	−0.004	−0.032	+0.231	−0.064	
2-fluoroadenine	+0.431	+0.226	−0.070	+0.283	+0.176
adenine	+0.229	+0.214	−0.057	+0.264	+0.178
ΔE_T	+0.202	+0.012	−0.013	+0.019	−0.002

[a] CNDO/2 (Dobosh, 1970). [b] ΔE_T = E(fluorinated base) − E(normal base).

to contribute to chemical shifts in purines than in pyrimidines (Alderfer et al., 1971, 1972) since the ring currents of purines are generally larger than pyrimidines (Giessner-Prettre & Pullman, 1976). In order to eliminate these potential effects, we also determined the $\Delta\delta_F$ values in organic solvent (Me_2SO-d_6) for adenosine and 2-fluoroadenosine. However, both the slope and correlation coefficient of the $\Delta\delta_F$ vs. ΔE_T data in D_2O and Me_2SO-d_6 are essentially identical, indicating base-stacking effects are not contributing to the observed results. From these data (Figure 9) then it would appear that the C13 chemical shifts of the purine and pyrimidine bases respond quite differently to the introduction of the fluorine atom, since the slopes of the $\Delta\delta_F$ vs. ΔE_T plot are quite different. However, close inspection of the $\Delta\delta_F$ and ΔE_T values (Tables I and IV) for carbons located in β, γ, and δ positions to the fluorine atom does not substantiate an apparently different pyrimidine and purine response. In fact the ΔE_T values of all the α carbons are reasonably similar; only the $\Delta\delta_F$ of α carbons in the pyrimidines and purines are different. If the A2 value (Figure 9) of the purine is not included in the linear regression analysis, the calculated straight line has a slope and correlation coefficient of 108 and 0.976, respectively, values more consistent with those of the pyrimidines. A possible explanation for this may be related to the different arrangement of the ring nitrogens to the fluorine atoms in the pyrimidine and the purine ring system. In the purine, the N1 and N3 are in β positions with respect to the F2, while in the pyrimidine the N1 and N3 are γ to F5. This point will be discussed again later in this section.

The values of carbon–proton and carbon–fluorine coupling constants (Tables II and V) are in general agreement with published values of uracil and 5-fluorouracil (Tarpley & Goldstein, 1971) and fluorouridine (Triplett et al., 1977; S. L. Smith, personal communication). In the discussion to follow later in this section, the pyrimidines and purines will be critically compared in regard to their $J_{C,H}$ and $J_{C,F}$ values. It has been recognized for a long time that these values may be solvent dependent (Douglas & Dietz, 1967; Cox & Smith, 1969). Since the $J_{C,H}$ values of the purines (Table V) were obtained in Me_2SO-d_6 and the pyrimidines were obtained in aqueous solution, included for comparison are the $J_{C,F}$ values of 2-fluoroadenosines in Me_2SO-d_6 (Table II) and selected $J_{C,H}$ values of adenosine 3′-phosphate in aqueous solution (Table V). The only $J_{C,F}$ value that is significantly affected by solvent is $^1J_{C2,F2}$ (211.5 Hz in aqueous solution and 204.2 Hz in Me_2SO-d_6). The $J_{C,H}$ values for 3′-AMP in aqueous solution are essentially identical with those of adenosine in Me_2SO-d_6. Therefore, only $^1J_{C2,F2}$ is markedly solvent dependent, and for purposes of these discussions we will consider the other $J_{C,F}$ and $J_{C,H}$ values to be solvent dependent.

There are certain general features of $^1J_{C,H}$ in the purine and pyrimidine ring systems [$^1J_{C,H}$(purines) > $^1J_{C,H}$(pyrimidines)] that are seen in Table V. The $^1J_{C,H}$ value has been used as a measure of the hybridization of the carbon atom, where increasing $^1J_{C,H}$ values represent larger amounts of s character (Muller & Pritchard, 1959a,b; Newton et al., 1974). However, the $^1J_{C,H}$ value may also be dependent on ring size and substituents (Tori & Nakagawa, 1964). It is interesting to compare the $^1J_{C,H}$ values of the parent ring systems from which the nucleic acid bases are derived (pyrimidine and imidazole). In pyrimidine these values are 206 (C_2), 182 (C4 and C6), and 168 Hz (C5) and in imidazole 208 (C2, which is equivalent to C8 in the purine numbering system) and 190 Hz (C4 and C5) (Tori & Nakagawa, 1964). The similarity of some of the $^1J_{C,H}$ values in both the five- and six-membered ring systems indicates ring size is not responsible for the differences in the $^1J_{C,H}$ values observed in the purines and pyrimidines. This point will be discussed further later in this section.

The Tarpley and Goldstein study of 5-halogen substituents of uracil reveal that the absolute magnitude of $J_{C,H}$ values change in a predictable way with substituent electronegativity (E_X); thus, $^1J_{C,H}$ decreases with increasing E_X, $^2J_{C,H}$ increases with increasing E_X, and $^3J_{C,H}$ decreases with increasing E_X. It is immediately obvious (Tarpley & Goldstein, 1971; Table V) that these same trends are not rigorously followed when the 5-substituent is hydrogen and fluorine. In all cases, $^1J_{C,H}$ values are opposite the expected trend or invariant, while $^2J_{C,H}$ follows the predicted trend with larger values for the fluoro analogues. The effects on the $^3J_{C,H}$ values are mixed, where $^3J_{C2,H6}$ (pyrimidines) and $^3J_{C4,H8}$ and $^3J_{C5,H8}$ (purine) do not follow the predicted trend. These data do demonstrate the presence of four-bond coupling, which Tarpley & Goldstein

Table V: Carbon-Proton Coupling Constants[a] of the Base Moiety of Cytidine, Uridine, Adenosine, and Their Fluorinated Analogues

	C2			C4		C5			C6		
nucleoside	$^3J_{C2,H6}$	$^3J_{C2,H1'}$	$^4J_{C2,H5}$	$^2J_{C4,H5}$	$^3J_{C4,H6}$	$^1J_{C5,H5}$	$^2J_{C5,H6}$	$^4J_{C5,H1'}$	$^1J_{C6,H6}$	$^2J_{C6,H5}$	$^3J_{C6,H1'}$
cytidine	6.1	1.9	<0.2	1.6	9.4	175.4	2.9	0.9	182.7	4.5	3.3
5-fluorocytidine	6.4	1.7			6.0		4.2	0.8	183.5		3.4
uridine	8.0	2.3	<0.2	1.6	10.6	178.5	2.2	0.5	184.2	5.2	3.6
5-fluorouridine	8.1	2.1			7.1		4.9	1.0	184.1		3.6

		C4			C5			C8	
nucleoside	C2, $^1J_{C2,H2}$	$^3J_{C4,H2}$	$^3J_{C4,H8}$	$^3J_{C4,H1'}$	$^3J_{C5,H8}$	$^4J_{C5,H2}$	C6, $^3J_{C6,H2}$	$^1J_{C8,H8}$	$^3J_{C8,H1'}$
adenosine[b]	199.2[c]	12.1	4.7	3.6	11.3	1.3	11.2	213.3[d]	4.0[e]
2-fluoroadenosine[b]			4.8	3.2	11.5			214.2	4.1

[a] In hertz; in aqueous solution unless otherwise indicated. [b] In Me_2SO-d_6 solution. [c] In aqueous solution, 3′-AMP has a value of 202.7 Hz. [d] In aqueous solution, 3′-AMP has a value of 215.4 Hz. [e] In aqueous solution, 3′-AMP has a value of 3.9 Hz.

(1971) expected to see in uracil ($^4J_{C2,H5}$) and 5-fluorouracil ($^4J_{C2,F5}$) but did not observe. In the pyrimidines nucleosides (Tables II and V), this long-range coupling has only been clearly observed as $^4J_{C2,F5}$ (0.5 Hz) in 5-fluorouridine. The logical extension of the $J_{C,H}$ trends (odd $J_{C,H}$ decreases and even $J_{C,H}$ increases) with substituent electronegativity is to expect $^4J_{C,H}$ to increase with increasing E_X. Since $J_{C,F}$ values are generally larger than $J_{C,H}$ values and $^4J_{C2,F5}$ is 0.5 Hz, the lack of observed coupling for $^4J_{C2,H5}$ is not unexpected. A smaller $^4J_{C2,F5}$ in fluorocytidine than in fluorouridine is also consistent with smaller $^2J_{C4,F5}$ and $^2J_{C6,F5}$ and a larger $^1J_{C5,F5}$ in fluorocytidine. These values would suggest that the fluorine atom is appearing more electronegative in cytidine than uridine. However, this is not observed for $J_{C,H}$ values (Table V). Carbon–proton coupling constants are consistently smaller in fluorocytidine compared to fluorouridine, as well as in cytidine compared to uridine.

Comparison of C4 and C6 in uridines and cytidines and their coupling to the C5 substituent (H or F) reveals an interesting difference. In uridine and cytidine the $^2J_{C4,H5}$ values are similar (1.6 Hz), and their $^2J_{C6,H5}$ values are similar (5.2 and 4.5 Hz, respectively). The same comparison in the fluoro analogues indicates similar $^2J_{C6,F5}$ values (34.9 and 32.5 Hz, respectively) but very different $^2J_{C4,F5}$ values (26.1 and 14.6 Hz, respectively); thus, when contrasted in this way, the value of $^2J_{C4,F5}$ for fluorocytidine is much lower than expected. Additional insight may be obtained by four seemingly unrelated observations: (1) $^1J_{C,F}$ of both pyrimidines is greater than in 2-fluoroadenosine; (2) the $\Delta\delta_F$ value of C2 of the purine is much less than that of the C5 of the pyrimidines; (3) the $^3J_{C6,F5}$ values of the pyrimidines are similar while the $^3J_{C4,F5}$ values are different; and (4) $^3J_{C4,F2}$ and $^3J_{C6,F2}$ of 2-fluoroadenosine are very similar in value (18.9 and 19.8 Hz, respectively), and the $\Delta\delta_F$ values of C4 and C6 are similar. All of these can be understood by identifying carbons that are covalently bonded to *two* nitrogens. In the pyrimidines C2 and C4 are adjacent to two nitrogens and in adenosine C2, C4, C6, and C8. A second condition is to identify those carbon sets that are pseudosymmetrically related with respect to the fluorine. Thus in the pyrimidines, only C2 meets these conditions, and in adenosine, C2 is one set with C4 and C6 forming another. In the case of the first observation cited above, we reason that the two nitrogens have the effect of reducing the $^1J_{C,F}$ value (in these cases 20–30 Hz); a similar argument is used to explain the reduced $\Delta\delta_F$ value cited in observation 2. Observation 4 is consistent in that $^2J_{C4,F5}$ is less in cytosine (due to the two nitrogens) while the $^2J_{C6,F5}$ values are similar (both uracil and cytosine have one nitrogen). Observation 4 is understood since C4 and C6 satisfy both conditions indicated above. Although these may be only circumstantial evidence, they are internally consistent and point to a special role played by the nitrogen atoms in the fluoro analogues.

Some of the apparent inconsistencies in trends of $J_{C,H}$ and $J_{C,F}$ can be understood by evaluating the coupling constant as a signed parameter and considering the electronic effects of a nitrogen atom. It has been determined that $^1J_{C,H}$ values are positive (Buckingham & McLauchlan, 1963; Bernheim & Lavery, 1967; Spiesecke, 1968) and $^2J_{C,H}$ values are positive or negative (Weigert & Roberts, 1967; Tarpley & Goldstein, 1972). On the other hand, it has been determined that $^1J_{C,F}$ values are negative (Bernheim & Lavery, 1967) and $^2J_{C,F}$ values are usually positive (Weigert & Roberts, 1971).

The effects of the nitrogen atom in contrast to those of the carbon atom on the $^1J_{C,F}$ and $^1J_{C,H}$ values are seen by comparison of benzene, pyridine, and their fluoro analogues (Table III). The $^1J_{C2,H2}$ values of benzene (157.5 Hz) and fluorobenzene (−245.2 Hz) can be compared to the values of pyridine (177.6 Hz) and 2-fluoropyridine (−236.3 Hz). The nitrogen atom has similar effects on both $^1J_{C,H}$ and $^1J_{C,F}$ so that these values are *less negative* than in the benzenes. These effects are dependent on their position relative to the nitrogen atom, since $^1J_{C3,H3}$, $^1J_{C4,H4}$, and $^3J_{C3,F2}$ are similar to values found in the benzenes. The unusually large value for $^1J_{C4,F4}$ in 4-fluoropyridine has been explained (Muller & Carr, 1963; Doddrell et al., 1972) as the result of conjugation (back-donation of the fluorine electron increases the C–F bond order, which would increase $^1J_{C4,F4}$). The effect of nitrogen on both $^1J_{C,H}$ and $^1J_{C,F}$ values is in accord with the suggestion that the lone pair of electrons makes a positive contribution (produces an algebraically more positive value) to the coupling constant (Gil & Alves, 1969; Lichter & Wasylishen, 1975). Assigning the change of the $^1J_{C,H}$ and $^1J_{C,F}$ values to the lone-pair electron (LPE) effect of the nitrogen rather than to changes in hybridization (s character) of the carbon to which the hydrogen or fluorine is attached receives support from the studies of pyridines, fluoropyridines, and their respective pyridinium ions (Lichter & Wasylishen, 1975; Seel & Gunther, 1980). These data illustrate (Table VI) that the LPE effect exists only when the lone pair is free to delocalize into the ring system (cf. NH_2 vs. NH_3^+ and NO_2 substituents of *o*-fluorobenzene). It is also clear that both endocyclic and exocyclic nitrogen lone-pair donors contribute by algebraically increasing the $^1J_{C,F}$ values. A similar increase in $^1J_{C2,H2}$ in pyridine compared to that in benzene (Table III) is observed as noted earlier.

Table VI: Carbon–Fluorine Coupling Constants in Substituted Monofluorobenzenes[a] and in Fluoropyridines[b]

substituent	$^1J_{C,F}$ (Hz) ortho	meta
H	245.3	245.3
NO_2	264.4	250.9
NH_2	237.5	241.4
NH_3^+	248.6	247.5
OH	238.8	244.5
O^-	235.8	241.1
2-fluoropyridine	236.7	
3-fluoropyridine		255.4
2-fluoropyridine hydrochloride	263.4	
3-fluoropyridine hydrochloride		255

[a] Weigert & Roberts (1971). [b] Lichter & Wasylishen (1975).

The relative magnitudes of $^1J_{C,H}$ in related classes of aromatic heterocycles are now reasonably straightforward to predict: $^1J_{C,H}$(no adjacent nitrogen) < $^1J_{C,H}$(one adjacent nitrogen) < $^1J_{C,H}$(two adjacent nitrogens). Recall earlier in this section the $^1J_{C,H}$ values of pyrimidine and imidazole (carbon in parentheses): C5, no nitrogen atoms (168 Hz); C4, C6, and (C4 and C5), one nitrogen atom (182–190 Hz); C2 and (C2), two nitrogen atoms (206–208 Hz). It can also be seen by inspection of the $^1J_{C,H}$ values in Table V that a similar correlation exists for the nucleosides, where $^1J_{C5,H5}$ < $^1J_{C6,H6}$ < $^1J_{C2,H2}$ and $^1J_{C8,H8}$, thus providing a rationale for $^1J_{C,H}$ (purines) > $^1J_{C,H}$(pyrimidines). The $^1J_{C,F}$ values (Table II) can also be understood since $^1J_{C,F}$ values are negative values ($^1J_{C,H}$ values are positive). The nitrogen lone pair will make the same contributions to $^1J_{C,F}$ and $^1J_{C,H}$ values, so $^1J_{C,F}$ also will increase (become less negative): $^1J_{C5,F5}$ (−233 and −242 Hz) < $^1J_{C2,F2}$ (−211 Hz).

The longer range $J_{C,H}$ and $J_{C,F}$ values are more difficult to evaluate since their absolute signs are not known. In the case of 5-fluorocytidine, a significantly smaller $^2J_{C4,F5}$ was obtained compared to 5-fluorouridine although both have similar $^2J_{C6,F5}$

values. In the case of 2-fluoropurine (Thorpe et al., 1974), $^3J_{C4,F2}$ = 17.1 Hz and $^3J_{C6,F2}$ = 15.9 Hz while in 2-fluoroadenosine (Table II) $^3J_{C4,F2}$ = 20.2 Hz and $^3J_{C6,F2}$ = 21.2 Hz. These data suggest that nitrogens are affecting the absolute value of the coupling constant. However, it is not possible to say at this time if they are contributing an algebraically positive effect in these situations, since the signs of $J_{C,F}$ are not yet determined.

There are two additional long-range carbon–fluorine couplings in 2-fluoroadenosine ($^4J_{C5,F2}$ and $^5J_{C8,F2}$) and two carbon–proton couplings between the base and anomeric proton of the sugar observed in both the pyrimidines and purines. The $^4J_{C5,F2}$ value (4.0 Hz) of 2-fluoroadenosine is markedly larger than the $^4J_{C5,H2}$ value (1.3 Hz) and is larger than $^4J_{C2,F5}$ values in the fluoropyrimidines, perhaps reflecting the effect of the two nitrogens flanking C2 and one flanking C5. However, the $\Delta\delta_F$ values of C2 (pyrimidines) and C5 (purine) are unexpectedly similar. While the $\Delta\delta_F$ value of C5 (−1.688) is larger than that of C2 (−1.476) of uridine, which parallels the $^4J_{C5,F2}$ (4.0 Hz) and $^4J_{C2,F5}$ (0.5 Hz) values, the $\Delta\delta_F$ value of C2 (−1.857) of cytidine is larger than expected since the $^4J_{C2,F5}$ value (0.2 Hz) is so small. The longest range coupling observed is $^5J_{C8,F2}$ (2.6 Hz) in 2-fluoroadenosine. The significance and interpretation of this value are uncertain since the sign is unknown and no other five-bond couplings exist for comparison, but this value is unexpectedly large compared to the $\Delta\delta_F$ value of C8 (0.069 ppm).

The three-bond carbon–proton (H1′) couplings between the base and sugar are of special interest. In the pyrimidines these are $^3J_{C2,H1'}$ and $^3J_{C6,H1'}$ and in purines $^3J_{C4,H1'}$ and $^3J_{C8,H1'}$. The values of these coupling constants have been useful indicators of the conformation of the base with respect to the sugar (i.e., a measure of the dihedral angle χ) (Lemieux et al., 1972; Davies, 1976; Alderfer & Ts'o, 1976; Danyluk, 1979). In the case of the pyrimidines, these $^3J_{C,H1'}$ values are essentially invarient to base moiety, implying no detectable conformational differences exist among these four pyrimidine nucleosides. Also, the similarity of $^3J_{C4,H1'}$ and $^3J_{C8,H1'}$ in the adenosines indicates a similarity in their sugar–base angle, χ. The relationship $\Delta\Delta J = \Delta J_{obsd} - \Delta J_{free}$ has been used (Davies, 1978) to distinguish between anti and syn conformational preferences of the base moiety. The ΔJ is defined as ΔJ(pyrimidine) = $^3J_{C6,H1'} - {}^3J_{C2,H1'}$, ΔJ(purine) = $^3J_{C8,H1'} - {}^3J_{C4,H1'}$, and ΔJ_{free} = ~0.6 (uracil), ~0.9 (cytosine), and ~1.2 (adenine and guanine), where the ΔJ_{free} is that observed for free rotation of the glycosidic bond of model derivatives (Davies, 1978). Thus, a positive $\Delta\Delta J$ value indicates a higher population of anti conformers, while a negative $\Delta\Delta J$ value indicates a higher population of syn conformers. By use of the above values for ΔJ_{free} and the appropriate observed values from Table V, the calculated $\Delta\Delta J$ values for the pyrimidines are all *positive*, consistent with a preference for the anti conformation. In contrast the $\Delta\Delta J$ values for the purines are *negative*, indicating a preference for the syn conformers.

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A Carbon-13 Nuclear Magnetic Resonance Study of Aortic Lesions and Cholesteryl Ester Rich Lipoproteins from Atherosclerotic Rabbits†

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ABSTRACT: When rabbits are fed a diet supplemented with cholesterol, their plasma cholesterol levels increased markedly and they developed atherosclerosis. Most of the plasma cholesterol exists as cholesteryl esters in very low density and low-density lipoproteins. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra (at 48 °C) of lipoproteins, and of arterial lesions from cholesterol-fed rabbits, are dominated by well-resolved cholesteryl ester resonances. An analysis of their line widths shows that the cholesteryl esters are in a liquid state at this temperature. Calculations based on line widths and spin–lattice relaxation times show that the motion of the cholesteryl ester molecules is highly anisotropic; motion about the long axis of the cholesteryl moiety is 38–75 times faster than motion about the short axis. Spectra for the lipoproteins and arterial lesions show temperature-dependent line-width changes that are consistent with an order–disorder transition of the cholesteryl esters above physiological temperatures. The similarity of line widths and spin–lattice relaxation times for lipoproteins and arterial lesions indicates that their molecular organization and molecular dynamics are also similar and suggests that an appreciable fraction of the cholesteryl esters are derived from nonmetabolized lipoproteins. The phospholipid choline methyl resonance is the only one that is not the same in lipoproteins and lesions. The lipoprotein choline methyl resonance is relatively narrow (~12 Hz) at all temperatures studied, consistent with a fluid phospholipid monolayer. The same resonance for arterial lesions is 2.5 times broader. The increased line width is at least partially due to a more heterogeneous environment in the arterial lesions.

A range of biochemical and structural problems are involved in developing an understanding of disease states. For example, an understanding of atherosclerosis requires a characterization at the molecular level of normal and atherosclerotic arteries and of the structural and metabolic alterations that convert a normal artery into an atherosclerotic artery. In recent years much insight has been gained into the architecture of atherosclerotic lesions. Gross morphological differences between normal and atherosclerotic arteries of various species have been determined by electron microscopy (Bowyer et al., 1977; Veress et al., 1977; Haust, 1977; Minick et al., 1977; Gerrity et al., 1979; Weber et al., 1973). Transport of lipoproteins into the arterial wall (Hollander et al., 1977; Walton & Morris, 1977) and the abundance of arterial smooth muscle cells (Thomas et al., 1977 a,b; Stary, 1977; Holle et al., 1977) and of arterial glycosaminoglycans (Schneider et al., 1977; Toledo & Mourao, 1979) are all strongly affected by atherosclerotic complications.

Atheroslcerotic lesions are characterized by the presence of large quantities of extracellular, lipoprotein-derived cholesterol and cholesteryl esters in the intima and media of the arterial wall (Smith, 1974; VanGent & Eineis, 1977). Various investigators have studied the structure of these lipid deposits (Engelman & Hillman, 1976; Small & Shipley, 1974; Hamilton et al., 1979; Katz & Small, 1980). Engelman & Hillman (1976) have detected sharp, reversible thermotropic order–disorder transitions in human aortic samples containing fatty streak lesions. Transition temperatures ranged between 28 and 42 °C. The ordered state exhibited a sharp reflection corresponding to a Bragg spacing of about 35 Å, which is consistent with the presence of smectic[1] cholesteryl ester domains. No evidence was found for similar order–disorder transitions in normal aortae. Physical and chemical characterization of particles released from human atherosclerotic aortae by chemical methods reveal structural similarities with very low density lipoprotein (VLDL)[2] and low-density lipo-

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[1] The smectic liquid-crystalline state of cholesteryl esters is believed to consist of planar arrays stacked with a repeat distance of 36 Å. In the cholesteric liquid-crystalline state cholesteryl ester molecules are ordered in helices about an axis at right angles to the long molecular axis.

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