results support also the prediction of the allosteric model, recently proposed by Gill et al., that carbon monoxide ligation occurs first at $\alpha$ chains in the T state and then at the $\beta$ chains after the conformational transition to the R state.[18] Like CO binding which is also cooperative, $PMe_3$ binding is turning out to be a useful model for the physiologically important binding of $O_2$.

## Experimental Section

Adult human hemoglobin was prepared in the usual manner from fresh whole blood samples obtained from the local blood bank.[6] The $\alpha$ and $\beta$ subunits of human hemoglobin were separated and purified as described by Geraci et al.[19] A stock solution of sodium inositol hexaphosphate (IHP) (obtained from Sigma as the sodium salt) was added to the hemoglobin samples to a final concentration of 3.6 molar excess per Hb tetramer when required. Deoxygenated human hemoglobin solutions (1.5 mM) in the Tris-HCl buffer (0.1 M, pH 7.1) were introduced in argon-filled NMR tubes. A small amount of sodium dithionite ($\approx$1 mg) was injected in the deoxy sample to be sure it was completely deoxygenated. Small aliquots (10-60 $\mu$L) of standard phosphine solution were then added. Stock solutions containing 0.26 M $PMe_3$ were prepared by dissolving 40 mg of fresh $PMe_3$ in 2 mL of degassed water.

Visible and NMR spectra were observed concurrently at various stages of ligand binding. NMR samples were transferred anaerobically to a flushed cell usuable for optical absorbance measurements on samples with high absorbance. The percentage of ligation $\bar{Y}$ was determined by measurement at $\lambda_{max}$ = 535 ($\epsilon$ 22.8 $mM^{-1}$ $cm^{-1}$) and 560 nm ($\epsilon$ = 24.6 $mM^{-1}$ $cm^{-1}$) with extinction coefficients taken from the fully liganded form of the protein. The $^1H$ and proton decoupled $^{31}P$ NMR spectra were recorded in a pulse Fourier transform mode with a Brucker AM 300 WB spectrometer operating at 300 MHz for $^1H$ and at 121.49 MHz for $^{31}P$. In $^1H$ NMR experiments, lines were drawn along the sides of each resonance, giving an essentially triangular representation of the area of each resonance. The area was then measured with a planimeter. The probe temperature was maintained at 20 °C. $^1H$ and $^{31}P$ chemical shifts were respectively measured with respect to (trimethylsilyl)propanesulfonic acid and $H_3PO_4$ external standards. The chemical shift scales are defined as positive in the low-field direction with respect to the references.

Ultraviolet-visible spectra were recorded with a Jobin Hitachi spectrometer. The pH values of the samples were measured on a Bioblock Model 93301 pH meter equipped with a Schott electrode (A 90438).

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**Registry No.** $PMe_3$, 594-09-2.

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# NMR Studies of Nucleic Acids. Deuterium Isotope Effects on $^{13}C$ Chemical Shifts in Hydrogen-Bonded Complexes of Pyrimidines and Purines

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**Abstract:** $^{13}C$ NMR spectra were recorded for chloroform solutions of 2',3'-*O*-isopropylidene-5'-*O*-acetyladenosine (**1**), 2',3',5'-*O*-tribenzoyluridine (**2**), 2',3'-*O*-isopropylidene-5'-*O*-acetyluridine (**2a**), 2',3'-*O*-isopropylidene-5'-*O*-(*tert*-butyldimethylsilyl)guanosine (**3**), and 2',3'-*O*-isopropylidene-5'-*O*-(*tert*-butyldimethylsilyl)cytidine (**4**) in which the imino hydrogens were partially exchanged with deuterium. Upfield two-bond deuterium isotope effects (DIE) on $^{13}C$ chemical shifts were detected under conditions of slow exchange as multiple peaks for the appropriate resonances and ranged in magnitude from 40 ppb for the amino interaction with C2 in guanosine to 217 ppb for the imino interaction with C4 in the uridine self-association dimer. $^{13}C$ chemical shifts and DIEs for **2** were measured at 12 different concentrations from 219 to 231 K. The data were used in an iterative procedure to estimate chemical shifts at C2 and C4 for monomeric and dimeric forms of **2**, equilibrium constants and enthalpies for self-association, and the distribution of isomeric self-association dimers. Enthalpies for formation of hydrogen bonds to C2 and C4 in **2** were similar, $\Delta H$ = -1.8 kcal/mol. DIEs at C2 and C4 increased upon formation of a hydrogen bond to the carbonyl oxygens. The maximal increase for each center was estimated to be 90 ppb. Small increases were also observed in DIEs when nucleosides **1-4** were mixed with their complementary bases.

Hydrogen bonds are responsible for much of the specificity seen in the conformations of proteins[1] and nucleic acids,[2] in enzyme-substrate binding,[3,4] and in replication,[5] transcription,[6] and translation[7] of the genetic code. Of the many different techniques that have been employed to study hydrogen bonds during the past 20 years, NMR spectroscopy has proved to be especially useful. The chemical shifts of magnetically active nuclei, such as $^1H$, $^{13}C$, and $^{15}N$, often show substantial displacements when groups bearing these atoms engage in hydrogen bonding.[8-10] This information is useful for detecting specific hydrogen bonds and studying the thermodynamic properties of hydrogen-bonded complexes.

NMR techniques have been widely used to study the structures of nucleic acids. In a pioneering study, Katz and Penman[11]

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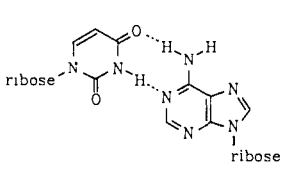
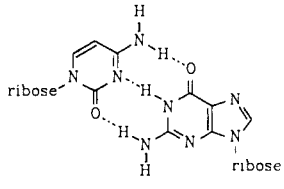
discovered large downfield shifts for the resonances of imino and amino protons in guanosine and cytidine upon formation of base pairs in dimethyl sulfoxide. Shortly afterward, Newmark and Cantor[12] used $^1H$ chemical shifts to obtain equilibrium constants and heats of formation for the same system. During the past 10 years, $^1H$, $^{13}C$, and $^{15}N$ NMR studies of hydrogen bonds in RNAs, DNAs, and DNA–protein complexes have provided a wealth of new information about the structures and stabilities of these systems.[13,14]

Substitution of a proton by deuterium can produce substantial changes in $^{13}C$ chemical shifts.[15] Although the deuterium isotope effect (DIE) is normally largest for directly bonded protons, significant perturbations are often seen for nuclei separated by two and three bonds. In some aromatic systems the effect has been found to operate over six bonds.[16] Amino and amide protons generate two-bond isotope effects to carbons attached to the nitrogen that range from approximately 0.1 ppm for amide carbonyl carbons[17,18] to up to 0.3 ppm for amine carbons.[19] When these protons are exchanging slowly and the nitrogen is partially deuteriated, a cluster of closely spaced peaks, each representing a different isotopically substituted species, appears at the normal carbon resonance position in a $^1H$-decoupled $^{13}C$ spectrum. The isotope effect on $^{13}C$ chemical shifts can be determined with a high degree of accuracy by simply measuring the differences between the positions of the individual peaks.

The magnitude of isotope-induced shifts often increases upon formation of hydrogen bonds.[15] In some systems, for example where phenolic or enolic hydroxyls form intramolecular hydrogen bonds, two-bond DIEs can be as large as 1.5 ppm. However, more typical values are in the 50–300 ppb range. Reuben[20] noted that the magnitudes of the isotope effects were directly proportional to the strengths of hydrogen bonds for enolic and phenolic hydroxyls and for aromatic amines and developed empirical correlations of two-bond DIEs versus hydrogen bond energy. Recently he extended the study to the imino and amino hydrogen bonds for the complementary base pairs guanosine (G)–cytidine (C) and adenosine (A)–uridine (U) in DMSO.[21] Two-bond DIEs were




A–U




G–C

then used to estimate the strengths of individual hydrogen bonds in the dimeric complexes. In addition, Reuben reported that the imino proton in uridine exchanged rapidly in DMSO and suggested that exchange was the result of a coordinated tautomerization of both partners in an uridine self-association dimer to the less stable hydroxyimine form.

We were concerned by those aspects of Reuben's study related to uridine. DMSO is a strong acceptor disrupting uridine hydrogen bonds in the U–U self-association complex and A–U dimers. Furthermore, we[9] and other[22,23] had observed the uridine imino proton in slow exchange over a wide range of solute concentrations in chloroform, a nonpolar solvent that promotes formation of intermolecular hydrogen bonds between nucleosides. We now report a study in chloroform of two-bond deuterium isotope effects on chemical shifts of carbons attached to imino and amino moieties in A, U, G, and C and in complementary base pairs.

## Experimental Section

**Materials.** 2′,3′-Isopropylidene-5′-*O*-acetyladenosine (**1**) and 2′,3′-isopropylidene-5′-*O*-acetyluridine (**2a**) were purchased from Sigma. Deuteriochloroform was purchased from Stohler Isotopes, and $D_2O$, from Cambridge Isotopes. [2-$^{13}C$]Uridine was obtained from Merck, Sharp, and Dohme Isotopes, and [4-$^{13}C$]uridine was available from an earlier study in this laboratory. All other compounds were purchased from Aldrich Chemical Co.

**Synthesis of Protected Nucleosides.** 2′,3′,5′-Tri-*O*-benzoyl[2-$^{13}C$]-uridine ([2-$^{13}C$]**2a**) and 2′,3′,5′-tri-*O*-benzoyl[4-$^{13}C$]uridine ([4-$^{13}C$]**2a**) were prepared from the labeled nucleosides by the procedure of Roberts and Poulter.[24] 2′,3′-Isopropylidene-5′-*O*-(*tert*-butyldimethylsilyl)-guanosine (**3**) and 2′,3′-isopropylidene-5′-*O*-(*tert*-butyldimethylsilyl)cytidine (**4**) were prepared from the 2′,3′-isopropylidene derivatives by the procedure of Ogilvie and co-workers.[25]

**Preparation of NMR Samples.** Samples containing protium and deuterium at exchangeable sites were prepared by addition of the nucleoside or mixtures of nucleosides, 0.5 mL of 99% deuterium oxide, and 0.5–1.0 mL of absolute ethanol to 1 mL of deuteriochloroform. The components were mixed by magnetic stirring for 4 h. The solution was dried over magnesium sulfate, and solvent was removed at reduced pressure. The resulting gum was placed under high vacuum (<0.02 mmHg) at room temperature for 24 h. The dry white foam was dissolved in deuteriochloroform and transferred by pipet to a dry Wilmad 528 NMR sample tube. Subsequent dilutions were made from the original sample.

**Acquisition of NMR Data.** $^1H$ NOE difference and $^{13}C$ NMR spectra were collected on Varian VXR-500 and XL-400 NMR spectrometers, respectively. Low-power broad-band proton decoupling was accomplished by WALTZ-16 modulation.[26] $T_1$'s were measured for the isotopic doublet at position 4 of 2′,3′-isopropylidene-5′-*O*-acetyluridine at 25 °C by the inversion/recovery method.[27] $T_1$ was 2.6 ± 0.2 s for the protonated carbon and 3.1 ± 0.1 s for the deuteriated carbon. An Ernst angle calculation[28] based on a measured 90° pulse of 14 μs, a 3-s acquisition time, and a 0.1-s delay between transients gave an optimized pulse width of 10.8 μs. A 9624.6-Hz spectral window was scanned, and 57 728 points were sampled for a digital resolution of 0.167 Hz (1.3 ppb). Widths at half-height for the isotopic doublets for C2 and C4 were ≤1.1 Hz. Probe temperature was calibrated to ±1 °C with the Varian TEMCAL program and a methanol standard. Samples were allowed to equilibrate for 15 min before acquisition of data, and probe temperature was controlled to ±0.1 °C. Routine $^1H$ NMR spectra were obtained at ambient temperature on a Varian XL-300 NMR spectrometer.

## Results and Discussion

**Nucleosides.** Two-bond deuterium isotope effects on $^{13}C$ chemical shifts were measured in chloroform, a nonpolar aprotic solvent with a low dielectric constant ($\epsilon^{213K} = 6.8$, $\epsilon^{243K} = 4.8$) that we[9,10] and others[8,18,22,23,29] used to successfully study base pairing at the nucleoside level. Partially deuteriated samples of 2′,3′-*O*-isopropylidene-5′-*O*-acetyladenosine (**1**), 2′,3′-*O*-isopropylidene-5′-acetyluridine (**2a**), 2′,3′-*O*-isopropylidene-5′-*O*-(*tert*-butyldimethylsilyl)guanosine (**3**), and 2′,3′-*O*-isopropylidene-5′-*O*-(*tert*-butyldimethylsilyl)cytidine (**4**) were prepared by dissolving the nucleosides in a mixture of chloroform, ethanol, and deuterium oxide. Solvent was removed at reduced pressure, and the residue was placed under vacuum (<0.02 mmHg) for 24 h to remove the last traces of water. The material was then dissolved in deuteriochloroform for NMR measurements. Samples prepared in this manner gave well-defined signals for

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Table I. $^2\Delta$ Deuterium-Induced $^{13}C$ Chemical Shifts[a]

| nucleoside | resonance | type | $\delta[^{13}C(^1H)]$ | DIE, ppb this study | DIE, ppb Reuben[21] |
|---|---|---|---|---|---|
| 1 | C6 | amino | 155.688 | 61 | 65 |
| 2 | C2 | imino | 149.664 | 87 | *b* |
| | C4 | imino | 163.724 | 121 | *b* |
| 3 | C2 | amino | 153.536 | 40 | 41 |
| | | imino | | 102 | 82 |
| | C6 | imino | 158.739 | 123 | 90 |
| 4 | C4 | amino | 165.650 | 72 | 73 |

[a] Measured in $CDCl_3$ at 219 K; concentration, 0.28 M. [b] Not observed.

amino and imino protons, and $^1H$-decoupled $^{13}C$ spectra had isotopically shifted resonances for carbons attached to amino and imino nitrogens. Absolute values of chemical shifts for protonated and deuteriated carbons varied slightly from sample to sample, but differences between the two were highly reproducible.

Some typical spectra are shown in Figure 1. In samples containing an excess of deuterium, we could unambiguously assign chemical shifts to protonated and deuteriated species on the basis of the relative intensities of the peaks. In each case, deuteriation produced an upfield shift. C2 and C4 in **2a** (Figure 1A) and C6 in **3** gave simple doublet patterns expected for carbons attached to protonated and deuteriated imino nitrogens. Two-bond deuterium-induced $^{13}C$ isotope effects were measured directly from the separation between the peaks. Deuterium-induced shifts produced triplet patterns at C6 in **1** and C4 in **4** (Figure 1B) where the carbons were directly attached to amino nitrogens. The isotope effect was additive in these cases, and the differences in $^{13}C$ shifts for $NH_2$, NHD, and $ND_2$ species were identical. The intensities of the three peaks were directly related to the relative abundance of each isotopic species. In nucleoside **3**, C2 is directly attached to both amino and imino nitrogens, and the combined deuterium isotope effects gave a doublet of triplets, where the imino DIE was greater than the amino DIE (Figure 1C). For all of the nucleosides studied to date, imino DIEs are larger than amino DIEs. The data for **1**–**4** are summarized in Table I.

DIEs for the amino moieties in **1**, **3**, and **4** measured by us were similar to those reported by Reuben for the corresponding underivatized nucleosides in DMSO.[21] We found, however, significantly larger DIEs at both C2 and C6 in G. In chloroform at a concentration of 0.28 M, the G–G self-association dimer is the predominate species, and the differences between our values and those in DMSO may reflect disruption of G–G pairs in the more polar solvent. The major discrepancy between the two studies was for U. In contrast to Reuben's findings in DMSO, we saw a well-defined peak for the uridine imino proton in **2a**. The signal broadened and eventually disappeared when protic solvents were added to the chloroform solution, but for dry samples, the imino resonance was observed over a wide range of temperatures.

Reuben could not measure DIEs for uridine in DMSO because the imino proton was in rapid exchange. As mentioned above, we saw a well-defined peak for the imino proton in $^1H$ spectra and isotopically shifted peaks for C2 and C4 in $^{13}C$ spectra. $^{13}C$ spectra of a 0.17 M solution of [4-$^{13}C$]**2** were recorded in chloroform at temperatures from 233 to 323 K. In each case, the C4 resonance appeared as a sharp doublet. The observed DIE was inversely proportional to temperature and decreased from 114 ppb at 233 K to 77 ppb at 323 K. These limiting values are similar to those obtained for dimeric and monomeric **2**, respectively, from our study of self-association described below and probably reflect an increasing proportion of monomer in the equilibrium mixture. The mechanism Reuben proposed for rapid exchange of the imino proton of uridine in DMSO involved the simultaneous tautomerization of the uracil moieties to the hydroxyimine form, followed by dissociation of the dimer and retautomerism to the imino form.[21] It seems unlikely to us that DMSO would selectively stabilize the minor tautomeric forms within the U dimer to the extent needed to explain the differences in exchange rates found in the two solvents, and we suggest that exchange occurred at the monomer level in the DMSO/methanol system used by Reuben.

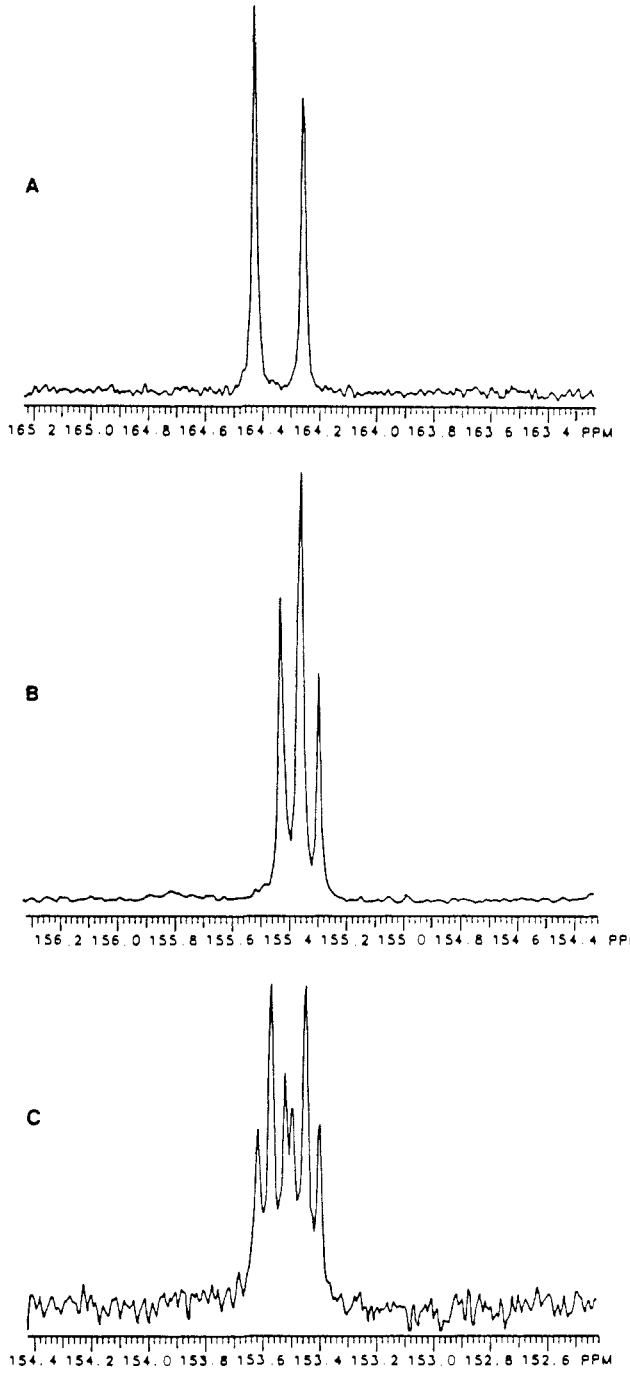


Figure 1. 100-MHz $^1H$-decoupled $^{13}C$ spectra of 0.28 M solutions of protected nucleosides in deuteriochloroform with half of the labile hydrogens exchanged by deuterium: (A) expansion about C4 of **2a**; (B) expansion about C6 of **1**; (C) expansion about C2 of **3**.

**Uridine–Uridine Self-Association.** Several studies of uridine and related derivatives indicate that the nucleoside forms dimeric self-association complexes in chloroform.[8,9,22,30-33] In the most stable structures, the monomeric units are linked by two hydrogen bonds. As shown in Chart I, three different dimers can be formed by utilizing the imino proton at N3 and the carbonyl moieties at C2 or C4 in hydrogen bonds. The monomeric units in the other dimers or higher polymers linked by a single hydrogen bond do not constitute a significant portion of the equilibrium mixture.

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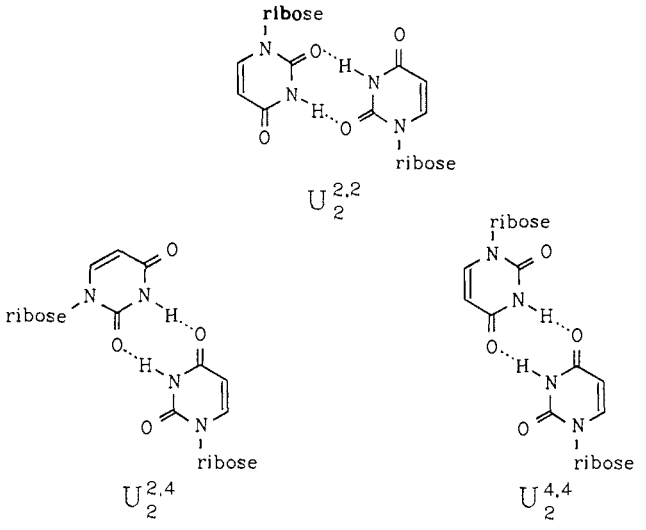
**Chart I.** Self-Association Dimers of Uridine




The formation of self-association dimers is easily detected by $^{13}C$ NMR. For example, Iwahashi and Kyogoku[8] measured $^{13}C$ chemical shifts for cyclohexyluracil as a function of concentration in chloroform and reported substantial downfield displacements for the C2 and C4 resonances upon formation of a hydrogen bond to the carbonyl oxygen.

In order to determine DIEs in monomeric and dimeric uridine, we measured deuterium-induced $^{13}C$ chemical shifts over a range of concentrations. To increase sensitivity at low concentrations, [2-$^{13}$C]- and [4-$^{13}$C]**2** were prepared from labeled uridine. The compounds were mixed in equal proportions, and $^{13}C$ chemical shifts of the labeled carbons were measured in chloroform at 12 different concentrations from 9.3 to 88 mM at each of five different temperatures. The results are available as supplementary material. For each set of measurements, downfield shifts were seen as concentrations were increased or temperature was decreased. These trends are consistent with an increase in the equilibrium concentrations of self-association dimers. Under these conditions the observed shifts of C2 and C4 are weighted averages of the monomer shifts and the shifts of the respective carbons in $U_2^{2,2}$, $U_2^{2,4}$, and $U_2^{4,4}$.

For uridine (U), the equilibrium expression for self-association can be written as

$$U + U \rightleftarrows xU_2^{2,2} + yU_2^{2,4} + zU_2^{4,4} \quad (1)$$

where

$$x + y + z = 1 \quad (2)$$

The equilibrium constant for self-association is

$$K = [U_2^{2,2}]^x[U_2^{2,4}]^y[U_2^{4,4}]^z/[U]^2 \quad (3)$$

where $f_m$ is the mole fraction of monomeric U and $f_d$ is the mole fraction of total dimeric U.

$$f_m + (x + y + z)f_d = 1 \quad (4)$$

The concentrations of individual species are

$$[U] = f_m U_0 \quad (5)$$

$$[U_2^{2,2}] = xf_d U_0/2 \quad (6)$$

$$[U_2^{2,4}] = yf_d U_0/2 \quad (7)$$

$$[U_2^{4,4}] = zf_d U_0/2 \quad (8)$$

where $U_0$ is the total nucleoside concentration. Upon substitution, eq 3 becomes

$$K_{app} = f_d/2f_m^2 U_0 \quad (9)$$

where $K_{app} = K/x^x y^y z^z$. Constants for the individual equilibria are

$$K^{2,2} = [U_2^{2,2}]/[U]^2 = xK_{app} \quad (10)$$

$$K^{2,4} = [U_2^{2,4}]/[U]^2 = yK_{app} \quad (11)$$

$$K^{4,4} = [U_2^{4,4}]/[U]^2 = zK_{app} \quad (12)$$

As mentioned above, under conditions of rapid exchange the observed $^{13}C$ chemical shifts at positions 2 and 4 are weighted averages of the monomer shifts and shifts of the respective carbons in $U_2^{2,2}$, $U_2^{2,4}$, and $U_2^{4,4}$. Iwahashi and Kyogoku[8] studied $^{13}C$ chemical shifts in uridine and several derivatives of uridine and discovered that the resonances for C2 and C4 moved downfield upon formation of self-association dimers. They also found that resonances for C5 and C6 did not vary substantially and concluded that formation of hydrogen bonds in uridine only significantly altered the chemical shift of the resonance for the carbonyl carbon directly involved. On the basis of these assumptions, the observed chemical shift, $\delta_{obs}$, is a weighted average of monomer, $\delta_m$, and dimer, $\delta_d$, shifts. However, in the self-association dimer, only one of the two carbonyl moieties is engaged in hydrogen bonding. Thus, the chemical shifts of C2 and C4 approach an apparent dimeric value, $\delta_d$(app), as [U] $\rightarrow \infty$, which is the intrinsic dimeric shift, $\delta_d$, corrected for the fraction of dimers with hydrogen bonds to C2 or C4 in the equilibrium mixture of $U_2^{2,2}$, $U_2^{2,4}$, and $U_2^{4,4}$. Thus

$$\delta_{obs}{}^{C2} = f_m\delta_m{}^{C2} + f_d\delta_d{}^{C2}(app) \quad (13)$$

and

$$\delta_{obs}{}^{C4} = f_m\delta_m{}^{C4} + f_d\delta_d{}^{C4}(app) \quad (14)$$

The apparent dimeric chemical shifts are

$$\delta_d{}^{C2}(app) = f_{C2}(\delta_d{}^{C2} - \delta_m{}^{C2}) + \delta_m{}^{C2} \quad (15)$$

and

$$\delta_d{}^{C4}(app) = f_{C4}(\delta_d{}^{C4} - \delta_m{}^{C4}) + \delta_m{}^{C4} \quad (16)$$

The fraction of total C2 carbonyls engaged in hydrogen bonding, $f_{C2}$, is determined by the relative amounts of $U_2^{2,2}$, in which both are bonded, and $U_2^{2,4}$, in which only one C2 carbonyl is hydrogen bonded. Thus

$$f_{C2} = x + y/2 \quad (17)$$

and in a similar manner $f_{C4}$, the fraction of C4 carbonyls engaged in hydrogen bonding, is given by

$$f_{C4} = y/2 + z \quad (18)$$

Although the observed chemical shift for C2 does not depend on $U_2^{4,4}$ directly, it indirectly measures the equilibrium between monomeric U and all three dimers because the relative proportions of $U_2^{2,2}$, $U_2^{2,4}$, and $U_2^{4,4}$ do not change with concentration. The same holds for C4. Thus, plots of C2 and C4 chemical shifts as a function of concentration should have the same form. The maximal $^{13}C$ shifts observed at high concentrations, where $f_d \rightarrow 1$, are, however, attenuated. Substitution of $f_m$ or $f_d$ in eq 13 and 14, where $f_m + f_d = 1$, gives general expressions (eq 19 and 20) for the mole fractions of monomer and dimer regardless of whether the equilibrium is monitored at C2 or C4.

$$f_m = (\delta_d(app) - \delta_{obs})/(\delta_d(app) - \delta_m) \quad (19)$$

$$f_d = (\delta_{obs} - \delta_m)/(\delta_d(app) - \delta_m) \quad (20)$$

Chen and Shirts[34] recently developed an iterative algorithm to calculate equilibrium constants for self-association ($K$), monomer chemical shifts, and dimer chemical shifts in a system undergoing rapid interconversion. Since $\delta_d$(app) measures total dimer concentration in equilibrium with monomeric U, substitution of eq

(34) Chen, J.; Shirts, R. B. *J. Phys. Chem.* **1985**, *89*, 1643–1646.

Table II. Monomeric and Apparent Dimeric $^{13}C$ Chemical Shifts and Self-Association Constants for **2**

| isotope | T, K | $^{13}C$ chemical shifts | | | | $K_{app}$, $M^{-1}$ | | |
|---|---|---|---|---|---|---|---|---|
| | | $\delta_m^{C2}$ | $\delta_d^{C2}$(app) | $\delta_m^{C4}$ | $\delta_d^{C4}$(app) | C2 | C4 | av |
| $^1H$ | 219 | 149.356 | 150.420 | 162.039 | 164.430 | 41 | 40 | 41 |
| $^2H$ | 219 | 149.296 | 150.331 | 161.966 | 164.298 | 40 | 40 | 40 |
| $^1H$ | 231 | 149.399 | 150.446 | 162.022 | 164.371 | 25 | 23 | 24 |
| $^2H$ | 231 | 149.346 | 150.365 | 161.948 | 164.238 | 24 | 23 | 24 |
| $^1H$ | 243 | 149.444 | 150.473 | 161.953 | 164.251 | 16 | 16 | 16 |
| $^2H$ | 243 | 149.385 | 150.385 | 161.871 | 164.109 | 16 | 16 | 16 |
| $^1H$ | 255 | 149.480 | 150.462 | 161.885 | 164.075 | 12 | 12 | 12 |
| $^2H$ | 255 | 149.422 | 150.370 | 161.816 | 163.940 | 12 | 12 | 12 |
| $^1H$ | 267 | 149.528 | 150.446 | 161.851 | 163.927 | 8.5 | 8.5 | 8.5 |
| $^2H$ | 267 | 149.472 | 150.364 | 161.788 | 163.801 | 8.3 | 8.4 | 8.4 |

19 and 20 into eq 9 and taking the square root of the resulting expression gives

$$\delta_{obs} = \delta_d(app) \pm \left[\frac{|\delta_d(app) - \delta_m|}{2K_{app}}\right]^{1/2}\left[\frac{|\delta_{obs} - \delta_m|}{[U_0]}\right]^{1/2} \quad (21)$$

For convenience during iterations, the second square root term is defined as

$$X = \left[\frac{|\delta_{obs} - \delta_m|}{[U_0]}\right]^{1/2} \quad (22)$$

Also from the Chen and Shirts derivation

$$f_d = \frac{(1 + 8K_{app}[U_0])^{1/2} - 1}{(1 + 8K_{app}[U_0])^{1/2} + 1} \quad (23)$$

Since $f_m = 1 - f_d$, substitution for $f_m$ in a general form of eq 13 gives

$$\delta_{obs} = \delta_m + f_d(\delta_d(app) - \delta_m) \quad (24)$$

and substitution for $f_d$ gives

$$\delta_{obs} = \delta_m + (\delta_d(app) - \delta_m)\left[\frac{(1 + 8K_{app}[U_0])^{1/2} - 1}{(1 + 8K_{app}[U_0])^{1/2} + 1}\right] \quad (25)$$

From eq 25 it can be seen that a plot of $\delta_{obs}$ versus $f_d$ will be linear for accurate values of $K_{app}$, and the intercept is $\delta_m$. When accurate values of $\delta_m$ are used in eq 22 to calculate $X$, a plot of $\delta_{obs}$ versus $X$ will also be linear (see eq 21) with an intercept at $\delta_d$(app) and a slope from which a value for $K_{app}$ can be calculated based on $\delta_d$(app) and $\delta_m$ from the preceding step.

We used a computer program to calculate $f_d$ (eq 23) for initial values of $K_{app}$ at different $[U_0]$'s. A linear regression of $\delta_{obs}$ versus $f_d$ (eq 23–25) gave estimates of $\delta_m$. These monomeric shifts were then used to calculate $X$ at given values for $[U_0]$, which were then used in a linear regression of $\delta_{obs}$ versus $X$ (see eq 21) to calculate $\delta_d$(app). This procedure was repeated with incremental changes in $K_{app}$ until the correlation coefficients for plots of $\delta_{obs}$ versus $X$ reached a maximal value. The values of $\delta_m$, $\delta_d$(app), and $K_{app}$ are assumed to be the iteratively converged values. A second iterative cycle with increments in $\delta_m$ was performed using the converged association constant in the first cycle and a trial value for $\delta_m$. Converged values for $\delta_m$, $\delta_d$(app), and $K_{app}$ based on C2 and C4 in **2** are listed in Table II, and examples of plots of $\delta_{obs}$ for C2 and C4 of protonated and deuteriated **2** with theoretical lines are given in Figure 2. The observed chemical shifts for **2** were referenced to the center line in deuteriochloroform, and $\delta_m$ and $\delta_d$(app) show a small temperature dependence between 219 and 267 K. Values for $K_{app}$ calculated from chemical shifts at C2 are similar to those from C4. Deuterium isotope effects at C2 and C4 are given in Table III.

In all cases the magnitudes of changes in chemical shifts and deuterium isotope effects with concentration were greater at C4 than C2. As pointed out in eq 15 and 16, $\delta_d$(app) is a composite of the intrinsic dimeric shift, $\delta_d$, for C2 or C4 and a term for the fraction of dimer bound at that position. There are no direct measurements of $\delta_d^{C2}$ or $\delta_d^{C4}$. However, from their study of $^{13}C$

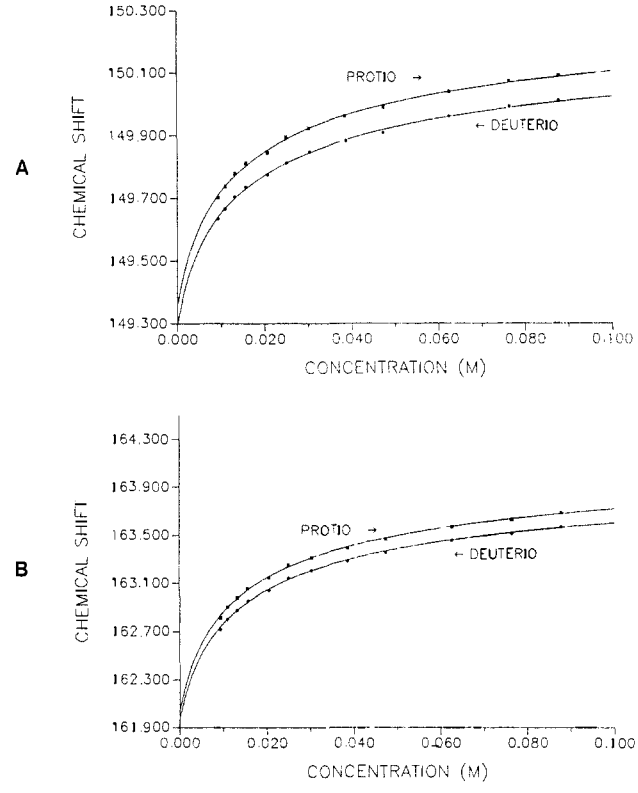



**Figure 2.** Plots of $^{13}C$ chemical shifts versus concentration for positions 2 (A) and 4 (B) of 2′,3′,5′-tri-*O*-benzoyluridine in deuteriochloroform at 219 K. Spectra were obtained on a Varian XL-400 spectrometer at 100 MHz utilizing WALTZ-16 low-power broad-band proton decoupling. Protio and deuterio refer to the imino hydrogen two bonds removed from the carbon resonance. The theoretical curve was calculated from eq 25 and converged values for $\delta_m$, $\delta_d$(app), and $K_{app}$.

Table III. Deuterium Isotope Effects on $^{13}C$ Chemical Shifts at C2 and C4 in **2**

| T, K | deuterium isotope effects, ppb | | | |
|---|---|---|---|---|
| | $DIE_m^{C2}$ | $DIE_d^{C2}$(app) | $DIE_m^{C4}$ | $DIE_d^{C4}$(app) |
| 219 | 60 | 89 | 73 | 132 |
| 231 | 53 | 81 | 74 | 133 |
| 243 | 59 | 88 | 82 | 142 |
| 255 | 58 | 92 | 69 | 135 |
| 267 | 56 | 82 | 63 | 126 |
| av | 57 ±3 | 86 ± 5 | 72 ± 7 | 134 ± 6 |

chemical shifts of a variety of different cyclohexyluridines during self-association in chloroform Iwahashi and Kyogoku[8] concluded that the magnitude of the downfield shifts for resonances at C2 and C4 upon formation of a hydrogen bond to the carbonyl oxygen were similar: i.e., $\Delta_\delta^{C2} = \delta_d^{C2} - \delta_m^{C2} \approx \Delta_\delta^{C4} = \delta_d^{C4} - \delta_m^{C4}$. Thus

$$f_{C2} \approx \Delta_\delta^{C2}(app)/(\Delta_\delta^{C2}(app) + \Delta_\delta^{C4}(app)) \quad (26)$$

and

$$f_{C4} \approx \Delta_\delta^{C4}(app)/(\Delta_\delta^{C2}(app) + \Delta_\delta^{C4}(app)) \quad (27)$$

**Table IV.** Differences between Monomeric and Apparent Dimeric $^{13}C$ Chemical Shifts for C2 and C4 in **2**

| isotope | $T$, K | $\Delta_\delta^{C2}$(app) | $\Delta_\delta^{C4}$(app) | $f_{C2}$ | $f_{C4}$ |
|---|---|---|---|---|---|
| $^1H$ | 219 | 1.064 | 2.391 | 0.31 | 0.69 |
| $^2H$ | 219 | 1.035 | 2.332 | 0.31 | 0.69 |
| $^1H$ | 231 | 1.047 | 2.349 | 0.31 | 0.69 |
| $^2H$ | 231 | 1.019 | 2.290 | 0.31 | 0.69 |
| $^1H$ | 243 | 1.029 | 2.298 | 0.31 | 0.69 |
| $^2H$ | 243 | 1.000 | 2.238 | 0.31 | 0.69 |
| $^1H$ | 255 | 0.982 | 2.190 | 0.31 | 0.69 |
| $^2H$ | 255 | 0.948 | 2.124 | 0.31 | 0.69 |
| $^1H$ | 267 | 0.918 | 2.076 | 0.31 | 0.69 |
| $^2H$ | 267 | 0.892 | 2.013 | 0.31 | 0.69 |

**Table V.** Differences between Monomeric and Apparent Dimeric Deuterium Isotope Effects for C2 and C4 in **2**

| $T$, K | $\Delta_{DIE}^{C2}$ | $\Delta_{DIE}^{C4}$ | $f_{C2}$ | $f_{C4}$ |
|---|---|---|---|---|
| 219 | 29 | 59 | 0.33 | 0.67 |
| 231 | 28 | 59 | 0.32 | 0.68 |
| 243 | 29 | 60 | 0.33 | 0.67 |
| 255 | 34 | 66 | 0.34 | 0.66 |
| 267 | 26 | 63 | 0.29 | 0.71 |
| av | 29 ± 3 | 61 ± 3 | 0.32 ± 0.02 | 0.68 ± 0.02 |

where $\Delta_\delta^{C2} + \Delta_\delta^{C4}$ is the maximal shift in the resonance of C2 or C4 upon formation of a hydrogen bond.

If one assumes that the strengths of the hydrogen bonds to C2 and to C4 are, respectively, the same in $U_2^{2,2}$, $U_2^{2,4}$, and $U_2^{4,4}$, the difference in free energy between $U_2^{2,2}$ and $U_2^{2,4}$ represents the difference between a C2 ($\Delta G^2$) and a C4 ($\Delta G^4$) hydrogen bond as does the difference between $U_2^{2,4}$ and $U_2^{4,4}$. Thus

$$\Delta G^4 - \Delta G^2 = -RT \ln K^{2,4} + RT \ln K^{2,2} = -RT \ln K^{4,4} + RT \ln K^{2,4} \quad (28)$$

and

$$K^{2,2}/K^{2,4} = K^{2,4}/K^{4,4} \quad (29)$$

Substitution of values for the equilibrium constants given in eq 10–12 gives

$$x/y = y/z \quad (30)$$

Solving eq 17 for $x$ and eq 18 for $z$ and substituting both expressions into eq 30 then gives

$$y^2 + 0.667y - 1.333 f_{C2} f_{C4} = 0 \quad (31)$$

Using values for $f_{C2}$ and $f_{C4}$ given in Table IV and eq 2, 17 (or 18), and 31 gives $x = 0.16$, $y = 0.30$, and $z = 0.54$. Although monomeric and apparent dimeric $^{13}C$ chemical shifts changed slightly with temperature, the fractions $f_{C2}$ and $f_{C4}$ remained constant, and the relative concentrations of the three self-association dimers did not change with temperature.

The magnitudes of deuterium-induced isotope effects on $^{13}C$ chemical shifts in **2** increased upon self-association, and $f_{C2}$ and $f_{C4}$ can also be calculated from DIEs in a manner similar to that described for chemical shifts. Although there is somewhat more scatter in the data, the mole fractions determined from DIEs shown in Table V are also invariant with temperature and the composition of the dimeric mixture is indistinguishable from that obtained from chemical shifts.

The equilibrium constants $K^{2,2}$, $K^{2,4}$, and $K^{4,4}$ given in Table VI were calculated from $K_{app}$ and the values for $x$, $y$, and $z$ obtained from $^{13}C$ chemical shifts. Since, as mentioned above, $f_{C2}$ and $f_{C4}$ were invarient with temperature, the relative proportions of the three self-association dimers were also invariant, and van't Hoff plots for $K_{app}$, $K^{2,2}$, $K^{2,4}$, and $K^{4,4}$ had similar slopes. Thus, the enthalpies for the equilibria are the same, and differences in the relative concentration of $U^{2,2}$, $U^{2,4}$, and $U^{4,4}$ reflect small differences in entropy (see Table VII). The enthalpy and entropy for self-association of **2** calculated from $K_{app}$ compare favorably

**Table VI.** Equilibrium Constants for Self-Association of **2**

| $T$, K | $K_{app}$ | $K^{2,2}$, $M^{-1}$ | $K^{2,4}$, $M^{-1}$ | $K^{4,4}$, $M^{-1}$ |
|---|---|---|---|---|
| 219 | 40 | 6.4 | 12 | 22 |
| 231 | 23 | 3.7 | 6.9 | 12 |
| 243 | 16 | 2.6 | 4.8 | 8.6 |
| 255 | 12 | 1.9 | 3.6 | 6.5 |
| 267 | 8.5 | 1.4 | 2.6 | 4.6 |
| 298 | 4.1[a] | 0.66[a] | 1.2[a] | 2.2[a] |

[a] Extrapolated values assuming $x = 0.16$, $y = 0.30$, and $z = 0.54$.

**Table VII.** Enthalpies and Entropies for Self-Association

| dimer | $-\Delta H$, kcal/mol | $-\Delta S$, eu |
|---|---|---|
| 2,2 | 3.6 | 13 |
| 2,4 | 3.6 | 12 |
| 4,4 | 3.6 | 11 |
| app | 3.7 | 9 |

**Table VIII.** Deuterium Isotope Effects on $^{13}C$ Chemical Shifts in AU and GC Complexes

| | | | | DIE,[a] ppb | | |
|---|---|---|---|---|---|---|
| complex | nucleoside | resonance | type | complex | nucleoside | diff |
| **1–2** | **1** | C6 | amino | 80 | 61 | 19 |
| | **2** | C2 | imino | 110 | 86 | 24 |
| | | C4 | imino | 181 | 121 | 60 |
| **3–4** | **3** | C2 | amino | 50 | 40 | 10 |
| | | | imino | 120 | 102 | 18 |
| | | C6 | imino | 130 | 123 | 7 |
| | | C4 | amino | 90 | 72 | 18 |

[a] Measured in $CDCl_3$ at 219 K. Total nucleoside concentration, 0.28 M.

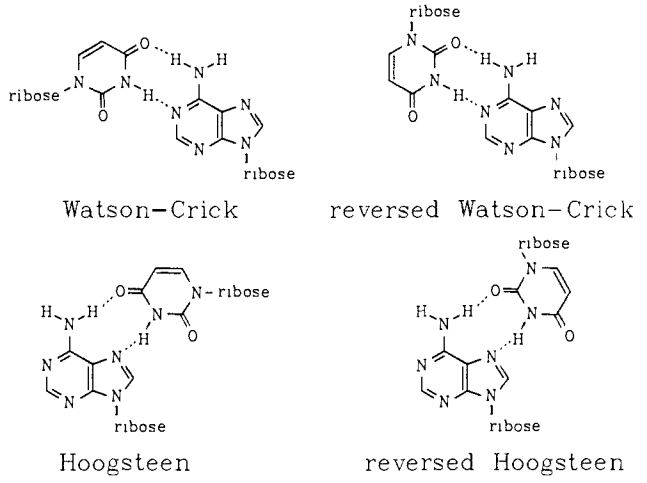
with values for cyclohexyluracil obtained by Kyogoku and coworkers[33] from infrared measurements ($\Delta H = -4.3$ kcal/mol, $\Delta S = -11$ eu) and by Hammes and Park[31] ($\Delta H = -4.0$ kcal/mol, $\Delta S = -14$ eu) from kinetic studies. Since each dimer has two hydrogen bonds, the enthalpy per hydrogen bond in the self-association dimers is 1.8 kcal/mol.

The intrinsic DIEs at C2 and C4 in monomeric **2** were 57 and 72 ppb, respectively. Both DIEs increased as the concentration of **2** increased to limiting apparent values of 86 ppb for C2 and 134 ppb for C4 (see Table III). If one assumes that the magnitudes in the increases in DIEs at both carbons are directly proportional to $\Delta H$ for hydrogen-bond formation, the apparent maximal values of 29 ppb for C2 and 61 ppb for C4 (see Table V) should reflect the fractional amounts of C2 hydrogen bonds ($f_{C2}$) and C4 hydrogen bonds ($f_{C4}$) since $\Delta H_{C2} \approx \Delta H_{C4}$. The fractional values calculated from DIEs (Table V) are identical within experimental error with those calculated from chemical shifts (Table IV). Thus, the maximal difference between intrinsic DIEs at C2 and C4 for free and hydrogen-bonded species is estimated to be 90 ppb, and the maximal DIEs at C2 and C4 are 147 and 224 ppb, respectively.

**Nucleoside Base Pairs.** Deuterium isotope effects were measured for chloroform solutions of **1–4** in the presence of a 3-fold excess of complementary nucleosides. To minimize concentration effects not associated with hydrogen bonding, the total nucleoside concentration was maintained at 0.28 M. In each case, DIEs increased for the amino and imino carbons of the nucleosides in the presence of their complements. The changes that occurred upon hydrogen bonding are given in Table VIII. In general, the DIEs did not show dramatic changes when nucleosides were mixed with an excess of the complementary base. Several factors combine to produce the low values, which do not reflect the change in DIE that occurs upon association of monomeric units. G, U, and, to a lesser extent, A self-associate at the concentrations used in this study. Thus, the differences listed in Table VIII are between complementary base pairs and self-association complexes that already have DIEs enhanced over monomeric values. The strong tendency for G to self-associate in chloroform probably

is the major cause for the very small changes seen for **3** upon addition of **4**.

Determination of DIEs for A–U complexes is further complicated by the formation of up to four complementary dimers. The two bases can pair to generate normal and reversed forms of Watson–Crick and Hoogsteen structures. NMR,[8] IR,[33] X-ray,[35] and theoretical[36] studies suggest that normal cyclic dimers hydrogen bonded through the C4 carbonyl of U are more stable, both in solution and in mixed crystals. Watson–Crick and Hoogsteen structures are thought to have similar stability. In a classic experiment Sanchez and co-workers[37] were able to distinguish between Watson–Crick and Hoogsteen interactions in tRNAs by a nuclear Overhauser experiment. Since internuclear dipolar relaxation between protons decreases with the inverse of the sixth power of the internuclear distance, transfer of saturation from one proton to its neighbors is highly dependent on geometry. They found that saturation of uridine imino protons selectively produced nuclear Overhauser effects (NOEs) at C2 protons in adjacent adenines for Watson–Crick structures and at the C8 protons for Hoogsteen structures in tRNAs.




We conducted NOE experiments with a mixture of **1** and **2a** to obtain evidence for Watson–Crick and Hoogsteen structures under conditions where the monomeric and dimeric structures are in dynamic equilibrium, and the results are shown in Figure 3. Figure 3C shows an $^1H$ NMR spectrum for a 3:1 mixture of **1** and **2a** in deuteriochloroform at 219 K with off-resonance decoupling at 10000 Hz downfield from the characteristic hydrogen-bonded imino signal at 13.3 ppm. A spectrum of the sample with on-resonance saturation of the imino peak is shown in Figure 3B, and the NOE difference spectrum is shown in Figure 3A. As expected, the imino signal decreased substantially in the on-resonance spectrum, and a large decrease was also seen in the signal for the amino moiety of **1**. Although the imino and amino signals are sharp, the rate of exchange between protons in the two moieties is sufficiently fast for transfer of saturation. In addition, substantial nuclear Overhauser enhancements were seen for the C2 and C8 protons in **3** at 8.0 and 8.3 ppm, respectively. The peaks from 1.0 to 6.1 ppm in Figure 3B,C have the same relative intensities. Thus, the high-field signals in the difference spectrum probably resulted from poor subtraction rather than NOEs. These experiments clearly reveal the presence of Watson–Crick and Hoogsteen forms. In addition, the substantial downfield shifts seen for C2 and C4 of **2a** in the presence of a 3-fold excess of **1** relative to the chemical shifts in monomeric **2a** indicate that the uridine moiety is paired in both normal and reversed modes. Therefore, chloroform solutions of **1** and **2a** contain substantial amounts of all four possible dimers, in agreement with previous $^1H$ chemical shift studies at low temperature by Iwahashi and co-workers.[38]

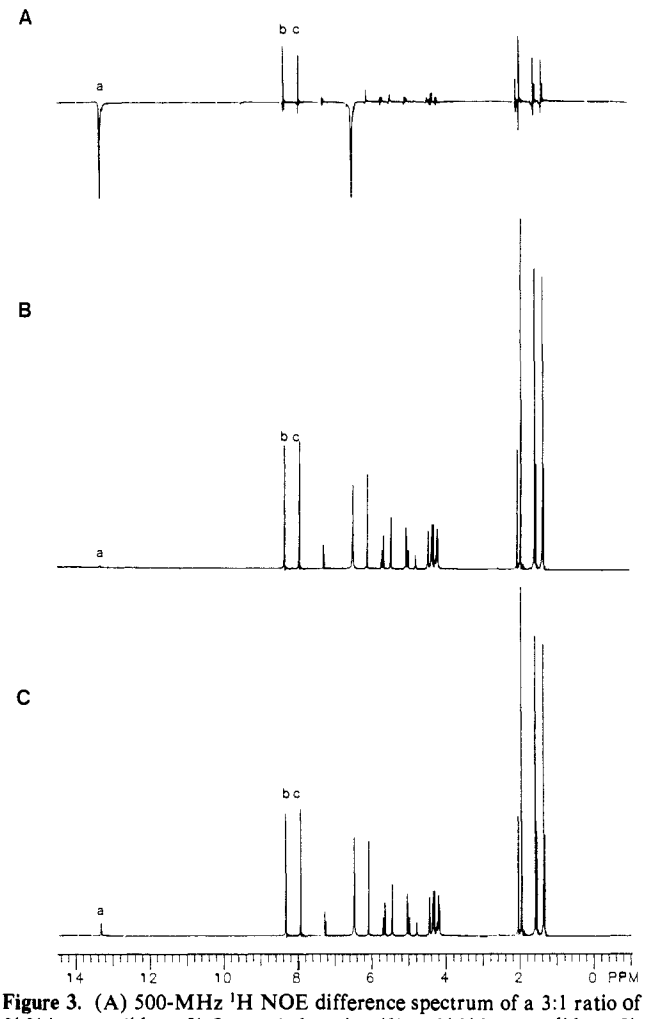



**Figure 3.** (A) 500-MHz $^1H$ NOE difference spectrum of a 3:1 ratio of 2′,3′-isopropylidene-5′-*O*-acetyladenosine (**1**) to 2′,3′-isopropylidene-5′-*O*-acetyluridine (**2a**) in deuteriochloroform. Total nucleoside concentration was 0.28 M. (B) $^1H$ spectrum following 5.0-s saturation of the 13.3 ppm imino proton of uridine. (C) $^1H$ spectrum following 5.0-s irradiation 10000 Hz downfield of the imino uridine proton. Peak a is the imino resonance; peaks b and c are from the C8 and C2 protons of adenosine, respectively.

The isotope effects for **2a** given in Table VII are, in analogy to the case for self-association, apparent values since in the presence of excess **1** only one of the two carbonyl moieties in **2a** is engaged in a two-centered interaction. The apparent DIE at C4 in **2a** is 181 ppb in the presence of an excess of **1**. This is only 30 ppb smaller than the estimated value of the intrinsic isotope effect at C4 for self-association of **2**. Unfortunately, the relative amounts of the four A–U dimers cannot be calculated from our data nor can the intrinsic DIEs for each species. Thus, it is not possible to relate the intrinsic DIE for a C4-amino–hydrogen bond in an AU dimer to that for a C4-imino interaction in a UU dimer.

In view of the problems associated with determining intrinsic two-bond DIEs on $^{13}C$ chemical shifts for systems with multiple equilibria, we question the quantitative and, in some cases, the qualitative value of the empirical correlations of DIEs with $\Delta H$'s reported by Reuben[21] for estimating strengths of hydrogen bonds between nucleosides, especially for comparisons among individual hydrogen bonds in AU and GC complexes. The study we conducted for self-association of **2** provides a calibration for deuterium isotope effects on $^{13}C$ chemical shifts involving interactions between imino carbonyls and imino protons. In this instance, an enhancement in the DIEs at C2 and C4 in uridine of 90 ppb is associated with $\Delta H = -1.8$ kcal/mol. However, additional ex-

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amples for imino carbonyls with the imino and amino hydrogens typically found in nucleic acids will be needed to determine whether there is a general quantitative correlation between $\Delta H$ and DIE for individual hydrogen bonds between nucleosides.

**Acknowledgment.** This work was supported by Grant GM32490 from the National Institutes of Health.

**Supplementary Material Available:** Table showing dependence of $\delta$ ($^{13}C$) at C2 and C4 of protonated and deuteriated **2** on temperature and concentration (1 page). Ordering information is given on any current masthead page.

# Solid-State $^{13}C$ NMR Characterization of Polyanilines

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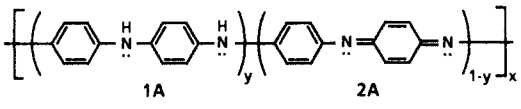
*Received April 1, 1988*

**Abstract:** $^{13}C$ solid-state nuclear magnetic resonance measurements are reported for the leucoemeraldine base, emeraldine base, and emeraldine hydrochloride forms of polyaniline in order to characterize the structures of these three distinct polymers. Chemical shift assignments are facilitated by use of the cross-depolarization technique to distinguish carbons with and without directly bonded hydrogens. Comparison of the spectra of emeraldine base with those of leucoemeraldine base and air-oxidized leucoemeraldine (which partially converts to emeraldine base) establishes that emeraldine base is essentially an alternating copolymer of reduced **1A** [$-(C_6H_4)N(H)(C_6H_4)N(H)-$] and oxidized **2A** [$-(C_6H_4)N{=}(C_6H_4){=}N-$] repeat units. The 8-12 ppm spectral line widths measured for both emeraldine base and leucoemeraldine base are attributed to local fluctuations in conformational and configurational geometries, a distribution in chain packing, and compositional defects. $^{13}C$ spin-echo measurements establish that the 60 ppm wide line from the conducting emeraldine hydrochloride is *inhomogeneously* broadened. It is postulated that this line width is due to local variations in charge density along the polymer backbone arising from polymer structural heterogeneity.

Polyaniline has been the subject of considerable scientific inquiry because of its unique electrical behavior and its potential as an environmentally stable conducting polymer. Conductivity exhibits a strong dependence on solution pH,[1-5] oxidation state[3,6] and water content.[3,7-10] Polyaniline is synthesized by the electrochemical or chemical oxidative polymerization of aniline and can exist as a number of unique structures, characterized by the oxidation state, i.e., the ratio of amine to imine nitrogens, and the extent of protonation. These different structures can be interconverted by acid/base or oxidation/reduction treatment. Six basic repeat unit building blocks have been proposed from which the different forms of polyaniline are composed.[1] They are designated by **1** = reduced or **2** = oxidized, followed by **A** = amine or imine base, **S′** = monoprotonated salt, or **S″** = diprotonated salt. The unprotonated forms of polyaniline consist of reduced base units, **1A**, and oxidized base units, **2A**, represented as follows




where the oxidation state of the polymer increases with decreasing values of $y$ ($0 \leq y \leq 1$). Claims have been made that polyaniline polymers having the following compositions (and others) have been isolated: the fully reduced leucoemeraldine base ($y = 1$), the half-oxidized emeraldine base ($y = 0.5$), and the fully oxidized pernigraniline base ($y = 0$).[1]

Structural characterization of polyanilines has been limited because, like most conducting polymers, they are largely insoluble in common organic solvents. Raman studies of films of emeraldine base have identified the presence of para-disubstituted benzene and quinone diimine moieties and, taken together with infrared spectra, have provided evidence for a head-to-tail polymerization of aniline, with no ortho incorporation of phenylenediamine groups.[11] The same conclusion was reached by comparison of the infrared spectrum of emeraldine base with that of a regiospecifically synthesized polymer.[12] Cross-polarization magic angle spinning $^{13}C$ nuclear magnetic resonance (CPMAS NMR) spectra also resolve localized benzenoid and quinoid ring structures,[13,14] indicating that extensive electron delocalization along the backbone does not occur. In this paper we extend these NMR results to demonstrate that emeraldine base is, in fact, an *alternating* copolymer of **1A** and **2A** units.

Protonation of the base forms of polyaniline leads to polymers whose conductivity depends upon the ratio of reduced and oxidized units as well as the extent of protonation. Of particular interest is the highest conductivity form, the emeraldine salt, which is the diprotonated emeraldine base. It has been postulated (although not universally accepted[15,16]) that under strong acid conditions

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