Proton Magnetic Resonance Studies of Specific Association of Nucleic Acid Constituent Bases in a Nonaqueous Solvent. Utility of DTBN Radical to Probe the Affinity of Hydrogen Bonding Involved in Complementary Base Pairs[1]

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Abstract: Proton nuclear magnetic resonance (at 220 MHz) studies on the effect of substituents to the hydrogen bonding of adenine (A), uracil (U), guanine (G), and cytosine (C) derivatives are reported. To prove the affinity of the hydrogen bonding in base pairs, we used the DTBN (di-*tert*-butyl nitroxide) radical-induced broadening effect on the imino N-H proton signal. The N-H proton signal of free U derivatives (six derivatives) in chloroform solution is broadened out quite sensitively at very small concentration of DTBN. In the case of U···A or G···C complementary base pairing, the N-H signal survives this broadening effect even at higher concentration of added DTBN radical. Among various base pairs, the N-H proton of the G···C system was most insensitive to DTBN. The "insensitivity" of the N-H signal to the DTBN-induced broadening effect is also shown to reflect qualitatively the strength of association between A and U derivatives which has been determined by Kyogoku et al. with the measurement of infrared spectra.

We are currently interested in use of stable radicals, such as DTBN[3] (di-*tert*-butyl nitroxide), in the nuclear magnetic resonance studies to probe hydrogen bonding[4-11] and charge transfer interaction[12-14] involved in organic and biologically important molecules. We have demonstrated that the DTBN···H-X hydrogen bonding, where X denotes oxygen, nitrogen, or carbon atom, induces a strong *upfield* contact shift for the X-H proton accompanied by signal broadening and a stereospecific *downfield* contact shift for other C-H protons.[10] These DTBN-induced contact shifts have been proved to provide fruitful information on the intrinsic nature of this hydrogen bonding in the closed-shell/open-shell bimolecular system and on structural elucidation around the proton donor group. The DTBN-induced broadening effect on the X-H proton signal has also been shown to be quite useful to differentiate the free X-H group which is exposed to DTBN radical from the solvated X-H group; intra- or intermolecularly strongly hydrogen bonded X-H proton is hardly affected by the presence of DTBN radical, while free X-H proton is susceptible to DTBN-induced broadening. This effect has been widely seen for cyclic peptides in which both the free and intramolecularly hydrogen bonded N-H groups are involved.[15]

In the present study we have performed an application of this technique to probe the affinity of the nucleic acid constituents, i.e., association between adenine (A) and uracil (U) derivatives, and between guanine (G) and cytosine (C) derivatives. In order to see specific hydrogen bonding between these constituents of nucleic acids, we used nucleic acid derivatives which are sufficiently soluble in chloroform and allow their interaction to be observed.[16,17] The nonaqueous solvent is chosen because the hydrophobic or stacking interaction is a minimum there. Another advantage of the use of this nonaqueous solvent is that DTBN is not strongly solvated and exposed to solute molecules, causing efficient relaxation of free N-H proton resonance.

The formation of selective hydrogen bonding between A and U and G and C derivatives in organic solvents has already been detected in the proton magnetic resonances by previous workers.[18-20] They observed specific shifts of the imino and amino proton signals on the addition of complementary base derivatives. In this paper we report specific DTBN-induced broadening effect of the imino N-H proton in U and G derivatives, which is shown to reflect the strength of the hydrogen bonding involved in A···U and G···C base pairs. The results are discussed in comparison with the association constants previously determined for the systems by infrared spectroscopy.[21]

Experimental Section

Materials. 9-Ethyladenine (A), six derivatives of uracil with a cyclohexyl group on the 1-N position (uracil (U), thymine (T), 5,6-dihydrouracil (di-U), 5-bromouracil (Br-U), 4-thiouracil (t-U), and 5-fluorouracil (F-U), and 2′,3′-benzylidene-5′-trityl derivatives of guanosine (G) and cytidine (C) were used in this study. These were purchased from the Cyclo Chemical Co., Los Angeles, Calif., except for F-U. 1-Cyclohexyl-5-fluorouracil (F-U) was kindly supplied by Dr. T. Katsura (University of Tokyo). 9-Ethyladenine was purified by recrystallization in chloroform after removing the paramagnetic metal ion impurity with EDTA. Selectively deuterated 9-ethyladenine at the 8-C position was prepared by heating an alkaline deuterium oxide solution of ethyladenine in a sealed tube at 85 °C overnight. Chloroform-d_1 was obtained from E. Merck, Darmstadt, and was dried by passing through an aluminum gel column. All the samples of 0.04 and 0.02 M in C^2HCl_3 for NMR measurement were prepared in a drybox. Only 0.2 M solutions were examined for F-U and C because of their limited solubility in C^2HCl_3. DTBN radical was prepared by the usual method.[22]

^{1}H NMR Measurements. Proton magnetic resonance (^{1}H NMR) spectra were acquired with a Varian HR-220 spectrometer operating at 220 MHz at room temperature.

Results

Base Pairing Shifts. ^{1}H NMR studies of the associative interaction between adenine and uracil derivatives in chloroform solution have already been performed by Katz and Penman[18] and by Katz,[19] although the uracil derivatives examined in their studies were rather limited. We have made here a more comprehensive study on the association of A and U derivatives and G and C in chloroform solution. Especially, F-U···A, t-U···A, and G···C base pairs in C^2HCl_3 solution are investigated here for the first time.

In order to avoid the formation of trimer and other higher order polymers, we have performed ^{1}H NMR measurements

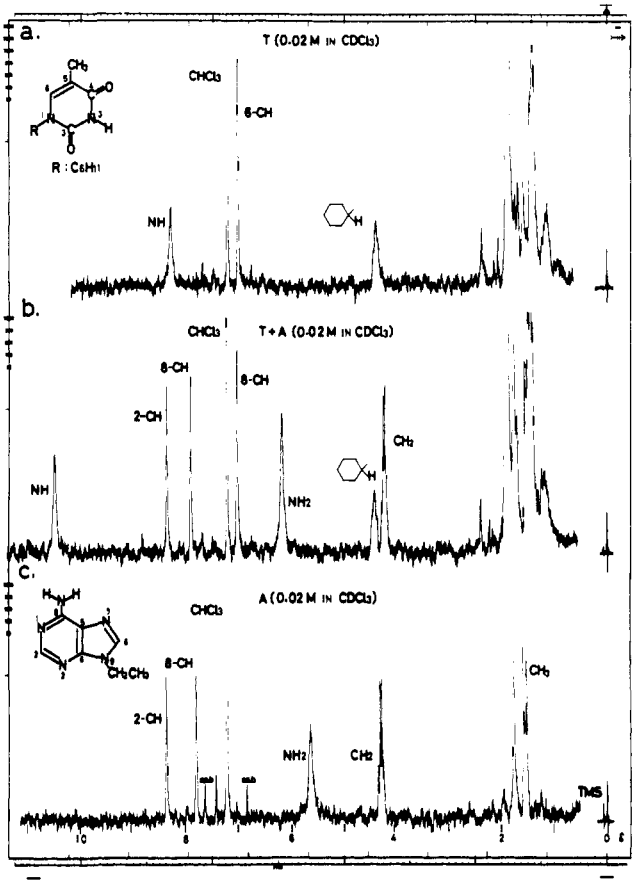

Figure 1. Proton NMR spectra of 1-cyclohexylthymine (T), 9-ethyladenine (A), and T + A in C^2HCl_3 (0.02 M): (a) T; (b) T + A; (c) A.

at concentrations as low as 0.04 and 0.02 M in C^2HCl_3 solution. Since the solubility of F-U and C was limited in C^2HCl_3, the 0.04 M solution of F-U and the 0.02 M solution of C were not examined here. The binary system of G + C, however, allowed us to prepare a 0.02 M solution.

Characteristic 220 MHz ^1H NMR spectrum of the complementary base pair T···A, in comparison with T and A alone, is illustrated in Figure 1, where A + T denotes the equal amount of each base (1:1) dissolved in C^2HCl_3 solution. Peak assignments for the 2-C and 8-C protons of A were confirmed by taking the spectrum of selectively deuterated 9-ethyladenine at the 8-position, and other peaks were readily assigned on the basis of their resonance intensities and signal patterns. This figure shows that the 3-N imino proton of T in the presence of A was most remarkably shifted downfield (484 Hz), as referred to the chemical shift of free 3-N proton. The 6-N amino proton of A also shifted in the same direction (149 Hz) in the presence of T. These downfield shifts indicate that strong association occurs by the formation of a hydrogen-bonded cyclic dimer including the 3-N imino (T) and 6-N amino (A) protons. In Table I the observed proton chemical shifts of A, U, and G derivatives alone and in the presence of complementary or noncomplementary bases are given. The base pairing shifts upon the formation of cyclic dimer are also included in this table (figures in parentheses). This table shows that the 3-N imino protons of U derivatives are strikingly shifted downfield by base pairing with A as was found in the T + A system. The characteristic downfield shift of the imino proton is increased in the order of di-U < U, T < t-U < Br-U < F-U, which is in agreement with the order of corresponding association constants with A reported by Kyogoku et al.[21] and by Katsura[23] with the exception of t-U. The hydrogen bonding shift of t-U was slightly larger than that of U or T, although the association constant reported for t-U is smaller than that for T or U. The downfield shifts of the 3-N protons are proportional to the order of their pK values.[21] Furthermore, the base pairing shifts for the 6-N amino proton of A also increase in the similar trend found in the 3-N imino proton of U derivatives, but here the shifts induced by the presence of t-U and T are almost the same. The exception of t-U is of some interest in perhaps providing an insight into the origin of base pairing shifts (an inductive or ring current effect) for these N-H protons.

On the other hand, the resonances of the 2-C and 8-C ring protons of A also shifted downfield by the formation of base pair with U derivatives and the shifts were characteristic of individual U derivatives, although analogous results have also been observed by Katz.[19] However, it should be noted that the order of these downfield shifts is not in agreement with that of the hydrogen bonding shifts for imino (U derivatives) or amino (A) protons, and the largest association shift was found in the t-U···A system. Moreover, the magnitude of the downfield shift for the 8-C proton was larger than that for the 2-C proton in each base pair. It is evident that not only the Watson-Crick type base pair[24] but also the Hoogsteen type[25] and their reverse types are present in a chloroform solution.[21,26,27] The ratio of these type base pairs may contribute to the magnitude of the downfield shift of the 8-C and 2-C protons.

We have also studied A + G and U + G binary systems in which no usual complementary base pair is formed. Specific shifts were not observed for 1-N imino (G) and 6-N amino (A, G) protons in the A + G pair, while the 2-C and 8-C protons exhibited upfield shifts. In the case of the Br-U + G system, a considerable downfield shift was observed for the 3-N imino proton of Br-U but no specific shift for 1-N (G) and other ring protons. This result is interesting from the viewpoint of the wobble concept which has been devised in the base pairing between codon and anticodon (see later for discussion).

DTBN-Induced Broadening of the Imino Proton Signal and Base Pairing Effect. Spectral perturbation by the addition of DTBN radical was examined in the above single and base pair systems. The addition of DTBN to the U or G solution caused substantial broadening of the imino proton signal due to the N-H···DTBN hydrogen bonding, while the signals of the other protons such as the C-H protons in the cyclohexyl groups were hardly affected. In the A + U or G + C binary system, however, the DTBN-induced broadening effect on these imino protons diminishes. For example, the N-H proton signal of F-U is broadened out at an appropriate concentration of added DTBN radical (Figure 2a). However, in the F-U + A (1:1) binary system, this N-H proton signal survives this serious broadening at the corresponding DTBN concentration (Figure 2b). For the G + C solution, the imino proton signal of G is hardly affected by the addition of DTBN radical even at the higher DTBN concentration where the free N-H signal is completely broadened out for the G solution. The "insensitivity" of the N-H signal to the DTBN-induced broadening effect is expected to correspond to the strength of the N-H···N hydrogen bonding involved in the complementary base pairs of nucleic acid constituents.

In order to compare and discuss quantitatively this "insensitivity" for different sets of base pairing, we have systematically examined the effect of added DTBN concentration on this signal broadening. The half-widths of the imino proton signals of U and G derivatives in the presence and absence of other bases are plotted against the DTBN concentration in Figures 3-5. The increment of line width was affected in proportion to the concentration of added DTBN radical. The slopes of these linear plots, referred to as $\delta(\Delta\nu_{1/2})$, represent the rate of increase of the half-width (Hz/M) on the addition of DTBN radical. They are characteristic of each system and could be used as a measure of the DTBN-induced broadening effect on the imino proton signal. The plots for U + A and

Table I. Observed Chemical Shifts of NH, NH_2, and Ring CH Protons of Uracil, Adenine (A), and Guanine (G) Derivatives in the Presence and Absence of Other Nucleic Acid Bases and Their Association Shifts[a]

System	Concn, M	Uracil derivatives			A			G
		3-NH	5-CH[b]	6-CH[b]	6-NH_2	2-CH	8-CH	1-NH
di-U	0.04	1965	582	750				
di-U + A	0.04	2082 (387)	575	743	1364 (100)	1850 (1)	1743 (14)	
U	0.04	1945	1264	1603				
U + A	0.04	2510 (565)	1266	1605	1412 (148)	1862 (13)	1764 (33)	
U	0.02	1868	1262	1602				
U + A	0.02	2352 (484)	1265	1603	1364 (126)	1858 (8)	1756 (29)	
T	0.04	1898		1549				
T + A	0.04	2475 (577)		1553	1426 (182)	1878 (29)	1782 (53)	
T	0.02	1828		1548				
T + A	0.02	2312 (484)		1550	1387 (149)	1870 (20)	1770 (43)	
t-U	0.04	2119	1432	1562				
t-U + A	0.04	2767 (648)	1418	1560	1430 (166)	1885 (36)	1810 (81)	
t-U	0.02	2085	1418	1565				
t-U + A	0.02	2630 (545)	1428	1570	1385 (147)	1880 (30)	1800 (73)	
Br-U	0.04	1957		1680				
Br-U + A	0.04	2843 (886)		1688	1460 (196)	1881 (32)	1790 (61)	
Br-U	0.02	1898		1863				
Br-U + A	0.02	2676 (778)		1680	1428 (190)	1880 (30)	1787 (60)	
F-U	0.02	1917		1612				
F-U + A	0.02	2727 (810)		1614	1433 (195)	1860 (10)	1766 (39)	
A	0.04			1264	1849	1729		
A	0.02			1218	1850	1727		
G	0.02						2695	
G + C	0.02						2987 (292)	
A[c]	0.02				1244	1866	1742	
G[c]	0.02							2681
Br-U[c]	0.02	1868		1667				
A + G	0.02				1248 (4)	1837 (−29)	1712 (−30)	2685 (4)
Br-U + G	0.02	1959 (91)		1660				2680 (−1)

[a] The chemical shifts are given in Hz downfield from tetramethylsilane. The association shifts are obtained from the difference of the corresponding chemical shifts in binary and unitary systems and given in parentheses. [b] The association shifts of 5-CH and/or 6-CH for uracil derivatives are very small; therefore, these values are not given in parentheses. [c] Different chemical shifts are observed in comparison with those in the above rows, which are plausibly caused by the different experimental conditions. However, these deviations do not make ambiguous the following discussions.

Table II. Summary of DTBN-Induced Broadening Effects on the Imino Protons[a] in the Presence and Absence of Complementary and Noncomplementary Bases and Their Relative Ratios[a]

Base	0.04 M			0.02 M		
	$\delta(\Delta\nu_{1/2})_0$[b]	$\delta(\Delta\nu_{1/2})_p$[c]	Rel	$\delta(\Delta\nu_{1/2})_0$[b]	$\delta(\Delta\nu_{1/2})_p$[c]	Rel
di-U + A	8	6	0.80			
U + A	24	12	0.50	39	18	0.45
T + A	36	14	0.40	49	17	0.35
t-U + A	51	31	0.60	64	42	0.65
Br-U + A	84	41	0.50	158	40	0.25
F-U + A				248	67	0.25
G + C				276	14	0.05
G + A				290	280	1.00
Br-U + A				195	190	1.00
G + C				290	310	1.05

[a] The DTBN-induced broadening effects $\delta(\Delta\nu_{1/2})$ are slopes obtained from the plot of the half-width of the imino proton signal $\Delta\nu_{1/2}$ vs. the concentration of DTBN [DTBN]. Relative ratios (Rel) are $\delta(\Delta\nu_{1/2})_p/\delta(\Delta\nu_{1/2})_0$'s. [b] Free base. [c] Binary system.

Br-U + A systems in Figure 3 indicate that the effect of DTBN radical on the line width of the imino proton signals is diminished by the presence of their complementary base, adenine. The phenomena can be seen more remarkably for F-U + A and G + C systems (Figures 4b and 4c). In the case of di-U + A, however, the effect of adenine to the DTBN-induced broadening of di-U is quite small (Figure 4a). Moreover, in the A + G and Br-U + G binary systems, the broadening of the imino proton signals induced by DTBN is hardly affected on the addition of noncomplementary base derivatives (Figure 5). In Table II are given the values of $\delta(\Delta\nu_{1/2})$ for the imino proton signals of U derivatives and G alone and in the presence of the partners at the given concentration of DTBN radical. The relative ratios of this signal broadness for the base pairing system with respect to that for free base are also presented in Table II. This ratio, which corresponds to "insensitivity" to DTBN-induced broadening, is expected to provide a measure of the strength of N-H···H hydrogen bonding. If the hydrogen bonding is quite strong, the N-H proton signal could suffer from the DTBN-induced broadening, leading to remarkable

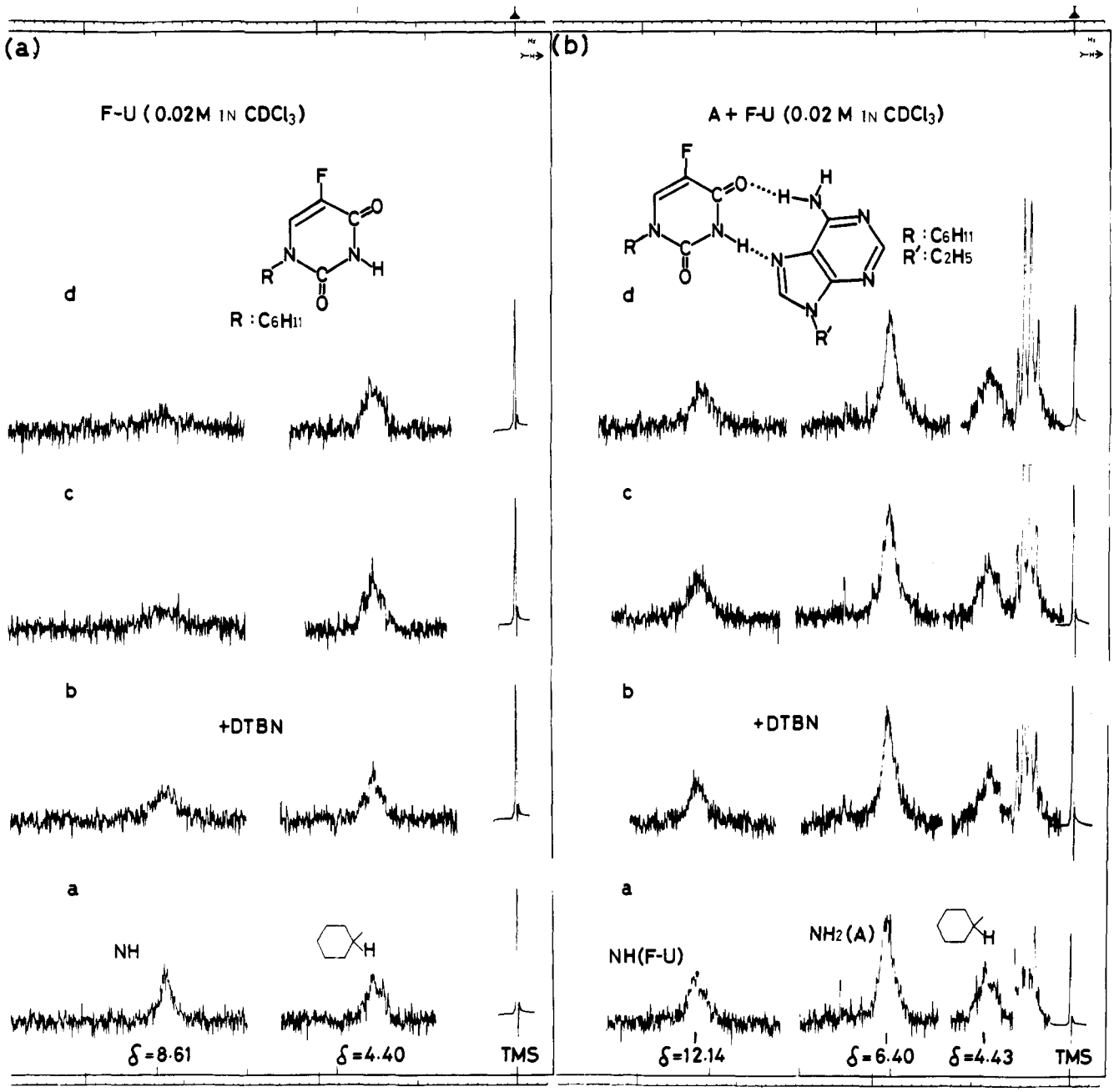

Figure 2. DTBN-induced broadening effects on the 3-N imino proton of 1-cyclohexyl-5-fluorouracil (F-U) in the presence and absence of 9-ethyladenine (A): (a) F-U alone; (b) F-U + A binary system.

change in the DTBN-induced broadening on going from free base to base pairing system.

Discussion

The studies on the specific association of 9-ethyladenine with 1-cyclohexyluracil derivatives have been made by means of infrared spectra,[21,27-29] ultraviolet spectra,[30-32] proton magnetic resonance spectra,[18-20] and vapor pressure osmometry,[33] where the association constants K_{AU} have been well established in conjunction with the higher order association K_{AU_2}, self-associations K_{A_2} and K_{U_2}. The major concern in this study is an application of DTBN-induced signal broadening effect to probe the affinity of the intermolecular hydrogen bonding in the A + U and G + C base pairings.

We have demonstrated in our previous paper[8] that the upfield X-H proton contact shifts accompanied by signal broadening induced by the X-H···DTBN hydrogen bonding follow the acidity of the X-H proton donating groups in various organic molecules. Here we are concerned with the effect of DTBN radical on the imino proton signal of di-U, U, T, t-U, Br-U, and F-U in relation to their pK values associated with the N-H group in these uracil derivatives. However, DTBN-induced broadening of the imino proton signal is too serious for us to follow the upfield contact shift. Thus we are concerned with the DTBN-induced boradening effect as a measure of the affinity of N-H···DTBN hydrogen bonding.

Inspection of Table II reveals that the order of DTBN-induced broadening effect for the imino protons ($\delta(\Delta\nu_{1/2})_0$) (see footnote a in Table II) is di-U(8) < U(24) < T(36) < t-U(51) < Br-U(84) for the 0.04 M solution and U(36) < T(49) < t-U(64) < Br-U(158) < F-U(248) for the 0.02 M solution. These trends agree qualitatively with the order of pK values, di-U(11) > T(9.9) > t-U(8.2) > Br-U(7.8) > F-U(7.5),[23] except for U and T, where these pK values were taken from those of related molecules and the corresponding nucleosides.[21] The comparison of $\delta(\Delta\nu_{1/2})_0$'s with p$K$ values is summarized

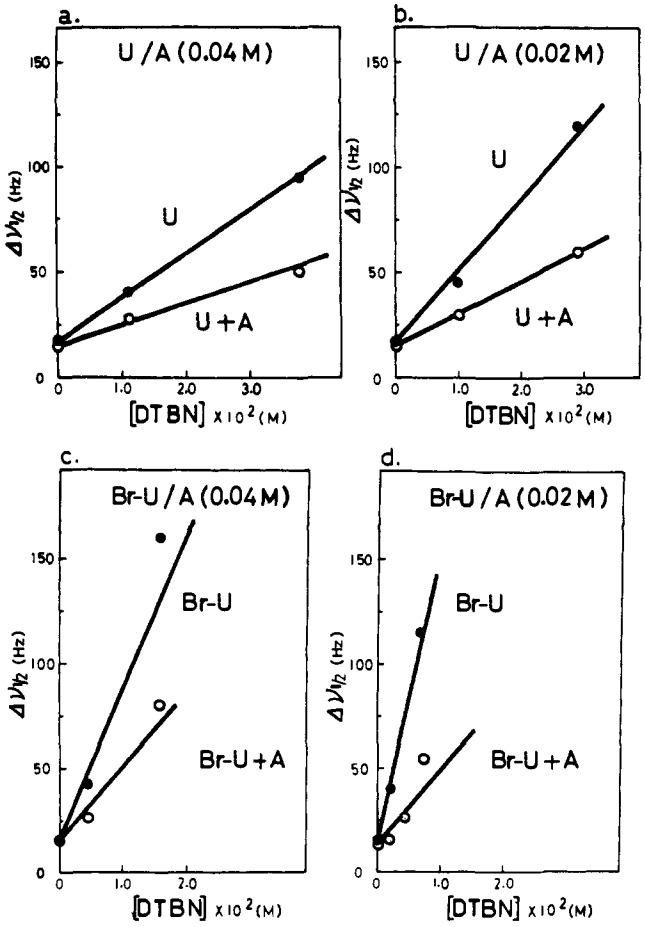

Figure 3. Plots of $\Delta\nu_{1/2}$ of imino proton in uracil derivatives vs. DTBN concentration in the presence and absence of the complementary base: (a) U and U + A (0.04 M); (b) U and U + A (0.02 M); (c) Br-U and Br-U + A (0.04 M); (c) Br-U and Br-U + A (0.02 M).

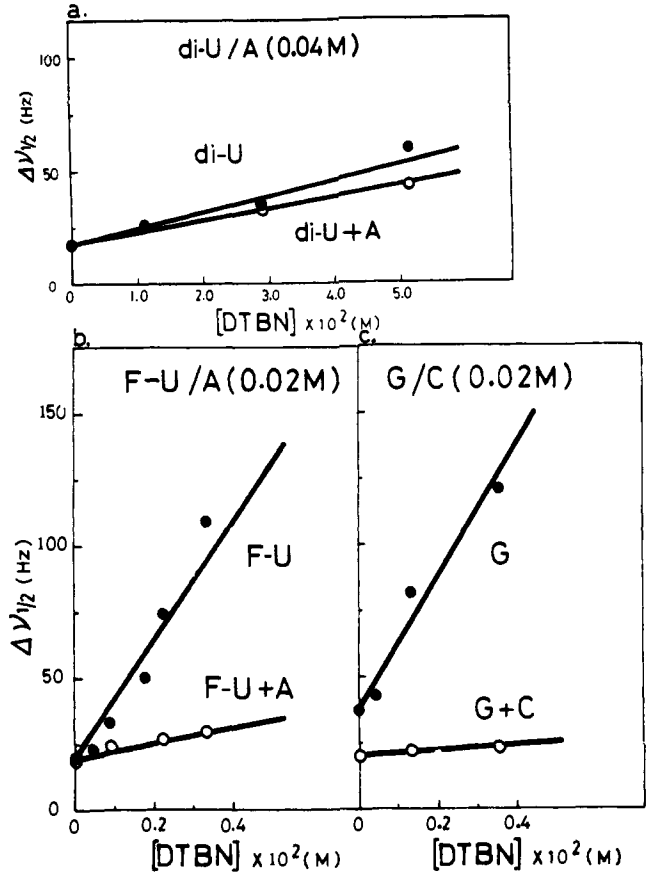

Figure 4. Plots of $\Delta\nu_{1/2}$ of imino proton in uracil and guanine derivatives vs. DTBN concentration in the presence and absence of the complementary bases: (a) di-U and di-U + A (0.04 M); (b) F-U and F-U + A (0.02 M); (c) G and G + C (0.02 M).

Table III. Comparison of DTBN-Induced Broadening Effects on the Uracil Imino Protons with Their pK_a Values of Corresponding Nucleosides

	Uracil derivatives					
	di-U	T	U	t-U	Br-U	F-U
Broadening effect[a]						
0.04 M	8	36	24	56	84	
0.02 M		49	39	64	158	248
pK_a	11	9.9	9.4	8.2	7.8	7.5

[a] Slopes obtained from the plot of $\Delta\nu_{1/2}$ vs. DTBN concentration; $(\delta(\Delta\nu_{1/2})_0$'s in Table II.

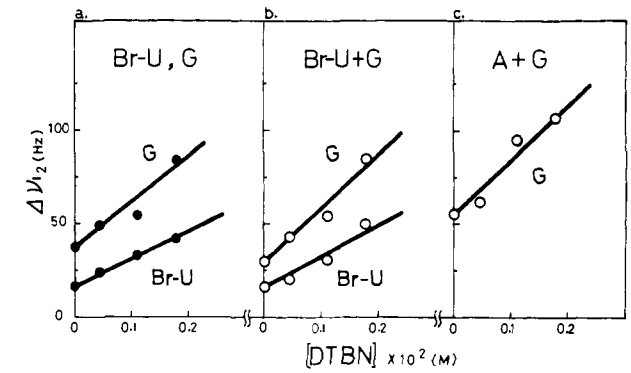

Figure 5. Plots of $\Delta\nu_{1/2}$ of imino proton vs. DTBN concentration in the presence and absence of noncomplementary bases: (a) Br-U and G (0.02 M); (b) Br-U + G (0.02 M); (c) A + G (0.02 M).

in Table III. It is tempting to conclude from the above discussion that the DTBN-induced broadening effect $(\delta(\Delta\nu_{1/2})_0)$ for the imino proton is possibly best interpreted in terms of the acidity of the uracil imino proton and this "spin probe" technique using DTBN as a relaxation reagent could serve as a useful tool to follow qualitatively the acidity of the N-H group in these uracil derivatives.

In the binary system where the A + U or G + C base pair is formed, the broadening effect $(\delta(\Delta\nu_{1/2})_p)$ of the uracil imino proton is substantially decreased, compared with the case of free bases (see Table II). In Table II are given the relative values of the broadening effect $(\delta(\Delta\nu_{1/2})_p/\delta(\Delta\nu_{1/2})_0)$ for the binary system with respect to that of the free base. These values may represent the "insensitivity" of the imino proton to DTBN radical in going from free base to the base pairing system. The strength of hydrogen bonding in A + U or G + C base pairs is expected to be reflected by decrease of this broadening effect.

For di-U, there is a quite small change in the DTBN-induced broadening effect of the imino proton when equimolar A is added to the solution of di-U and therefore the relative value of the broadening effect amounts nearly to unity (0.80). This implies that the amount of the hydrogen bonded pairs between di-U and A is quite small at the concentration and the imino proton of di-U easily interacts with DTBN radical. On the other hand, the relative broadening effects $(\delta(\Delta\nu_{1/2})_p/\delta(\Delta\nu_{1/2})_0)$ for Br-U + A and F-U + A are 0.25 and 0.25 for the 0.02 M solution, respectively. It is striking that the corresponding value for the G + C pair is 0.05, implying that the guanine imino proton is not exposed to the DTBN radical in

Table IV. The Decrease of DTBN-Induced Broadening Effects on the Imino Protons in the Presence of Other Bases, Hydrogen Bonding Shifts, and Association Constants

Bases with imino proton / Other base	di-U / A	t-U / A	U / A	T / A	Br-U / A	F-U / A	G / C	Br-U / G	G / A
Decrease of DTBN induced broadening effect[a]									
(0.04 M)	0.80	0.60	0.50	0.40	0.50				
(0.02 M)		0.65	0.45	0.35	0.25	0.25	0.05	1.00	1.05
Hydrogen bonding shift, Hz									
(0.04 M)	387	648	556	577	886				
(0.02 M)		545	490	484	776	810	292	91	4
Association constant, M^{-1} [b]	30	90	100	130	240		10^4–10^5		

[a] The relative ratios listed in Table II. [b] From ref 21 and 29.

the presence of a cytosine derivative. It is found in Table II that the pairing effect of DTBN-induced broadening of the imino proton mentioned above is increased, namely, the relative ratios are decreased, in the order of di-U(0.80) > U(0.45) > T(0.35) > Br-U(0.25), F-U(0.25) > G(0.05). This is in agreement with the order of the association constants K_{AU}, di-U(30) < t-U(90) < U(103) < T(130) < Br-U(240) (see Table IV). Quite a large association constant K_{GC} of 10^4–10^5 M^{-1} is also reflected by the largest pairing effect for G. In contrast to this, the relative ratios for A + G and Br-U + C binary systems are close to unity (Table II). The association between these nucleic acid bases cannot be detected by DTBN in these systems. It is, therefore, likely that the DTBN-induced broadening effect for the imino protons of U or G derivatives in the complementary or noncomplementary base pairing is qualitatively correlated with their association constants. This will be substantiated by the following arguments.

The addition of DTBN radical into the solution containing only an uracil derivative causes substantial broadening of the imino proton signal. The line widths of the protons increase in proportion to the amount of added DTBN radical as shown in Figures 3–5. This may allow us to use eq 1 to represent the observed line width, namely, the reciprocal T_2 (transverse relaxation time),

$$\Delta\nu_{1/2} \equiv \frac{2\pi}{T_2} = 2\pi\left(\frac{f}{T_{2_p}} + \frac{1-f}{T_{2_0}}\right) \approx 2\pi\left(\frac{f}{T_{2_p}} + \frac{1}{T_{2_0}}\right) \quad (1)$$

where T_{2_0} and T_{2_p} are the transverse relaxation times of the imino protons in free base and hydrogen bonded one to DTBN radical respectively and f is the fraction of the complex.

The slopes obtained from the plots of $\Delta\nu_{1/2}$ vs. [DTBN] correlate to the concentration of U···DTBN complex formed in the following equilibrium,

$$\text{U} + \text{DTBN} \overset{K}{\rightleftarrows} \text{U···DTBN}$$

$$K = [\text{U···DTBN}]/[\text{U}][\text{DTBN}] \quad (2)$$

where K is the equilibrium constant between U and DTBN and [U] and [DTBN] are the concentrations of nonbonded U and DTBN, respectively. When the complementary base (A) is present in the above system, the equilibrium reaction between A and U competes with reaction 2 and is expressed as follows:

$$\text{U} + \text{A} \overset{K_{AU}}{\rightleftarrows} \text{U···A}$$

$$K_{AU} = [\text{U···A}]/[\text{U}][\text{A}]$$

$$\text{U} + \text{DTBN} \overset{K}{\rightleftarrows} \text{U···DTBN}$$

$$K = [\text{U···DTBN}]'/[\text{U}][\text{DTBN}] \quad (3)$$

In eq 3 the concentration of U···DTBN in eq 2 is decreased to ([U···DTBN]′) to some extent by the formation of A···U base pairs. Under our experimental condition of [DTBN] ≪ [U] and [A], the ratio R of the fraction of U···DTBN hydrogen bonded complex in the presence and absence of the complementary base can be expressed in the following relation:

$$R = \frac{f'}{f} = \frac{[\text{U···DTBN}]'}{[\text{U···DTBN}]} = \frac{[\text{U}]_0 - [\text{U···A}]}{[\text{U}]_0} \times \frac{K[\text{U}]_0 + 1}{K([\text{U}]_0 - [\text{U···A}]) + 1} \quad (4)$$

where $[\text{U}]_0$ is the initial concentration of dissolved U. Furthermore, with the use of the conditions $K \leq 1$ and $[\text{U}]_0 = 0.04$ or 0.02 M, the last term of eq 4 is approximated to unity and we obtain,

$$R \simeq 1 - \frac{[\text{U···A}]}{[\text{U}]_0} = 1 - r_{\text{pair}} \quad (5)$$

where r_{pair} indicates the fraction of hydrogen bonded complex formed between nucleic acid bases. This assumption is valid since K is smaller than 1 in an organic solvent.[7] Following the above discussions, the relative ratio Rel in Table II is expressed as

$$\text{Rel} = \frac{\delta(\Delta\nu_{1/2})_p}{\delta(\Delta\nu_{1/2})_0} = \frac{f'}{f} \quad (6)$$

Thus the ratios of the slopes (Rel) discussed in the preceding section are quantitatively correlated with the fraction of base pairs r_{pair} as demonstrated in eq 4 and 5.

From the above consideration, the ratio of the slopes $(\delta(\Delta\nu_{1/2})_p/\delta(\Delta\nu_{1/2})_0)$ can be used as a measure of the association between nucleic acid bases. The utility of the measure is demonstrated in Table IV, where the relative ratios studied in the base pair systems are compared with the corresponding association constants obtained from the IR method. This table shows that the relative ratios given in the first row increase on going from di-U to F-U, and these are compared with the association constants listed in the last row. For example, the relatively large value (R = 0.80) was experienced for the di-U + A base pair system, namely, the r_{pair}'s in eq 5 are small, which may be attributed to the small association constant $K_{\text{di-U,A}}$. This is the case for weak association between nucleic acid bases. By contrast, the smallest R's obtained for the G + C system clearly show that the strong association occurred between G and C.

Table IV also gives the hydrogen bonding shifts of the imino protons induced on the addition of equimolar complementary base derivatives. Inspection of this table reveals that t-U exhibits fairly larger shifts than U and T, while the association of the t-U constant is smaller than those of U and T. The shift of the imino proton of G in the G + C system is smaller than expected from its large association constant 10^4–10^5 M^{-1}. Thus the magnitude of the hydrogen bonding shift does not always predict the strength of association in a base pair. The

shifts seem to be more related to the pK values of the imino protons.

The effect of pairing to the DTBN-induced broadening is not observed for A + G and Br-U + G systems. In the infrared study, formation of weak hydrogen bonding between A and G was detected.[28] Although the imino and amino proton signals of A and G do not shift on mixing, their downfield shift due to hydrogen bonding might be canceled by the upfield shift induced by the stacking interaction, since the 2-C and 8-C protons of adenine manifest upfield shifts. On the other hand, a small downfield shift of the Br-U imino proton was observed for the Br-U + G system. This shift implies the formation of the additional hydrogen bonding between Br-U and G, though it was not detected in the infrared study.[21,28] The interaction between U and G is expected as wobble pairing in the codon–anticodon interaction.[34] The effect of these inter-base bondings to the DTBN-induced broadening must be too small to be detected under the present experimental conditions.

In summary, we have demonstrated here the utility of the DTBN radical to probe the affinity of hydrogen bonding involved in complementary base pairs of nucleic acid constituents. Specific hydrogen bonding and bonding sites in base pairs can be detected by the DTBN-induced broadening effect in the ^{1}H signals of the imino group. Furthermore, the strength of association in base pairs can also be predicted by this method in parallel with infrared spectroscopic method, although the amount of hydrogen bonding shift cannot be used for the prediction. This facile method seems to serve as a potential tool to gain insight into strength of hydrogen bonding frequently encountered in nucleic acids. It now remains to be seen whether the method used here will be equally appropriate for the system in aqueous solution as well. We are now trying to use DTBN as a spin probe for the detection of base pairing in aqueous solution.

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References and Notes

(1) Part 13 of the series, "Studies on the Nuclear Magnetic Resonance of the Paramagnetic Solution Involving Stable Free Radical". Taken in part from the Ph.D. Thesis of T.I. submitted to Kyoto University.
(2) (a) Kyoto University; (b) Osaka University.
(3) Abbreviations used are: DTBN, di-*tert*-butyl nitroxide; A, 9-ethyladenine; U, T, di-U, Br-U, t-U, and F-U, 1-cyclohexyluracil, -thymine, -5,6-dihydrouracil, -5-bromouracil, -4-thiouracil, and -5-fluorouracil, respectively; G and C, 2′,3′-benzylidene-5′-tritylguanosine and -cytidine, respectively.
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