A Spectroscopic Study of Hydrogen-bonds Involving the 2-Thiouracil Residue

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Infrared absorption spectra, ultraviolet absorption spectra, phosphorescence spectra, phosphorescence lifetimes, and phosphorescence excitation spectra have been examined of 1-propyl-2-thiouracil in various solvents, including CCl_4, CH_3OH, CCl_4+CH_3OH, and CCl_4+9-ethyladenine. For 1-cyclohexyluracil, and for 2-*S*-propyl-2-thiouracil, similar infrared spectroscopic examinations were also made. The results obtained are as follows: (1) In CCl_4, the 2-thiouracil residue forms a hydrogen-bond complex with the adenine residue, in which the C^4=O is involved in the hydrogen-bond. (2) The binding of 2-thiouracil and adenine is much stronger than that of 2-thiouracil and CH_3OH; the 2-thiouracil·methanol binding is stronger than the uracil·methanol binding. (3) The hydrogen-bonding of the 2-thiouracil residue (with CH_3OH or with adenine) causes an elevation of the lowest singlet excited state S_1, which is probably an $^1(n\pi^*)$, from 2.5×10^4 cm^{-1} to 2.6×10^4 cm^{-1}, and the lowest triplet excited state T_1, which is probably an $^3(n\pi^*)$, from 2.4×10^4 cm^{-1} to 2.6×10^4 cm^{-1}. (4) The hydrogen bonding of the 2-thiouracil residue causes a great enhancement of the phosphorescence intensity. On the basis of these observations, a discussion is given on the nature of the inter-base hydrogen-bonds involving 2-thiouracil residue.

2-Thiouracil is a modified base, closely related with a number of naturally occurring nucleic acid constituents. Yeast $tRNA_3^{Glu}$, for example, has 2-thiouridine-5-acetic acid methyl ester as the first letter of its anti-condon triplet.[1] The thymidine residue in the TψC sequence is partly replaced by 2-thiothymidine residue in the tRNAs of *Thermus thermophilus*, an extremely thermophilic bacterium.[2] In an attempt to characterize the hydrogen-bondings involving the 2-thiouracil residue, we have made a detailed spectroscopic examination of 1-propyl-2-thiouracil, which is soluble both in polar and nonpolar solvents.

Experimental

Materials and Methods. 1-Propyl-2-thiouracil was prepared by a direct alkylation of 2-thiouracil (from Tokyo-Kasei Co.) with propyl bromide, following the method previously given for N-1 alkylation of uracil.[3,4] 1 Mol of 2-thiouracil and 2/3 mol (or less) of propyl bromide were mixed together in a small amount of dry dimethyl sulfoxide with 1 mol of potassium carbonate, and the slurry was kept at room temperature for 48 h under vigorous stirring. After removing K_2CO_3 by filtration, the clear solution was concentrated *in vacuo*, and an excess of ethanol was added. The precipitates, unreacted 2-thiouracil, was discarded after filtration. This process was repeated three times or more until the 2-thiouracil is adequately removed from the solution. The final filtrate was subjected to a silica gel chromatography with CCl_4/*t*-BuOH(10:1) as the eluent. Two fractions corresponding to 1-propyl-2-thiouracil and to 2-*S*-propyl-2-thiouracil were obtained. The yields of the above products were about 3% and 20%, respectively. Both were purified by recrystallization from ethanol. No 3-propyl derivative was produced. The 1-propyl-2-thiouracil sample, thus obtained, gave a single spot in a thin layer chromatography: R_f 0.82 with $CHCl_3/C_2H_5OH$=9/1. Melting point, 187 °C.

The ultraviolet absorption spectrum of 1-propyl-2-thiouracil was found to be identical to 1-ethyl-2-thiouracil in neutral and pH 12 solutions. The 1-ethyl-2-thiouracil sample was provided by Professor D. Shugar of Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Poland, to whom we are indebted. The product was also identified by its mass spectrum (M^+(*m/e*): Found 170, Calcd, 170) and elementary analysis. Found: C, 49.34; H, 6.09; N, 16.52; O, 9.37; S, 18.78%. Calcd for $C_7H_{10}N_2OS$: C, 49.39; H, 6.21; N, 16.46; O, 9.40; S, 18.83%.

2-*S*-Propyl-2-thiouracil obtained as described above was identified by infrared absorption spectrum,[5] ultraviolet absorption spectrum,[5] melting point (117 °C), and elementary analysis. Found: C, 49.24; H, 5.97; N, 16.50; O, 9.16; S, 18.59%. Calcd for $C_7H_{10}N_2OS$: C, 49.39; H, 6.21; N, 16.46; O, 9.40; S, 18.83%.

1-Cyclohexyluracil was prepared by Dr. T. Katsura, Research Institute for Polymer and Textiles, Tsukuba, and was kindly given us by him. Its infrared and ultraviolet absorption spectra were found to be identical with what are known.[6,7]

9-Ethyladenine is a gift of Professor T. Miyazawa, Department of Biochemistry and Biophysics, Faculty of Science, University of Tokyo. Its identification was made by the infrared and ultraviolet absorption spectra.[6,7]

All the solvents used were carefully purified by distillation and then mostly by an activated alumina column. Bases were dissolved in these solvents by the use of a sonicator and by heating at 40 °C with hot air. For phosphorescence measurements, the solutions were carefully degassed in a vacuum line.

Infrared absorption spectra were observed by the use of a Digilab FTS-14 Fourier transform spectrophotometer. The disturbance due to the atmospheric water vapour was removed by filling the inside of the instrument with dry air. Each of the absorption curves in Figs. 1, 2, and 4 was recorded with resolution=4 cm^{-1} and number of scans=240. Each of the sample solutions was placed in one of the following three sealed cells: one with KBr windows and with 1 mm path, another with CaF_2 windows and 1 mm path, and that with CaF_2 windows and 50 μm path. The actual optical path lengths of these cells were determined by examining interference patterns. Ultraviolet absorption spectra were examined by the use of a Hitachi 124 spectrophotometer. Phosphorescence was observed with a Hitachi MPF-4 fluorometer with a phosphoroscope. Spectral slit width was set at 10 nm. Phosphorescence decay curves were obtained by the use of a nitrogen laser of 20 kW in the peak power

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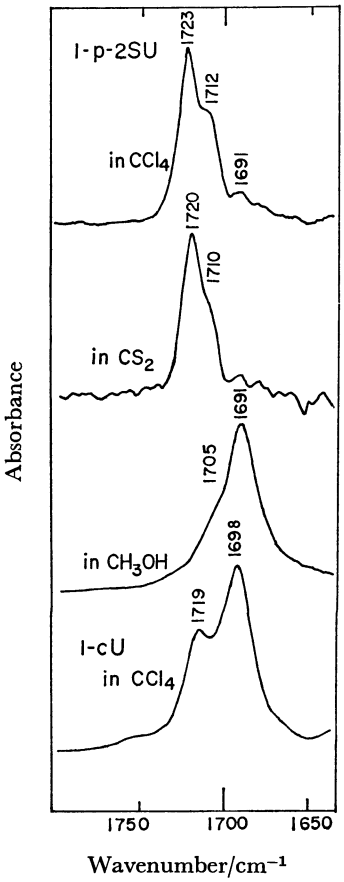

Fig. 1. Infrared absorption spectra of 1-propyl-2-thiouracil (1-p-2SU) in various solvents. 1.3×10^{-3} M in CCl_4; 9.2×10^{-4} M in CS_2, and 8.0×10^{-3} M in CH_3OH, and of 1-cyclohexyluracil (1-cU) 1.6×10^{-3} M in CCl_4.

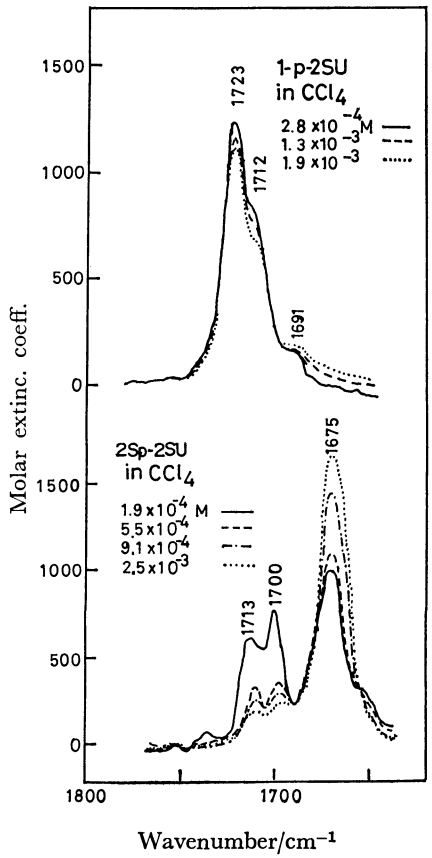

Fig. 2. Infrared absorption spectra of 1-propyl-2-thiouracil and 2-*S*-propyl-2-thiouracil (2Sp-2SU) in the solution of CCl_4 at various concentrations.

and 8 ns in duration time. This was combined with a monochromator, photomultiplier (HTV R106 UH) and Tektronix 465 (100 MHz) oscilloscope. Decay curves were also observed with another apparatus with two electromagnetic shutters placed before and after the light beam (313 nm from a Hg lamp) goes into the sample,[8] through the courtesy of Professor Minoru Kinoshita, Dr. Noriko Iwasaki and Dr. Nobuyuki Nishi, Institute for Solid State Physics, University of Tokyo.

Infrared Absorption Spectra

Carbonyl Bands of Free Base. As is shown in Fig. 1 (top), 1-propyl-2-thiouracil shows two absorption bands at 1723 and 1712 cm^{-1} in the carbonyl stretching region, in its dilute CCl_4 solution (concentration 1.3×10^{-3} M). In dilute CS_2 solution it shows two bands at 1720 and 1710 cm^{-1}. 1-Cyclohexyluracil, which has two carbonyl groups at 2- and 4- positions, shows also two bands in the 1700 cm^{-1} region, at 1719 and 1698 cm^{-1}.

The appearance of two (instead of one) carbonyl bands for 1-propyl-2-thiouracil, free from any hydrogen bonds, may be ascribed to a Fermi resonance, although there is no strong evidence established for supporting this idea.

Self-association. When the concentration of 1-propyl-2-thiouracil in CCl_4 is increased from 2.8×10^{-4} M to 1.9×10^{-3} M, a lowering of the absorption intensities of the 1723 and 1712 cm^{-1} bands takes place while their intensity ratio remains unchanged. In addition, a new band appears at 1691 cm^{-1} (see Fig. 2). These are considered to be caused by a formation of a hydrogen-bond dimer of two 2-thiouracil residues, such as Fig. 3 (d). The 1691 cm^{-1} band is assigned to such a dimer. On the basis of an intensity measurement of the 1723 and 1691 cm^{-1} bands, and by postulating only one form of dimer, the equilibrium constant of the dimerization has been estimated to be about 40 M^{-1} in CCl_4 at 34 °C.

2-*S*-Propyl-2-thiouracil shows three bands (at 1700, 1713, and 1675 cm^{-1}) in its dilute (1.9×10^{-4} M) CCl_4 solution (see Fig. 2, lower). The former two are assignable to the carbonyl bands of free base, while the last one is assignable to a dimer band. The equilibrium constant of dimerization of 2-*S*-propyl-2-thiouracil has been found to be about 100 times as great as that of 1-propyl-2-thiouracil (see Fig. 3 (f)).

Hydrogen-bond Complex with Methanol. When methanol is added to a dilute CCl_4 solution of 1-propyl-2-thiouracil, the 1723 and 1712 cm^{-1} bands become weaker and two new bands appear at 1708 and 1693 cm^{-1}, which become stronger with the methanol concentration (see Fig. 4, top). Both of the 1708 and 1693 cm^{-1} bands are attributed to a binary complex of 1-propyl-2-thiouracil and methanol bound through

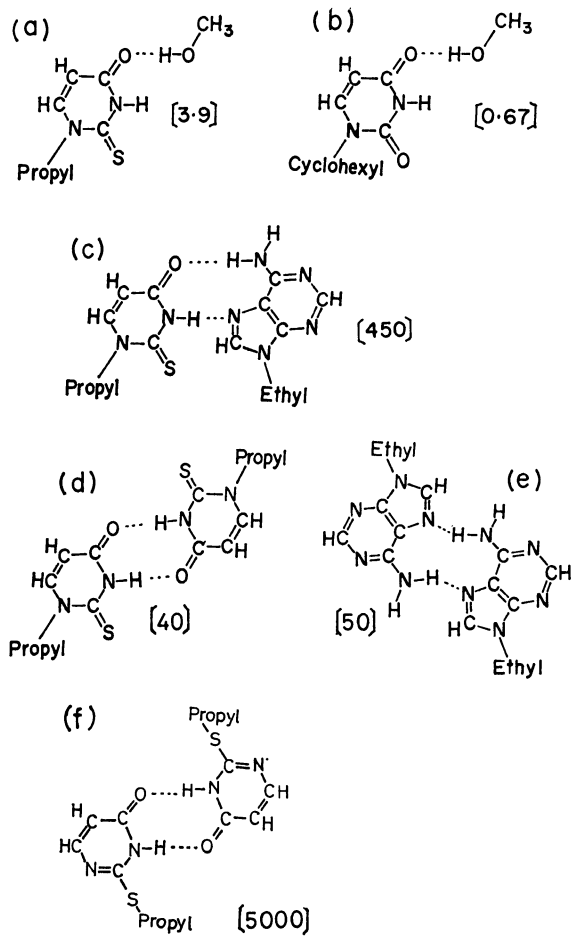

Fig. 3. Possible structures of hydrogen-bonded complexes involving uracil, 2-thiouracil, and/or adenine residues. The figure in bracket indicates the equilibrium constant in M^{-1} of the association reaction of each complex in CCl_4 at 34 °C. The equilibrium constants have been calculated by the method of Refs. 6 and 14.

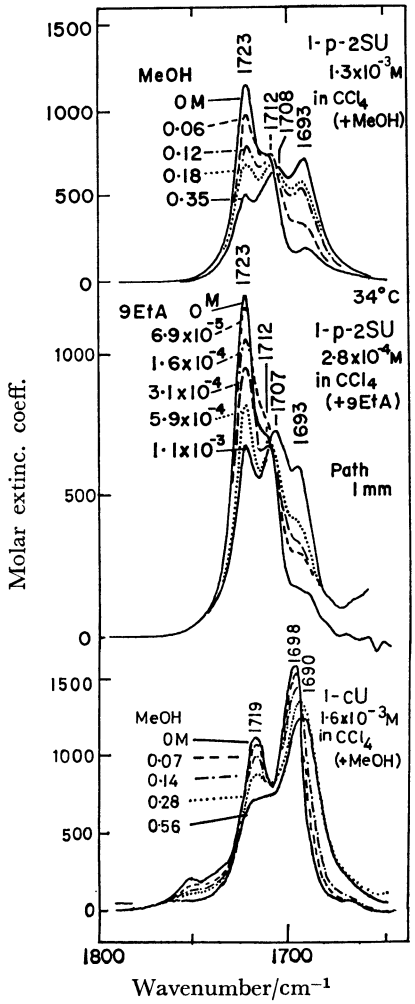

Fig. 4. Infrared absorption spectra of 1-propyl-2-thiouracil in the solutions of CCl_4+CH_3OH and in the solutions of CCl_4+9-ethyladenine and of 1-cyclohexyluracil in the solutions of CCl_4+CH_3OH.

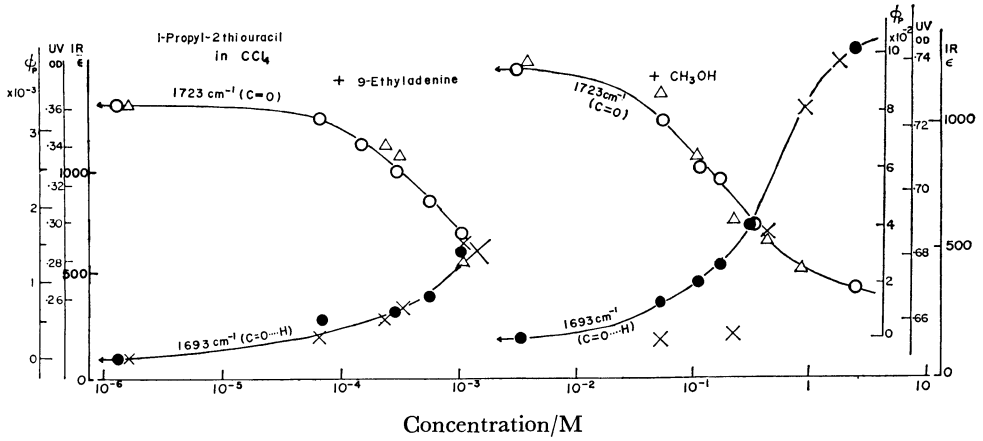

Fig. 5. The absorption- and phosphorescence-intensities of 1-propyl-2-thiouracil in CCl_4 plotted against the concentration of the proton-donor molecule (methanol or 9-ethyladenine) in the solvent.
(○ and ●): Infrared absorption intensities; concentration of 1-propyl-2-thiouracil, temperature of the solution, and optical path length are all what are given in Fig. 4, (△): ultraviolet absorption intensity at 302.5 nm, read from the curves given in Fig. 7, (×): phosphorescence intensity at 410 nm, read from the curves given in Fig. 8; the arrows in this figure show intensities of 1-propyl-2-thiouracil in pure CCl_4.

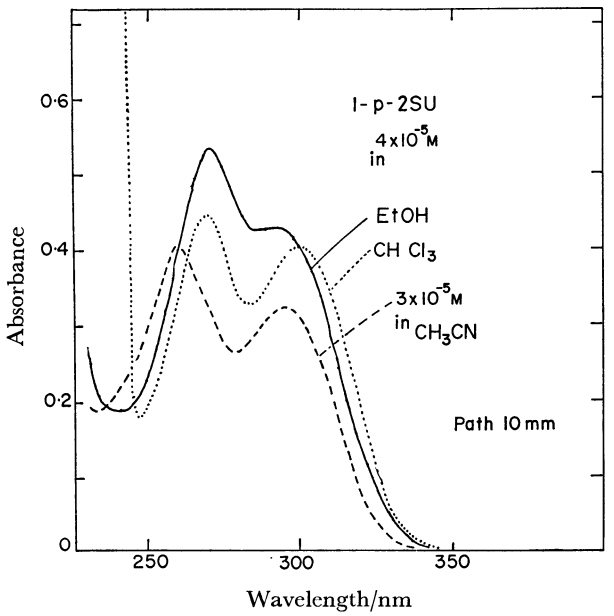

Fig. 6. Ultraviolet absorption spectra of 1-propyl-2-thiouracil in various solvents.

the O–H···O hydrogen bond between the methanol OH and position-4 carbonyl of the 2-thiouracil residue (see Fig. 3 (a)). By assuming that only one form of complex is involved in the methanol-2-thiouracil interaction, the equilibrium constant for that complex formation has been estimated from the intensity measurements of the carbonyl bands. This is found to be 3.9 M^{-1} in CCl_4 at 34 °C. This value is to be compared with the equilibrium constant, 0.67 M^{-1}, for the complex formation (Fig. 3 (b) and Fig. 4, bottom) between methanol and 1-cyclohexyluracil, which was determined in a similar way in dilute CCl_4 solution at 34 °C.

Association with Adenine. If 9-ethyladenine, instead of methanol, is added to the dilute CCl_4 solution of 1-propyl-2-thiouracil, the complex formation is caused more easily. As may be seen in Figs. 4 (middle) and 5, the lowering of the 1723 cm^{-1} band (free C=O) and the elevation of the 1693 cm^{-1} band (hydrogen-bonded C=O) take place for 9-ethyladenine at a concentration 100 times as low as that for methanol. This fact may be taken as indicating that a binding (such as Fig. 3 (c)) of 2-thiouracil and adenine residues is much greater than that of 2-thiouracil and methanol. The association constant of the former pair has been determined to be 450 M^{-1} in CCl_4 at 34 °C.

Ultraviolet Absorption Spectra

Solvent Effect. 1-Propyl-2-thiouracil shows two strong absorption peaks at 268 and 300 nm in $CHCl_3$, while in ethanol these two come closer, at 272 and 295 nm (Fig. 6).

Complex with Methanol. On adding methanol to a dilute (7×10^{-4} M) CCl_4 solution of 1-propyl-2-thiouracil, two absorption peaks (see Fig. 7 upper) at 269 and 301 nm (molar extinction coefficient are 12800 and 10400 respectively) are replaced by other

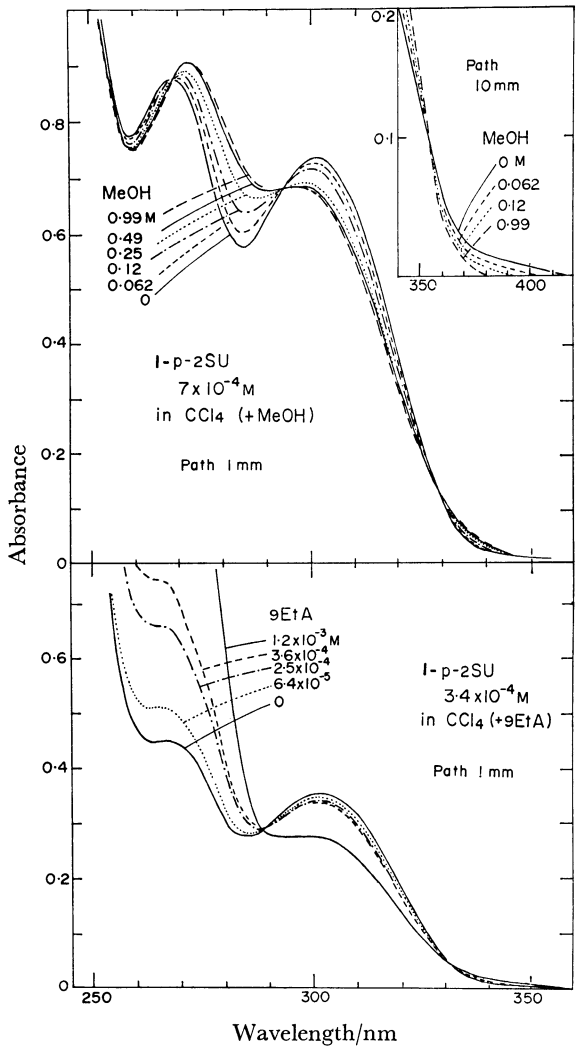

Fig. 7. Ultraviolet absorption spectra of 1-propyl-2-thiouracil in the solutions of CCl_4+CH_3OH (upper) and in the solutions of CCl_4+9-ethyladenine (lower).

two strong peaks at 272 and 298 nm. There appear isosbestic points not only at 268 and 294 nm, but also at 329 and 352 nm. Thus, there must be a very weak absorption band at about 400 nm in dilute CCl_4 solution, and this is shifted to a shorter wavelength on forming a complex with methanol. This weak band (molar extinction coefficient $\fallingdotseq 10^2$) is assignable to an n-π^* transition, and the two strong bands to π-π^* transitions.

The lowering of the 301 nm peak (or the elevation of the 269 nm peak) takes place on adding methanol in parallel with the lowering of the 1723 cm^{-1} band (see Figs. 4 and 5). Therefore, these ultraviolet spectral changes are attributed to the change from free 2-thiouracil base to the hydrogen-bonded complex which is the same as what was observed in our infrared spectroscopic examination. On the basis of a series of absorption intensity measurements, the association constant is calculated here to be 5.5 M^{-1} (which is comparable to 3.9 M^{-1}, obtained in the infrared absorption measurements).

By a similar experiment on a dilute methylcyclohexane solution of 1-propyl-2-thiouracil with ethanol,

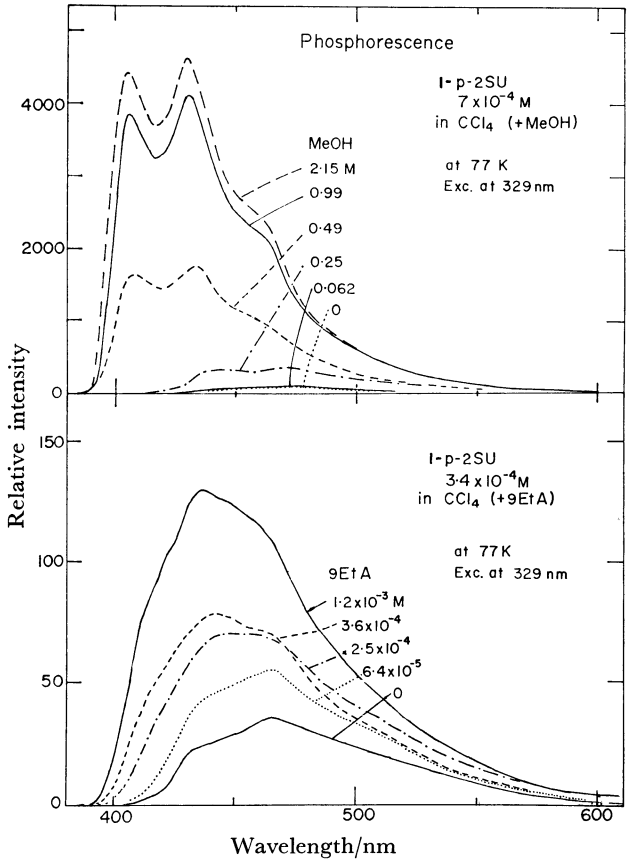

Fig. 8. Phosphorescence spectra of 1-propyl-2-thiouracil dissolved in the solutions of CCl_4+CH_3OH (upper) or dissolved in the solutions of CCl_4+9-ethyladenine (lower) and then cooled by liquid nitrogen. Sector: fast (about 10000 rpm).

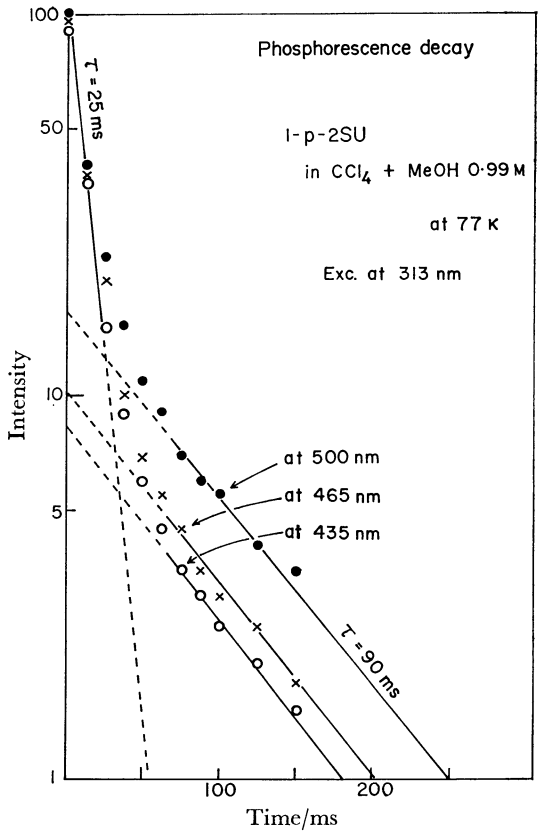

Fig. 9. Phosphorescence decay curves of 1-propyl-2-thiouracil dissolved in the solutions of CCl_4+CH_3OH and then cooled to the liquid nitrogen temperature. τ is the life time, which is the time required for bringing the intensity from 1 to 1/e.

the association constant of 1-propyl-2-thiouracil and ethanol is estimated to be 4.7 M^{-1}. When 2,2,2-trifluoroethanol, which is considered to be a stronger proton donor than ethanol (or methanol), is added to the dilute CCl_4 solution of 1-propyl-2-thiouracil, the complex bands appear at 270 and 291 nm. Here, the equilibrium constant for the complex formation is found to be 7.4 M^{-1}.

Complex with 9-Ethyladenine. Next 9-ethyladenine was added to the dilute CCl_4 solution of 1-propyl-2-thiouracil to observe ultraviolet absorption spectrum of the thiouracil–adenine complex examined in the infrared spectroscopy. Here a spectral change similar to that observed when CH_3OH was added instead of 9-ethyladenine was observed (see Fig. 7, lower). The isosbestic points appear at 289 and 331 nm. The decrease in the absorption intensity at 300 nm, which is considered to correspond to the decrease in the amount of free 2-thiouracil residue, goes in parallel with the lowering of the 1723 cm^{-1} band (see Figs. 4 and 5).

Phosphorescence

At liquid nitrogen temperature, 1-propyl-2-thiouracil shows only a weak phosphorescence (excited at 329 nm) with peaks at 445 and 465 nm in CCl_4. The phosphorescence is also very weak in 2-methylpentane and in methylcyclohexane. In all of these nonpolar solvents, the quantum yield ϕ_P was estimated to be 10^{-3}, on the basis of a comparison with the known phosphorescence of naphthalene.[9] While, in acetonitrile $\phi_P=0.02$, and in methanol and in ethanol $\phi_P=0.1$. In all of these three polar solvents, the phosphorescence maxima are found at 408 and 432 nm. When methanol is added stepwise into a dilute CCl_4 solution of 1-propyl-2-thiouracil, a stepwise increase in phosphorescence intensity is observed at 408 and 432 nm (Fig. 8, upper). When 9-ethyladenine is added, a similar increase in phosphorescence intensity at 408 nm (shoulder) and 432 nm (peak) is observed, except that it is observed at much lower concentration of 9-ethyladenine than that of methanol (Fig. 8, lower).

Phosphorescence lifetime of 1-propyl-2-thiouracil in pure CCl_4 was found to be 90 ms. Figure 9 shows phosphorescence decay curves at various wavelengths for 1-propyl-2-thiouracil in a mixed solvent $CCl_4+0.99$ M CH_3OH. On the basis of our infrared spectroscopic study, roughly 80% of the 1-propyl-2-thiouracil molecules in the solution are supposed to be involved in a hydrogen-bond complex (with CH_3OH) and remaining 20% are free. While, the phosphorescence decay curves indicate that the emission from this consists of two components: one with lifetime=25 ms at liquid N_2 temperature) which predominates at 435 nm and the other with lifetime=90 ms whose

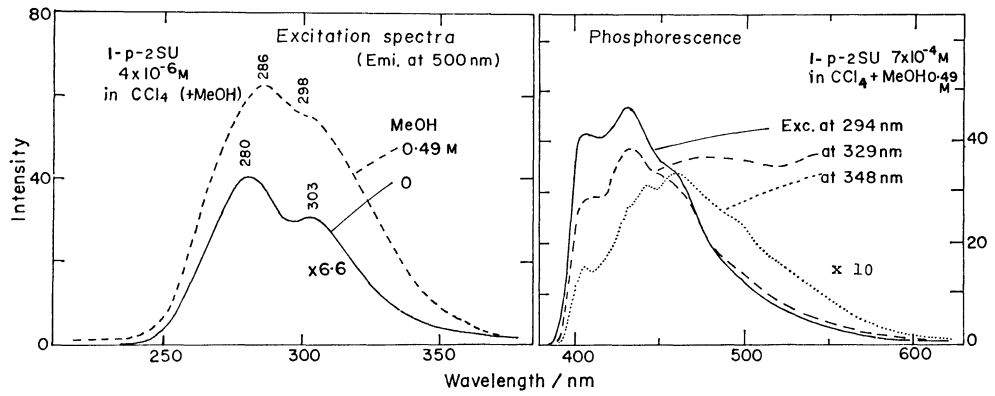

Fig. 10. (Left) Uncorrected excitation spectra of phosphorescence of 1-propyl-2-thiouracil dissolved in CCl_4 with and without CH_3OH. Sector: fast (about 10000 rpm). (Right) Phosphorescence spectra of 1-propyl-2-thiouracil in CCl_4+CH_3OH, where half of the thiouracil molecules are involved in a hydrogen-bond complex and the other half are free, with various excitation wavelengths. Sector: slow (about 1000 rpm).

contribution is only 17% (at zero time) at 500 nm and 8% at 435 nm.

The phosphorescence excitation spectrum of the free 1-propyl-2-thiouracil molecule has its maxima at 280 and 303 nm (Fig. 10, left). On going from a pure nonpolar solvent (CCl_4) to a mixed solvent ($CCl_4+0.49$ M CH_3OH) the excitation spectrum shows a change which is similar to the corresponding change in the ultraviolet absorption spectrum; *i.e.*, a blue shift of the longest wavelength peak and a red shift of the second longest wavelength peak. In the mixed solvent of $CCl_4+0.49$ M CH_3OH, about 50% of the 1-propyl-2-thiouracil molecules are involved in a hydrogen-bond complex (with CH_3OH) and 50% is free, on the basis of our infrared spectroscopic study. Figure 10 (right) shows phosphorescence spectra of 1-propyl-2-thiouracil in such a solvent with various excitation wavelengths (294, 329, and 348 nm). This figure indicates that the phosphorescence component with longer lifetime is located in the longer wavelength region ($\approx$500 nm), and that the longer wavelength excitation (348 nm) causes the longer lifetime component.

Discussion

It has been shown in our present study that, in an interaction of the 2-thiouracil residue with a residue having a proton-donating power, the position-4 carbonyl can act as a strong proton-acceptor.

Secondly, it has been found that the proton accepting power of the position-4 carbonyl of 2-thiouracil residue is greater than that of uracil residue. This is supported by an infrared spectroscopic study in the NH stretching region (3000 cm^{-1}) made by one of us, Higuchi.[10] This finding may be associated with some biological significance. Thus, it is in consistence with the fact[11] that a trinucleotide diphosphate codon $s^2U_pU_pU$ or $U_ps^2U_pU$ causes an *in-vitro* ribosome binding of *Escherichia coli* $tRNA^{Phe}$ (whose anticodon is G_pA_pA) more efficiently than a codon U_pU_pU. The finding may also be associated with the fact that a thermal stability of *Thermus thermophilus* HB8 tRNA is acquired by replacing the ribothymidine in its TψC loop by 2-thiothymidine.[2] It is true that an X-ray structure analysis for yeast $tRNA^{Phe}$ showed that the ribothymidine in TψC sequence binds to 1-methyladenosine with the position-2 carbonyl in the hydrogen bonding.[12] We suspect, however, that the position-4 carbonyl (instead of the position-2 carbonyl) is involved in the corresponding hydrogen bonding in the HB8 tRNA. We like to point out that, such a hydrogen bonding is quite possible if the 1-methyladenosine takes a *syn* conformer instead of *anti* (like in yeast $tRNA^{Phe}$), and that such a small variation in the bonding scheme is not improbable among different tRNA molecules.

It has been shown that the binding between 2-thiouracil residue and adenine residue is much stronger than the binding between 2-thiouracil residue and methanol. This fact indicates that there must be a second hydrogen bond between the 2-thiouracil and adenine residues in addition to the hydrogen bond between the position-4 carbonyl of the 2-thiouracil and the position-6 amino group of adenine. The second hydrogen bond may be formed between the position-3 imide of 2-thiouracil and position-7 nitrogen of adenine but it may also be formed between the position-3 imide of 2-thiouracil and position-1 nitrogen of adenine.

We have shown in the present work that hydrogen bonding of 2-thiouracil residue with alcohol or with adenine causes a marked enhancement of the phosphorescence. This fact suggests that phosphorescence may be a useful probe of the intramolecular environment of the 2-thiouracil residue in a biological system. In pursuing this line, however, it is desirable to discuss on the nature of the excited singlet and triplet states of this residue (especially these states with hydrogen bonding) slightly more in detail.

The ultraviolet absorption spectrum suggests (see Fig. 7) that 2-thiouracil residue in nonpolar solvent has an $n\rightarrow\pi^*$ level (S_1) at 2.5×10^4 cm^{-1} (its 0—0 transition is probably around 400 nm) and two $\pi\rightarrow\pi^*$ levels (S_2 and S_3) around 2.9×10^4 cm^{-1} (340 nm). When its position-4 carbonyl is brought into an intermolecular hydrogen bonding, S_1 is elevated by about 1300 cm^{-1}, S_2 is elevated by about 400 cm^{-1}, while

Table 1. A classification of phosphorescence of 2-thiouracil residue

	Transition frequency cm^{-1} (nm)	Intensity	Lifetime ms	Proposed assignment of the triplet state
I	24000 (410)	Weak	90	$^3(n\pi^*)$, free
II	26000 (385)	Strong	25	$^3(n\pi^*)$ with H-bonds

S_3 is lowered by about 300 cm^{-1}. On the basis of Strickler-Berg relation, [13] the radiative lifetime of S_1 is estimated to be 10^{-6} s (from the $S_1 \leftarrow S_0$ absorption intensity) and of S_2 10^{-8} s at room temperature. The fluorescence quantum yield ϕ_F is very low; it is less than 10^{-4}. Therefore, from the relation $\phi_F = k_F/(k_F + k_{nr}^F + k_{isc})$, k_{nr}^F and/or k_{isc} must be as high as 10^{10}, where k_F ($\fallingdotseq 10^6$) is the $S_1 \rightarrow S_0$ fluorescence rate constant, k_{nr}^F is the $S_1 \rightarrow S_0$ internal conversion rate constant, and k_{isc} is the $S_1 \rightarrow T_1$ intersystem crossing rate constant. Because phosphorescence is appreciable at liquid nitrogen temperature, k_{isc} is supposed to be high here.

In interpreting the observed phosphorescence spectra, we propose to classify them into two, as shown in Table I. The weak phosphorescence of 1-propyl-2-thiouracil residue in pure CCl_4 (see Fig. 8, upper) with lifetime 90 ms ($k_p \fallingdotseq 11\ s^{-1}$) is spectrum I. When methanol is added to the CCl_4 solution, a stronger phosphorescence appears first at the slightly shorter wavelength with nearly the same lifetime. Its intensity becomes stronger with the methanol concentration. A further addition of methanol to the solution results in the appearance of a new phosphorescence, at about 1600 cm^{-1} higher frequency than spectrum I. This is spectrum II. The phosphorescence lifetime of spectrum II is shorter *i.e.*, 25 ms ($k_p = 40\ s^{-1}$) at 77 K. There would be no problem to attribute spectrum I to the free 2-thiouracil residue. We speculate here that the intensity of spectrum I, which is due to the free molecule, becomes higher by a change of the environment, *i.e.*, from matrix without methanol to that with some amount of methanol. On the other hand, the fact that the spectrum II become stronger with methanol concentration is attributable to the increase of the amount of 2-thiouracil hydrogen-bonded to methanol. In other words, we postulated here a dynamic equilibrium among free triplet-state 2-thiouracil residue $^3(2SU)^*$, a hydrogen-bonded complex $^3(2SU)^* \cdot CH_3OH$ and the free CH_3OH molecules.

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