Studies on Prototropic Tautomerism in Neutral and Monoanionic Forms of Pyrimidines by Nuclear Magnetic Resonance Spectroscopy

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NMR methods have been applied to evaluation of prototropic tautomerism, N(1)H ⇌ N(3)H, in several selected pyrimidines, viz. the neutral forms of isocytosine and 2-alkylthiopyrimidone-4, and the monoanionic forms of uracil, 5-fluorouracil and 4-thiouracil.

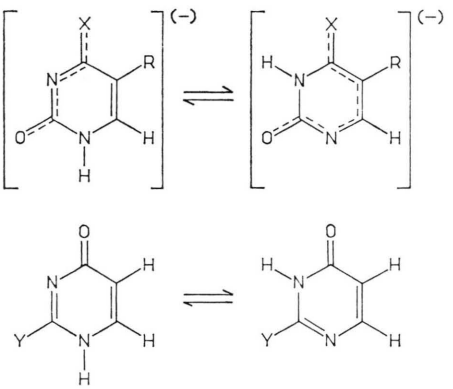
The predominant tautomeric species of the neutral forms could be estimated only qualitatively from ^1H chemical shifts. For the monoanionic forms this procedure was not applicable, for reasons which are discussed in detail.

For the monoanionic form of uracil, ^{13}C chemical shifts of C(5) provided a suitable criterion for quantitative estimation of the populations of the two known tautomeric species. However, the potential scope of this procedure appears somewhat limited.

By contrast, the values of the vicinal proton-proton coupling constants, $J(5,6)$, provided both necessary and adequate criteria for quantitative evaluation of the tautomer populations for all the neutral and monoanionic forms. The results were in satisfactory agreement with those obtained by optical spectroscopic methods. In some instances the results obtained in this way may be more reliable than those derived from optical methods.

The range of applicability, and utility, of NMR methods to studies on protropic tautomerism in pyrimidines are critically assessed.

Considerable attention has been devoted to investigations on the tautomerism of natural purines and pyrimidines, and of many of their analogues, to a large extent because of the biochemical and genetic significance of this phenomenon. A good deal of the information in this field has been derived with the aid of UV and IR spectroscopy, extensively reviewed by Elguero *et al.* [1], and most widely applied in solution studies [2–4]. Investigations on such tautomerism in the gas phase, and their relevance to solution studies, have been recently reviewed by Beak [5]. NMR methods have also been applied, with particular utility in studies on keto-enol and amino-imino tautomerism of such compounds as uracil, cytosine, and some of their derivatives and nucleosides [1, 6–8]. Additional development include the use of ^{13}C chemical shifts for evaluation of tautomer populations in purines and purine nucleosides [9, 10], and of ^{15}N chemical shifts for establishment of site(s) of protonation on the aglycones of nucleosides [11].



Scheme 1. Prototropic tautomeric equilibria N(1)–H ⇌ N(3)–H in monoanionic (upper) and neutral (lower) forms of pyrimidines. The N(1)–H tautomers are shown at the left and the N(3)–H tautomers to the right. R=H or F, X=O or S, Y=NH_2 or alkylthio.

We present below the results of attempts of apply NMR methods to the quantitative evaluation of prototropic tautomerism, N(1)–H ⇌ N(3)–H, in the neutral forms of some pyrimidines, and the monoanionic forms of others (see Scheme 1). The compounds selected for this study were amongst those previously investigated by optical spectro-

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Abbreviations: UV, ultraviolet; IR, infrared; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; DSS, sodium 2,2 dimethyl, 2 silapentane sulfonate; TMS, tetramethylsilane.

scopic methods, so as to permit of a direct comparison of the range of application of the two procedures.

As will now be shown, one of the interesting findings is the hitherto unreported use of vicinal proton-proton coupling constants to measurements of equilibrium tautomer populations in solution. As in the case of optical methods, this was based on the use of the mono N(1)- and N(3)-methyl derivatives constituting the two fixed tautomers in each system.

Experimental

Uracil was a product of Waldhof Pharmacia (Stuttgart, GFR), 5-fluorouracil was obtained from Hoffman-LaRoche (Zurich, Switzerland), and 4-thiouracil was synthetized according to Ikehara *et al.* [12].

1-Methyl, 3-methyl and 1,3-dimethyl uracils were prepared as elsewhere described [13], as were 1-methyl [14] and 3-methyl 4-thiouracils [15]. The corresponding methylated 5-fluorouracils were obtained by scaling up a previous method [2], based on dimethylsulphate treatment of 5-fluorouracil in neutral aqueous medium, and crystallization of the products from aqueous methanol to give 1-methyl-5-fluorouracil (m.p. 256 – 258 °C), 3-methyl-5-fluorouracil (m.p. 196 – 197 °C) and 1,3-dimethyl-5-fluorouracil (m.p. 132 – 134 °C). The synthesis of 2-butylthiopyrimidone-4 has been elsewhere described [16]. The 1-methyl and 3-methyl derivatives of 2-ethylthiopyrimidone-4 were kindly provided by Dr. P. H. Bell of Lederle Laboratories (American Cyanamid Co., Pearl Harbour, N. Y.). Isocytosine, and its two N-methylated derivatives, were prepared according to Brown and Teitei [17].

Purity of all compounds was additionally checked by paper and thin-layer chromatography, as well as by their UV absorption spectra.

Aqueous solutions (in ^2H_2O) of the monoanionic forms of compounds were prepared by addition of conc. NaOD (>99.7 mol % ^2H from Merck, Darmstand, GFR) to give pD ~12 for uracil and its N-methylated derivatives, and of conc. ammonia to give pD ~10.5 for 4-thiouracil, 5-fluorouracil and their methylated analogues. These pD values were selected on the basis of the known pK values for monoanion formation for the foregoing compounds.

^1H and ^{13}C NMR spectra were recorded on JEOL JNM-4H-100 and Bruker-90 instruments at a common probe temperature of 22 °C, using ~0.4 M solutions in ^2H_2O (>99.7 mol % ^2H, from Merck) for the monoanionic forms with DSS as internal standard, or in DMSO-d_6 (99.5 mol % ^2H, from Merck) for neutral forms with TMS as internal standard.

Results and Discussion

The signals originating from the H(5) and H(6) protons are doublets characteristic for an AB system which may be treated as an AX system. The distances between the doublet components represent reasonably accurately the coupling constants $J(5-6)$. The chemical shifts of H(5) and H(6), and the coupling constants $J(5-6)$, for the various compounds and their methylated derivatives are collected in Tables I and II.

^1H Chemical shifts

As was to be anticipated, all of the pyrimidines examined exhibited characteristic differences between the H(5) and H(6) chemical shifts of the N(1)-methyl and N(3)-methyl derivatives. If the H(5) and H(6) chemical shifts of these methylated derivatives were to correspond to those for the appropriate tautomer, *i. e.* N(1) – H and N(3) – H, they could be used directly to calculate the populations of the two tautomeric forms. Under conditions of rapid tautomeric exchange, the chemical shifts of H(5) and H(6) for the non-methylated derivatives would represent the weighted means of the corresponding chemical shifts for the methylated fixed tautomers. The existence of rapid exchange between tautomers is, of course, obvious from the existence of single doublets for H(5) and H(6) of all compounds.

From Table I it is clear that, in some instances, the chemical shifts of non-methylated derivatives, which consist of an equilibrium mixture of two tautomers, are outside the range of those for the methylated, fixed, tautomeric forms. It follows that the chemical shifts of the N-methylated forms do not correspond to those for the corresponding natural tautomers; and this must be due to the effect, on the chemical shifts of H(5) and H(6), of the magnetic anisotropy of the $N-CH_3$. Attempts were made to evaluate the magnitude of this effect by measuring the changes in chemical shifts due to N-methylation in the neutral forms of uracil and 5-fluorouracil (Table II). Application of these data to correct the observed chemical shifts of H(5) and H(6) in the monoanionic forms did not, however, lead to the expected changes in the range of chemical shifts. The magnitudes of the estimated corrections are of the same order as the differences in chemical

Table I. Chemical shifts of H-5 and H-6, and coupling constants $J(5,6)$, for various pyrimidines and their N-methylated derivatives, in neutral or monoanionic forms, and the calculated populations of N_1-H and N_3-H tautomers, as compared to those calculated from UV spectroscopy (data from refs. 2, 3, 16, 21).

	Chemical shifts [ppm ± 0.01]		$J(5,6)$ [Hz ± 0.05]	Tautomer populations [%] by NMR		by UV	
	H-5	H-6		N_1-H	N_3-H	N_1-H	N_3-H
Monoanionic forms [a]							
Uracil	5.70	7.57	6.80	52	48	51	49
1-Methyluracil	5.71	7.44	7.32				
3-Methyluracil	5.74	7.66	6.26				
4-Thiouracil	6.53	7.68	5.84	16	84	27 (25)	73 (75) [c]
1-Methyl-4-thiouracil	6.55	7.34	6.89				
3-Methyl-4-thiouracil	6.69	7.61	5.64				
5-Fluorouracil	—	7.52	4.96	61 (75)	39 (25) [d]	63	37
1-Methyl-5-fluorouracil	—	7.56	5.86				
3-Methyl-5-fluorouracil	—	7.69	3.53				
Neutral forms [b]							
Isocytosine	5.52	7.52	6.68	31	69	25	75 [e]
1-Methylisocytosine	5.50	7.28	7.45				
3-Methylisocytosine	5.60	7.50	6.33				
2-Butylthiopyrimidone-4	6.05	7.81	6.32	0	100	0	100 [f]
1-Methyl-2-ethylthiopyrimidone-4	5.81	7.68	7.53				
3-Methyl-2-ethylthiopyrimidone-4	6.16	7.81	6.30				

[a] In D_2O, at appropriate *p*D, *vs* internal DSS.
[b] In DMSO-d_6, *vs* internal TMS.
[c] Values in brackets are from infrared spectroscopy.
[d] Figures in brackets correspond to corrections obtained on the assumption that the effect of an N-methyl substituent on chemical shifts is the same as for the neutral forms.
[e] In 50% aqueous dioxane.
[f] In $CDCl_3$.

Table II. Chemical shifts of H-5 and/or H-6 (in ppm *vs* internal TMS) and coupling constants $J(5,6)$ (in Hz) for the neutral forms of uracil, 5-fluorouracil and their N-methylated derivatives in DMSO, and the observed effects of N-methylation on these values.

Compound	Chemical shifts [ppm]		$J(5,6)$ [Hz]	Effect of N-methylation on		
				Chemical shift [ppm]		$J(5,6)$ [Hz]
	H-5	H-6		H-5	H-6	
Uracil	5.46	7.39	7.70	—	—	—
1-Methyluracil	5.52	7.61	7.82	+0.06	+0.22	+0.12
1,3-Dimethyluracil	5.66	7.66	7.78	+0.20	+0.27	+0.08
5-Fluorouracil	—	7.73	6.17	—	—	—
1-Methyl-5-fluorouracil	—	8.08	6.82	—	+0.35	+0.65
3-Methyl-5-fluorouracil	—	7.81	5.57	—	+0.08	−0.60
1,3-Dimethyl-5-fluorouracil	—	8.13	6.20	—	+0.40	+0.03

shifts for the two tautomeric species; hence the necessity of accurate measurements of these corrections. The corrections evaluated for the neutral forms of uracil and 5-fluorouracil are not of sufficient accuracy to apply to the monoanionic forms, or to the neutral form of isocytosine. It consequently appears that the use of proton chemical shifts data alone for measurements of the tautomeric constants, K_T, for the compounds embraced in this study, is not applicable. Nonetheless the values of the chemical shifts of H(6) of the unsubstituted forms are closer to the values of the N-methyl derivatives corresponding to the predominant tautomen, so that the chemical shift of H(6) does indi-

cate which tautomer predominates. It should be noted that difficulties associated with the use of chemical shifts for quantitative evaluations of tautomeric constants have been reported by others [18].

^{13}C chemical shifts

Attention was then directed to the use of ^{13}C chemical shifts, using as a model system uracil (and its two N-monomethyl derivatives). From the results, shown in Table III, it will be noted that only

Table III. ^{13}C chemical shifts (in ppm *vs* internal dioxane) for the monoanionic forms of uracil and its N-methyl derivatives.

Compound	CH_3	C-2	C-4	C-5	C-6
Uracil	—	94.7	106.7	33.5	84.1
1-Methyluracil	−29.7	94.2	110.9	35.0	79.8
3-Methyluracil	−39.0	113.9	124.4	32.0	77.5

in the case of C(5) does the chemical shift for the uracil monoanion fall in a range intermediate between those for the anions of the 1-methyl and 3-methyl derivatives. The resulting calculated proportions of the N(1) − H and N(3) − H forms in the uracil monoanion is 1 : 1, in agreement with results obtained by optical methods. However, the fact that the chemical shifts of only one carbon reflect the known tautomeric properties of the uracil monoanion raises some questions as to the general applicability of this procedure (see Concluding Remarks) below.

Coupling constants

From Table I it will be noted that, for all five pyrimidine system investigated, the values of the coupling constants $J(5-6)$ are different for the N(1)-methyl and N(3)-methyl derivatives. Those for the N(1)-methyl derivatives are larger than for the corresponding N(3)-methyl analogues by 1.0 − 1.2 Hz. For the N-methylated 5-fluorouracil derivatives, the value of $J(5F-6H)$ for the N(1)-methyl derivative is 2.3 Hz higher than that for the N(3)-methyl analogue.

Under conditions of rapid tautomeric exchange, the coupling constants of a mixture of two tautomers in equilibrium should be the mean weighted value of the coupling constants for the individual tautomers and their relative populations. Assuming that the coupling constants of the individual fixed tautomers N(1)-methyl and N(3)-methyl have the same values as those for the equilibrium tautomers N(1) − H and N(3) − H, the relative populations of the two tautomeric forms were evaluated for the five pyrimidine systems investigated (Table I). It will be noted from the table that the coupling constants for the equilibrium mixtures of tautomers of the individual pyrimidines do, in fact, fall in the range between the coupling constants for the two, fixed, N-methylated forms of each. The resulting error in the determination of the populations of the two forms derives largely from the error in measurement of coupling constants (statistical error, ~0.05 Hz), and from the assumption of equality of coupling constants for an N-methylated fixed tautomeric form and the corresponding natural N − H tautomer. The latter may be evaluated approximately, by comparison of the coupling constants for the neutral form with those for the neutral forms of the N-methylated derivatives, so as to estimate the effect of a given N-methyl on the coupling constants (see Table II).

The results for 5-fluorouracil and its N-methylated derivatives point to additivity of the changes in $J(5-6)$ due to N_1 and N_3 methylation. It is pertinent to recall that such additivity of the influence of substituents in the benzene ring has been noted experimentally [19], and predicted theoretically [20]. Note from Table II that an N_1 methyl group increases the value of $J(5-6)$ in uracil by 0.12 Hz and in fluorouracil by 0.65 Hz, whereas an N_3 metyl substituent decreases $J(5-6)$ in uracil by 0.04 Hz and in 5-fluorouracil by 0.60 Hz. For uracil and 4-thiouracil anions, and the neutral forms of 2-butylthiopyrimidone-4 and isocytosine, where calculations of tautomer populations are based on measurements of proton-proton coupling constants, the corrected results differ only minimally (0 − 4%) from those obtained without introducing corrections for the N-methyl substituents. By contrast, in the case of 5-fluorouracil, where measurements are based on fluorine-proton coupling constants, the corrected results differ appreciably from the uncorrected, leading to an increase in the population of the predominant form N(1) − H of 14%.

Comparison with Results from Optical Spectroscopy

Monoanionic forms: In the case of uracil, the results agree with those obtained by UV spectroscopy to within 5% [1]. With 4-thiouracil the NMR results differ by 10% from those obtained by optical methods. This may be due to the lower ac-

curacy of the optical spectroscopic methods in this instance, resulting from the following: (a) the UV absorption spctra of the two thiouracil monoanions fully overlap, so that differential spectroscopy at the limit of resolution had to be employed to estimate the tautomer populations [3], and (b) the estimates from IR spectroscopy were based on the integral intensities of the C(5) = C(6) bands in 4-thiouracil and 1-methyl-4-thiouracil, with accompanying errors resulting from the fact that these bands were not adequately resolved.

In the case of 5-fluorouracil monoanions, the NMR results uncorrected for the influence of N-methyl substituents on $J(5,6)$ are in agreement with those obtained by UV spectroscopy [2]. Introduction of this correction gives results somewhat different from those obtained by UV. However, it must be borne in mind that the corrections applied to the monoanions were those obtained for the neutral form, although it is to be anticipated that the sign of the correction will be similar in the two cases. It would therefore follow that the N(1) – H tautomer is predominant, to the extent of about 70%.

Neutral forms: In the case of 2-butylthio-4-oxopyrimidine, the NMR results were obtained in a solvent different from that previously employed with UV spectroscopy [16]. However, in non-aqueous media both methods point to the presence of only one tautomeric species, *viz.* N(3) – H.

For isocytosine the UV results were obtained in solutions of various polarities (aqueous dioxane), the population of the N(3) – H tautomer increasing from 50% in aqueous medium to 100% in pure dioxane [21] and pointing to the marked influence of the dielectric constant of the solvent on the populations of the two tautomeric species. At room temperature the dielectric constant of water is 80.3 and of dioxane 2.2. For 50% aqueous dioxane, with a dielectric constant of about 40, the population of the N(3) – H tautomer is about 75%. In DMSO, with a dielectric constant of 46.5, the NMR method gives a value of 70%, hence in reasonably good agreement with the value from optical methods in aqueous dioxane. It also follows that, in different solvents with similar dielectric constants, the populations of the two tautomeric species are similar.

Concluding Remarks

For all five tautomeric pyrimidines examined here, the N(1) – H form exhibits a higher value of the coupling constant $J(5-6)$ than the corresponding N(3) – H tautomer. Since the values of these coupling constants are dependent on the electron density distribution in the region where the coupled nuclei are located, it is to be anticipated that the foregoing regularity is related to the type of charge distribution in each of the tautomeric forms.

From the infrared spectra of the monoanionic forms it is known that the N(1) – H tautomer contains the C(5) = C(6) double bond, whereas in the N(3) – H form this bond loses its double-bond character because of charge delocalization (see Scheme 1). For the neutral form of 2-butyl-thiopyrimidone-4 the change in location of the C(5) = C(6) band frequency, of the N(3) – H tautomer, relative to that for the N(1) – H, is due to its strong coupling to the C(4) = O and C(2) = N(1) bonds [16].

It is the bond-order which characterizes the properties of a given bond. It is to be anticipated that the bond order of the bonds in the tautomer N(1) – H will be higher than for the N(3) – H monoanion, and this is supported by the results of calculations by the CNDO/2 method for the uracil and 4-thiouracil monoanions [22]. Attempts to correlate the bond order of pyrimidine ring bonds with the coupling constant $J(5-6)$ have demonstrated a linear dependence of one on the order [23], with the higher bond order corresponding to the higher coupling constant. It follows that the observer changes in coupling constants are in accord with the proposals regarding the electronic structures in the vicinity of the C(5) = C(6) bond deduced from the IR spectra.

From the foregoing, and the reasonably close agreement of the results obtained with the use of coupling constants, it is clear that this procedure may be applied to measurements of prototropic tautomer populations in a wide variety of pyrimidine analogues. In some instances, *e.g.*, 4-thiouracil monoanion, the results may be more accurate than those obtained by optical methods. In general, however, this procedure should be considered as complementary to the optical methods. The NMR method also permits of measurements within the concentration range embraced by optical methods, as well as above this range by an order of magnitude. It may consequently supplement optical methods in cases where solubility considerations are important, both at low or high concentrations.

The use of the NMR technique also extends the range of useable solvent systems to those, *e. g.*, pyridine, dimethylformamide, which are unsuitable for UV or/and IR spectroscopy, a factor of some importance in studies on the role of the dielectric constant or other properties of the solvent system on tautomeric equilibria [24]. It should be noted, in this connection, that the 1 : 1 ratio of this two uracil monoanion tautomers by ^{13}C chemical shifts at a concentration of about 1 M is the same as that obtained by the use of UV and IR spectroscopy in the concentration range $10^{-4} - 10^{-2}$ M.

The possible range of application of ^{13}C chemical shifts calls for special comment. One of the major disadvantages of this method is the large amount of substance required in view of the low natural ^{13}C abundance. In our opinion this a serious limiting consideration. An additional drawback is the fact that the two uracil monoanion tautomers could be distinguished, and their populations evaluated, from the chemical shifts of only the C(5) carbon (see Table III). It is even questionable whether the ^{13}C chemical shifts could have, in this instance, been used to deduce the presence of two tautomeric species in the absence of the earlier UV and IR data on which this conclusion was based.

Similar considerations apply to the extensive data of Chenon *et al.* [9, 10] showing that the chemical shifts of certain carbons in purines and purine nucleosides lie outside the range of those in the N-methyl derivatives with fixed tautomeric forms. Prototropic tautomer populations [N(7) – H ⇌ N(9) – H] were evaluated from the chemical shifts of C(4) and C(5), and keto-enol (or thione-thiol) tautomerism from the chemical shift of C(6), both based on the following considerations. Chemical shift corrections due to a methyl substituent were most marked for carbons closest to the site of tautomerism; while measurements on a variety of analogues provided reasonable corrections for the chemical shifts of C(4) and C(5). An examination of their results also shows that the differences in chemical shifts, for a given carbon, between two tautomeric forms is most pronounced for a carbon adjacent to the site(s) of tautomerism.

Corrections due to methyl substituents were, in fact, usually small (~1 ppm), and did not significantly affect estimations of tautomer populations. Some doubts therefore exist as to whether it is justified to ignore the chemical shifts of the other carbons in calculations of tautomer populations, *e. g.*, C(8) in the case of the protomeric equilibrium N(7) – H ⇌ N(9) – H. From the data presented by the authors, we have found that it is, in fact, possible to calculate corrections on the basis of their assumptions. From the diagram of these authors, showing the chemical shifts of the individual carbons of the purines and N-methyl purines investigated, it can be seen that, in the absence of appropriate corrections, the tautomers populations calculated from the chemical shifts of the various carbons differ quantitatively. Hence, if the corrections are not large and do not appreciably affect the results, the selection of only some specific carbon(s) to estimate tautomer populations may not give accurate results.

Attention should, on the other hand, be drawn to the possible utility of the ^{13}C chemical shift data for evaluations of electronic charge distributions and bond orders in both purines and pyrimidines. This should be feasible in those instances where tautomeric populations have been independently established by optical methods. Such electronic charge distributions have hitherto been estimated by theoretical calculations, *e. g.*, for the two thymine monoanions [25].

Finally, the use of vicinal proton-proton coupling constants for determining tautomer populations might profitably be extended by the use of vicinal ^{1}H, ^{13}C coupling constants. For the pyrimidines reported on above, the necessary information regarding tautomer populations should be forthcoming from the coupling constants $^{1}H(6)$, $^{13}C(2)$ and $^{1}H(6)$, $^{13}C(4)$, as well as coupling constants via two bonds, *e. g.* $^{1}H(6)$, $^{13}C(5)$. In the case of N(7) – H ⇌ N(9) – H tautomerism in purines, one might profit from the couplings $^{1}H(8)$, $^{13}C(4)$ and $^{1}H(8)$, $^{13}C(5)$, whereas keto-enol (or thione-thiol) tautomerism could be established from the couplings $^{1}H(2)$, $^{13}C(6)$ and $^{1}H(2)$, $^{13}C(4)$. The use of proton-carbon couplings does, of course, suffer from the disadvantage (see above) that it requires inordinately high concentrations for recording of the necessary ^{13}C spectra. On the other hand it would serve as an excellent test for the procedures based on analyses of chemical shifts.

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