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An N.m.r. Investigation of Proton Mobility in Substituted Uracils

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The n.m.r. spectra for a series of fifteen substituted uracils have been observed in deuterated dimethyl sulfoxide (d_6-DMSO). The interpretation of these spectra provides a method of estimating the extent of tautomerization and proton mobility in this series. It has been shown that the spectral position of the amide proton correlates linearly with both the Hammett σ-constant and group dipole moment of the substituents at the 5-position, and that the proton at C_6 also correlates linearly with the group dipole moment of the 5-substituent. These results have been discussed with reference to the hydrogen bonding properties of the uracils, and their behavior in biological systems.

Introduction

At the present time, the literature on the replacement of naturally occurring pyrimidine bases of the desoxyribonucleic acid (DNA) of microörganisms by unnatural analogous pyrimidines is growing at an increasing rate. Many investigators in this field attribute this replacement to the similarity in steric properties of the pyrimidine bases. However, there is reason to believe that tautomeric structures and proton mobility in the pyrimidines should be considered to be at least as important as the steric factor, since hydrogen bonding between the purine–pyrimidine bases is of the utmost importance for maintenance of the physiologically active helical structure of the DNA molecule. It is the purpose of this communication to describe some fundamental studies by the methods of nuclear magnetic resonance (n.m.r.) spectroscopy of the tautomeric structures of many of the biologically important pyrimidines in order that a more complete understanding might be gained about the mechanism of pyrimidine replacement as influenced by the lability of protons on the pyrimidine nucleus.

Experimental

All spectra were obtained at 40 megacycles using a Varian model 4300B high-resolution n.m.r. spectrometer equipped with a field homogeneity control unit. Samples were prepared by dissolving the pyrimidines in dimethyl sulfoxide (DMSO) under dry nitrogen gas because of the hygroscopic character of the solvent. In cases where there are no high field proton peaks due to the solute, ordinary DMSO was used, but where there was a possibility of overlapping of the

TABLE I

Compound	Amide peaks with respect to TMS[a]	H_6 peaks with respect to TMS[a]	H_5 peaks with respect to TMS[a]	Substituent peaks with respect to TMS[a]	$J_{R_5-R_6}$	$J_{NH_1-R_6}$
6-Azauracil[b]	−498.7	−308.4	.....	.....	..	..
5-Bromouracil[b,c]	−472.3 (N_3) −462.8 (N_1)	.....	−327.7	.....	..	6.0
5-Chloro-6-methyluracil[b]	[e]	.....	.....	−103.3 (−CH_3)	..	0.0
5-Chloro-6-methyluracil[d]	−471.8	.....	.....	−103.3 (−CH_3)	..	.0
Cytosine[b,c]	[e]	−238.0	−308.4	.....	7.1	.0
5-Fluorouracil[b]	−471.9 (N_3) −448.4 (N_1)	.....	−324.1	.....	6.1	.0
5-Fluorouracil[d]	−472.9 (N_3) −442.6 (N_1)	.....	−323.7	.....	6.1	5.9
5-Iodouracil[b]	[f]	.....	−329.2	.....	..	0.0
5-Iodouracil[d]	−469.2	.....	−329.2	.....	..	3.9
5-Methylcytosine[b]	[e]	.....	−325.6	− 93.7 (−CH_3) −385.2 (−NH_2)	0.0	0.0
6-Methyluracil[b]	−448.1	−227.0	.....	− 94.7 (−CH_3)	0.8	.0
5-Nitro-6-methyluracil[b]	[e]	.....	.....	−106.2 (−CH_3)	..	.0
5-Nitro-6-methyluracil[d]	−486.1	.....	.....	−106.3 (−CH_3)	..	.0
5-Nitrouracil[b]	[f]	.....	−368.0	.....	..	.0
5-Nitrouracil[d]	−483.8	.....	−368.0	.....	..	.0
Orotic acid[b]	−466.4 (N_3) −448.8 (N_1)	−254.3	.....	−260.1 (−COOH)	..	..
Orotic acid[d]	−465.9 (N_3) −449.2 (N_1)	−254.6	.....	−269.1 (−COOH)	..	
2-Thiouracil[b]	−506.6	−247.6	−311.2	.....	7.6	0.0
2-Thiouracil[d]	−509.4	−247.8	−311.2	.....	7.6	5.6
Thymine[b,c]	−453.4 (N_3) −438.4 (N_1)	.....	−303.4	− 81.7 (−CH_3)	1.1	5.6
Uracil[b,c]	−452.5	−232.9	−309.6	.....	7.7	5.7
Uracil-5-carboxylic a.[b]	[f]	.....	−344.6	.....	..	0.0
Uracil-5-carboxylic a.[d]	−493.6	.....	−344.6	−236.0 (−COOH)	..	[g]

[a] External TMS capillary. [b] Neutral DMSO solution. [c] Spectra in previous report.[1] [d] Acidified by passing one bubble of anhydrous HCl through the sample. [e] Peak too broad for even rough calibration. [f] Peaks altogether unobservable. [g] The doublet separation, 5.1 ± 0.6 c.p.s., was not included in the table since the other coupling constants are measured to within 0.2 c.p.s. accuracy.

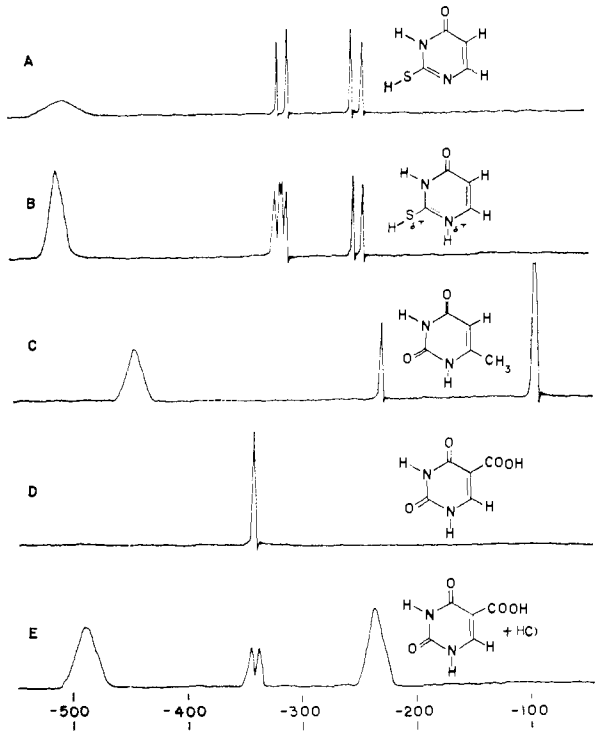


Fig. 1.—N.m.r. spectra of some substituted uracils: A, 2-thiouracil in neutral d_6-DMSO; B, 2-thiouracil in d_6-DMSO which has been acidified with anhydrous HCl; C, 6-methyluracil in neutral d_6-DMSO; D, uracil-5-carboxylic a, in neutral d_6-DMSO; E, uracil-5-carboxylic a in acidified d_6-DMSO.

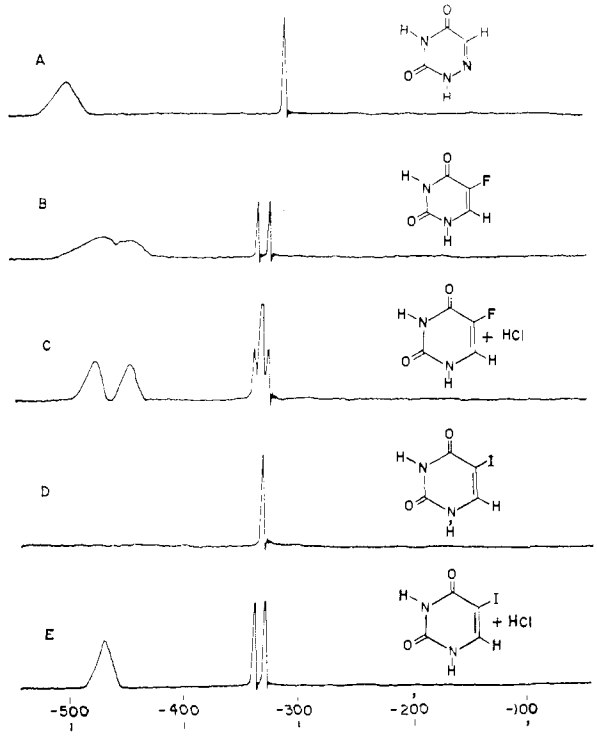


Fig. 2.—N.m.r. spectra of some substituted uracils: A, 6-azauracil in neutral d_6-DMSO; B, 5-fluorouracil in neutral d_6-DMSO; C, 5-fluorouracil in acidified d_6-DMSO; D, 5-iodouracil in neutral d_6-DMSO; E, 5-iodouracil in acidified d_6-DMSO.

solvent peak with those of the solute, fully deuterated dimethyl sulfoxide (d_6-DMSO) was used.[1] In each case the samples were transferred to 5-mm. sample tubes containing tetramethylsilane (TMS) reference capillaries, and calibrations were performed by the usual side-band modulation technique using a Krohn-Hite model 440-B audiooscillator which was checked against a Hewlett-Packard model 522-B electronic counter.

In cases where the labile protons were undergoing intramolecular exchange at such a rate that observation of the peaks due to these protons was impossible, a small bubble of anhydrous HCl was passed through a fine capillary tube into the sample solution. Careful control was required at this step, since an excess of HCl appears to cause partial degradation of the bases. The neutral samples can be stored at room temperature for many months without change in their spectra. However, once the solution is acidified with HCl, decomposition of the product usually occurs within a few hours to a few days if stored at room temperature.

Concentration studies were also conducted in solutions diluted from complete saturation down to 12.5% saturation. In the compounds thus studied, no appreciable change was observed in the position of the peaks.

The compounds used in this study were the commercially available materials, used without further purification. The n.m.r. spectra sufficed as a test of the purity of the compounds investigated.

Spectra of the Substituted Pyrimidines.—Figures 1, 2, 3 and 4 contain the spectra of the pyrimidines studied. The abscissa gives the chemical shifts in cycles per second (c.p.s.) relative to an external TMS capillary at approximately 20° using DMSO as a solvent. These parameters are also listed in Table I.

The broad peaks occurring below −430 c.p.s. from TMS are due to amide protons. The peaks in the vicinity of −230 to −250 c.p.s. from TMS are due to C_5 protons with

(1) J. P. Kokko, J. H. Goldstein and Leon Mandell, *J. Am. Chem. Soc.*, **83**, 2909 (1961).

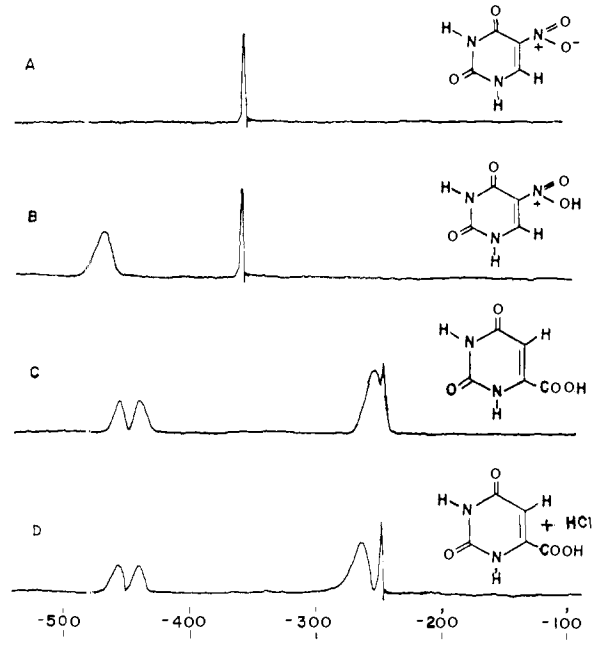


Fig. 3.—N.m.r. spectra of some substituted uracils: A, 5-nitrouracil in neutral d_6-DMSO; B, 5-nitrouracil in acidified d_6-DMSO; C, orotic acid in neutral d_6-DMSO; D, orotic acid in acidified d_6-DMSO.

the exception of 6-azauracil where the $-H_5$ is obviously at a much lower field due to the presence of the nitrogen at the six position, and the peaks from −300 to −350 c.p.s. are due to $-H_6$ protons. The above assignments are based

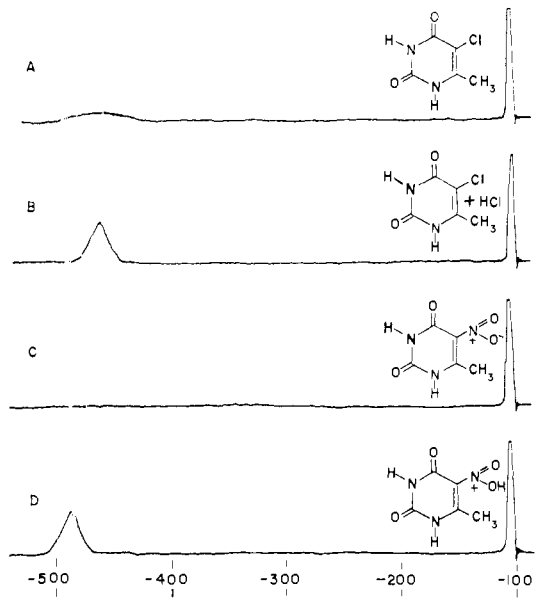
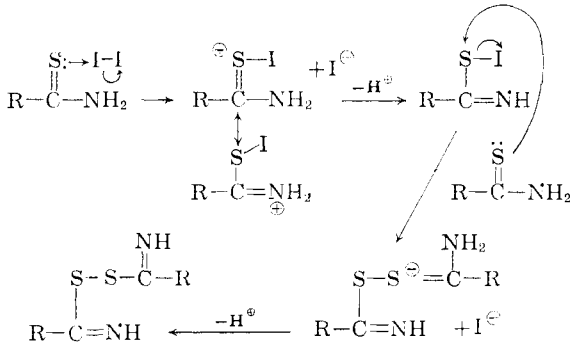


Fig. 4.—N.m.r. spectra of some substituted uracils: A, 5-chloro-6-methyluracil in neutral d_6-DMSO; B, 5-chloro-6-methyluracil in acidified d_6-DMSO; C, 5-nitro-6-methyluracil in neutral d_6-DMSO; D, 5-nitro-6-methyluracil in acidified d_6-DMSO.

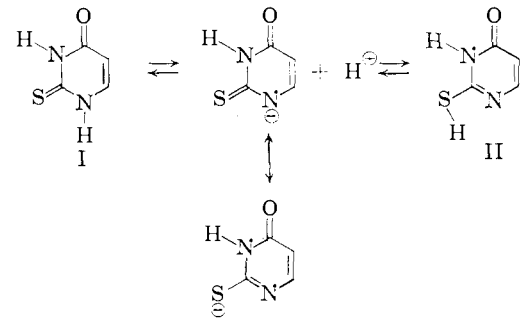
on the same reasoning as that employed in our previous study of pyrimidine derivatives.

Discussion

2-Thiouracil and its derivatives are an important group of drugs in the treatment of hyperthyroidism, *i.e.*, over supply of thyroid hormones. This over functioning of the gland can result from various causes, for example, adenoma of the thyroid gland. Although the mechanism of its action is not established, it is known that some step in the iodination is inhibited,[2-4] even though iodide is still accumulated in the gland.[4] It is also known that thioamides can reduce free iodine[5,6] thus leaving iodide which is not physiologically active.[6] The mechanism for this process possibly involves

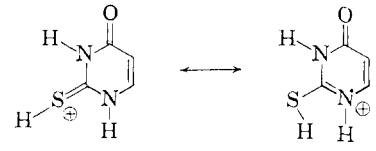


The spectrum of 2-thiouracil does not show separate N–H and S–H proton peaks, nor is there any coupling between the C_6 and the amide proton. The observed doublet due to C_6 is the result of coupling with the C_5-proton.



I II

Acidification of 2-thiouracil would be expected to produce III (see below), by protonation of I on sulfur and II on nitrogen in which the above tautomerism is no longer possible; this is evidenced by the splitting of the $-H_6$ into a quartet in the acidified material.



III

It is not unreasonable to believe that thiouracil might act in the same manner as thioamides since, from these spectral studies, it has a structure and reactions completely analogous to thioamides.

It has also been reported that thiouracil is capable of inhibiting[7] phage growth and replacement of uracil in RNA.[8] If it is accepted that adenine is hydrogen-bonded to uracil, as is currently believed, then it is still possible for the adenine and 2-thiouracil to hydrogen bond together providing the –S–H does not sterically hinder the bonding.

In **6-methyluracil** the very presence of N–H peaks as well as its position, approximately the same as that in thymine, suggests very strongly that the tautomeric situation does not differ significantly from that of thymine.

Uracil-5-carboxylic acid is interesting in that its carboxylic acid substituent induces enolic structure even though it is a relatively weak electronegative group. The labile amide protons are not visible in the spectrum of this compound and produce no splitting of the H_6 peak prior to acidification, presumably as a result of the tautomeric exchange process. When HCl is bubbled through the solution, the amide proton becomes "fixed" and the usual splitting of the $-H_6$ peak occurs. The new large peak at approximately -236 c.p.s. from TMS possibly represents the average position of the carboxylic acid proton in DMSO undergoing exchange with the small amount of water present. Solutions of the carboxylic acid including a small amount of water, but no HCl, also show a peak at about -230 c.p.s. If the carboxylic acid

(2) C. C. MacKenzie and J. B. Mackenzie, *Endocrinol.*, **32**, 185 (1943).

(3) E. B. Astwood, J. Sullivan, A. Bissell and R. Tyslowitz, *ibid.*, **32**, 210 (1943).

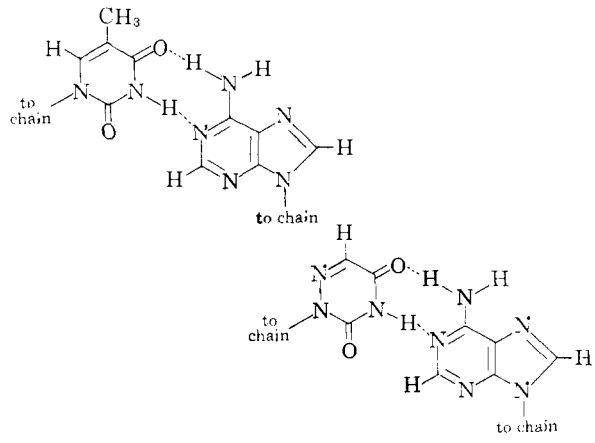
(4) J. E. Vanderlaan and W. P. Vanderlaan, *ibid.*, **40**, 403 (1947).

(5) E. A. Werner, *J. Chem. Soc.*, **101**, 2166 (1912).

(6) W. H. Miller, R. O. Robbin and E. B. Astwood, *J. Am. Chem. Soc.*, **67**, 2201 (1945).

(7) R. Jeener, C. Hamers-Casterman and N. Mairesse, *Biochim. Biophys. Acta*, **35**, 166 (1959).

(8) R. Hamers, *ibid.*, **21**, 170 (1956).



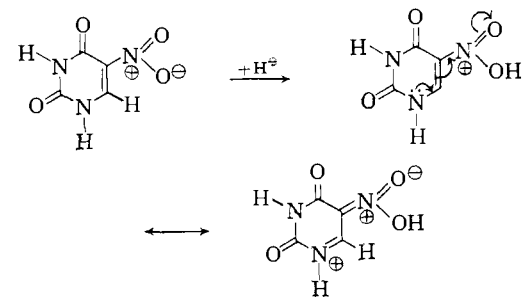
a, thymine–adenine b, 6-azauracil-adenine

Fig. 5.—Possible hydrogen bonding between 6-azauracil and adenine.

substituent is on the C_6-position (orotic acid) then a diketo structure is present.

6-Azauracil has recently acquired pharmacological importance in the treatment of various leukemias. The mechanism by which this action is exerted is not known, but it has been postulated that the azauracil is first converted into nucleotide derivatives before its inhibitory effect on the rapidly proliferating cells is evidenced.[9,10] Later work has indicated that in *E. coli*, K-12, the azauracil does not even reach the cytoplasm of the intact cells and is not incorporated into the nucleic acids.[11] From the viewpoint of such factors as tautomerism and steric hindrance there is no reason to believe that azauracil would not replace the uracil of RNA or the thymine of DNA. The lability of the N_3 proton of thymine does not differ significantly from that of uracil, and the lability of N_3 proton of 6-azauracil falls in the same range, although slightly more so, as do the N_3 protons of thymine and uracil (Table I). Figure 5 shows the possibility of similar intermolecular hydrogen bonding in thymine and 6-azauracil. Thus, it might seem that the physical properties of 6-azauracil would permit it to be incorporated into the nucleic acid structure, but the fact that this does not happen indicates factors other than those considered here are possibly involved. Perhaps the azauracil-containing nucleotides competitively antagonize the function of the uridine nucleotides.[11]

5-Nitrouracil before acidification exhibits only one sharp peak which obviously means that the labile protons are undergoing exchange at such a rate as to eliminate coupling effects with $-H_6$. It is interesting to note, however, that when the solution is acidified the amide peaks appear as expected, but the protons are still too labile for observable coupling with $-H_6$. This is not unexpected, for the proton addition probably takes place as pictured in the process below in which there is shown the "acinitro" character of the molecule



Substitution of a methyl group as, for example, in 5-nitro-6-methyluracil, does not affect the tautomeric behavior significantly.

The halogenated uracils are widely used in experimental bacteriology and 5-fluorouracil has even been used in clinical trials against metastatic neoplasias of various kinds.[12,13]

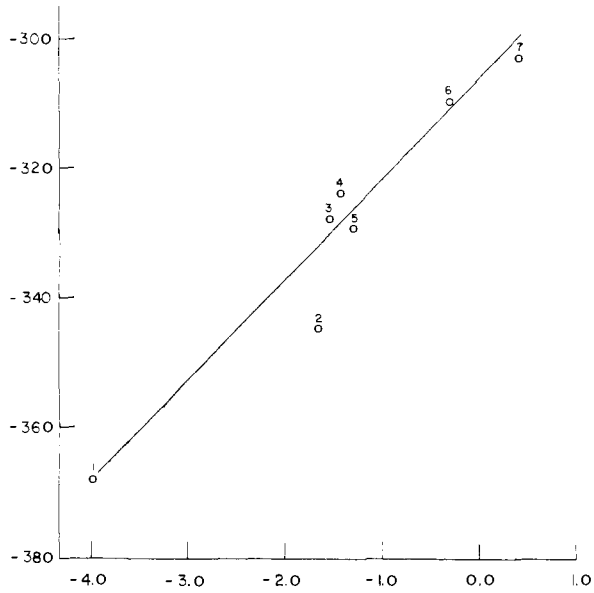


Fig. 6.—Plot of group dipole moments of the substituents at the 5-position *vs.* the chemical shift of $-H_6$ in c.p.s. from TMS in the substituted uracils. The substituents are: 1, $-NO_2$; 2, -COOH; 3, -Br; 4, -F; 5, I; 6, -H; 7, $-CH_3$. The group dipole moments are from C. P. Smyth ("Dielectric Behavior and Structure," McGraw-Hill Book Co., Inc., New York, N. Y., 1955), group dipole moment of -H is from C. P. Smyth (*J. Phys. Chem.*, **41**, 209 (1937)).

Of the halogenated uracils, bromouracil becomes incorporated in the DNA molecule to the greatest extent,[14,15] with chlorouracil replacing thymine more than does iodouracil in *E. coli* (strain 1).[15] whereas this order is reversed in *E. coli* (15T).[14] 5-Fluorouracil is not capable of replacing thymine in DNA,[16] but 28–47% of uracil can be replaced by 5-fluorouracil in RNA.[17] These results suggest

(9) R. E. Handschumacher, *Biochim. Biophys. Acta*, **23**, 428 (1957).
(10) R. Schindler and A. D. Welch, *Science*, **125**, 548 (1957).
(11) Y. Takagi and N. Otsuji, *Biochim. Biophys. Acta*, **29**, 227 (1958).
(12) M. J. Brennan and V. K. Vaitkevicius, *Cancer Chemother. Rep.*, **6**, 8 (1960).
(13) P. E. Perillie and S. C. Fine, *Conn. Med.*, **24**, 415 (1960).
(14) D. B. Dunn and J. D. Smith, *Biochem. J.*, **67**, 496 (1957).
(15) S. Zamenhof, B. Reiner, R. deGiovanni and K. Rich, *J. Biol. Chem.*, **219**, 165 (1956).
(16) S. Zamenhof, *Ann. N. Y. Acad. Sci.*, **81**, 784 (1959).
(17) M. P. Gordon and M. Staehelin, *Biochim. Biophys. Acta*, **36**, 351 (1959).

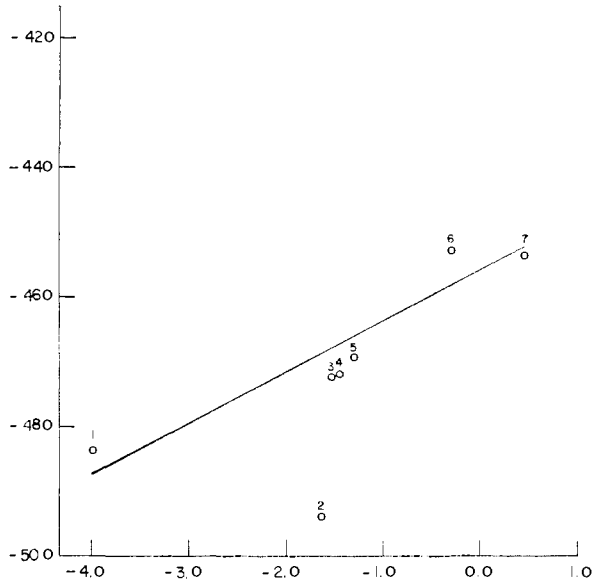


Fig. 7.—Correlation of group dipole moment of the substituents at the 5-position *vs.* the chemical shift of the N_3 protons in c.p.s. from TMS in the substituted uracils. The group dipole moments are obtained from the same source as in Fig. 6, and the number of assignments of the substituents are the same. The chemical shifts of the proton occurring at the lowest field are plotted since this resonance frequency is due to the N_3 proton.[1] If only one peak occurs this chemical shift is plotted, and in cases where it is necessary to acidify the medium before resonance frequencies become apparent this chemical shift is plotted.

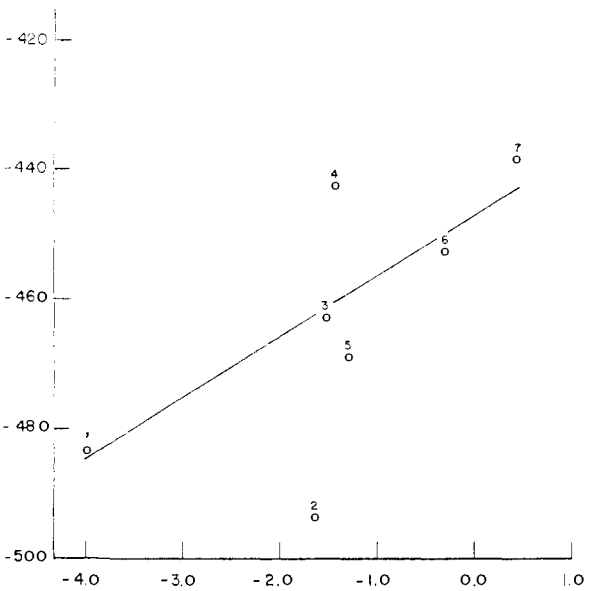


Fig. 8.—Correlation of group dipole moments of the substituents at the 5-position *vs.* the chemical shift of the N_1 protons in c.p.s. from TMS in the substituted uracils. The group dipole moments are obtained from the same source as in Fig. 6, and the number assignments of the substituents are the same. The chemical shifts of the protons at the highest field of the amide protons are plotted as this resonance frequency is due to the N_1 protons.[1] If only one peak occurs this chemical shift is plotted, and in cases where it is necessary to acidify the solution before resonance frequencies become apparent this chemical shift is plotted.

that it is not necessarily the size of the pyrimidine molecule which determines its capacity for replacement of thymine, but that the controlling factor may be the presence or absence of an enzyme capable of converting pyrimidine analogs to triphosphates.[18] For example, *E. coli* has a kinase which is capable of converting 5-bromouridylate to the triphosphate, whereas this enzyme is lacking in dioxyuridylate.[18] On the basis of both the appearance of low field (NH) region of the spectrum as well as the observed coupling between N_1H and C_6H it appears that 5-bromouracil has a definitely greater tendency to exhibit keto structure than do the other 5-substituted uracils, although 5-fluorouracil also contains some keto structure. The reason for this behavior is not altogether clear, but it may be that in 5-bromouracil there is just the correct balance between inductive and mesomeric effects to account for the observed behavior.

Prior to acidification the $-H_6$ peak of 5 FU is a doublet split by 6.1 c.p.s. through coupling with the fluorine at the 5-position. This splitting is slightly less than that reported for H–F couplings in substituted benzene compounds.[19] (If the splitting were due to $-H_5$ then the coupling constant would probably be more than 7 c.p.s.) On acidification a triplet is observed which arises from the superposition of two doublets. Under similar conditions the $-H_6$ peak of 5-iodouracil changes to a doublet due to fixing of amide protons which permits coupling to become observable.

Correlations with Other Molecular Properties.—In view of the proposed role of proton mobility in determining the mutagenic behavior of the substituted pyrimidines, and the unmistakeable evidence from the n.m.r. spectra of the influence of the nature of the substituent upon proton mobility, it is pertinent to inquire whether any fundamentally sound generalizations may be offered to account for the observations. From a relatively simple, although general, point of view the substituent effect might be expected to correlate with some appropriate over-all measure of electron-attracting or releasing capacity, for example, the group dipole moments of the various substituents. The choice of this criterion follows from a previous observation[20] that chemical shifts at both β-positions (*cis* and *trans*) of a series of monosubstituted ethylenes exhibit a linear relationship to the group moments of the substituent, whereas no equally satisfactory correlation with electronegativities has been obtained.

Figure 6 is a plot of the H_6 chemical shifts in the uracils *vs.* the group moments for seven substituents at the C_5-position. All the points fall reasonably close to a straight line, except that for –COOH which

(18) M. J. Bessman, I. R. Lehman, J. Adler, S. B. Zimmerman, E. S. Simms and A. Kornberg, *Proc. Natl. Acad. Sci. U. S.*, **44**, 633 (1958).

(19) H. S. Gutowsky, C. H. Holm, A. Saika and G. A. Williams, *J. Am. Chem. Soc.*, **79**, 4596 (1957).

(20) G. S. Reddy, C. E. Boozer and J. H. Goldstein, *J. Chem. Phys.*, **34**, 700 (1961).

is not too surprising in view of the acidity and hydrogen bonding properties of this group, as well as the fact that the carboxyl group moment is directed at an angle to the carboxyl-ring bond and has the possibility of rotation.

Figure 7 shows a similar plot for the N_3 protons. Again, except for -COOH, the linear correlation is quite apparent, and it can be seen that the three halogens (Br, F and I) form a close cluster of points. However, in the graph for the N_1 protons, shown in Fig. 8, the F point is now well removed from the straight line. (The -COOH point will hereafter be ignored.)

However, a reasonable explanation can be offered to account for the seemingly anomalous behavior of fluorine. The lone-pair electrons of F, N_1 and the C_5–C_6 double bond form an extended conjugated system. Because of its small size, F is a more effective electron donor to the conjugated system than are the other halogens; hence it is understandable that the N_1 proton should be considerably displaced to high-field in 5-fluorouracil relative to 5-bromouracil and 5-iodouracil. (An analogous explanation accounts for the small effect of F on the acidity of benzoic acid, as compared with the other halogens.) On the other hand, N_3 is cross-conjugated with F and should thus experience a primarily inductive effect from the C_5-substituent.

Another type of correlation is shown in Fig. 9 where the average values of the two N–H shifts are plotted against Hammett σ-constants for the substituents at the 5-position. The σ-values are those for *meta* substituents, since this is the appropriate choice here. Again the -COOH point is irregular, which is not too surprising, but the linear correlation for the remaining substituents is excellent. This result again reflects the fact that the influence of 5-substituents (except-COOH) on the N–H environment follows a course predictable in advance from criteria that have proved successful in other situations.[21]

From the point of view taken in this investigation, the mobilities of the hydrogen-bonding protons

(21) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Holt, Rinehart and Winston, Inc., New York, N. Y., 1960.

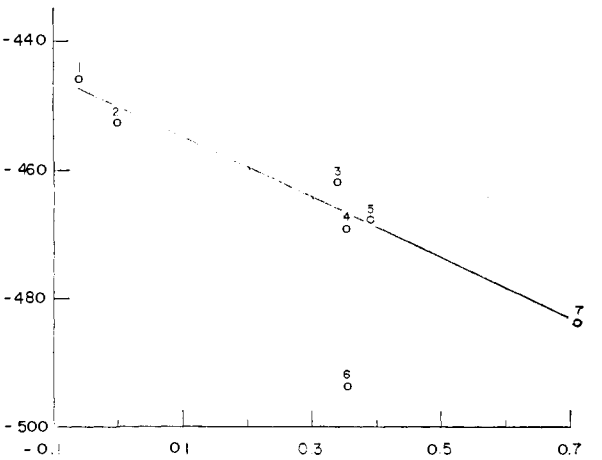


Fig. 9.—Plot of Hammett σ-constants of the substituents at the 5-position *vs.* the average values of the two amide proton shifts. The substituents are: 1, $-CH_3$; 2, -H; 3, -F; 4, -I; 5, -Br; 6, -COOH; 7, $-NO_2$. The Hammett σ-values used were calculated by Dr. C. E. Boozer. In cases where acidification was necessary before resonance frequencies became apparent this chemical shift was plotted.

appear to be the most characteristic single feature of the pyrimidine derivatives. Since 5-fluorouracil is somewhat unusual in the series for its antimetabolitic behavior, the somewhat exceptional behavior of its N_1 proton appears to be worthy of further consideration in the effort to understand its role in biological systems.

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Studies on Sphingolipids. VII. Synthesis and Configuration of Natural Sphingomyelins[1]

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Both enantiomorphs of *erythro*-sphingomyelin have been synthesized. The synthesis involves acid-catalyzed ring scission of the suitably substituted *cis*-oxazoline I and the optical resolution of II. Acylation of the latter and phosphorylation of III with β-chloroethylphosphoryl dichloride followed by treatment of the β-chloroethylphosphates of 3-O-benzoylceramides (IV) with trimethylamine leads to the sphingomyelins (VI). DL-*threo*-Dihydrosphingomyelin has been prepared similarly from the *trans*-oxazoline I. Natural sphingomyelin has been found identical with the D-*erythro*-enantiomorph.

The generally accepted view that the asymmetric carbon atoms in sphingomyelin have the *erythro* configuration is largely based on analogy with the cerebrosides whose structure has been firmly established.[2,3] However, the surprising finding

(1) Supported in part by a grant from Mr. Samuel Rothberg of Peoria, Ill.

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(3) D. Shapiro and H. M. Flowers, *J. Am. Chem. Soc.*, **83**, 3327 (1961).