about the histidyl C_α–C_β bond sufficiently rapid, only an average of *trans* and *gauche* couplings would appear. If the tyrosyl side chain occupies the space over the diketopiperazine ring, preventing rotation about a full circle, oscillation between the two remaining conformations might still result in time-averaging the observed couplings. It is noteworthy that completely averaged values are only observed in trifluoroacetic acid at the higher temperatures; oscillation of the imidazolymethyl side chain must therefore be considerably inhibited, and more inhibited in water, which seems reasonable, than in trifluoroacetic acid.

c-L-Valyl-L-tyrosyl. Only single temperature measurements of the spectra of this peptide are available at present, but it appears, from data in Tables VI and VII, that the valyl β-hydrogen is shifted to higher field by a noticeably smaller amount than one would estimate if conformation IA were as favored as in the other peptides. This is especially so in dimethyl sulfoxide and trifluoroacetic acid. In its version of conformation IA, the valyl peptide must have all rotation about the C_α–C_β bond removed, since a γ-methyl and the aromatic ring cannot simultaneously occupy the space over the diketopiperazine ring. In contrast, leucyl and histidyl residues have two C_α–C_β rotamers allowed to them in the IA conformation. The additional restriction in the valyl case may account for reduced stability of the folded form.

L-Tyrosyl-L-tyrosyl. The four β and two α protons of this peptide give rise to a single ABX pattern with the chemical shifts indicated in Table VI. This spectrum does not suggest major contributions from conformers of type IA. The two kinds of β protons differ by 0.7 ppm; the more shielded has an apparent coupling to the α proton of 8 Hz; the less shielded, one of 4 Hz. If conformations of type IA were important and persisted long enough to prevent averaging of this nonequivalence, there would be separate resonance patterns for the two kinds of methylene. As just indicated, this is not observed. Therefore, it seems that a preferred conformation is likely to be one in which each hydroxyphenyl tends to associate with one amide group, sharing the space over the diketopiperazine ring in such a fashion that the two β-methylenes have identical environments.

The same conclusion may be drawn for *c*-L-phenylalanyl-L-phenylalanyl, which has a similar nmr spectrum.

Acknowledgment. This work was supported by research grants from the National Science Foundation, GB 4514, and the National Institute of General Medical Sciences, U. S. Public Health Service, GM 14069. The initial observations were made by K. K. during a stay in the laboratory of Professor Melvin Calvin, University of California, Berkeley, in 1965; we wish to express gratitude for the facilities extended to us at that time.

Steric Control of Geometrical Isomerism in Cytosine Cations. A Nuclear Magnetic Resonance Study

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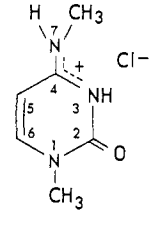
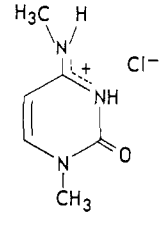
Abstract: Proton nmr spectra are reported for several methyl derivatives of cytosine hydrochloride, with emphasis on the geometrical isomerism resulting from hindered rotation about the bond joining the amino group to the ring. A definitive assignment is made of the spectra of the two geometrical isomers of 1,7-dimethylcytosine hydrochloride. Regularities are pointed out in the spectral changes resulting from introduction of methyl substituents and from temperature variation.

In a recent detailed nmr study of some derivatives of 1-methylcytosine, Becker, Miles, and Bradley[1] demonstrated that the rotation of the amino group in various species is restricted, and that this restriction leads to the observation of two isomers of 1,7-dimethylcytosine hydrochloride (or hydriodide) (I).

The spectrum of 1,7-dimethylcytosine hydrohalide showed a long-range spin coupling of 0.7 Hz[2] between the protons attached to atoms in the 6 and 7 positions in more abundant isomer. The spectra of the two isomers were assigned to IA and IB on the assumption that the all-*trans* arrangement of the protons in IB would lead to an observable long-range coupling $J_{6H,7H}$, while in IA $J_{6H,7H}$ would be less than 0.1 Hz.



IA



IB

We present here independent experimental evidence that this assignment is correct.

(1) E. D. Becker, H. T. Miles, and R. B. Bradley, *J. Am. Chem. Soc.*, 87, 5575 (1965).
(2) 1 Hz = 1 cps.

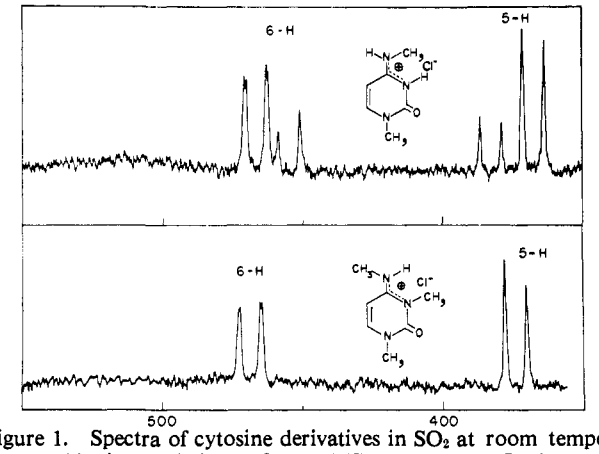


Figure 1. Spectra of cytosine derivatives in SO_2 at room temperature. Abscissa scale in Hz from TMS at 60 MHz. In the upper spectrum a pair of doublets is seen for both 6-H and 5-H since both *cis* and *trans* geometrical isomers are present, but only the more abundant isomer shows a 0.7-Hz splitting of the 6-H peak. The lower spectrum has only one doublet at 6-H and 5-H since steric interference of methyl groups precludes the formation of one geometrical isomer. The presence of a 0.7-Hz splitting of 6-H in this molecule confirms the assignment of an all-*trans* 6H,7H coupling in both molecules.

Experimental Section

The nmr spectra were recorded on a Varian A-60 spectrometer with a variable-temperature probe. The temperature measurements, using the chemical shifts of methanol and ethylene glycol as standards, were assumed to be accurate within ±1°. Each spectrum was calibrated by means of audio-frequency side bands, and the frequencies of sharp peaks are accurate to within ±1 Hz. The spectrometer was modified to permit the frequency scale to be expanded so that 10 Hz covers the full width of the chart.[3] Sulfur dioxide solutions were prepared as described previously.[1]

1,5-Dimethyl- and 1,5,7-trimethylcytosine were prepared by the reaction of 1,5-dimethyl-4-methoxypyrimidone-2[4] with methanolic ammonia and aqueous methylamine, respectively, at 100–120°: 1,5-dimethylcytosine, mp 319–320° subl (*Anal.* Calcd for $C_6H_9N_3O$: C, 51.78; H, 6.52; N, 30.19. Found: C, 52.17; H, 6.60; N, 30.87); 1,5,7-trimethylcytosine, mp 177–178° subl (*Anal.* Calcd for $C_7H_{11}N_3O$: C, 54.89; H, 7.24; N, 27.43. Found: C, 54.72; H, 7.16; N, 27.24). 1,3,5- and 1,3,7-trimethylcytosines were prepared by methylation with methyl iodide of 1,5- and 1,7-dimethylcytosines, respectively, at room temperature. The resulting hydriodides were treated with sodium hydroxide to produce the free bases, which were recrystallized from ether–hexane mixtures: 1,3,5-trimethylcytosine, mp 77.0° (*Anal.* Calcd for $C_7H_{11}N_3O$: C, 54.89; H, 7.24; N, 27.43. Found: C, 54.94; H, 7.51; N, 27.06); 1,3,7-trimethylcytosine, mp 78.5–79.5° (*Anal.* Found: C, 54.98; H, 7.15; N, 27.75).

The hydrochlorides were prepared by dissolving the bases in concentrated hydrochloric acid, evaporating the acid, and recrystallizing from methanol–acetone mixtures. The following analyses were performed: 1,5-dimethylcytosine hydrochloride (*Anal.* Calcd for $C_6H_{10}N_3ClO$: C, 41.04; H, 5.74; N, 23.93; Cl, 20.19. Found: C, 40.82; H, 5.90; N, 23.80; Cl, 19.96); 1,5,7-trimethylcytosine hydrochloride (*Anal.* Calcd for $C_7H_{12}N_3ClO$: C, 44.33; H, 6.38; N, 22.15; Cl, 18.99. Found: C, 44.30; H, 6.32; N, 21.72; Cl, 18.68); 1,3,5-trimethylcytosine hydrochloride (*Anal.* Found: C, 44.16; H, 6.39; N, 22.14; Cl, 18.64); 1,3,7-trimethylcytosine hydrochloride (*Anal.* Found: C, 44.13; H, 6.43; N, 21.59; Cl, 18.23).

Our attempts to prepare 1,5,7,7- and 1,3,5,7-tetramethylcytosine were unsuccessful but yielded two interesting examples of steric inhibition of chemical reactions. 1,5-Dimethyl-4-methoxypyrimidone-2 heated at 100° with dimethylamine in either aqueous or anhydrous methanolic solution gave exclusively 1,5-dimethyluracil rather than the desired tetramethylcytosine. Since the anhydrous reaction mixture did not contain enough water to account for the yield of uracil, steric hindrance of the 5-methyl group must have promoted cleavage of the O–CH_3 bond instead of the usual displacement reaction. Similarly, 1,5,7-trimethylcytosine failed to produce 1,3,5,7-tetramethylcytosine hydriodide upon treatment with methyl iodide in absolute methanol. The only product isolated, in low yield, was 1,5,7-trimethylcytosine hydriodide.

(3) We thank B. L. Shapiro for suggesting this modification and E. Lustig for advice on the necessary minor changes in the instrument.

(4) W. Schmidt-Nickels and T. B. Johnson, *J. Am. Chem. Soc.*, **52**, 4511 (1930).

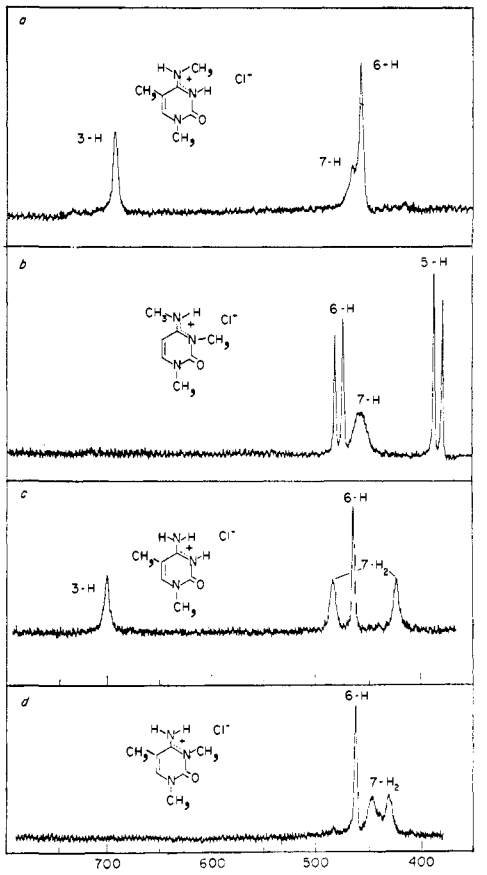
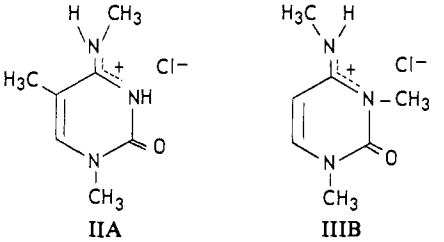


Figure 2 Spectra of cytosine derivatives in SO_2 at −60°. Abscissa scale in Hz from TMS at 60 MHz.

Results and Discussion

Stereoisomerism. We have employed steric interference of methyl groups to control the stereochemistry of the cytosine cations and so provide independent evidence that the splitting observed in the 6-H proton signal of I results from a five-bond coupling of a 7-H proton in the all-*trans* configuration (*i.e.*, structure IB). A bulky substituent in either the 3 or the 5 position should greatly enhance the restriction of the rotation of the methylamino group and force the 7-CH_3 group away from the introduced substituent. Thus, the hydrochlorides of 1,5,7- and 1,3,7-trimethylcytosines should exist solely as the geometrical isomers IIA and IIIB rather than as the isomers where the methylamino



IIA IIIB

group is rotated 180°. Space-filling Pauling–Corey–Koltun models of these compounds indicate that the presence of the other isomers can safely be ruled out. The nmr spectra of II and III are entirely in accord with this expectation (see Figures 1b and 2a); in fact,

Table I. Chemical Shifts and Spin–Spin Coupling Constants for Cytosine Derivatives in SO_2 at −60°

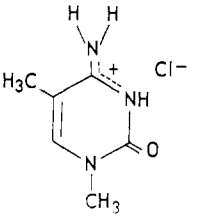
Compound	Chemical shifts, ppm[a]								Coupling constant, Hz		
	1-CH_3	3-CH_3	5-CH_3	7-CH_3	5-H	6-H	3-H	7-H	$J_{5H,6H}$	$J_{7CH_3,7H}$	$J_{5CH_3,6H}$
1,5-Dimethylcytosine hydrochloride (IV)	3.52	...	2.14	...	...	7.71	11.62	7.03, 8.04	...	...	1.0
1,3,5-Trimethylcytosine hydrochloride (V)	3.55	3.65	2.16	...	...	7.70	...	7.18, 7.44	...	...	1.0
1,5,7-Trimethylcytosine hydrochloride (IIA)	3.55	...	2.13	3.30	...	7.57	11.48	7.70	...	5.3	1.1
1,3,7-Trimethylcytosine hydrochloride (IIIB)	3.58	3.60	...	3.25	6.32	7.89	...	7.55	7.9	5.1	...

[a] From TMS.

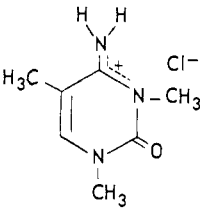
spectra of II and III in DMSO solution show no indication of a second isomer even at 160°.

The chemical shifts and coupling constants observed for these and related cytosines in SO_2 solution at −60° are listed in Table I. The frequency ranges observed for individual protons are in close agreement with those reported previously,[1] and the assignments in Table I follow from those given in the earlier study.

Figure 1 shows the low-field part of the spectra of I and of IIIB in SO_2 at 20°. The observation of a 0.7-Hz, five-bond coupling in the 6-H signal of IIIB, as well as in the more abundant isomer of I, clearly establishes the latter as the geometrical stereoisomer IB as suggested by Becker, Miles, and Bradley.[1]



IV



V

splitting at normal scanning speeds. The 5-CH_3,6-H coupling is thus easily observable in the 5-methyl peak, whereas the 6-H peak looks broad and irregular. With a very slow scanning speed of 0.02 Hz/sec it is possible to extract some information from the 6-H region, as

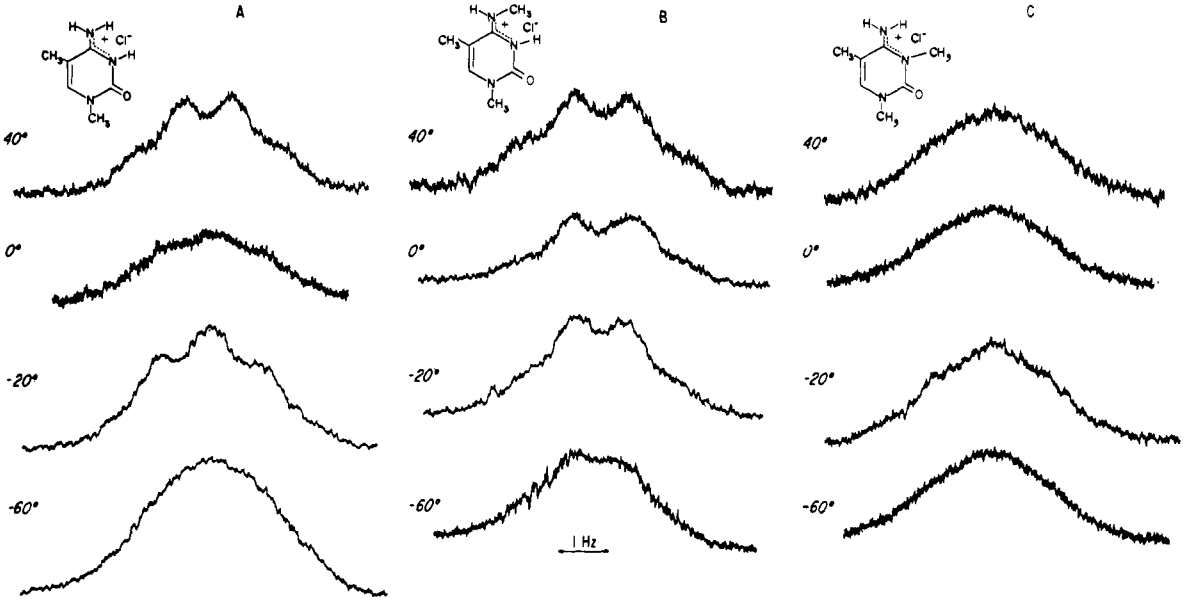


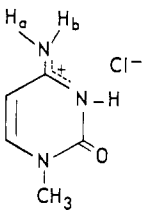
Figure 3. Spectra due to 6-H in cytosine derivatives in SO_2, obtained at 60 MHz.

Further confirmation of this assignment was obtained from a study of the fine structure in the 6-H signal of 1,5-dimethyl-, 1,3,5-trimethyl-, and 1,5,7-trimethylcytosine hydrochlorides (IV, V, and II, respectively). The presence or absence of a five-bond 6-H,7-H coupling in these compounds is more difficult to establish since the 5-CH_3,6-H coupling gives the 6-proton region a quartet structure with a splitting of about 1 Hz. The 7-H peaks are too broad, because of the influence of the quadrupole moment of N_7, to allow observation of a coupling as small as 0.7 Hz. The nitrogen in position 1 likewise causes some broadening of the 6-H peak making it difficult to observe any shown in Figure 3. The 6-H peak of 1,5-dimethylcytosine hydrochloride (IV) measured at 40° shows a quartet structure (see Figure 3A) due to coupling with the 5-methyl group (J = 1.0 Hz). By −20° the appearance of the spectrum changes markedly, the central minimum being replaced by a maximum. If 6-H is coupled to four other protons (one of the amino protons and the three methyl protons) with coupling constants of the same magnitude, the 6-H signal would be a quintet of relative heights 1:4:6:4:1. The −20° spectrum of Figure 3A is consistent with this analysis, since the weak outer peaks of the quintet may be undetectable. Some distortion or broadening of the

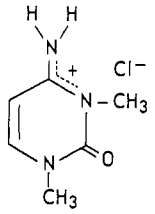
peaks would be anticipated since the expected value of $J_{6H,7H}$ is 0.7 Hz, while $J_{5CH_3,6H}$ = 1.0 Hz. The disappearance of the 6-H,7-H coupling at 40° is due to rapid exchange of H_b.

In contrast to this the 6-H peak of 1,5,7-trimethylcytosine hydrochloride (IIA) retains its quartet structure (J = 1.1 Hz) down to −20° (see Figure 3B). The splitting of the 7-CH_3 peak due to coupling with 7-H all through the measured temperature range shows that the quartet structure is not merely the result of rapid exchange of the 7-proton. Failure to detect splitting of the 6-H proton by 7-H when the latter is constrained to a *cis* configuration further supports the view that these protons must be all-*trans* in order to show a measurable splitting.

We have remeasured the spectra of 1-methyl- and 1,3-dimethylcytosine hydrochlorides (VI and VII, respectively) at slow scanning speeds in unsuccessful attempts to observe a long-range coupling between 6-H and 7-H_b. The 6-H peaks are broader than the 5-H peaks ($\Delta\nu_{1/2} \simeq$ 1.3 Hz *vs.* $\simeq$ 0.7 Hz), but this difference may be due solely to quadrupole broadening from N_1. The spectrum of the 6-proton of 1,3,5-trimethylcytosine hydrochloride (V) at −20° shows poorly resolved fine structure, probably indicative of long-range coupling. Possible explanations for the different behavior of these compounds were suggested in a previous paper.[1]



VI



VII

The −60° curves in Figure 3 show that fine structure is lost. Although the resolution of the spectrometer deteriorates slightly at this low temperature, the 6-H, 5-CH_3 coupling can nevertheless still be observed in the 5-methyl signal at −60°. A similar loss of detail was observed in the 6-H peak in the more abundant isomer (IB) of 1,7-dimethylcytosine hydrochloride.[1,5]

Chemical Shifts of NH_2 Protons. In the hope that methyl substitution might cause regular changes in the chemical shifts of H_a and H_b, we have examined the pattern of chemical shifts for the eight different compounds available (counting the stereoisomers IA and IB as separate compounds). The assignments of the 7-H lines to H_a and H_b have been firmly established in the foregoing section for IA, IB, IIA, and IIIB. Each of the remaining four compounds contains an NH_2 group, so that there are two possible assignments of the H_a and H_b chemical shifts for each case, or a total of 2^4 distinct possibilities. We have tabulated these 16 cases, together with the known values for IA, IB, IIA, and IIIB, and have then calculated the effects of various methyl substituents from differences in chemical shifts for appropriate pairs. One of the 16 calculations is summarized in Table II; this one results in systematic behavior of chemical shifts for both H_a and H_b, as to sign and approximate magnitude. Twelve of the 16 calculations resulted in no over-all systematic behavior, with frequent contradictions in sign of the methyl effects. The four remaining showed behavior such as that depicted in Table II, with either no inconsistencies in sign or only one contradiction where 0.07 was found in place of an expected small negative shift.[6] The significant point emerging from these computations is that all four of the consistent sets require the assignments of H_a and H_b for IV and VII as given in Table III. Thus we believe there is good, though not conclusive, evidence for the assignments of H_a and H_b in IV and VII, but we are unable to make a choice on the basis of chemical shifts for V and VI. A tentative assignment for VI, based on magnitudes of splittings found in the $^{15}NH_2$ lines, was given previously.[1]

Table II. Effect of Methyl Substitution (in ppm) upon the Chemical Shifts of Other Protons in 1-Methylcytosine Hydrochlorides Measured in SO_2 at −60° [a]

		7-H_a	7-H_b	3-H	5-H	6-H
		Effect of 5-Methyl				
1–1,5	VI–IV	0.80	−0.59	−0.18	...	0.11
1,3–1,3,5	VII–V	0.82	−0.19	...	...	0.10
1,7A–1,5,7	IA–IIA	0.95	...	−0.39	...	0.13
		Effect of 3-Methyl				
1–1,3	VI–VII	−0.17	0.20	...	−0.11	0.02
1,5–1,3,5	IV–V	−0.15	0.60	...	...	0.01
1,7B–1,3,7	IB–IIIB	...	0.48	...	−0.07	0.01
		Effect of 7-Methyl				
1–1,7A	VI–IA	−0.82	...	0.35	...	0.12
1,5–1,5,7	IV–IIA	−0.67	...	0.14	...	0.14
1–1,7B	VI–IB	...	−0.58	−0.10	0.03	−0.08
1,3–1,3,7	VII–IIIB	...	−0.30	...	0.08	−0.09

[a] The numerical values are obtained by subtracting the chemical shift (ppm) for the indicated proton (*e.g.*, 6-H) in two molecules lacking and possessing a particular methyl group (*e.g.*, 5-methyl), as indicated by the notations 1–1,5 or VI–IV. Thus a positive number denotes an upfield shift on methyl substitution. The chemical shifts for I, VI, and VII were reported previously.[1]

Table III. Assignment of Chemical Shifts to Amino Protons[a]

Compd	H_a	H_b	Compd	H_a	H_b
IA	8.65	...	IV	7.03	8.04
IB	...	8.03	V	(7.18)[b]	(7.44)[b]
IIA	7.70	...	VI	(7.83)[b]	(7.45)[b]
IIIB	...	7.55	VII	8.00	7.25

[a] Values are given in ppm from internal TMS at −60° in SO_2 solution. See discussion in text for reliability of assignments. [b] These assignments may be reversed. See text.

(5) Some broadening of the 6-H lines might be expected from coupling to N_1, which relaxes at an intermediate rate because of its quadrupole moment. However, reduction of temperature normally increases the rate of quadrupolar relaxation so that at low temperature N_1 would be effectively "decoupled" from 6-H, and the 6-H, signal would sharpen, not broaden, as a result. Experimental confirmation that nitrogen quadrupolar relaxation does not contribute to the breadth of the 6-H signal at low temperature was obtained when we found no observable change in the proton resonance spectrum at −55° upon irradiation with high radiofrequency power in the ^{14}N resonance frequency region.

(6) To obtain a more quantitative measure of the self-consistency of these results, we have calculated for each of the 16 combinations of assignments a quantity R determined as follows. The average deviation of the two or three values contained within each block (*cf.* Table II) is determined, and R is then the sum of these six average deviations. For the 12 "inconsistent" assignments R ranged from 1.84 to 2.82, while the four "consistent" assignments had values of R of 0.64, 0.80, 0.88, and 1.04.

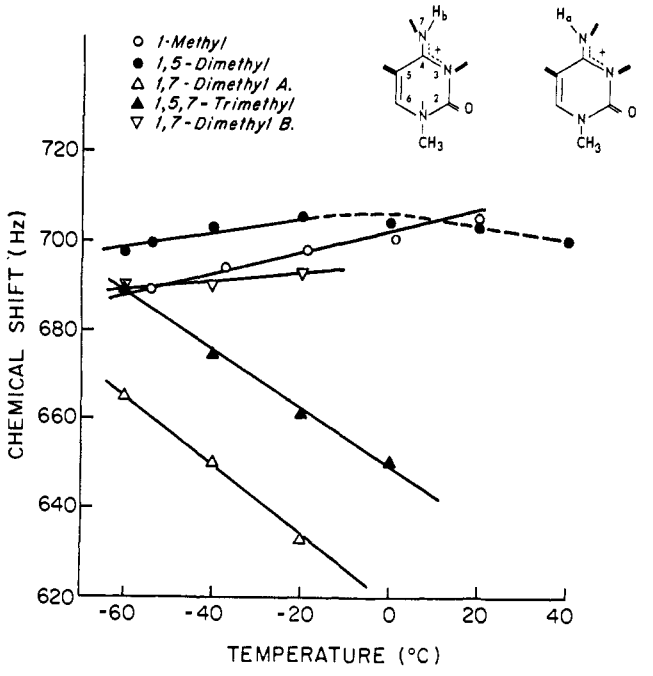


Figure 4. Chemical shift of 3-H in cytosine hydrochlorides in SO_2; scale in Hz from TMS at 60 MHz.

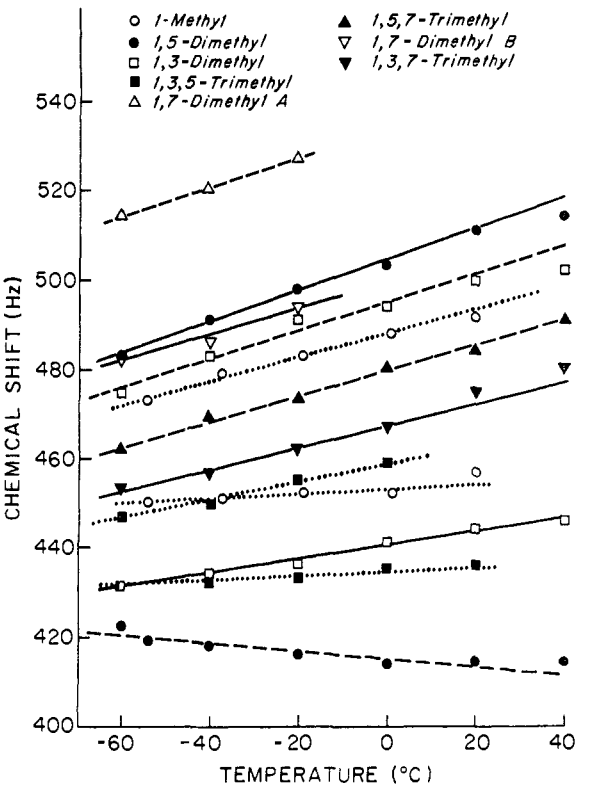


Figure 5. Chemical shift of amino protons in cytosine hydrochlorides in SO_2. Solid lines refer to H_b, broken lines to H_a, as indicated in Figure 4. Dotted lines are used where the assignment is inconclusive.

Table II also shows for comparison shifts in the frequencies of the protons attached to the ring.

Table II, as well as the other three consistent assignments, shows that methyl substitution in the 3 or 5 position causes an upfield shift (0.2 to 0.9 ppm) in the frequency of the 7-proton *cis* to it. This shift has the proper direction and approximate magnitude to be attributed to the magnetic anisotropy of the C–C bond of the methyl group.

The effect of temperature variation upon the frequency of the NH peaks of several methylcytosine hydrochlorides in SO_2 is plotted in Figures 4 and 5. It can be seen from Figure 5 that the general effect of decreasing the temperature is an upfield shift of the amino protons. One would expect the opposite shift from hydrogen bonding to the solvent. The presence of ion pairs furnishes a possible explanation in that a closer average approach of the anion at lower temperature could partially neutralize the positive charge on the cation and lead to an upfield shift. Fraenkel[7] has shown that in anilinium salts the chemical shifts of the aromatic protons are critically dependent upon the distance between the centers of positive and negative charges in the ion pairs.

The behavior of the 3-H frequency is consistent with the assignment of structure to the geometrical isomers IA and IB. In Figure 4 it can be seen that 3-H of IA moves downfield with decreasing temperature in the same manner as IIA, whereas the 3-proton of IB behaves more like other derivatives, which do not have a 7-methyl group in the immediate vicinity of 3-H.

Acknowledgment. We thank R. B. Bradley for maintenance of the spectrometer and help in recording the spectra, and H. K. Miller for assistance in sample preparation.

(7) G. Fraenkel, *J. Chem. Phys.*, 39, 1614 (1963).